SplinterCell System: Degradation of Cellulose Through a Cell-Free TxTl System

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Wood splinters are a nuisance that individuals frequently encounter. Currently, the most prevalent method of extraction is using common household tweezers; however, this process can be painful and susceptible to bacterial infection. In order to resolve this dilemma, we propose a minimally invasive enzymatic cream that has the ability to degrade splinters. Within the cream, cellulolytic enzymes, endoglucanase and beta-glucosidase, degrade the lignin and cellulose of wood into monosaccharides that the body can easily absorb. Our previous research in this domain produced negative results, attributable to design flaws in our past system. The new system uses an *Escherichia coli* cell-free transcription-translation (TxTl) system for a more consistent protein expression, as well as green fluorescent protein (*GFP*) reporting to verify protein expression. Thus far, when the TxTl system was tested with the *GFP* gene, a fluorescent glow was detected. This result signifies that this system is a viable approach to successfully expressing the two other cellulolytic enzymes. Moving forward, we hope to test and assemble the rest of the system and hopefully provide the public with a pleasant alternative method of removing wood splinters.

Keywords: Splinter, Enzymes, *Escherichia coli*, Green Fluorescent Protein, TxTl, Endoglucanase, Beta-Glucosidase, Cell-free expression

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Background

Afflicting the lives of nearly every individual, splinters are a frequent and painful injury. Their removal requires the use of tweezers, which generally are ineffective and painful. Splinters can carry a variety of pathogens and if left unattended, an infection can occur. For example, rose gardeners are susceptible to splinters from rose thorns which are often coated with the fungus *Sporothrix schenckii*. The resulting fungal infections can lead to diseases such as Sporotrichosis, which mainly infects the skin. While there is anti-fungal medicine available, the symptoms can persist for years. A splinter can also lead to discharge, increased pain, redness, swelling, or red streaking (Chan et al. 2003).

Therefore, the main objective of our experiment aimed to create an alternative method to

remove wood splinters without the pain and ineffectiveness of tweezers. In order to accomplish this task, we propose to create an enzymatic cream that utilizes a cell-free expression system to degrade wooden splinters into monosaccharides such as glucose. Our system is specifically focused on degrading wood splinters, as wood is more likely to trigger an immune response than other materials such as glass or metal. This is due to the fact that wood is an organic material and therefore is more likely to carry microorganisms.

Based on previous research, the splinter system utilizes two enzymes to degrade cellulose into glucose: endoglucanase and beta-glucosidase. The endoglucanase first breaks down cellulose into cellobiose, a disaccharide. Then, the beta-glucosidase further degrades the cellobiose into glucose, which the body can safely digest it. Both of these enzymes are found naturally within fungi and bacteria such as *Escherichia coli* (Gupta et al. 2012).

There are two main approaches to express the desired enzymes within the cream. The first method is by transforming bacteria with the genes that code for the enzymes, thereby creating a wood-digesting bacteria. But, due to public concerns over the safety of inserting bacteria into the skin, this method is not viable for marketing. The other course is through a cell-free expression system, called TxTl. This system has the ability to conduct both transcription and translation, allowing it to correctly assemble the protein product of a gene. The TxTl is mostly composed of RNA polymerase, primary sigma factors and amino acid mix. This system has been

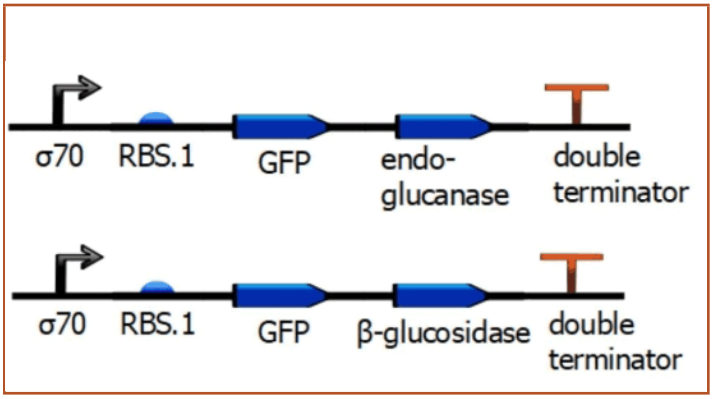

Figure 1. Proposed system for the enzymatic cream. *There are two sections of the system, one with the endoglucanase and one with the beta-glucosidase. Sigma 70 is the promoter specific to the Txtl system and it binds to the RBS.1 start codon when activated. Then the GFP is expressed, helping to signal that the system is correctly function and that the endoglucanase or beta glucosidase was successful expressed. The system ends with double terminator.*

demonstrated to produce a high yield of proteins, which could prove to be beneficial in this system. Although only the GFP component of the system was tested in the subsequent experiments, the ultimate goal is to assemble an enzymatic cream that has the ability to digest wood splinters (Garamella et al. 2016). The proposed system is illustrated in Figure 1, which depicts two parallel sections that start with a Sigma 70 promoter and ends with a double terminator. Each system contains either the endoglucanase or beta-glucosidase. Both systems also contain a GFP to demonstrate that the system is functioning correctly.

Materials and Methods

DNA Minipreparation and Plasmid Digest

In the initial experiment, the deoxyribonucleic acid (DNA) plasmid was isolated from *E. coli.* This process was completed with the Omega Bio-Tek E.Z.N.A Plasmid DNA Kit, which purifies the *E. coli* culture and aids in DNA extraction. First, the *E. coli* culture was incubated overnight in a 20 mL culture tube, allowing for the colony to grow. Then, the water bath and heat block were set to 55 ºC and 70 ºC, respectively. The elution buffer, solution 1, solution 2, solution 3, DNA wash buffer and HBC buffer were also obtained. The elution buffer was then heated in the heat block, increasing the yield of the DNA extraction.

Next, the *E. coli* culture was placed in a centrifuge and spun at a rate of 10000 rotations per minute (RPM) for 1 min, the resulting media was then discarded with a micropipette. The suspension was then transferred into a new 1.5 mL microcentrifuge tube, where 250 µL of solution 2 was added obtain clear lysate solution. After the addition of solution 2, 350 µL of solution 3 was pipetted into the tube, creating a white precipitate. The suspension was then centrifuged at 13000 RPM for 10 min, forming a pellet at the bottom of the test tube that contained all the components of the *E. coli* cells, excluding the DNA. The clear supernatant, incorporated with the DNA, was transferred into a HiBind® DNA Mini column with a two mL collection tube. The column was centrifuged at the maximum speed for one minute and the liquid was removed from within the collection tube. Then, 500 µL of a diluted HBC Buffer was added to the column and centrifuged again at the maximum speed for another one minute and the filtrate was once again discarded. Afterwards, 700 µL of DNA Wash Buffer was transferred to the column and centrifuged at the maximum speed for 30 s. Subsequently, the filtrate was discarded and the previous step was repeated. Finally, the column was centrifuged at maximum speed for 2 min to remove any trace ethanol. Once the precipitate was dry, the column was placed in a 1.5 mL centrifuge tube and 100 µL of Elution Buffer was added to the column. The column was

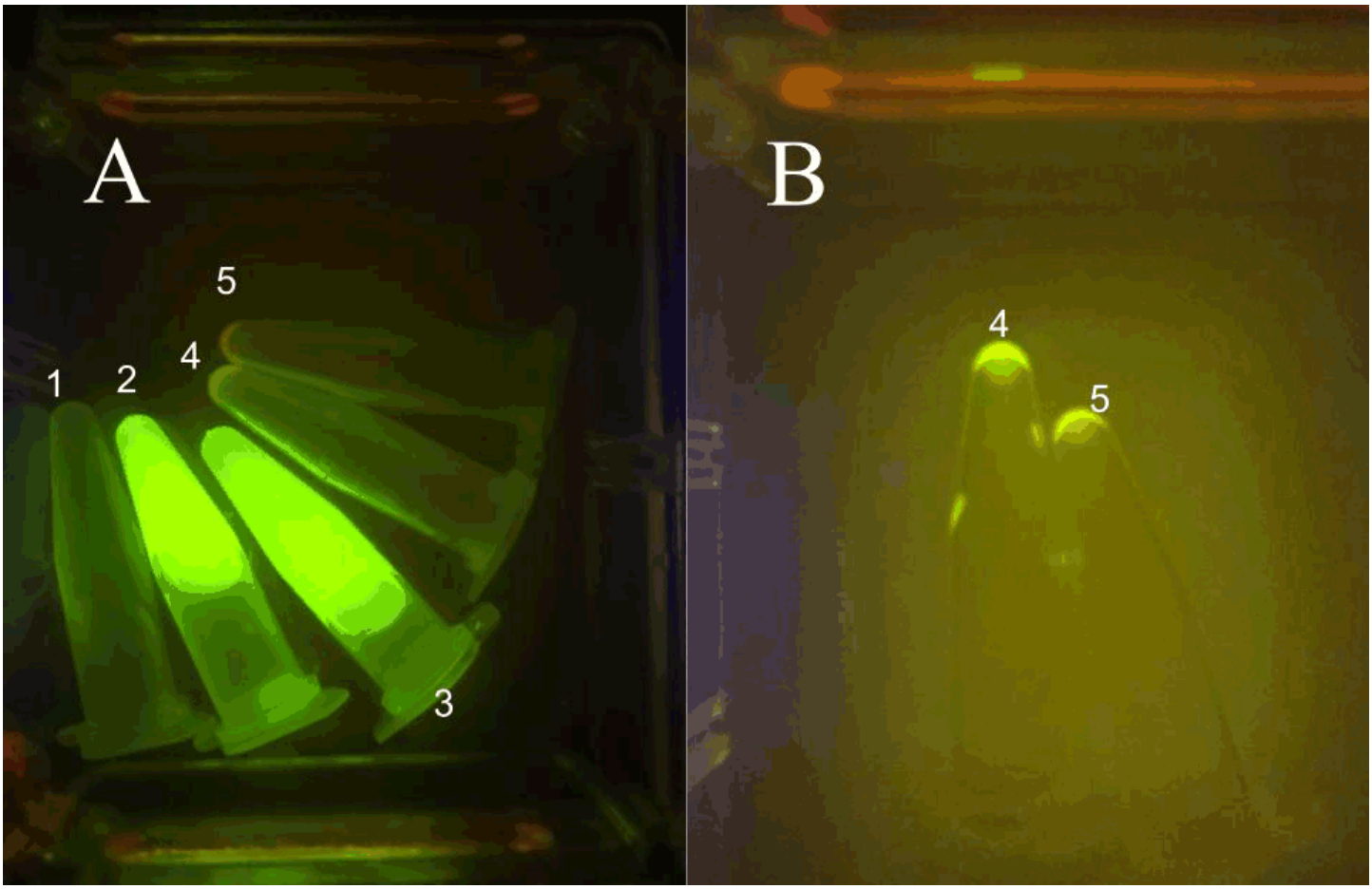

Figure 2. TxTl system successfully expresses the protein product of the GFP gene. *A) The bottom three centrifuge tubes represent controls that can be compared to the GFP expression. Each of the three tubes contain distilled water and various quantities of highlighter fluid. The leftmost tube contained the lowest concentrated highligher solution, increasing in concentration until the third tube. The other two tubes in the panel contain the TxTl system. The top tube is a negative control containing water and TxTl system and the second tube from the top encapsulates the GFP gene along with the TxTl system. B) This panel provides a clearer image of the experimental and negative control test tubes. The tube containing the GFP gene is on the left and the negative control is on the right. Illuminated by an ultraviolet light, it is evident that the experimental tube exhibits a green fluorescence. In the panel, it seems as if the negative control is also illuminated, but in reality, it is an reflection of the light being emitted from the experimental tube.*

then rested and centrifuged, both for exactly one minute. The final eluted DNA was stored at -20 ºC.

Once the DNA plasmid was successfully purified from *E. coli* pellet, the specific genes that code for endoglucanase and beta-glucosidase were isolated by completing a DNA digestion. First, 0.5 µL of the *EcoRI* restriction enzyme and the *PstI* restriction enzyme were added to a 1.5 mL centrifuge tube. Afterwards, the DNA plasmid was added to the tube, along with a buffer. Finally, nuclease-free distilled water was added, bringing the solution up to its total volume. The solution was mixed together ensuring homogeneity and was incubated at 37 ℃. Then. the solution can be analyzed to determine whether the digest was successful.

TxTl System and GFP Expression

In this portion of the experiment, the ability of the TxTl system to express the protein products of genes was examined. The *GFP* gene was utilized due to its ability to give off visual stimulus when its protein product is created, simplifying the protein assay. First, the heat block was preheated to 29 ºC. Then, the myTxtl® Sigma 70 Master Mix, template DNA, and P70a-deGFP plasmid were thawed and stored on ice. The P70a-deGFP plasmid encodes for an engineered version of a *GFP* gene that helps visualize the protein under ultraviolet light. Once thawed, the myTXTL® Sigma 70 Master Mix was vortexed and pipetted slowly to ensure the homogeneity of the mixture. In general, a single myTXTL® reaction is constructed by pipetting 9 µL of Sigma 70 Master Mix and a variable amount of template DNA into a centrifuge tube sitting on ice. In this experiment, 9 µL of the Sigma 70 Master Mix and 3 µL of the P70a-deGFP plasmid was pipetted into a tube, resulting in a final volume of 12 µL. Afterwards, the negative control was constructed by replacing the 3 µL of P70a-deGFP with nuclease-free

distilled water. Due to the fact that water does not react with the TxTl system, no qualitative change was visible. Once the two centrifuge tubes were prepared, both were vortexed and centrifuged in order to amass the solution at the bottom of the test tube. After centrifugation, the two tubes were incubated at 29 ºC for 16 h.

After the incubation period, the two centrifuge tubes were placed underneath an ultraviolet light along with three highlighter controls and a negative control. The expression of the P70a-deGFP was evaluated qualitatively through visual estimation of the fluorescence, comparing the intensity of green pigment from the GFP expression to three highlighter controls at varying concentrations. The comparison between the experimental tube and negative control was used to determine whether the TxTl system correctly expressed the GFP gene.

Laboratory and Environmental Safety

Throughout both experiments, DNA miniprep/digest and TxTl expression, protective equipment was worn by each individual of the participating groups. Specifically, each student wore a pair of goggles to protect the eyes, latex gloves to protect the hands and a lab coat to protect their overall body. In this experiment, a nonpathogenic strain of *E. coli* K-12 strain was used. It is found in the human gastrointestinal tract and thereby is safe for experiments. Since none of the materials used in either experiments were dangerous to the homeostasis of the body, neither a fume hood nor masks were worn by any of the students. Also, highlighter was used for the negative control for testing the TxTl system with GFP because it is nontoxic and safe for humans. Environmentally, the students did not pour any liquid besides water down the normal sink drain as it could harm the piping and the environment that it would arrive at.

Results

Positive Expression of GPF in the TxTl System

At the completion of the initial lab, which assessed the expression of the *GFP* gene using the TxTl system, the resulting data was positive. This conclusion was primarily ascertained by the qualitative comparison between the experimental centrifuge tube (Tube 4) and the negative control tube (Tube 5), which is visible in section A and B of Figure 2. When the tube encapsulating the *GFP* gene and TxTl system was exposed to ultraviolet light, a fluorescent green glow could be visually detected. However, when the negative control, which was distilled water and the TxTl system, was introduced to the ultraviolet light, no fluorescent was identified. Therefore, the visual difference between the experimental and the negative control tube illustrates the correct functionality of the TxTl system. Figure 2 suggests that both the experimental and the control expressed a fluorescent glow. However, in reality, the light coming from the control is a reflection of fluorescence being emitted from the experimental. The tubes would ideally have been photographed separately in order to prevent the visual contamination.

The tube containing the TxTl system and the GFP gene was also compared to three highlighter controls. Tube 3 contained the highest concentration of highlighter and Tube 1 had the lowest concentration. The concentration for the tube was determined by the number of times that the highlighter was dipped into the distilled water. The intensity of the expression from the experimental tube was lower than both Tube 2 and Tube 3, based on visual estimations. This helps to determine the strength of the TxTl system, acting as a basic quantitative assay for the GFP protein expression.

Unknown Results of Miniprep and Digest of the Enzymatic DNA

The second experiment that was conducted was a miniprep and a DNA digest of *E. coli* in order to isolate the specific genes that code for the two cellulolytic enzymes: endoglucanase and beta-glucosidase. Although the experiment was completed and a product was obtained, it is currently unknown whether the genes were correctly isolated from the rest of the *E. coli* plasmid. This is due to the fact that a gel electrophoresis with the isolated DNA has not yet been conducted. After loading the gel, electricity would be run through it and banding would appear. The banding resulting from the electrophoresis would be compared to the DNA ladder to determine the approximate base pair (bp) length of the bands. Once that value is qualitatively determined, it will be compared to the predicted band length of the two enzymes. For the endoglucanase and the beta-glucosidase, the anticipated base pair lengths are 1233 bp and 2280 bp, respectively. If the predicted and the experimental bands are comparable, it could be concluded that the isolation was successful. The gels could also be sliced into slivers at where the banding occurs and sent off to a lab for sequencing. Then, the results of the sequencing could be compared to the predicted sequence of nucleotides to determine whether the isolation was successful.

Discussions

Although the degree of success of the miniprep and digest of the DNA is currently unknown, the result of the experiment testing the expression of the TxTl system with the *GFP* gene was successful. This was determined by an observable fluorescence under ultraviolet light. This observation visually illustrates that the system

is functioning and is correctly producing the protein product of the *GFP* gene. The conclusive results of this lab allows for further testing of the TxTl system, especially with the endoglucanase and beta-glucosidase.

Due to the success of the initial experiments, we aim to proceed with testing and constructing the SplinterCell system. The first experiment that would be conducted is the aforementioned gel electrophoresis of the genes that code for the two enzymes. If their presence is confirmed within the gel, then the two gene would be tested with the TxTl system to determine whether they can be expressed. Subsequently, the genes would need to be purified through polyhistidine-tags and Ni^{2+} columns. Afterwards, the quality of purification can be determined by conducting a Western blot with the two enzymes. Also, a sodium dodecyl sulfate - polyacrylamide gel electrophoresis or a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) could be conducted. The SDS-PAGE uses the sodium dodecyl sulfate to help isolate the protein products of the two cellulolytic enzymes with in the gel. Then the gel can be sequenced to determine whether the system was successful. To determine the quantity of the protein expression, we intend to conduct enzymatic assays of the two enzymes. In order to assay the endoglucanase, the enzyme would be incubated with carboxymethyl cellulose and the rate at which one micromole of sugar is reduced every minute would be measured. For the beta-glucosidase, the cellulolytic enzyme would be incubated with p-nitrophenyl-b-D-glucopyranoside and the measurement would be quantified in a similar manner to endoglucanase assay (Gupta et al. 2012) Assuming that all of the components produce positive results, the entire SplinterCell system can be assembled, inserting the *GFP* gene, along with the chosen promoter and double terminator. The system depicted in Figure 1 could then be placed within a cream and begin development for public use and tested for adverse effects on the homeostasis of the body.

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