Lab Report

SplintErase: Lignocellulose Degradation Using an Extracellular TxTl system

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Wood splinters present a problem: they can cause pain and infection and are hard to remove. People are impacted daily by wood splinters, however, there is no optimal solution to combat them. The existing solution includes tweezers, but these often fail to remove the entire splinter. To resolve this issue, an extracellular method of expression called TxTl was utilized to degrade lignocellulose into compounds that the body can absorb. Lignocellulose is made of three components: cellulose, hemicellulose, and lignin. For the cellulose degradation, endoglucanase, beta-glucosidase, and exoglucanase are utilized. Xylanase is needed to degrade the hemicellulose, and blue copper laccases are used for lignin degradation. Based on our group's previous research into the system design, we decided to focus on testing the degradation of cellulose using solely endoglucanase and beta-glucosidase. After transforming the enzymes using an *Escherichia coli* chassis, we conducted a filter paper assay and found that there was a lack of filter paper degradation. In the future, our group plans to test the entire enzymatic system, incorporate it with a TxTl system, and improve the degradation of lignin employing blue copper laccases in conjunction with oxidative mediators.

Keywords: TxTl, *Escherichia Coli*, enzymes, assay

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Background

Wood splinters are painful; they penetrate multiple layers of an individual's skin and are difficult to remove. The predominant solution involves tweezers, but pieces of splinters are often left behind, possibly leading to bacterial infection as many wood splinters carry pathogens. The originally planned form of expression is called TxTl, which is a cell-free protein expression system. Our aim is to use specific enzymes to degrade ligninocellulose, the main component of wood, into glucose, which the body can safely absorb. Ligninocellulose has three main parts: cellulose, hemicellulose, and lignin. Hemicellulose is a heteropolymer that is degraded through an enzyme called xylanase. Lignin is the main component of wood, making up 25 to 30 percent of the cell wall and is theoretically degraded by blue copper laccases (Christopher et al. 2014). However, this lab experiment focused only on the cellulose degradation aspect of the TxTl system, involving two major enzymes, namely endoglucanase and beta-glucosidase.

Endoglucanase is the first step in the degradation of cellulose as it hydrolyzes cellulose into cello-oligosaccharides. The second enzyme, exoglucanase, randomly cleaves the cello-oligosaccharides into glucose and cellobiose, which is a disaccharide. The beta-glucosidase is the final step as it hydrolyzes the cellobiose into glucose monomers by breaking specific internal bonds, which should theoretically be absorbed into the body as a safe product of the reaction (Blanchette 1995). Endoglucanase is part of the cellulose family, has a high affinity for compounds like cellulose, and is known to hydrolyze solid or soluble substances. Endoglucanase cleaves glucose chains, making it ready for exoglucanase, which has a scaffold structure (Rodriguez 2005). Cellobiose inhibits the function of endoglucanase and exoglucanase, making beta-glucosidase necessary (Rodriguez 2005). We found a new enzyme for the degradation of hemicellulose: xylanase. Xylanase, unlike endoglucanase, is able to act on hemicellulose, hydrolyzing it and allowing the cellulolytic enzymes to access and degrade the cellulose (Parisutham 2012).

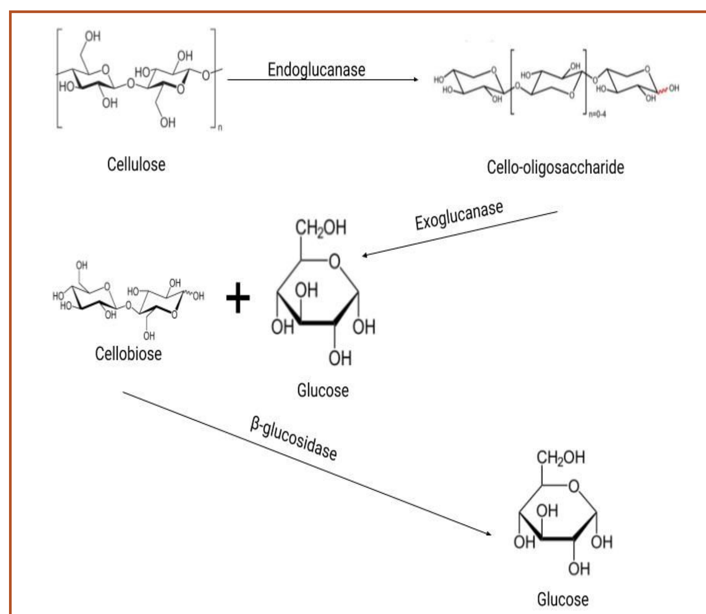

Figure 1. *System for cellulose degradation: The conversion of cellulose to glucose monomers. Cellulose, with the help of endoglucanase, converts into a cello-oligosaccharide, which turns into cellobiose and glucose with the aid of exoglucanase. These, together with beta-glucosidase, turns into glucose monomers, which the body can absorb.*

Materials and Methods

E. coli transformation with endoglucanase and beta-glucosidase

In the initial experiment, two cellulolytic enzymes, beta-glucosidase and endoglucanase, were produced using competent cells. First, competent *E. coli* cells from New England Biolabs were thawed from a -80 °C freezer in an ice bucket. Any unused cells were discarded as the cells could not be refrozen. Then, 10 mL of distilled water was pipetted into wells containing endoglucanase and beta-glucosidase plasmids from iGEM along with a red dye. The mixture of plasmids and water was pipetted into two 1.5 mL tubes, labeled endoglucanase and beta-glucosidase, respectively. Subsequently, 50 µL of competent cells were pipetted into each of the 1.5 mL tubes, and the solution was stirred with the pipette. The tubes were put on ice for 30 min. A water bath was warmed to 42 °C and the tubes were placed onto a floating tube rack. The tubes were heat shocked for 45 seconds and immediately returned to the ice for 5 mins. to allow for the absorption of the foreign DNA into the competent cells. The tubes were removed from the water bath, and 950 µL of Super Optimal Broth with catabolite suppression media (SOC) were pipetted into each tube, allowing the bacteria to recover. Then, 5 mL of chloramphenicol antibiotic were added to each tube to eliminate any untransformed bacteria. The tubes were placed in a rotator within an incubator at 37 °C for 1 h in order to confirm that the bacteria had absorbed the DNA. Four LB agar plates were obtained; two plates were named endoglucanase and the other two beta-glucosidase, and 100 mL of bacterial solution was pipetted onto each respective plate. Hockey stick spreaders were used to evenly spread the bacteria. Lastly, the plates were incubated for 14-18 h at 37 °C and the growth of bacterial colonies was observed.

Afterward, a cellulose assay was performed. First, 500 µL of the LB were pipetted into two 1.5 mL tubes labeled for endoglucanase and beta-glucosidase, respectively. A toothpick was used to select individual bacterial colonies and immerse these in their respective falcon tube. The tubes were incubated for 14-18 at 37 °C to allow multiplication of the colonies. Next, four falcon tubes were obtained and labeled endoglucanase, endoglucanase and beta-glucosidase, beta-glucosidase, and negative control. Then, 5 Then, 5 mL of mM citrate solution and 0.5 ml of LB were added to the transformed cells in the falcon tubes to preserve the secreted enzymes. A piece of Whatman Filter paper was rolled up and placed into each tube. The falcon tubes were placed in a 50 °C bath for 60 mins. to stimulate enzyme production. The tubes were removed from the water bath and placed on a tube stand. Glucose test strips were obtained and labeled endoglucanase, beta-glucosidase, endoglucanase and beta-glucosidase, negative control, and positive control. A glucose solution was prepared to obtain a positive control. The strips were placed into each of their respective falcon tubes. Color changes were observed in the glucose test strips, based on each solution's glucose concentrations.

Laboratory and environmental safety

Throughout both experiments (transformation of *E.coli* and the filter paper assay), protective clothing was worn

by the researchers; specifically, protective goggles for eye protection, latex gloves for hand protection, and lab coats to protect the whole body were worn by each individual. In the experiment, a harmless, non-pathogenic strain of *E.coli*, K-12, was used. Additional safety measures such as fume hoods and masks were not used due to the harmless nature of the materials utilized in both experiments. In addition, all of the materials we used posed no environmental threat and were disposed of through regular sink drains and trash cans.

Results

Initially, we transformed competent cells to determine the expression of the endoglucanase and beta-glucosidase enzymes. The transformed cells were grown in LB media, and there was successful growth of bacterial colonies for both beta-glucosidase and endoglucanase, as evidenced by the chloramphenicol resistance of the resulting competent cells. (see Figure 1). Using a filter paper assay, we then tested whether the combination of endoglucanase and beta-glucosidase could degrade cellulose into glucose, which can safely be absorbed by the body. We also tested endoglucanase and beta-glucosidase separately. The transformed colonies were used to inoculate an overnight broth culture and the enzymes were extracted onto filter paper. The filter paper containing the enzyme was placed in a glucose solution and then

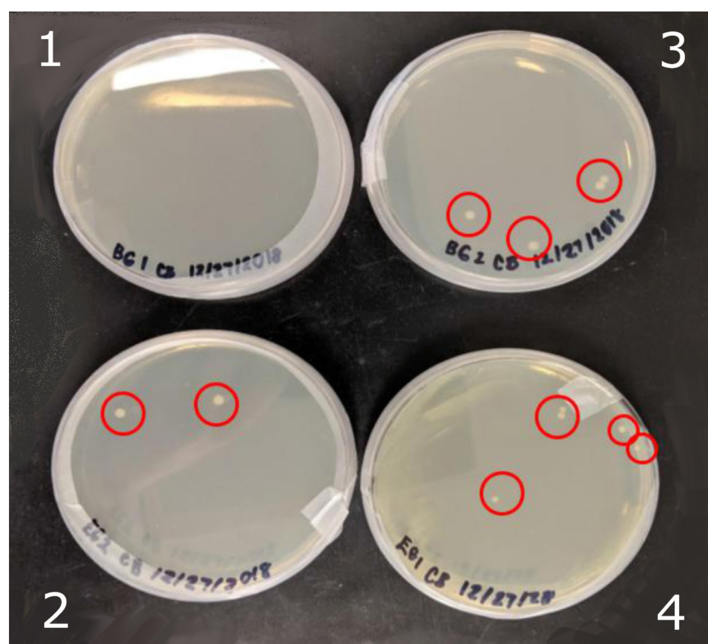

Figure 2. *This image of transformed* E. coli *grown on LB media shows successful growth in the presence of chloramphenicol, circled in red. The top two plates contain beta-glucosidase, and the bottom two plates have endoglucanase. The plate 1 unexpectedly had no growth, while the other beta-glucosidase plate had three successful areas of growth. Endoglucanase plates 2 and 4 showed growth.*

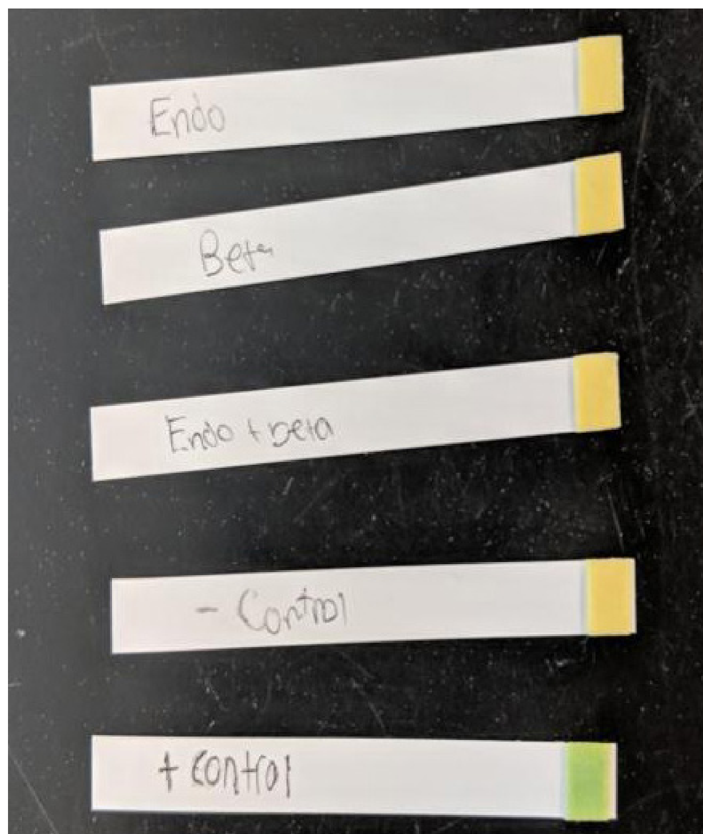

Figure 3. *This image shows the result of the filter paper assay. A green color is visible in the positive control glucose test strip, indicating that glucose was present. The endoglucanase, beta-glucosidase, endoglucanase and beta-glucosidase, and negative control strips are all the same color, illustrating that there was no color change after being dipped into the respective falcon tubes.*

tested with glucose test strips, which are highly sensitive at 100 mg/dL. The negative control consisting of water and filter paper, displayed no presence of glucose, which was expected because there were no enzymes present to degrade the paper into glucose (see Figure 2). The positive control showed that the glucose test strips worked properly and turned green when exposed to glucose. As expected, neither enzymes functioned in the separate tests as both are necessary to degrade cellulose. However, unexpectedly, the combination of both enzymes also did not produce glucose from the degradation of filter paper. We can infer that the filter paper did not degrade from the absence of glucose and, after further research, we found that we need to add other intermediate enzymes in order to accomplish degradation.

Discussions

In this experiment, we attempted to hydrolyze cellulose into its beta-glucose monomers through the sequential use of endoglucanase and beta-glucosidase collected from the transformed competent cells. In order to test

the collective effect of these enzymes on the degradation of cellulose, we conducted a filter paper assay. However, none of the glucose test strips, except for the positive control, showed a color change. Therefore, it can be inferred that at least one of these two cellulases failed to degrade their respective substrates.

Due to the negative results produced by the filter paper assay, we plan to test for intermediates, which include cellobiose and cello-oligosaccharides. It is also possible that the glucose concentration furnished by the enzyme degradation was insufficient to change the color of the glucose test strips (see Figure 3). Therefore, in the future, we may employ more sensitive methods of glucose determination, including utilization of GBPs (glucose-binding proteins) bound to two fluorophores (Pickup et al. 2013). This system would be able to detect micromolar concentrations of glucose. In order to ensure that enzymes are being produced, we could employ a Western Blot assay. Since cellulose is a 2-20 nm molecule, we could employ a lysis buffer or bacterial expansins to ensure that the enzymes diffuse through the cell wall and efficiently access the substrate. As mentioned above, the filter paper is processed and thus may include additional compounds that impede catalysis, so it might be better to use pure cellulose powder in our assay (see Figure 2). Although our experiment produced negative results, our efforts may strengthen the understanding of the inherent properties of these enzymes and provide insights as to the conditions at which they perform optimally. Furthermore, these three cellulase enzymes display relatively low specific activities *in vitro*; this could be attributed to the lack of cellulosomes, which are scaffold-like complexes in plant cells that position the three enzymes so as to optimize degradation (Zhang 2009). Efforts to mimic these *in vivo* biological systems may help to improve the efficiency of our Splinterase system.

Future experimentation

In order to efficiently degrade lignocellulose complexes, both lignin and hemicellulose need to be degraded through the incorporation of blue copper laccases and xylanases, respectively, into the endoglucanase and beta-glucosidase enzyme mixture. Blue copper laccases, along with lignin peroxidase and manganese peroxidase, are capable of degrading lignin (Qasemian et al. 2011). Blue copper laccases are copper-containing phenol oxidases that are used for the degradation of lignin; they are derived from white rot fungi, which degrade lignin into carbon dioxide and water (Blanchette 1995). Blue copper laccases perform this through a series of redox reactions as they have four copper atoms at the catalytic site, which mediate the process (Christopher et al. 2014). A recent scientific breakthrough is mediators, chemical compounds that are continuously oxidized by laccase and reduced by the substrate (Christopher et al. 2014).This will be further investigated in future experimentation. Laccases are copper-containing oxidases, cofactors that oxidize substances with the aid of mediators: chemical compounds that act as electron carriers between enzyme and substrate. Fortunately, fungal laccases act on both the intracellular and extracellular levels, thereby providing a greater likelihood of substrate degradation. Finally, their enzymatic optimal temperatures are between 50 °C and 70 °C. Thus, laccases and the other lignin-degrading enzymes show great potential in improving the Splinterase system to aid the degradation of the entire lignocellulose complexes present in wood, in conjunction with the previously investigated cellulases.

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