3.1.1.2 Bacterial cellulose biosynthesis

Cellulose was produced by bacteria from the *Acetobacter xylinum (A. xylinum)* or *Acetobacter Hansenii* (*A. Hansenii*) strains using glucose as carbon source in the laboratory scale and will be scaled up by using of waste of food industry such as potato starch. This section describes the steps required to produce cellulose from bacteria in detail.

(1) Preparation of wet bacteria growth medium

Hestrin-Schramm (HS) medium used for *A. xylinum* samples consists of the following materials (per 1 L of solution): 20 g glucose, 5 g vegetable peptone, 5 g yeast extract, 2.7 g sodium phosphate and 1.5 g citric acid.

Chemicals are weighed (with an error tolerance of <1%) and dissolved in deionized (DI) water. Glucose is kept separately while the rest of the chemicals are dissolved together. The solutions are then sterilized in an autoclave for 30 minutes at 121 °C. It is necessary to keep the glucose separated from the rest of the chemicals to avoid Maillard Reactions from taking place at high temperatures during sterilization. Once removed from the autoclave the solutions are cooled, then they are combined and either used or stored at 4 °C.

(2) Preperation of a sterile workstation

A portion of a workbench is sectioned off for sterilization prior to any bacteria work to avoid contamination of the bacteria. All supplies required for the task at hand are collected prior to sterilization are brought within the designated sterile area. This minimizes the potential of bringing any outside microbial activity into the sterile area during work with *acetobacter* samples. Anything that will come into direct contact with acetobacter samples (i.e. pipettes, test tubes, bottles, etc.) are assured to be sterile. Disposables are checked to make sure they are still in their factory sealed guaranteed sterile packaging, while reusable items are sterilized in an autoclave for 20 minutes at 121 °C. Items that will be in the sterile workstation but will not come into direct contact with *acetobacter* samples are sprayed with a 70% ethanol solution in water to clean and sterilize them. These precautions are taken prior to any of the following bacterial cellulose production procedures.

(3) Reviving freeze-dried bacteria samples

Freeze dried *A. Xylinum* strain (ATCC 53582) are contained within a vial sealed by cotton within a sealed glass container. 5 mL of wet growth medium are prepared in a sterile culture tube to revive the bacteria. The glass seal of the freeze-dried sample is broken, and the cotton seal is removed. The freeze-dried sample is added to the 5 mL of growth medium to form a culture solution. The culture solution is placed at an angle in a culture tube rack and fastened to a rotating stage in an incubator. The temperature in the incubator is set to 26 °C and the rotating

stage is set to 250 rpm (Figure 3-1-2). The culture solution is left under these conditions for 4-5 days until the solution becomes slightly translucent, indicating a high bacteria population. It is necessary to mix the culture solution using a vortex periodically over the course of this process to break down any cellulose agglomerations that form while the bacteria culture is growing.

Figure 3-1-2 Reviving Freeze Dried Bacteria Samples in an orbital incubator shaker.

(4) Bacteria freeze stock preparation

A 40% glycerol solution in water is prepared and sterilized in an autoclave for 30 minutes at 121 °C. 0.5 mL of the glycerol solution is combined with 0.5 mL of fully grown bacteria culture solution. This is mixed several times by pipetting to ensure an even mixture, and then placed in an ultra-low temperature freezer at -80 °C for future use.

(5) Single colony growth from culture plates

Growth medium is prepared as in section (1), except this time 15 g of agar (per 1 L of solution) is added to the glucose prior to sterilization in the autoclave for 30 minutes at 121 °C. The glucose and agar solution is combined with the solution containing the remaining chemicals while the solutions are still hot (>50 °C). The growth medium is then quickly poured into sterile petri dishes. A blow torch is used to flame the side of the bottle containing the culture solution, creating a positive pressure, and forcing the air out of the bottle to keep contaminants from entering the bottle, and also to pop any bubbles that form on the surface of the growth medium when it is poured into the petri dishes so that there is a nice uniform surface for bacteria samples to grow on. The growth plates are left out over night to solidify and then stored at 4 °C. Solidified plates are introduced to *acetobacter* samples either from either wet culture solution or freeze stock. A sterile inoculating loop is dipped in an *acetobacter* sample and that sample is then streaked across a

culture growth plate. Inoculated growth plates are kept in the incubator at 26 °C for 4-5 days until single acetobacter colonies are visible on the surface of the plate. The plates are then stored at 4 °C for future use of the single colonies.

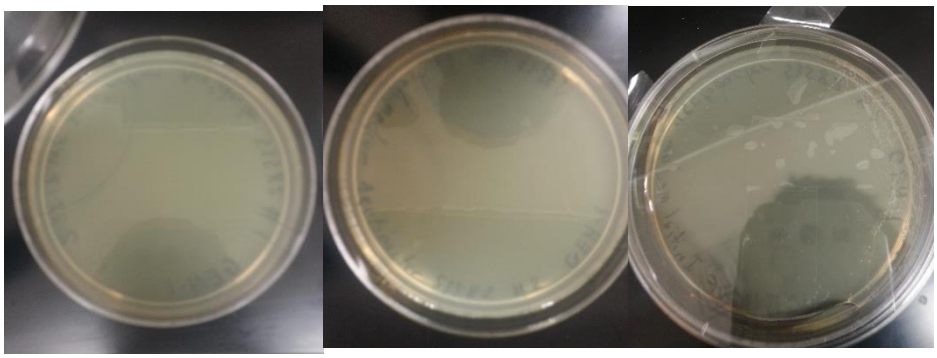

Figure 3-1-3 Acetobacter Culture Plate Growth at Day 0, Day 1 and Day 4 (from left to right).

(6) Preparation of *A. xylinum* cultures for cellulose production

A single colony of *A. xylinum* is taken from a culture plate with an inoculating loop and placed in a culture tube with 5 mL of growth medium. This culture solution is placed at an angle in a culture tube rack and fastened to a rotating stage in an incubator. The temperature in the incubator is set to 26 °C and the rotating stage is set to 250 rpm. The culture solution is left under these conditions for 4-5 days until the solution becomes slightly translucent, indicating a high bacteria population. 1 L of growth medium is prepared as in section (1) and is placed in a sterile 9"×13" baking pan to maximize the surface area of the air-solution interface where cellulose pellicles typically form. The 5 mL of concentrated *A. xylinum* culture solution is added to the 1 L of growth medium and is placed in an incubator at 26 °C and covered with aluminum foil to shield the culture from ambient light. After 2 weeks, a cellulose pellicle is formed and is removed from the culture solution. The pellicle is purified as described below and the culture solution is discarded.

(7) Purification of bacterial cellulose

Cellulose pellicles first removed from the culture solution are composed of a cellulose hydrogel containing many components of the solution, mainly bacteria and chemicals trapped within the cellulose network. These pellicles are opaque and generally a white-yellow color. The pellicles are washed several times by hand with water and then placed in a 1 wt.% sodium hydroxide solution and heated to 80 °C for one hour. The pellicles are then removed from the sodium hydroxide solution and are again washed by hand several times. The pellicles are then placed in DI water and heated to 80 °C for one more hour, and then removed and washed by hand with DI water several times. This process kills any excess bacteria and removes much of the contaminants from the bacterial cellulose pellicles. The pellicle is then cut into approximately 1" square pieces and placed in a large DI water bath for dialysis for 2 days. After 2 days, highly

pure, highly transparent cellulose pellicles remain (Figure 3-1-4). 1 liter of solution yields approximately 7.5 grams of cellulose after 2 weeks.

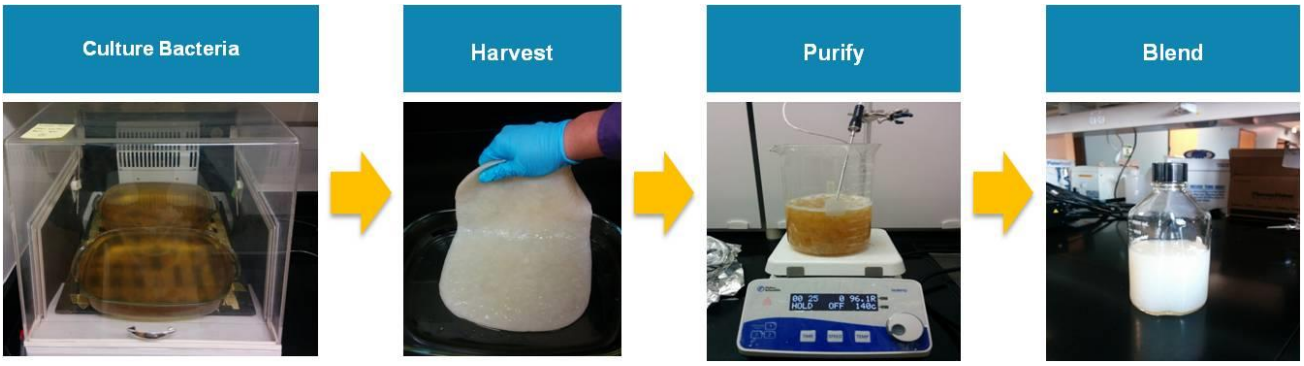

Figure 3-1-4 Procedure of producing bacterial cellulose

(8) Production of bacterial cellulose modified by sodium carboxymethyl cellulose

Cellulose is secreted by acetobacter through holes along the sides of the bacteria. These individual fibers quickly from hydrogen bonds with the fibers secreted through neighboring holes and assemble into ribbon-like structures with cross sectional areas of 10 nm × 50 nm. The addition of sodium carboxymethyl cellulose (CMC) with molecular weight (MW) of 90k to the growth medium of acetobacter can reduce the cross-sectional area of the secreted fibers by quickly bonding to the individual fibers as they are secreted before they have the chance to assemble into a ribbon-like thick cellulose fiber.

Preparation of *acetobacter* cultures for modified cellulose production by CMC is done in the same way except this time 15 g of CMC is added to 1L of glucose solution before sterilization in an autoclave. Each of the remaining steps is repeated exactly as described above. The resulting modified fibers have much smaller thickness, with cross-sectional areas as small as 6 nm × 6 nm. The cellulose pellicle with CMC addictive shows more transparent appearance than the one without CMC (Figure 3-1-5). 1 liter of solution yields approximately 6.5 grams of cellulose after 2 weeks.

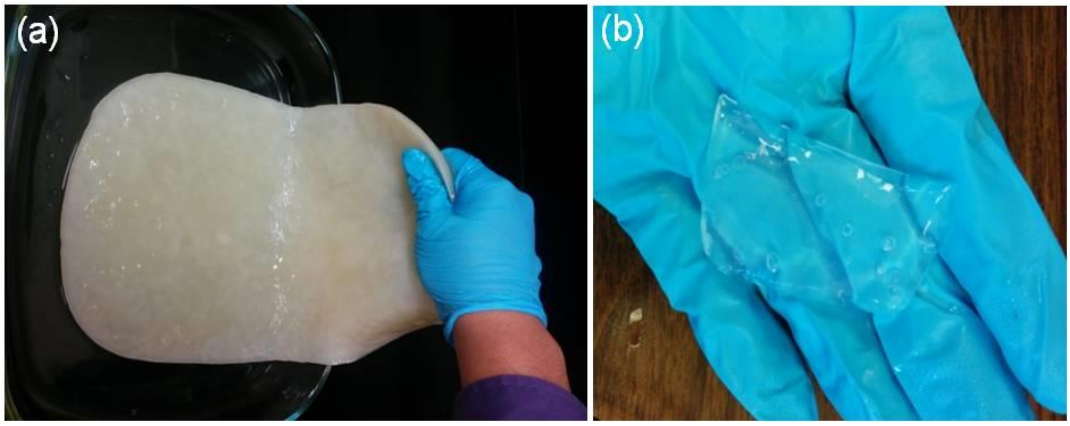

Figure 3-1-5 Comparison of BC pellicles without and with CMC