Production of a Fire-Resistant Housing Insulation Using Bacterial Cellulose and Transformed Escherichia coli BL 21

Acree McDowell, Sana McMurray, Nico Zuffoletti

Renaissance High School, Charlottesville, Virginia, United States

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Housing insulation is the material in any structure that acts as a thermal barrier, provides heat dampening around electrical components and serves as a fire retardant. Often, housing insulation is treated with chemicals in order to make it fire resistant. Unfortunately, during a fire, these chemicals off-gas, creating an unnecessarily toxic environment for victims and first responders. This research seeks to develop a housing insulation that is environmentally safe and non-carcinogenic while still serving as an effective fire retardant. The original concept of a fire-retardant protein came from the University of Mingdao's 2015 iGEM project. They developed a protein, which is rich in nitrogen and phosphorus, that proved to be effective in diffusing a combustion reaction. This current research will insert the gene that encodes this protein into a plasmid and transform the plasmid into *Escherichia coli* BL21. The extracted protein will be used in conjunction with bacterial cellulose, an environmentally friendly by-product in the production of kombucha, that will be pulped, dried, and aerated until it reaches the desired consistency to be used as insulation. After being combined with the protein, the insulation will be tested for its fire-resistant properties.

Keywords: *E. coli* BL21, bacterial cellulose, kombucha, fire-retardant insulation

Authors are listed in alphabetical order. Please direct all correspondence to the team mentor, Anna Minutella (aminutella@renaissanceschool.org).

The Renaissance School's synthetic biology team of 2017's project goal is to create a non-carcinogenic, environmentally friendly alternative form of housing insulation. The various choices of housing insulation today are as follows: fiberglass, spray polyurethane foam, polystyrene, and cellulose. Although these may be effective as housing insulation, some cause health and environmental issues, while others are quite costly. Therefore, to improve the construction of safe, environmentally friendly, and cheap housing insulation, bacterial cellulose (BC) will be used. BC is a byproduct of kombucha and is produced by vinegar bacteria. To create a consistency similar to current cellulose housing insulation, the BC will be separated from the fermented tea, pulped and aerated. BC is naturally flammable and consequently does not meet the requirement for fire-retardant housing insulation. To make the BC-based insulation fire-retardant, it will be combined with *E. coli* that is genetically engineered to be fire-retardant.

Research Question

If bacteria, specifically *E. coli* BL21, are transformed with the plasmids pGEX-2T containing the SR protein gene and pET-29b containing the SRPK protein gene and the resulting proteins are extracted from the bacteria and combined with pulped bacterial cellulose, then will the product of this procedure prove to be an effective and non-toxic form of housing insulation?

Hypothesis

If bacterial cellulose can reach the desired consistency to be used as insulation and used in conjunction with an extracted fire-retardant protein, then a more environmentally friendly and efficient housing insulation could be produced.

Proteins and Vectors

The two genes, SR and SRPK, both yield proteins. The serine-rich (SR) protein and serine-rich protein kinase (SRPK) genes were found in a protein database by the University of Mingdao. Their 2015 iGEM team's project was to produce a fire-retardant protein using those two genes. For the highest achievable level of fire retardancy, proteins must be high in nitrogen and phosphate, which the SR protein is due to the amino acids, serine and arginine, in its RS domains (Long, 2009).

To be able to express the SR protein, the SR gene is inserted into a vector (e.g. a plasmid). The SR gene circuit, of 1556 base pairs, will be inserted into the plasmid pGEX-2T through transformation. PGEX-2T is an ampicillin-resistant plasmid, meaning that if properly transformed, the bacteria will survive on ampicillin and other bacteria will be killed. This plasmid is a commercially produced plasmid and contains a glutathione S-transferase (GST) fusion tag on both ends of the sequence. When using affinity chromatography, the GST fusion tag aids the extraction of the desired protein. It is also runs under a ptac promoter, which is a combination of the trp and lac operon, and regulated by a lac repressor (LacI) that is induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) (Addgene, n.d.).

The SRPK adds stability to and is a kinase for the SR protein. Specifically, the SRPK will phosphorylate the RS domains. SRPK is controlled by the pET29b (Mingdao, 2015). The pEt-29b vector has a high expression level and is resistant to kanamycin. Its base pair number is circa 5200 (Addgene, n.d.). Similar to the GST fusion tag of the plasmid pGEX-2T, the plasmid pET29b contains a 6XHis tag. The 6XHis tag is fused with SRPK gene at the C-terminus location. The SRPK gene can be modified to be regulated by a lac repressor and operated by a pt7 promoter. This vector is kanamycin-resistant.

Both SR and SRPK will be transformed in the the *E. coli* strain BL21. This particular strain of *E. coli* is hard to transform, but it has a high transcription rate. *E. coli* BL21 has a t7 RNA polymerase, a polymerase that catalyzes RNA formation in the 5′⇨3′ direction, and which is controlled by the lac repressor Lac1 (Mingdao, 2015).

Understanding the Lac Repressor and Operon

The lac repressor, in conjunction with the lac operon, are two components that are commonly used for creating recombinant proteins in *E. coli*. When glucose is unavailable as an energy source in *E. coli*, the lac operon allows for the transportation and metabolism of lactose as an alternative energy source. The lac repressor, or LacI, acts as a lactose sensor: it regulates the transcription of the lac operon and only allows for transcription to occur when lactose is present. Thus, without lactose present in the environment, the transcription of the operon is blocked by the lac repressor (Khan Academy, 2016).

LacI binds in a specific way that blocks the T7 RNA polymerase from being able to bind to the promoter sequence. The T7 promoter is only able to match with RNA polymerase from T7 bacteriophage. For transcription to happen, the LacI protein must be removed from the operator DNA sequence that sits in front of the desired gene. When LacI is absent, the T7 promoter can then be recognized by the T7 RNA polymerase. LacI is able to sense the presence of lactose and IPTG, which imitates lactose structurally. When there is no lactose, the lac repressor (LacI) binds to the operator on DNA, blocking the T7 RNA polymerase's access to the promoter site; however, if lactose binds to the LacI, it induces change in the protein's structure so that it is no longer capable of binding to the operator DNA sequence. This system prevents transcription of your gene from being faulty. IPTG is often used because it is a better inducer than lactose and is not used or broken down by the cell. This helps ensure that the IPTG concentration remains constant. When the lac repressor has "fallen of", RNA polymerase is able to begin transcription, transcribing in high numbers. Then, once the T7 RNA polymerase binds to the T7 promoter, upstream of the desired gene, the desired gene can now be transcribed (Quora Answers, 2011).

Understanding GST

GST is a tripeptide that is often put into expression vectors for the production of recombinant proteins, which are encoded by a gene. GST is highly soluble after translation. Recombinant proteins with the GST tag often have greater expression and solubility than recombinant proteins without it. Reduced glutathione (GSH) can capture pure GST or GST-tagged proteins through the enzyme substrate binding reaction, when immobilized through its sulfhydryl group (Thermo Fisher Scientific, 2016a).

Understanding HIS

The string of six to nine histidine residues is often used, in vectors, for the production of recombinant proteins, resulting in the addition of a 6xHis or poly-His tag, to the N- or C- terminus of a recombinant protein. Under specific buffer conditions, the His-tagged proteins can be purified because of the string of histidine residues that is able to bind various immobilized metal ions (Thermo Fisher Scientific, 2016b).

Insulation

There are many choices when it comes to insulation. The choices can be broken down into four different categories: fiberglass, spray polyurethane foam, polystyrene and cellulose. Fiberglass is the most common form of insulation used today. It is an inorganic material that can either be installed by hose or as batting. It is inexpensive and non-combustible, but dangerous. Its fibers cause irritation when it comes in contact with the skin, eyes and lungs. If inhaled the fiberglass particles can disrupt cell division and cause cancer, according to the Occupational Safety and Health Administration (OSHA), International Agency for Research on Cancer (IARC) and the National Toxicology Program (NTP).

Spray polyurethane foam (SPF) is a very effective insulator. According to NIORA (2004), WRA and OSHA, it has been the leading cause of work-related asthma (U.S. Department of Labor, n.d.). It is also chemically dangerous for human contact and highly flammable.

Polystyrene has similar properties to SPF. It is applied by spray and has a higher r-value than the other insulations listed. It is a known respiratory irritant and neurologically damaging. In liquid form prior to application, it is highly combustible (U.S. Department of Labor, 2017).

Cellulose insulation is the oldest form of insulation. Cellulose insulation comes in many forms such as straw, hemp, wool, cotton or paper. Cellulose insulation is generally comprised of recycled materials. The practice of using straw is no longer practiced due to straw's high flammability. To make cellulose insulation, the hemp, wool, cotton and paper fibers are shredded and formed into a pulp that can be sprayed or batted. With the exception of wool, all of these materials are flammable, and usually treated with sodium borate, boric acid or ammonium sulfate (Gromicko and Shepard, n.d.), which are considered safe for human contact. There is some concern that the ink in the recycled paper could produce toxic gases when ignited.

Cellulose is a well-known organic material. It is a carbohydrate polymer and it is easily obtained due to its natural abundance. It is the primary element in most plant structures, such as cotton and wood. However, it is difficult to extract pure cellulose from these substances, as the makeup of this substance in plants is complicated and the cellulose is built into the structure of the plants that synthesized it (Esa, Tasirin, & Rahman, 2014). Interestingly, organisms other than plants, such as fungi and bacteria, are also able to produce forms of cellulose, but these different forms exhibit drastically different chemical and physical properties than most types of plant-derived cellulose. Bacterial or microbial cellulose is a form of cellulose that is produced by certain types of aerobic bacteria (Keshk, 2014). Specifically, this bacteria is from the species *Acetobacter xylinum*, which is a type of gram-negative, aerobic "vinegar" bacteria. These bacteria are used to ferment different types of sugars, and acetic acid is the product of this fermentation. Another result of this process is microbial cellulose (Kongruang, 2008). While the structure of bacterial cellulose maintains a "chemically equivalent structure [to] plant cellulose" according to Esa, Tasirin, and Rahman (2014), microbial cellulose exhibits physical properties that are not similar to those of plant-based cellulose. Nonetheless, while some of its physical properties are not the same as normal plant cellulose, bacterial cellulose does have many similarities to plant-derived cellulose. Its chemical structure is chemically identical and, like cotton, bacterial cellulose is produced in the form of fibers, but the fibers that make up microbial cellulose are much smaller. Cotton fibers are approximately ten times as wide as the fibers that make up bacterial cellulose (Yoshinaga, Tonouchi, & Watanabe, 1997). This type of fibrous makeup gives bacterial cellulose the potential to act as an effective insulation.

Materials and Methods

Restriction enzyme digest with EcoRI

One µL of the EcoRI (New England Biolabs) was added to a centrifuge tube. One µg of the plasmid pGex-2T was added. Also, 5 µL of 10X Cutsmart buffer (New England Biolabs) was added. Then, approximately 43 µL of distilled water was added to raise the reaction up to 50 µL. The reaction was incubated at 37°C for 15 minutes. Then, 10 µL of gel loading dye was added to the reaction and it was stored in the freezer.

Gel electrophoresis with products of restriction enzyme digests

For the restriction enzyme digest sample (a digest using plasmid PGEX-2t and the restriction enzyme EcoRI), a 1.2% agarose concentration was used. In this procedure, 25 µL of TAE buffer was measured into a graduated cylinder and poured into a beaker. On a scale, 0.3 g of agarose was measured. The agarose was then added to the TAE buffer in the beaker with 2.5 µL of gel stain. The entire mixture was then gently mixed using the tip of the pipet. It was then microwaved for 45 s until the agarose was dissolved into the TAE buffer. A gel box was lined with masking tape across both open edges to cover any areas in which the gel could leak out prior to setting. The comb was then inserted into the empty gel box, and the liquid agarose and TAE buffer mixture was poured into the prepared gel box. It was allowed to rest for approximately five minutes until it was firm. Then, the prepared gel box was placed into the container in which the digest would be run and covered with more TAE buffer.

Six µL of DNA ladder was added to the first column on the left in the gel. Then, 4.8 µL of gel loading dye was mixed with 1.2 µL of the DNA sample in a microcentrifuge tube. This mixture was subsequently added to the second column on the left in the gel. The gel was subjected to 100 volts over a 45-minute time period. It was then analyzed and stored in a plastic bag in the refrigerator. This procedure was later repeated, but the results of the EcoRI digest were tested in the second column from the left on the gel, and the results of a BamHI digest were tested in the fourth column from the left. The ladder was placed in the far left column.

Transformation

For the transformation, 200 µL of CaCl2 solution was pipetted into a microcentrifuge tube, then placed on ice. An inoculating loop was used to scrape up an entire patch of DH10 alpha cells, while not scraping up any of the agar on the petri dish. The cells were then swirled in the tube of cold CaCl2. Then they were gently flicked, inverted and placed on ice. These competent cells were put aside while 5 µL of the plasmid pGEX-2T was pipetted into a microcentrifuge tube. 100 µL of the competent cells was pipetted into the microcentrifuge tube containing the 5 µL of pGEX-2T. The other 100 µL of competent cells was stored on ice. The microcentrifuge tube was left in to sit in ice for five minutes. Then, the bottom of a petri dish was properly labeled with the date, strain of bacteria and plasmid that were used in the transformation lab.

After five minutes the microcentrifuge tube was put in a rack and heat shocked in a 42°C waterbath for 90 seconds exactly. Then, the rack with the microcentrifuge tube was removed from the bath and was put on a table in room temperature. A measurement of 0.5 mL of room temperature lysogeny broth (LB) was added to the microcentrifuge tubes, then the tube was inverted to mix the contents. A sterilized spreader was then used to spread 250 µL of the transformation mixes onto a

petri dish of LB+ ampicillin agar. Then, the plate was incubated overnight at 37°C.

Miniprep

600 uL of bacterial culture grown in LB medium was added to a 1.5 mL microcentrifuge tube. Then, 7X lysis buffer was added to the tube and mixed by inverting the tube 4-6 times. After being inverted the tube was incubated for 1-2 minutes. 350 µL of cold neutralization buffer was added and thoroughly mixed. Then, the tube was centrifuged for 2-4 minutes at less than or equal to 11,000 xg. A Zymo-Spin IIN column (Zymo Research) was placed in a collection tube and the supernatant of previous ste was transferred into the Zymo-Spin IIN column. The Zymo-Spin IIN was centrifuged for 15 seconds at more than or equal to 11,000 xg. The flow-through was discarded and the Zymo-Spin IIN was returned to the same collection tube. Then, 200 µL of Endo-Wash Buffer (Zymo Research) was added the the column and centrifuged for 1 minute at more than or equal to 11,000 xg. 400 µL of Zyppy Wash Buffer (Zymo Research) was added to the column and centrifuged for 1 minute at more than or equal to 11,000 xg. Then the column was transferred into a clean 1.5 mL microcentrifuge tube then 30 µL of Zyppy Elution Buffer (Zymo Research) was directly added to the column matrix and incubated for one minute at room temperature. Then, that was centrifuged for 30 s at more than or equal to 11,000 xg to elute the plasmid DNA.

Method for kombucha production and processing

For the production of kombucha the following was done. First, eight cups of distilled water were heated to 150°F. Eight black tea bags were then placed in the heated water in a shallow bowl. The tea was allowed to steep and cool to a temperature range of 65-80°F. One cup of white sugar was added to tea and thoroughly mixed. White distilled vinegar was added to the tea to bring the pH down to a range of 3-2.5. Once the tea was at the desired pH, the culture was put in the solution. The culture and solution were left at room temperature for two weeks. During this time, the culture grew a thick film on top of the solution. After the incubation period, the film was removed and processed with a food processor into a pulp. The pulp, or processed cellulose, was spread over a fine mesh and allowed to dry. The dried cellulose was removed from the mesh, and the cellulose was ready for protein treatment.

Results and Discussion

Gel electrophoresis with products of restriction enzyme digests

During analysis, the gel that had both the ladder and the digest with EcoRI was placed on a light box over blue LED light. In the column on the far left, several different bands of DNA (from the ladder) were visible (Figure 1). This was the only column on the gel that had visible bands. The second gel (the one with both the EcoRI and BamHI digest results) was analyzed, and there were no visible results.

These two rounds of gel electrophoresis did not maintain the results that were expected prior to completion of the procedure. The first gel, which had 6 µL of the DNA ladder and 6 µL

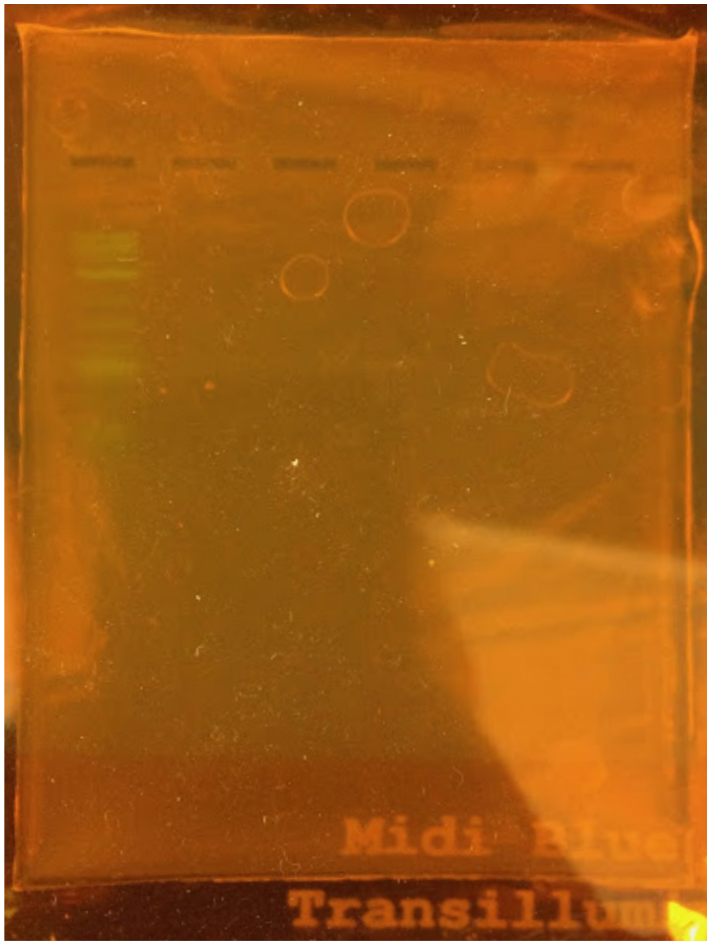

Figure 1. No bands were observed for the EcoRI digested DNA. The DNA ladder is visible in the well on the left.

of the EcoRI digest sample, did not show anything but the ladder. However, the ladder was spread out across the gel and was clearly visible, so there are many possibilities about what could have happened with the digest. This round of gel electrophoresis was completed in order to test the results of the restriction enzyme digest, but there could have been errors in either the restriction enzyme digest procedure or the gel electrophoresis procedure. Potentially, there were errors in both. One reason for why the results were not visible on the gel is that the digest might not have produced a sufficient concentration of DNA for there to be significant results in the gel that were visible to the naked eye. The reaction was diluted with distilled water until it was 50 µL, but only one µg of DNA was added to the entire reaction. This could be cause for the band to not be visible on the gel under the blacklight.

Also, it is possible that there were problems with the electrophoresis machine. It could have run the gel for too long and caused the DNA to run off the edge of the gel, but this problem is more likely to have happened with the second gel, as there was not even a visible ladder in those results. Also, it is possible that there was also too low of a concentration of DNA in both the EcoRI digest and the BamHI digest results, but that would not explain the lack of a ladder on the second gel.

Transformation and Mini Prep

During analyzation, the petri dish was taken out of the incubator. The petri dish showed a large colony of bacterial growth with a lawn-like appearance. The miniprep was successfully completed.

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