From Fungi to Fuel:

Not a Rotten Idea

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Abstract

White rot fungi have proven to contain enzymes that make them capable of mineralizing lignin into carbon dioxide. Lignin, a molecule found in some plant cell walls, impedes processing of cellulosic ethanol and requires the use of chemicals for breakdown. This study confirms the ability of white rot fungus to break down lignin and explores introducing that ability into bacteria to biodegrade lignin on a large scale for the production of cellulosic ethanol. The fungus was chopped and ground with extraction buffer using a mortar and pestle. The slurry was spun in a centrifuge, combined with p-Nitrophenyl glucopyranoside (representing cellulose) and then stop solution. Coloring confirmed the breakdown of cellulose and therein the presence of ligninolytic enzymes. Representing a genetic transfer from eukaryote to prokaryote, the gene coding for the glowing protein, pGLO, was incorporated into E. coli bacterial cells as DNA from fungi could be. Finally, Green Fluorescent Protein, or GFP, was extracted from the bacterial cells just as lignin degrading enzymes could be for use in the production of cellulosic ethanol.

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Background

Society has been in need of better sources of sustainable energy for many years. Between limited access to petroleum-based fuels and the unavoidable byproducts related to most renewable sources, the struggle to maintain a green planet has only become more challenging over time. Biofuels, such as ethanol and biodiesel, have been in the process of improvement and integration in hopes that they can help to alleviate stress on the world's supply of energy in the near future. Although it appeared to be a clean alternative at first glance, the use of ethanol for fuel posed problems. Ethanol is "a renewable fuel made from various plant materials" known as biomass ("AFDC Ethanol", 2009). It is made of the same chemical formula found in alcoholic drinks: C_2H_5OH ("AFDC Ethanol", 2009). The biomass that ethanol is comprised of is simply combined starch and sugar based feedstock, corn crops especially, which raised an issue in itself ("AFDC Ethanol", 2009). In order to produce ethanol, potential food had to be sacrificed. World hunger was already a growing threat because of increasing populations worldwide, and using food that could be used to feed the hungry to make fuel could only worsen the issue. However, ethanol can be made from plant matter that the human body cannot digest.

Ethanol made from otherwise useless biomass is called cellulosic ethanol. Possible sources include wood ships, sawdust, switch grass, miscanthus, non-edible crop material, cuttings from lawn and tree care, and other organic waste materials ("Students", 2008). According to

researcher Caleb Dulaney, a Mississippi State University biological engineering undergraduate, "the plant matter left over after harvesting (alone) can provide an almost unlimited resource for biofuel manufacturing" ("Students", 2008). The ability to replace ethanol with cellulosic ethanol has made it no longer necessary to use up valuable crops for the purpose of power. However, there are reasons that woody biomass cannot be digested by the human body, and these same reasons also make it more difficult to chemically process into fuel.

Woody biomass contains lignocellulose in the cell walls of the plant matter, a compound containing cellulose, hemicellulose, and lignin (Wood & Kellogg, 1988). Cellulose, hemicellulose, and lignin are all complex molecules found in plant matter that are difficult to break down when intertwined as they are in lignocelluloses. (See diagram 1). Within molecules of lignocellulose, lignin is found covalently bonded to hemicellulose, surrounding the cellulose molecules (Wood & Kellogg, 1988; Kirk & Ferrell, 1987). Lignin is responsible for the sturdy nature of the bases of most plants and is not found in plants of lower taxonomic ranking (Kirk & Ferrell, 1987). Evolutionally different plants contain different forms of lignin (Kirk & Ferrell, 1987). Lignin is also what makes wood and other tough plant types difficult to process. Wood and other vascular tissues usually consist of about twenty to thirty percent lignin, making it the second most available renewable resource on the planet, but it gets in the way more than anything else (Kirk & Ferrell, 1987). Once the lignin is broken down, cellulose and hemicellulose can be converted into sugars for ethanol production, however this is extraordinarily difficult to accomplish because of the nature of lignin ("Students", 2008).

Lignin is a complex molecule consisting of smaller hydrocarbons held together by very strong carbon-oxygen bonds ("Students", 2008; Wood & Kellogg, 1988). (See diagram 2). In order to

produce ethanol, these complex molecules must first be broken down into the smaller hydrocarbons they contain, called monomers and dimers, so those hydrocarbons can be used to create the alkanes and alcohols that are necessary in the production of ethanol (Barras, 2008). The only way to successfully break down lignin is to break the carbon-oxygen bonds that hold it together. Although this has been done many different ways, there has never been a reliable method that does not require the use of harmful chemicals or large amounts of energy and does not produce problematic waste products ("Students", 2008). Lignin has always broken down in an unpredictable manner and produced many different compounds, only some of which could be used to make biofuels, which made the production of cellulosic ethanol highly inefficient (Barras, 2008). Recent breakthroughs have offered new ideas that represent the next step in mastering lignin.

Fungi are decomposers; they can be found on many organic materials. White rot fungi, however, are the only fungi capable of decomposing wood (Bajwa & Arora, 2009). They use lignocellulose as a carbon source, breaking the carbon-oxygen bonds in lignin and returning the carbon to the atmosphere as carbon dioxide (Bajwa & Arora, 2009). They also use the cellulose in lignocellulose for food. The scientific community suspected the presence of lignin degrading enzymes in white rot fungi and in 1982, the first lignin degrading enzyme was uncovered (Kirk & Ferrell, 1987). White rot fungi do indeed produce ligninolytic enzymes that allow them to break down lignin and the other compounds in lignocellulose. The fungi "invade the lumens," or the cavities enclosed by cell walls, "of wood cells, where they secrete enzymes that degrade lignin and other wood components" (Kirk & Ferrell, 1987, p. 2). The enzymes involved include ligninases or lignin-degrading peroxidases, Manganese peroxidases, phenol-oxidizing enzymes,

H_2O_2-producing enzymes, and phenyloxidase or laccase (Kirk & Ferrell, 1987). These enzymes have been found in many species of fungi, although they vary in combination and concentration (Kirk & Ferrell, 1987). Peroxidases and hydrogen peroxide are also secreted by the fungi and help to catalyze the process (Bajwa & Arora, 2009).

Fungi that appeared to be most effective in the breakdown of lignin exhibit the highest production rates of ligninolytic enzymes (Kirk & Ferrell, 1987). *Phanerochaete Chrysosporium* yields the best rates of lignin decomposition (Kirk & Ferrell, 1987). Environmental factors play a significant part in the ability of a fungus to biodegrade lignin as well and not all fungi operate optimally in the same environment. In a study done by the School of Environmental Sciences at the University of Guelph in Canada, optimal production of lignin peroxidase by two highly efficient species of fungi, *P. chrysosporium* and *Polyporus sanguineus*, was reached in two greatly varied culture conditions (Aust & Benson, 1993). Both environmental and genetic factors have been investigated because of their relevance to the development of cellulosic ethanol. Research has shown that the species of white rot fungus *P. chrysosporium* cultured in highly aerobic environments performs better than all other species and possible environments (Kirk & Ferrell, 1987). The genes from *P. chrysosporium* should then be used for experimentation on the topic and environmental factors should be imitated.

Once isolated, ligninolytic enzymes and processes from this species can be introduced into strains of bacteria. The goal is to use bacteria to degrade lignin in biomass on a large scale, eliminating the necessity of toxic chemical processing and making cellulosic ethanol as a biofuel more efficient and environmentally responsible. Bacterial degradation has been intensely

researched lately as a more economical substitute for fungi in laboratories as producers of lignin degrading enzymes. Streptomyces have been studied most extensively for this purpose.

A species of white rot fungus was used to decompose a cellulose derivative, implying that the fungus was capable of lignin degradation as a method of accessing the cellulose molecule which it uses for energy . This supports the potential of white rot fungi in the development of cellulosic ethanol. Then, a jellyfish gene carrying plasmid, pGLO, coding for Green Fluorescent Protein, or GFP, was inserted into the DNA of bacterial cells, simulating the eukaryote to prokaryote transfer that would be necessary to transfer genetic material from fungi to bacteria. Finally, GFP was isolated from the bacteria, representing the isolation of lignin degrading enzymes from bacterial cells for industrial use.

Methods

Part One: Confirmation of the Existence of Lignin Degrading Enzymes in a Fungal Sample

Approximately 1.5 grams of white rot mushroom were weighed out and placed in a mortar. Two milliliters of extraction buffer was added for every gram of mushroom, making a total of three milliliters of extraction buffer added to the contents of the mortar. The contents of the mortar were then ground with the pestle to produce slurry. Cheesecloth was then stretched over the opening of a small beaker. The slurry was strained through the cheesecloth until all liquid had been forced through the cheesecloth. The slurry was scooped into a one and one-half milliliter centrifuge tube and the solid particles were collected into a pellet at the bottom of the tube by spinning at ten thousand rotations per minute for two minutes. Six fifteen milliliter test tubes were labeled “1 min”, “2 min”, “3 min”, “4 min”, “6 min”, and “8 min,” respectively and five hundred microliters of stop solution were pipetted into each. A fifteen

milliliter test tube was labeled “rxn tube” and 3 milliliters of substrate (p-Nitrophenyl glucopyranoside) were added into it. The substrate had a pH of five. Two hundred fifty microliters of mushroom extract were pipetted into the fifteen milliliter test tube containing the three milliliters of substrate. Five hundred microliters of the enzyme extract/ substrate mixture were pipetted out and added to each test tube containing the stop solution at the given intervals of time. A base with a pH of 9.5 was then added to indicate the presence of p-Nitrophenol, causing a color change from clear to yellow. All pipettes used were sterile (“Biofuel,” n.d.).

Part Two: The Transfer of Genetic Information from Eukaryote to Prokaryote and Protein Extraction

The presence of lignin degrading enzymes in white rot fungi, especially *P. chrysosporium*, has been confirmed. A map of the genome for *P. chrysosporium* has also been formulated; however, it was not available to the public at the time of this investigation. Therefore, DNA from a jellyfish coding for the production of Green Fluorescent Protein in the form of a plasmid, pGLO, was transferred to bacterial cells, simulating the same eukaryote to prokaryote transfer that would take place if transferring DNA from fungi to bacteria for protein production. The plasmid that contained the desired segment of jellyfish DNA also contained a gene that causes resistance to ampicillin. The purpose of this is to allow for two methods of confirmation that the plasmid was effectively transferred into the bacterial cell.

This procedure involves the use of rehydrated pGLO plasmids from jellyfish DNA in place of genes coding for the production of lignin degrading enzymes from white rot fungi, especially *P. chrysosporium* because a map of the genetic code for *P. chrysosporium* was not available to the

public at the time of this investigation, although it had been created. One closed micro centrifuge tube was labeled "+pGLO" and another "-pGLO" and placed in a foam tube rack. The tubes were opened and, using a sterile pipette, two hundred fifty microliters of transformation solution ($CaCl_2$) was transferred into each tube. The tubes were placed on ice in a small beaker. A sterile loop was used to pick up 5 large colonies of E. coli from a starter plate. Only colonies that were separate, uniformly circle, and smooth around the edges were selected. The sterile loop was then immersed into the transformation solution at the bottom of the +pGLO tube. The loop was spun between index finger and thumb until the entire colony was dispersed in the solution. The tube was placed back in the tube rack in the ice. This process was repeated for the –pGLO tube.

A new sterile loop was immersed into the pGLO plasmid DNA stock tube and a loop full was withdrawn. The loop full was mixed into the cell suspension of the +pGLO tube. The plasmid was not added to the –pGLO tube. Both the –pGLO and the +pGLO tubes were closed and returned to the rack on ice and allowed to sit for ten minutes.

While the tubes sat on ice, four LB nutrient agar plates were labeled (on the bottom, not the lid) as follows:

1) LB/amp plate 1: +pGLO
2) LB/amp/ara plate: +pGLO
3) LB/amp plate 2: -pGLO
4) LB plate: -pGLO

"Amp" stood for ampicillin and "ara" stood for arabinose.

Using the foam rack as a holder, both the +pGLO and –pGLO tubes were transferred into a forty-two degrees Celsius water bath for exactly fifty seconds. After fifty seconds, both tubes were placed back on ice quickly for best results. They were incubated on the ice for two minutes.

The rack containing the tubes was removed from the ice and placed on the table. A tube was opened and, using a new sterile pipette, two hundred fifty microliters of LB nutrient broth were added to the tube and the tube was reclosed. This process was repeated for the remaining tube with a new sterile pipette. The tubes were incubated at room temperature for ten minutes. The tubes were gently flicked while closed to mix and re-suspend the bacteria.

Using good sterile technique, one hundred microliters of the transformation and control suspensions were pipetted onto the appropriate nutrient agar plates. A sterile loop was used to spread the suspensions evenly around the surface of the LB nutrient agar by quickly sliding the flat surface of a new sterile loop back and forth across the plate surface without pressing too deeply into the agar. The plates were placed in an incubator set at thirty-seven degrees Celsius and left for the night. The next day, the fluorescence on the agar plates was examined qualitatively using a UV light source.

A sterile loop was used to scrape a healthy scoop of colonies from the Lb/amp/ara plate and transfer the colonies to a micro tube containing three hundred microliters of Laemmli sample buffer. The solution was thoroughly mixed by spinning the loop with the thumb and forefinger, leaving no visible clumps of bacteria in the tube. The contents of the tube were examined under UV light (“PGLO Bacterial”, n.d.).

Results

Part One: Confirmation of the Existence of Lignin Degrading Enzymes in a Fungal Sample

In this procedure, the substrate p-Nitrophynyl glucopyranoside, a cellulose derivative, represented cellulose within lignocellulose of plant cell walls. Cleavage of p-Nitrophynol from glucose produces a yellow color in basic solution. This simulates the cleavage of cellulose through enzymatic reactions that occurs naturally in white rot fungi. (See diagram 3). All of the timed trials turned yellow once exposed to the base. (See table 2). The yellow coloring of each test tube confirmed that the p-Nitrophynol was cleaved from the glucose by the enzymes in the white rot fungi.

Part Two: The Transfer of Genetic Information from Eukaryote to Prokaryote and Protein Extraction

The LB plate with bacteria negative for pGLO (noted as LB/amp –pGLO) exhibited normal bacterial growth. The LB plate with ampicillin and bacteria negative for pGLO (noted as LB/amp –pGLO) contained no growth. The LB plate with ampicillin and bacteria positive for pGLO (noted as LB/amp +pGLO) exhibited normal bacterial growth. The LB plate with ampicillin, arabisole, and bacteria positive for pGLO (noted as LB/amp/ara +pGLO) exhibited growth of bacteria that glow green when exposed to UV light. (See table 2). The GFP was then extracted from the bacterial cells. Success was confirmed using UV light.

Conclusions/ Recommendations/ Extensions

The cleavage that took place in the p-Nitrophenyl glucopyranoside during the first stage of the procedure is representative of that which would take place when cellulose from within lignocellulose is broken down into glucose molecules. The fact that the enzymes in the white rot fungus were capable of separating the glucose in the substrate means that they were

breaking down the cellulose within the substrate. Lignin intertwines itself with hemicellulose to surround the cellulose molecules, so if the cellulose was broken down it can be assumed that the fungus also contained enzymes for breaking down lignin and hemicellulose. The fact that all timed tests turned yellow also shows that the enzymatic reaction happened very quickly, occurring within one minute.

In the second part of the experiment, the LB plate with -pGLO bacteria was the control. It was simply a standard nutrient agar plate that allows almost anything to grow with an unaltered strain of bacteria on it that exhibited growth. The LB/amp plate with –pGLO bacteria showed the inability of unaltered bacteria to grow on a plate with the antibiotic ampicillin. The LB/amp plate with +pGLO bacteria exhibited growth despite the presence of ampicillin, which means that the bacteria absorbed the plasmid coding for ampicillin resistance. The bacteria on this plate did not glow green under ultraviolet light, though. The reason for this is that they were not provided with the sugar they need to actively produce GFP, even though their DNA contained pGLO. Finally, the LB/amp/are plate with +pGLO bacteria had growth and the bacteria glowed when exposed to ultraviolet light because of the available arabisole.

In summary: the sample of white rot fungus decomposed the cellulose derivative, implying that lignin could be was broken down by the fungus. This confirmation allowed the potential of white rot fungi in the development of cellulosic ethanol to be supported. Then pGLO, which codes for the production of the protein GFP, was successfully incorporated into the genome of E. coli so the bacteria glowed when exposed to UV light. This procedure represented the possible transfer of genes that code for lignin degrading enzymes from *P. chrysosporium* to bacteria. Finally, pGLO was extracted from the bacterial cells just as lignin degrading enzymes

could be. Those enzymes could then be used for the degradation of lignocellulose in biomass as they were in the first of the three procedures. With access to the *P. chrysosporium* genome, it would be possible to first isolate the desired segment of DNA with restriction enzymes, then transfer that segment into E. coli in the form of a plasmid, and then extract that enzyme and use it in place of the expensive and toxic chemicals typically required for the breakdown of lignocellulose for cellulosic ethanol production.

The future of biofuel might rest in the abilities of white rot fungus, and that future should be explored as extensively as possible; it's not a rotten idea.

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Appendices

Diagram 1: Structure of lignocellulose

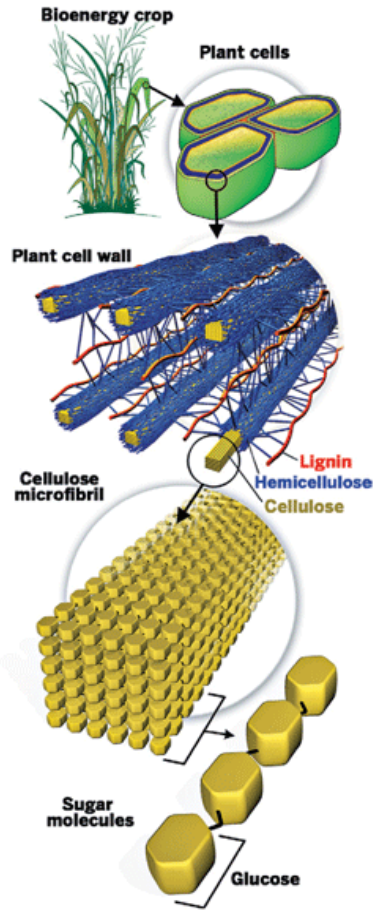


Diagram 2: Simplified Structure of Lignin

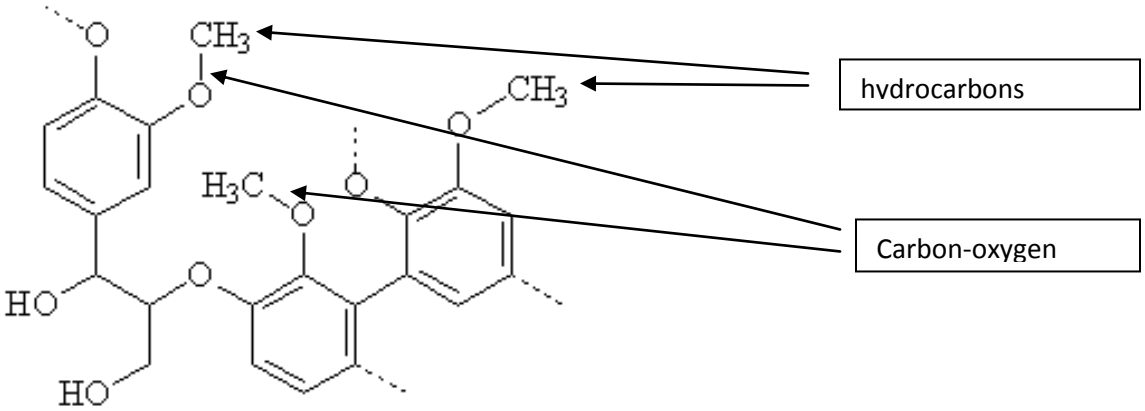


Diagram 3: breakdown of p-Nitrophenyl glucopyranoside as opposed to cellulose

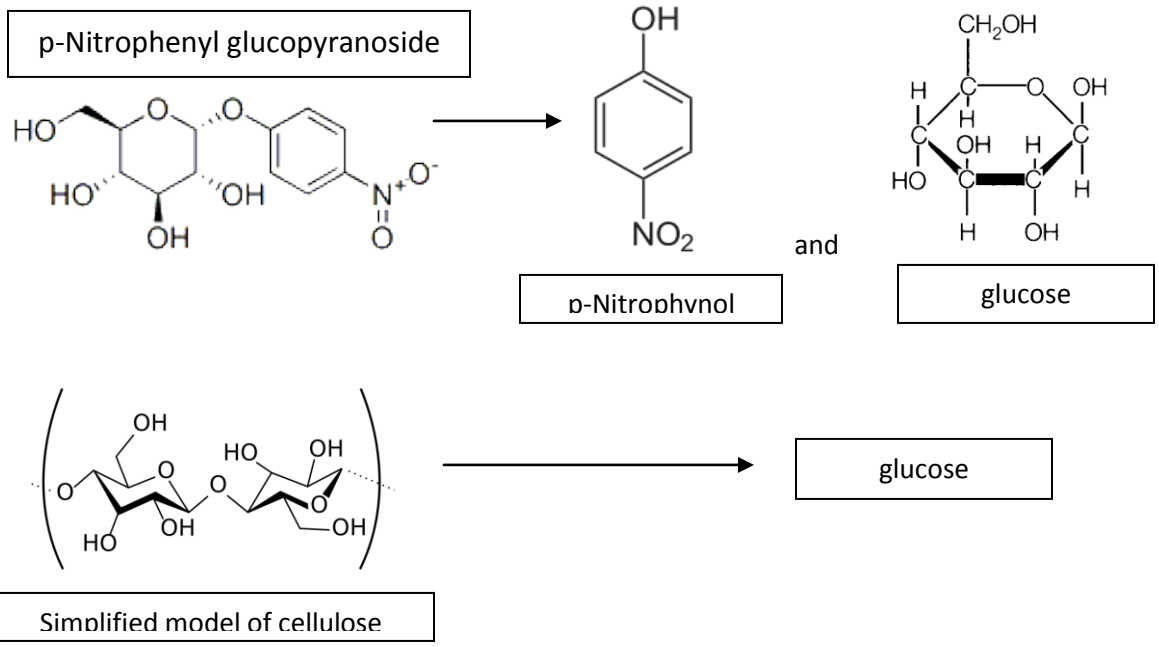
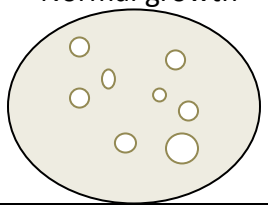
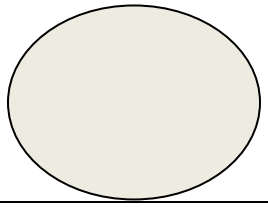
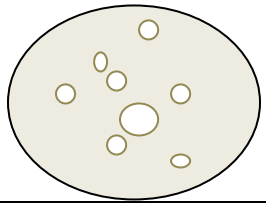


Table 1: Results for Part 1

time trial	1 min	2 min	3 min	4 min	6 min	8 min
color	yellow	yellow	yellow	yellow	yellow	yellow

Table 2: Results for Part 2

	-pGLO bacteria	+pGLO bacteria
LB Plate	Normal growth 	Not tested
LB/amp Plate	No growth 	Normal growth 
LB/amp/ara Plate	Not Tested	Growth and GFP Production