Methods for Producing Cellulose Using the Bacterium *Gluconacetobacter xylinus*

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Cellulose is a biologically useful substance that has a great potential within commercial bioengineering. We are specifically interested in using cellulose as a medium to create artificial seaweed which will allure the parasite, *Perkinsus marinus*, preventing it from attacking native oysters of the Chesapeake Bay. Here, we will address a procedure adapted from the 2014 Imperial College iGEM team, which describes the creation of the growth media and subsequent propagation of the *Gluconacetobacter xylinus*. We found an easy and reliable technique to culture the *G. xylinus* to produce a thick sheet of cellulose within two to three weeks.

Keywords: *Gluconacetobacter xylinus*, Cellulose

Watch a video introduction by the authors at https://youtu.be/L2qhLXG95P4

Cellulose is an extracellular matrix produced by bacteria such as *Gluconacetobacter xylinus*. It is a chain of β-D-glucose molecules and these polysaccharides make up the cell walls of plants. When cellulose is produced by *G. xylinus*, it forms in ribbons in the extracellular matrix; these ribbons intertwine and fuse with each other to produce a sheet of cellulose.

The cellulose floats due to its low density; the matrix, once formed, will retain a good bit of water. Since it is bacterial cellulose, it will be stronger, more malleable, and more pure (having little to no other molecules) than plant cellulose (Bielecki et al. 2005).

The tensile strength of the cellulose produced by *G. xylinus* (purely bacterial cellulose) was compared to that of "blended bacterial cellulose" (part plant, part bacterial) by the 2014 Imperial College iGEM team. Since non-blended bacterial cellulose had a tensile strength of 29.9 ± 6.4 MPa and blended bacterial cellulose had a tensile strength of 208 ± 80 MPa, it was determined that non-blended bacterial cellulose can withstand more stress than blended cellulose, therefore showing it to be the better candidate for filtering applications ("Mechanical Testing" d.t).

Possible applications include binding catalysts of particular reactions onto strips of the cellulose to stimulate the reaction and binding proteins that will bind to specific harmful chemicals in order to serve as a sort of filter.

This protocol was adapted from the one presented by the 2014 Imperial College iGEM Team ("Protocols" d.t). It describes how to safely prepare the specialized growth media, incubate the culture, and prepare subcultures.

Laboratory Safety

Laboratory safety has an important role in every experiment. Safety procedures are completed in order to avoid contamination of the specimens and to prevent any injury.

Materials

- Goggles
- Gloves
- Bleach
- Permanent Marker

Procedure

1. Wear gloves and goggles at all times.
2. Do not consume food or beverages while performing the experiments.
3. Avoid breathing, sneezing, or coughing on the media or

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culture.

4. Keep the culture covered when not in use.
5. Carefully label all of your samples so you know what you're working with.
6. Do not touch the tip of the inoculating loops.
7. Properly clean up and dispose of materials after experiment; this would involve bleaching anything that made contact with bacteria and storing those materials in a hazardous waste bag. The waste will be picked up by a hazardous waste company.

Preparing Hestrin Schramm Media

G. xylinus was found to grow best in liquid Hestrin Schramm (HS) media. Media is essential for the growth of bacteria. Prepare 500 mL HS media as follows.

Materials

- Dextrose (10 g)
- Yeast extract (2.5 g)
- Trypticase Peptone (2.5 g)
- Sodium Phosphate Dibasic (Na_2HPO_4) (1.35 g)
- 0.1 M Citric acid (40 mL)
- Distilled water (460 mL)
- Agar (if making HS-agar plates) (7.5 g)
- Petri Dishes (if making HS-agar plates)
- Antibiotics (as necessary)
- 0.2 µm filter if filter sterilizing the media
- Gluconacetobacter (Bacteria)

Procedure

1. Add 250 mL dH_20 to 10 g dextrose in one bottle.
2. Combine 210 mL dH_20 with the following reagents in a second bottle.
   - 40 mL 0.1 M citric acid
   - 2.5 g yeast extract (0.5% w/v)
   - 2.5 g trypticase peptone (0.5% w/v)
   - 1.35 g Na_2HPO_4 (0.27% w/v)
   - 7.5 g of agar if making HS-agar plates

   (Note 1)
3. Autoclave or filter sterilize both bottles and combine (Note 2).
4. If preparing agar plates, pour the molten agar solution (< 50°C) into the petri dishes and allow to solidify before using.

Notes

1. Liquid media produces much better culture growth than agar. We tested both and found that cultures grown in agar grow very slowly and eventually dry out.
2. Autoclaving glucose separately from amino acids avoids Maillard reaction, which can result in the formation of toxic byproducts in the media.

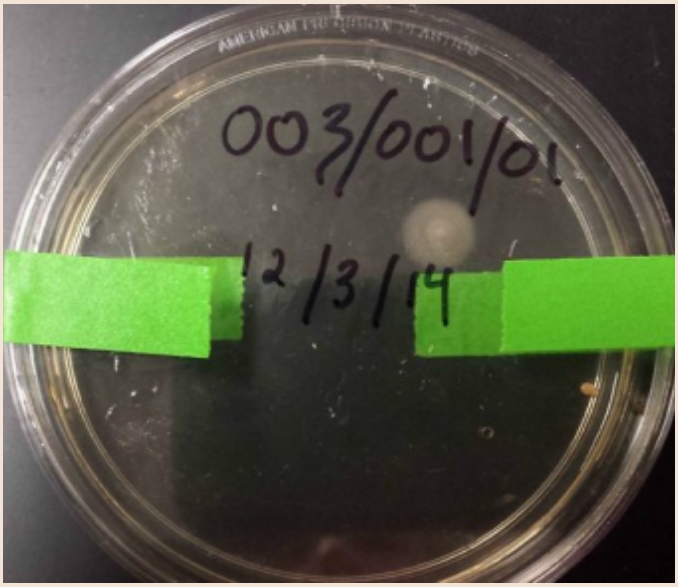

Figure 1. *This image illustrates what the inoculated plate should look like once the media has been inoculated with gluconacetobacter.*

Inoculating Media with Gluconacetobacter

Inoculation is the process of transferring bacteria from one sample to another. Transferring the bacteria allows for an additional plate of agar to grow a colony (Figure 1).

Materials

- Agar plate with prepared media
- Inoculating loop
- Incubator
- Parafilm

Procedure

1. Retrieve inoculating loop from packaging without touching the top of the loop in order to avoid contamination.
2. Incubate plates at 30°C inverted by inoculating the gluconacetobacter onto the media using an inoculating loop.The colonies will appear in 48-72 h.
3. Incubate liquid HS-cultures at 30°C standing.
4. Place the plates in the incubator upside down to avoid condensation dripping into the media/culture.
5. Store at 4°C until needed.

Subculturing the Bacteria

Subculturing is needed to transfer the cellulose from one media tube to another tube. This helps increase the amount of cellulose so that it may be used for further experimentation. Placing the cellulose in a new tube gives it a source of nutrients, which prolongs its lifespan.

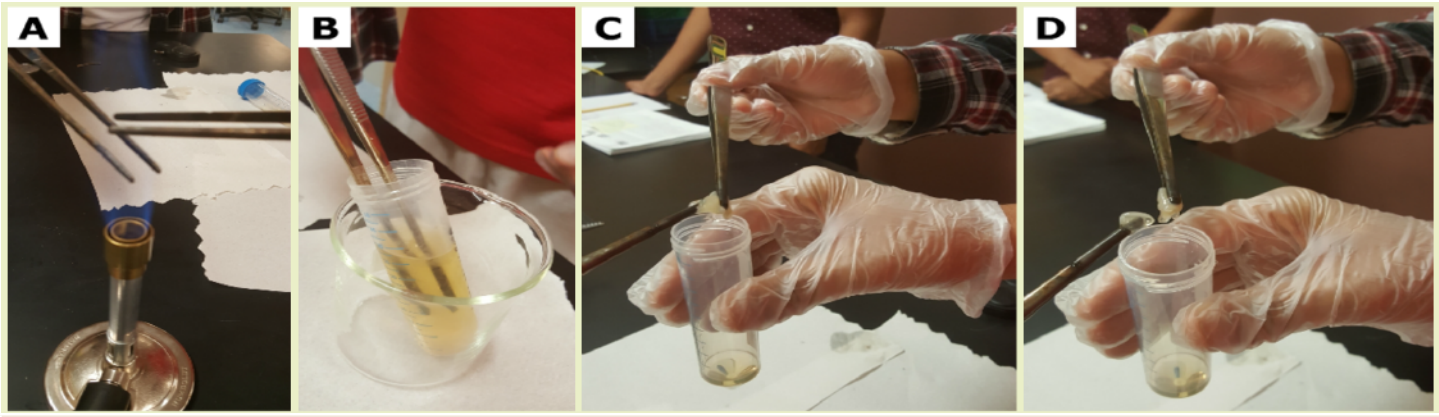

***Figure 2**. Subculturing G. xylinus can be accomplished by (A) flame-sterilizing two pairs of forceps, (B) cooling them to room temperature in a sterile solution, and (C-D) using them to rip off a piece of the old cellulose to transfer to a fresh media.*

Materials

- Forceps
- Tongs
- Bunsen Burner
- Striker
- 50 mL conical tubes
- 5 mL HS media
- Serological pipette and pipettor

Procedure

1. Heat up the forceps (Figure 2A).
2. Cool forceps down by immersing in sterile media (Figure 2B).
3. Use the forceps to rip a piece of cellulose off of a previously cultured sample (Figure 2C-D; See Note 1).
4. Use the cellulose to inoculate and culture three fresh tubes of 5 mL HS media with an inoculating loop.
5. Label and incubate the tube at room temperature (~25°C) without shaking (Note 2) for two weeks (Note 3).
6. At the end of this period fresh cellulose will be floating on the top of the media as seen in Figure 3.

Notes

1. Forceps are needed to pull apart the culture because the cellulose forms a dense, tough sheet.
2. Standing culture result in low growth rate, but avoid the formation of non-cellulose producing mutants, which have been reported to appear in shaking cultures.
3. Leaving a sample of cellulose alone to grow for 4-5 weeks causes it to become harder and denser, making it more difficult to work with.

Safely Disposing of *G. xylinus* Cultures

The experiment is never over until the lab has been returned to how it looked before the experiment started. Some bacteria are hazardous. If they are not disposed of properly, harmful effects may result.

Materials

- 10% Bleach
- Plastic tub with lid

Procedure

1. Place all tools and cultures used into a tub of 10% bleach and let it sit for at least 24 h.
2. Dispose of disposable tools and cultures in hazardous waste.
3. Wipe down all counters and surfaces that were used in the lab with a bleach solution.

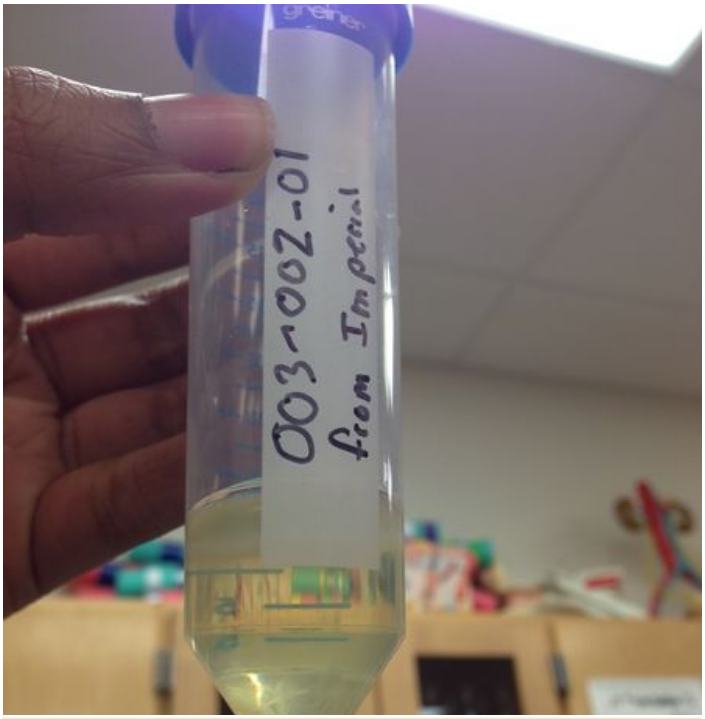

***Figure 3.** In this picture, a thin disc of cellulose has formed and is floating at the top of the liquid media. The very clear distinction between the media and the cellulose disk is proof that the cellulose was formed correctly and is contaminant free. The Tuscarora Bioengineering Team will now be able to culture more of this cellulose for use in future experiments.*

Discussions

Overall, we found these procedures to be very easy. The bacteria form a thin, but visible, cellulose disk after growing for just two weeks at room temperature. When subculturing, we had some difficulty ripping particularly thick samples of cellulose; the more time between subcultures, the more difficult it was to tear the cellulose disks.

Although we have not yet tested it, the bacteria can likely be used to grow larger sheets and unique shapes, depending on the size and shape of the container it is grown in. We also anticipate that it will be possible for us to kill the bacteria with a 0.1 M NaOH solution without harming the cellulose. This will allow us to produce cellulose sheets without the risk of introducing *G. xylinus* into the environment. The Imperial College iGEM team used this approach when preparing their cellulose sheets.

Our team plans to use this as the vehicle to remove pathogenic protists that are partially responsible for the decline of oysters in the waters of the Chesapeake Bay (Amin, et al. 2016). Our team plans to synthesize and produce a protein consisting of a galectin receptor that will bind to the protist, P. marinus, spores and a Cellulose Binding Domain that will bind to the cellulose sheets. These sheets can be grown in strips to make an artificial seaweed that will, in theory, act as filter when put in infected waters.

Acknowledgements

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