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Bacterial cellulose spheres that encapsulate solid materials

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TITLE:

Bacterial cellulose spheres that encapsulate solid materials.

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KEYWORDS:

Bacterial cellulose, agitation, spheres, encapsulation, biomaterial, controlled release, kombucha

SUMMARY:

This protocol presents an easy, inexpensive method of forming bacterial cellulose (BC) spheres. This biomaterial can function as an encapsulation medium for solid materials, including biochar, polymer spheres, and mine waste.

ABSTRACT:

Bacterial cellulose (BC) spheres have been increasingly researched since the popularization of BC as a novel material. This protocol presents an affordable and simple method for BC sphere production. In addition to producing these spheres, an encapsulation method for solid particles has also been identified. To produce BC spheres, water, black tea, sugar, vinegar, and bacterial culture are combined in a baffled flask and the contents are agitated. After determining the proper culture conditions for BC sphere formation, their ability to encapsulate solid particles was tested using biochar, polymer beads, and mine waste. Spheres were characterized using ImageJ software and thermal gravimetric analysis (TGA). Results indicate that spheres with 7.5 mm diameters can be made in 7 days. Adding various particles increases the average size range of the BC capsules. The spheres encapsulated 10 – 20% of their dry mass. This method shows low-cost sphere production and encapsulation that is possible with easily obtainable materials. BC spheres may be used in the future as a contaminant removal aid, controlled release fertilizer coating, or

soil amendment.

INTRODUCTION:

Bacterial cellulose (BC) has been noted for its potential industry use due to its mechanical strength, high purity and crystallinity, water retention abilities, and intricate fiber structure[1–4]. These characteristics make BC a favorable biomaterial for a variety of applications, including biomedical, food processing, and environmental remediation uses[1]. Formation of a BC film can be done with single organism cultures or mixed cultures like those used for kombucha[5], a fermented tea beverage. Brewing kombucha relies on a "Symbiotic Culture Of Bacteria and Yeast", commonly known as a SCOBY. Using this symbiotic culture of organisms, a similar technique is employed to create BC spheres. This biomaterial may be employed to help isolate environmental contaminants and anchor agricultural amendments like biochar to achieve more efficient crop production.

Previous literature has discussed how the characteristics of BC produced in agitated conditions compare to BC produced statically. A stationary culture results in a film that forms at the liquid-air interface, while a shaken culture results in varying BC particles, strands, and spheres suspended within the liquid[6]. Many studies have referenced the claim that commercial production of BC is more feasible in the dynamic conditions[6,7], providing rationale for applying this paper's method. Additionally, various investigations on the structure and properties of BC spheres have been conducted. Toyosaki et al.[6] compared baffled and smooth-walled Erlenmeyer flasks in their agitated BC production. A study by Hu and Catchmark[4] determined conditions for BC spheres that were used as guidelines for the current BC sphere production process, and their results indicate that the sphere size does not continue to increase after 60 hours. A review of BC production by Mohammad et al.[1] indicates that shaking the BC culture ensures even oxygen supply and distribution, which is necessary for successful BC growth. Holland et al.[8] have studied the crystallinity and chemical structure of BC using X-ray diffraction and Fourier transform infrared spectroscopy. It is assumed BC capsules will exhibit similar characteristics and will investigate structural properties with future research. Studies have also explored the beneficial effects of using BC to produce improved biocomposites. Using epoxy-resin as a base, researchers have showed that the addition of BC improves material characteristics like fatigue life, fracture toughness, and tensile and flexural strength[9,10]. As shown by past and current research, many are interested in commercializing BC use.

Many researchers have investigated bacterial cellulose in controlled release systems, and the method described here generates capsules that could be utilized as controlled release systems. Much of this research focuses on controlled release in the biomedical field, as well as some exploration in controlled release fertilizer (CRF) administration. Based on the success of BC's controlled release of amoxicillin[11], lidocaine[12], and ibuprofen[13], BC may exhibit similar delivery characteristics with other substances, such as a pelletized fertilizer. An overview of CRF's by Shaviv and Mikkelsen[14] acknowledges that CRF's are more efficient, save labor, and generally cause less environmental degradation than conventional fertilizer application. Bacterial cellulose may work as a favorable encapsulating material for CRF's. Fertilizers may leach out of BC membranes or discharge as BCbiodegrades[15,16]. BC's high water swelling capacity can also act as

a beneficial soil amendment[17–19] because both fertilizer nutrients and moisture may release into the ground through application of BC spheres. With these traits, a CRF formed by BC sphere encapsulation may have an advantage over other fertilizer coating materials that could have negative effects during their production and disposal stages. Adapting BC into a fertilizer coating may further improve CRF technologies. By lowering fertilizer release rate, crops will have sufficient time to uptake the fertilizer and prevent excess runoff into bodies of water, thereby reducing eutrophication and unoxygenated zones. Similar slow-release fertilizers have been prepared and piloted using polymer coatings[20].

Unlike protocols outlined in previous research, this one focuses on uniform, cohesive sphere production rather than high cellulose yield. Moreover, BC encapsulation of other solids has been studied with cellulose films, but not spheres[21]. By expanding the research on bacterial cellulose spheres, further steps can be made to produce BC commercially, which is beneficial because of BC's environmentally safe features. This method of BC sphere fabrication utilizes inexpensive, readily available culinary ingredients. After the initial assembly, BC spheres begin to form within 2 days without interference. Producing BC spheres through this strategy requires little space and has an edible by-product, the fermented tea 'kombucha'. Encapsulation techniques mentioned in other studies include coatings formed through the phase inversion technique[22,23], matrix formation[24], spray drying[25], and direct encapsulation during synthesis[26]. The direct encapsulation method outlined in this manuscript is useful for those who desire an easy, inexpensive process that utilizes readily available materials.

Through this research, a successful protocol for BC sphere production and encapsulation was created. BC spheres can encapsulate solid particles of biochar, mine tailings, and polystyrene microbeads within their individual structures. Although not widely used in industry yet, BC is a practical, sustainably made, and naturally occurring material that could be used for future applications.

PROTOCOL:

1. Creation and maintenance of bacterial cellulose starter culture

1.1. Obtain a starter culture of bacterial cellulose, approximately 50 g, in the form of a SCOBY. It can be purchased commercially (e.g., from Cultures for Health). Place the SCOBY into a 1 L beaker, covered with a paper towel.

1.2. Boil 700 mL of deionized water, transfer it to a separate vessel from the one containing the SCOBY, and add 85 g of sucrose.

1.3. Once the sucrose has dissolved, add 2 bags of black tea (4.87 g). Steep the tea for 1 h, then carefully remove the tea bags using a stir rod.

1.4. Add 200 mL of distilled white vinegar to the tea. Let the mixture cool to 25 °C. Once cooled, add 700 mL of the room temperature tea to the beaker containing the SCOBY.

CAUTION: Adding the acidic tea while it is too hot may harm the organisms in the SCOBY.

1.5. Cover the beaker with a paper towel and secure it with an elastic band, then place the beaker in a storage area that maintains a temperature of 25 °C. This vessel is commonly referred to as stock culture or a hotel.

1.6. To keep the SCOBY healthy, maintenance is required about 2x a month.

1.6.1. Using gloved hands to hold back the SCOBY mats, drain the liquid from the hotel into a separate beaker. In the container with the liquid, add enough acidic tea for a total of 700 mL of solution.

1.6.2. Dissolve 65 g of sucrose in the container with the acidic tea. While waiting for the sucrose to dissolve, carefully rinse the SCOBY mats in DI water.

1.6.3. Once the sucrose is fully dissolved, the liquid can be added to the beaker containing the rinsed SCOBY mats. Cover the beaker and return it to the incubation area.

2. Production of bacterial cellulose spheres

NOTE: Exercise caution when working with boiling water. Ensure glassware can withstand the boiling water temperatures, is free of defects, and is the appropriate size. A schematic describing the production of BC spheres is given in **Figure 1**.

2.1. Boil 350 mL of deionized water using a tea kettle. Transfer the hot water to a 500 mL beaker. Dissolve 42.5 g of granulated sucrose into the hot water using a stir rod.

2.2. When the sucrose is fully dissolved, steep 1 bag of black tea (2.54 g) in the flask containing the sucrose and water for 1 h. After this, remove the tea bag with a stir rod, taking care to avoid breaking open the tea bag, and then dispose of it in the trash.

2.3. Add 100 mL of distilled white vinegar to the beaker, and then thoroughly stir the mixture. Transfer 80 mL of the acidic tea mixture to a 250 mL baffled flask. Allow the tea mixture to cool to room temperature, 20 – 25 °C.

NOTE: At this point, the mixture can be left to cool overnight or until prepared for the next step.

2.4. Once the liquid temperature is at room temperature (20 – 25 °C), add 20 mL of microbial starter culture liquid to the baffled flask. This liquid can be obtained from a SCOBY hotel. Cover the flask with parafilm.

2.5. Place the baffled flask on an orbital shake table and set the speed to 125 rotations per minute (rpm). Allow the mixture to shake for 3 days in a room or incubator with a temperature from 20 – 25 °C to produce BC spheres.

NOTE: If irregular shapes form in the flask contents or if cellulose clumps stick to the walls of the flask, they should be removed to prevent further irregular BC masses from forming. Use tweezers to remove unwanted BC masses, including thin strings, rings, tubular shapes, and other clearly non-spherical shapes.

2.6. Once the spheres have formed, gently pour them from the flask and analyze, dispose, or use them in a way not outlined in this paper.

3. Using bacterial cellulose spheres to encapsulate particles or contaminants

3.1. Follow **steps 2.1-2.5** above.

3.2. After shaking for 3 days, add about 0.01 g of finely ground particulate matter to the baffled flask. Appropriate solids include biochar (260 ± 140 µm), mine waste (350 ± 140 µm), and polystyrene microbeads (3 µm) and data for these materials are outlined in the **Representative Results** section. Please see the attached **Table of Materials** for further descriptions of biochar, mine waste, and microbeads.

3.3. Cover the flask again with the parafilm and place it back on an orbital shaker, again at the same speed and ambient temperature (20 – 25 °C) for 3 more days. Remove the BC encapsulated particles for analysis, disposal, or other uses.

[Place Figure 1 here]

REPRESENTATIVE RESULTS:

BC spheres have the fastest growth rate during the first 48 h of culture (**Figure 2**). **Figure 2** also shows how the spheres tend to reach a maximum average size and then remain constant. In this experiment, the spheres reached an average diameter of 7.5 ± 0.2 mm. Although the BC spheres never completely deteriorate within the 10 day growth period, they did start to form tendrils that extend off the main body of the sphere around the eighth day. This can be seen in **Figure 1E**, most noticeably on the large sphere on the top left.

Applying the encapsulation method outlined in this paper results in an average of 57 ± 4 bacterial cellulose spheres ranging in diameter from 3 to 12 mm (**Figure 3**). It can also be seen in **Figure 3** that the addition of solids to BC spheres does not have a consistent effect on sphere size or frequency. The orbital shaking speed, ambient temperature, and formation of irregular particles seem to be the main factors that affect shape, size, and frequency of spherical particles. **Figure 4** shows how too high of a room temperature and improper removal of irregular masses can change the BC from an intact sphere (**Figure 4B**) to stellate particles (**Figure 4A**) or stringy clumps (**Figure 4C**).

To determine the fraction of encapsulated solids in the BC spheres, a thermal gravimetric analysis was done on four different samples of BC. The four samples tested were BC, BC with biochar, BC with polystyrene microbeads, and BC with mine waste. **Figure 5** shows how the individual samples behaved when exposed to a high temperature in nitrogen gas. It can be seen from the dashed line representing spheres BC and mine waste that 18.7% of that sample was mine waste by weight, revealing successful encapsulation. The dotted line shows that 14.5% of that sample contained biochar. These percentages were calculated by subtracting the plain BC mass percent from the mass percent of samples with added solids. Because BC and polystyrene decompose at similar temperatures, derivative mass curves were deconvoluted to separate the decomposition of polymer from that of cellulose (**Figure 6**). This analysis shows that 13% of the mass loss in this sample corresponds to the thermal degradation of polystyrene. Because the thermal degradation of neat polystyrene leads to a mass loss of approximately 100%[27], it is estimated that all 13% of the mass of the sample corresponds to encapsulated polystyrene beads. **Figure 7** shows that the blue polystyrene microbead solution resulted in blue BC (**Figure 7D**). These dried BC masses are the samples that were used for TGA.

FIGURE AND TABLE LEGENDS:

Figure 1. Bacterial cellulose sphere fabrication and encapsulation of solid particles. Step 1 involves combining bacterial stock culture with black tea, sugar, and vinegar media in a baffled flask. The disks in the stock culture represent BC mats. Then, the baffled flask is placed on an orbital shake table for 3 days. The middle step shows solids being added to the flask once BC spheres have formed. The flask is shaken for 3 more days. In the final step, BC spheres have continued increasing in size and encapsulated the solid particles.

Figure 2. Bacterial cellulose growth. (**A)** Diameter of bacterial cellulose capsules over time; photographs of bacterial cellulose capsules at (**B**) 1 day, (**C)** 3 days, (**D**), 7 days, and (**E**) 10 days. Bacterial cellulose was grown at 20 – 25 °C in a baffled Erlenmeyer flask on an orbital shaker at 125 rpm. Images of bacterial cellulose spheres were taken with a Gel Doc XR and size analysis was performed using ImageJ. Data in panel A is represented as mean with error bars denoting the standard deviation ($n \geq 8$). Scale bars represent 10 mm.

Figure 3. Size distribution of capsules at 7 days. With (**A**) no added solids; (**B**) biochar; (**C**) plastic microbeads; and (**D**) solid mine waste. Bacterial cellulose was grown at temperatures ranging from 20 – 25 °C in a baffled Erlenmeyer flask on an orbital shaker at 145 rpm. Growth media contained 0.0101-0.0114% additives. Images of bacterial cellulose spheres were taken with a Gel Doc XR and size analysis was performed using ImageJ.

Figure 4. Possible outcomes from optimal and suboptimal experiments. (**A**) Bacterial cellulose stellate particles formed at 30 °C and 140 rpm; (**B**) BC spherical orb formed at 20 – 25 °C and 125 rpm; and (**C**) BC globules formed at 20 – 25 °C and 140 rpm when irregular shapes are not removed from the flask as they form. Black and white images were taken with a Gel Doc XR and the color photo was taken with a Surface Pro. All images were analyzed using ImageJ and all scale

bars represent 10 mm.

Figure 5. Fraction of encapsulated solids. (**A**) Thermal gravimetric traces of capsules; with (**B**) no added solids; (**C**) biochar; (**D**) plastic microbeads; and (**E**) mine waste. Prior to TGA, samples were dried on a paper towel for 3 days to remove excess water. Thermal gravimetric analyses were performed with heating ramp of 4 °C/min to 800 °C in nitrogen gas. Images of bacterial cellulose spheres were taken with a gel doc. The red arrows point to encapsulated solid particles. Scale bars represent 10 mm.

Figure 6. Mass percent of encapsulation as determined by comparison of differential TGA profiles of (**A**) BC with polystyrene microbeads and (**B**) plain BC. The differential TGA profile of plain BC can be fitted with four Gaussian curves that appear in nearly identical magnitudes in the BC with polystyrene beads. However, a fifth peak (shown in red) centered around the decomposition temperature of polystyrene also appears in the latter. This peak has been ascribed to thermal decomposition associated with the polystyrene beads. The area underneath, 13%, corresponds to percent mass loss associated with the polystyrene.

Figure 7. BC samples drying on a paper towel in a covered petri dish. (**A**) and (**B**) Plain bacterial cellulose; (**C**) BC with biochar; (**D**) BC with plastic microbeads; and (**E**) BC with mine waste. Image was taken with a Surface Pro and analyzed using ImageJ. Scale bar represents 1 cm.

DISCUSSION:

This protocol outlines BC sphere production and encapsulation methods that are easy to conduct and cost effective. Through various adjustments to the original protocol, an adequate process has been identified. Critical steps must be followed to ensure viable spheres. All the ingredients involved in BC formation play a key role in the health and durability of the spheres. The sucrose feeds organisms, the tea provides nitrogen, and the vinegar lowers the pH to optimal conditions to prevent undesired contaminants[28]. Another important variable in this method is the temperature. The tea must be cooled to room temperature (about 25 °C) before adding microbial starter culture. If the organisms are exposed to high temperatures, BC sphere growth may be inhibited. The temperature of the room in which the flask is shaking also affects sphere growth[3, 28, 29]. Shaking at room temperatures over 30 °C causes irregular BC shapes to form (**Figure 4A**). In the encapsulation process, a key step is to allow BC spheres to form before adding solid particles. This is due to the observation that the presence of foreign objects in the flask inhibited BC growth.

Different culture conditions affect the success of BC sphere production, as also shown by Hu and Catchmark[4]. BC formed best in baffled flasks on an orbital shake table. The presence of baffles accelerated sphere development compared to smooth-walled flasks[6]. Conventional stirring with a magnetic bar prevented sphere formation. Additionally, differing ratios of microbial starter culture and tea mixture influenced sphere generation and abundance. Initially, 3 mL of starter culture (2.10 mass percent of solution) was added to 140 mL of tea media. After continuing trials, the microbial starter culture amount was increased while decreasing the volume of the tea media. Final amounts used were 20 mL of microbial starter culture (20 mass %) and 80 mL of tea

mixture. For rotation speed, BC sphere formation was not successful when shaken at speeds below 100 rpm. Speeds of 125, 140, and 150 rpm produce spheres but have variance in sphere size, number, and shape, as reported previously[6,29].

As a BC formation process, agitated culture is preferable to static culture, as previously stated[2]. Compared to the methods explained in other studies, this one is less complicated and requires fewer materials. Other literature mention preparing a stock culture of BC by first fermenting a static or agitated medium and then harvesting the BC cells for inoculation in the main culture[3,4,6,28–30]. Some cell harvesting methods include vigorous shaking then filtration[30], blending then filtration[4], and centrifugation[3,29]. The BC cells incorporated in this production process are always available in the microbial starter culture containers, so cell harvesting is not necessary. Moreover, by contributing another method of BC sphere formation to the existing literature, commercial BC use is more attainable. This is beneficial because of BC's environmentally friendly material properties[29,31].

Although BC is an interesting and potentially valuable biomaterial, there are still challenges for its widespread use as previous studies indicate[18,32]. In this method, there are inconsistencies with BC sphere size and shape. Tubular and strand-like structures sometimes form in the media[2,18,32]. BC also sticks to the walls of the flasks, forming rings that sometimes become suspended in the liquid, and should be removed to prevent further irregularities from forming. While uniform spheres enable consistent scientific analysis, they may not be required for some industrial uses. Another challenge is the culture time, with the minimum duration being at least 2 days. To overcome the waiting period, manufacturers could produce spheres in staggered batches or a continuous flow reactor for a steady supply of BC spheres. Even given these challenges, BC spheres present an interesting method for sustainable production of bacterial cellulose and the ability to encapsulate various materials within the BC matrix.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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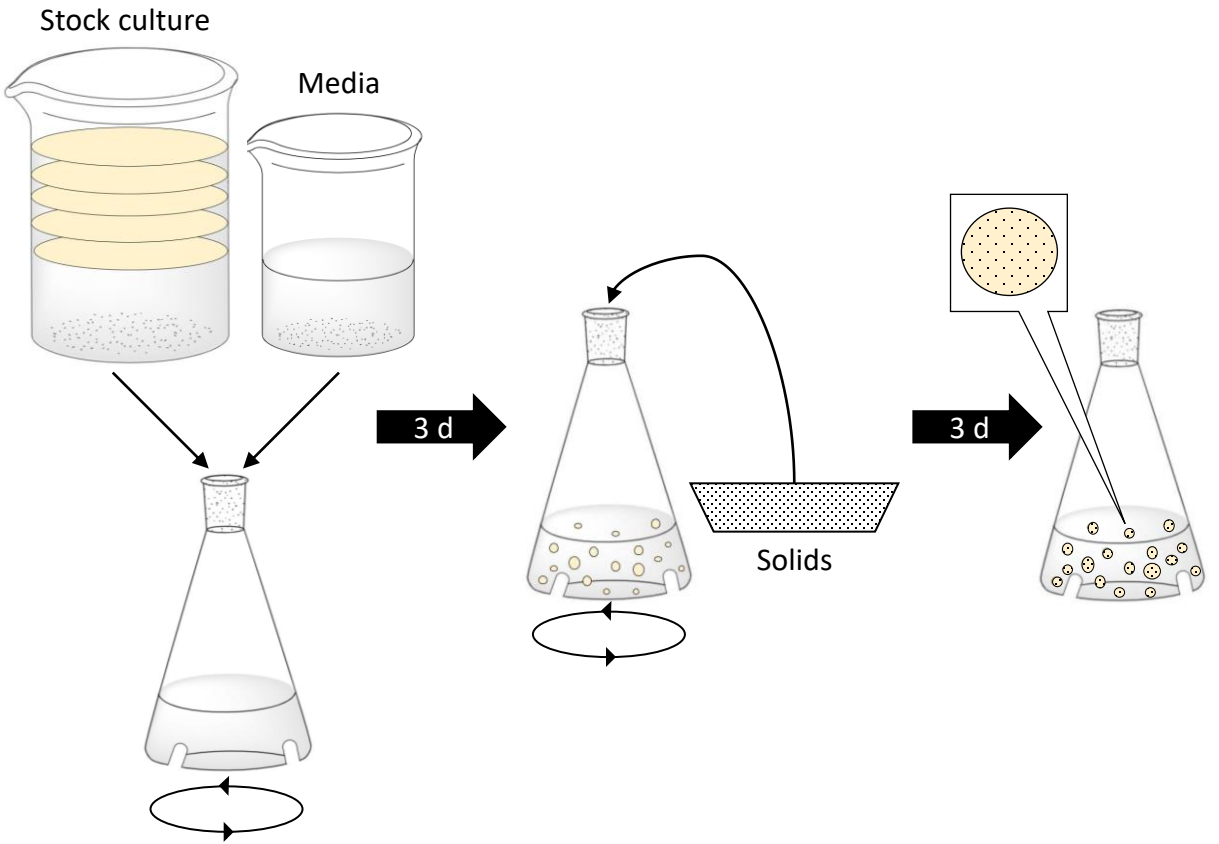

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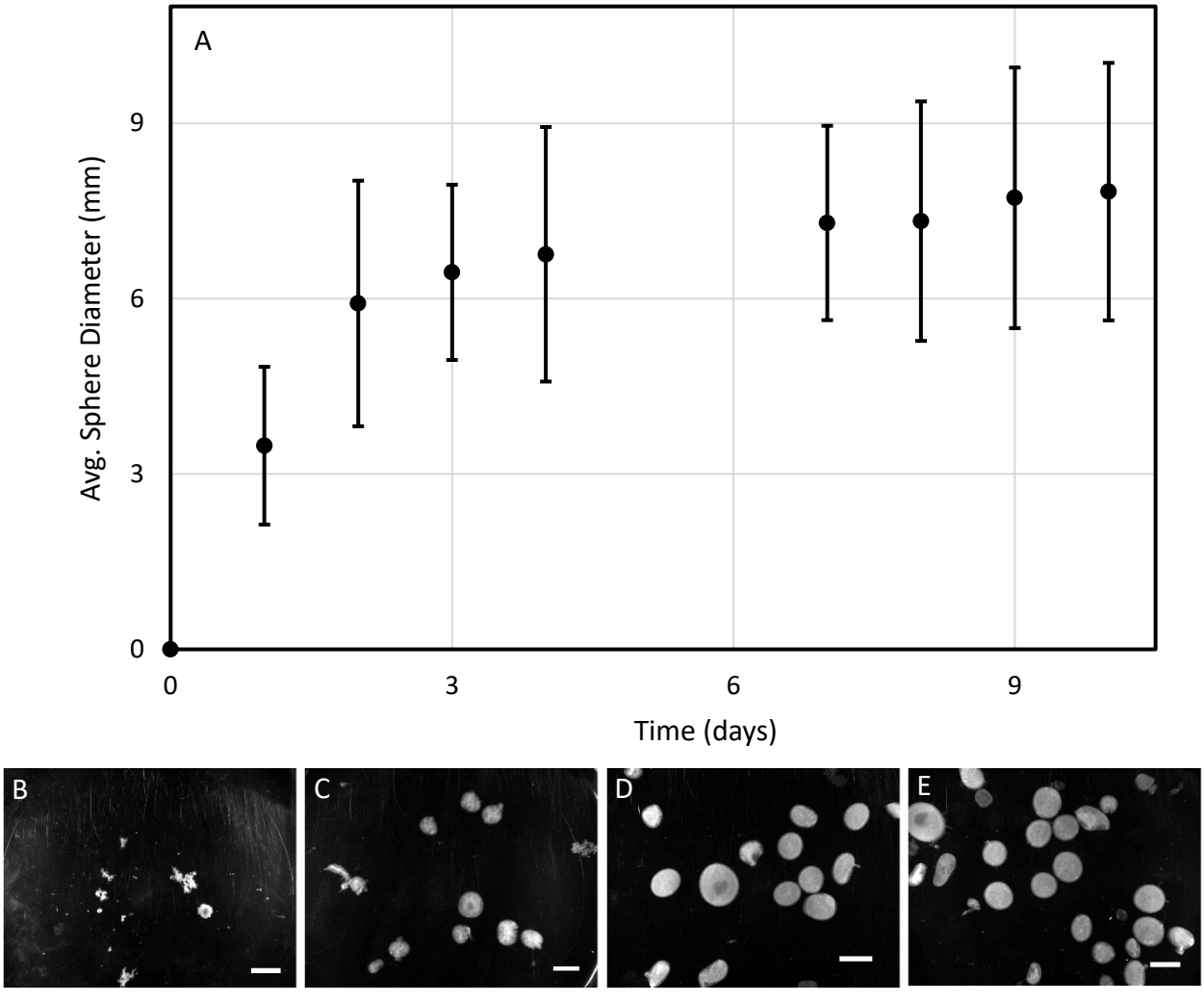

Figure 3

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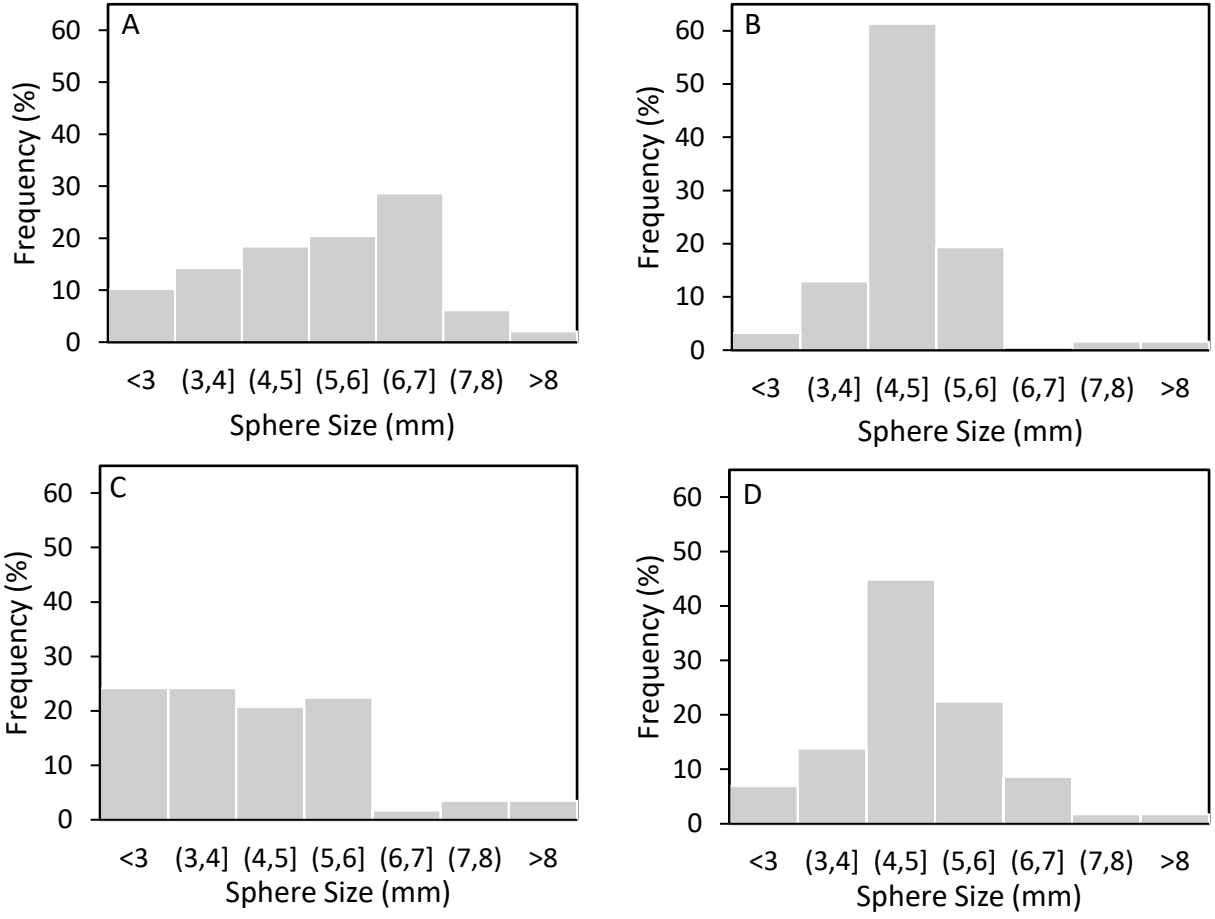

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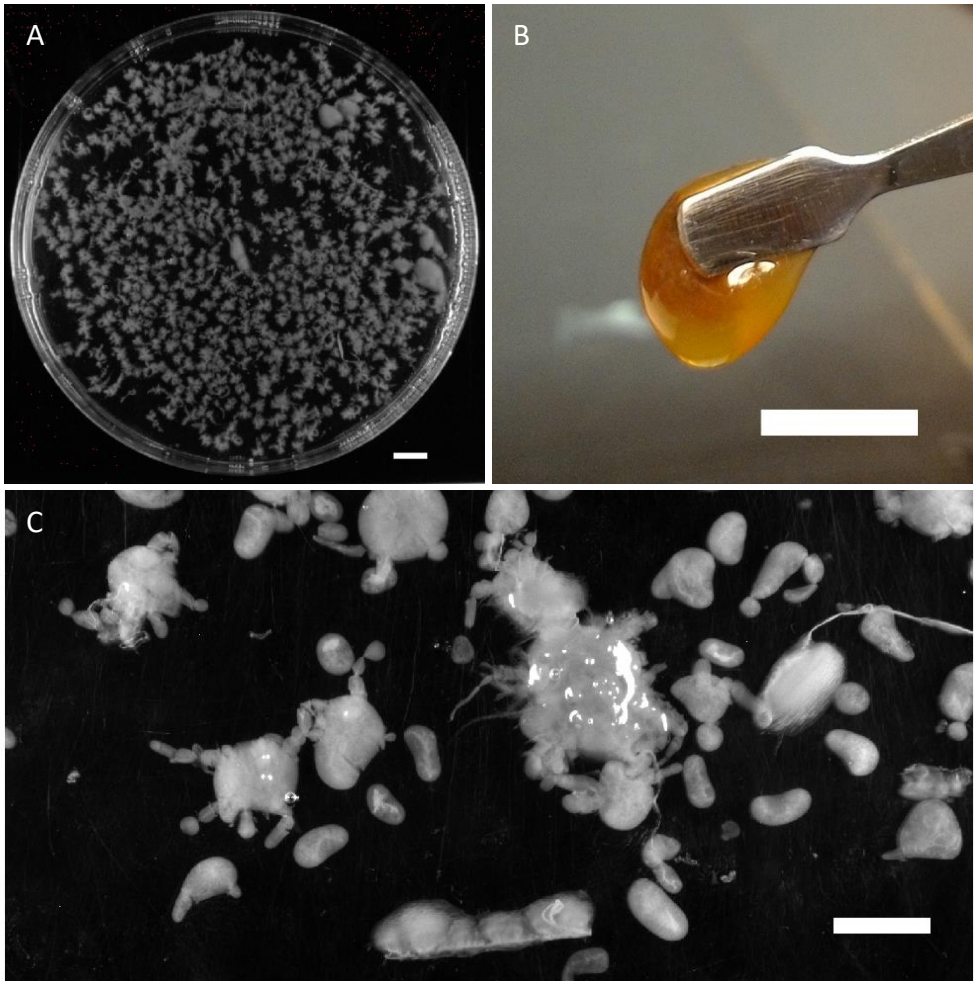

Figure 5

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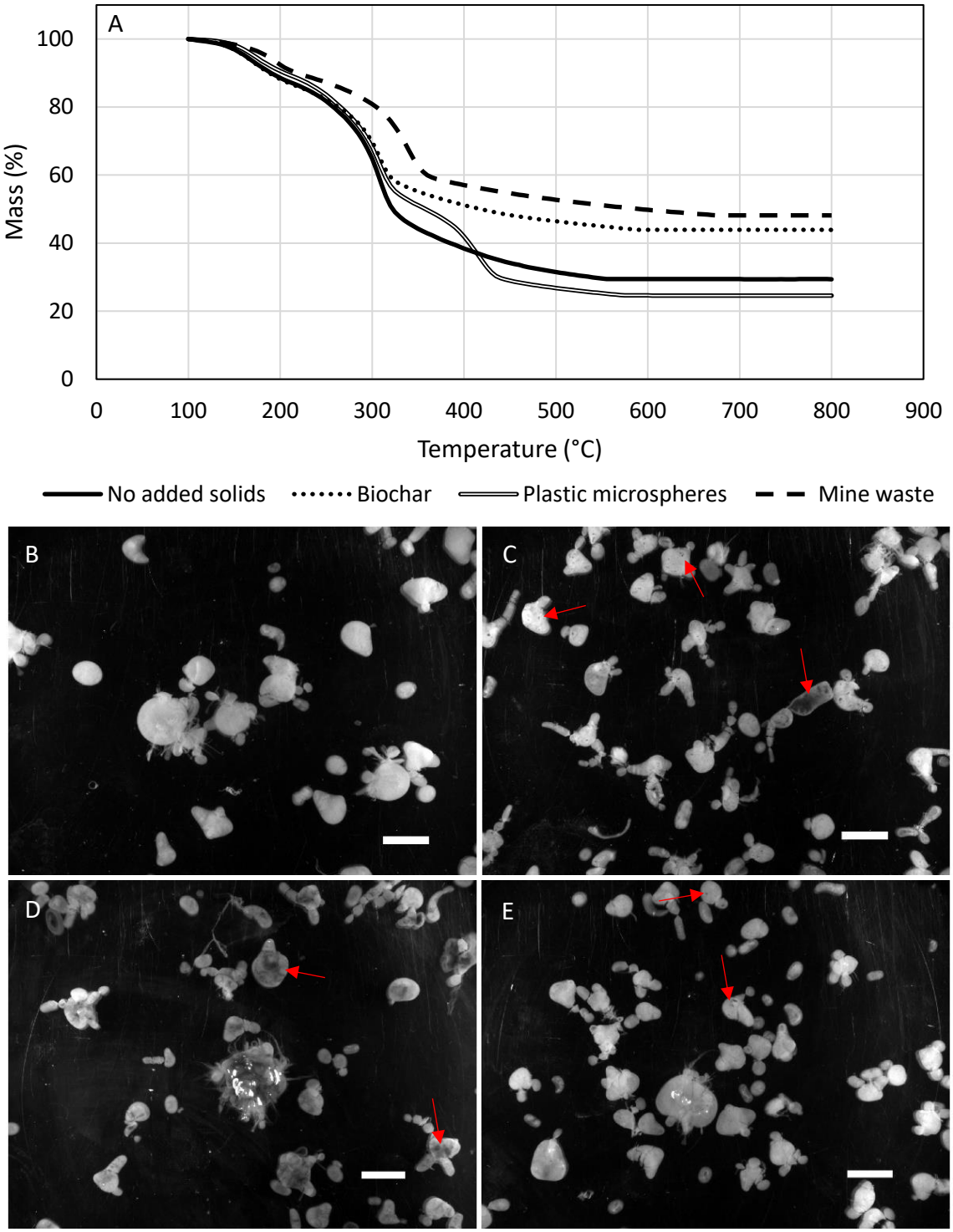


Figure 6

Click here to access/download;Figure;Microbial Cellulose Spheres JoVE Figure 6.pdf

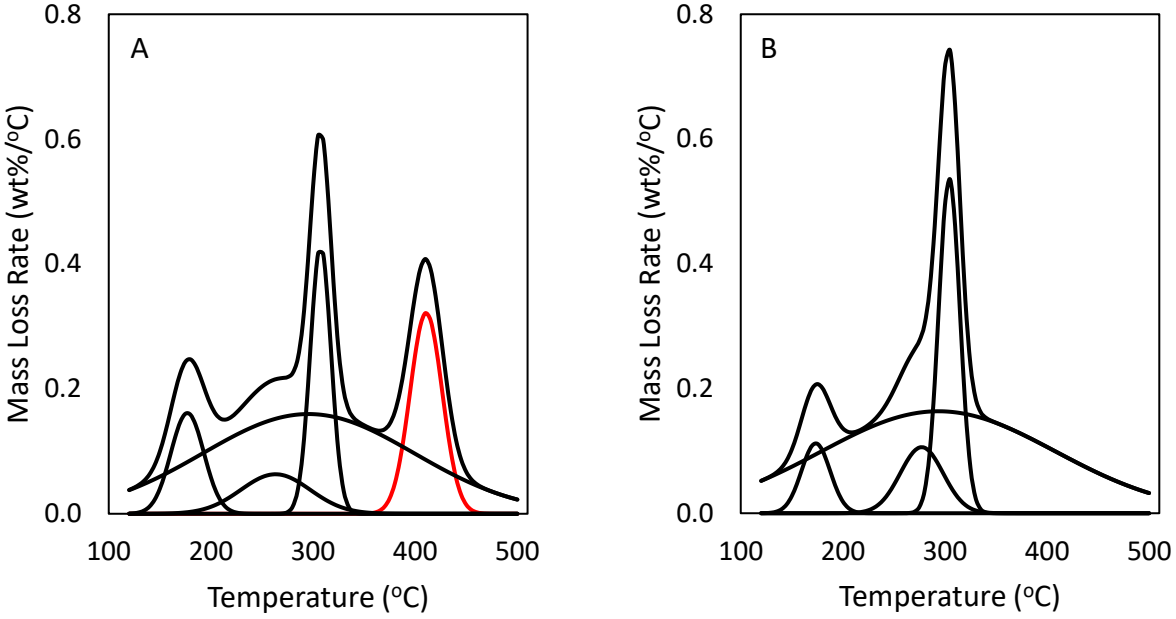
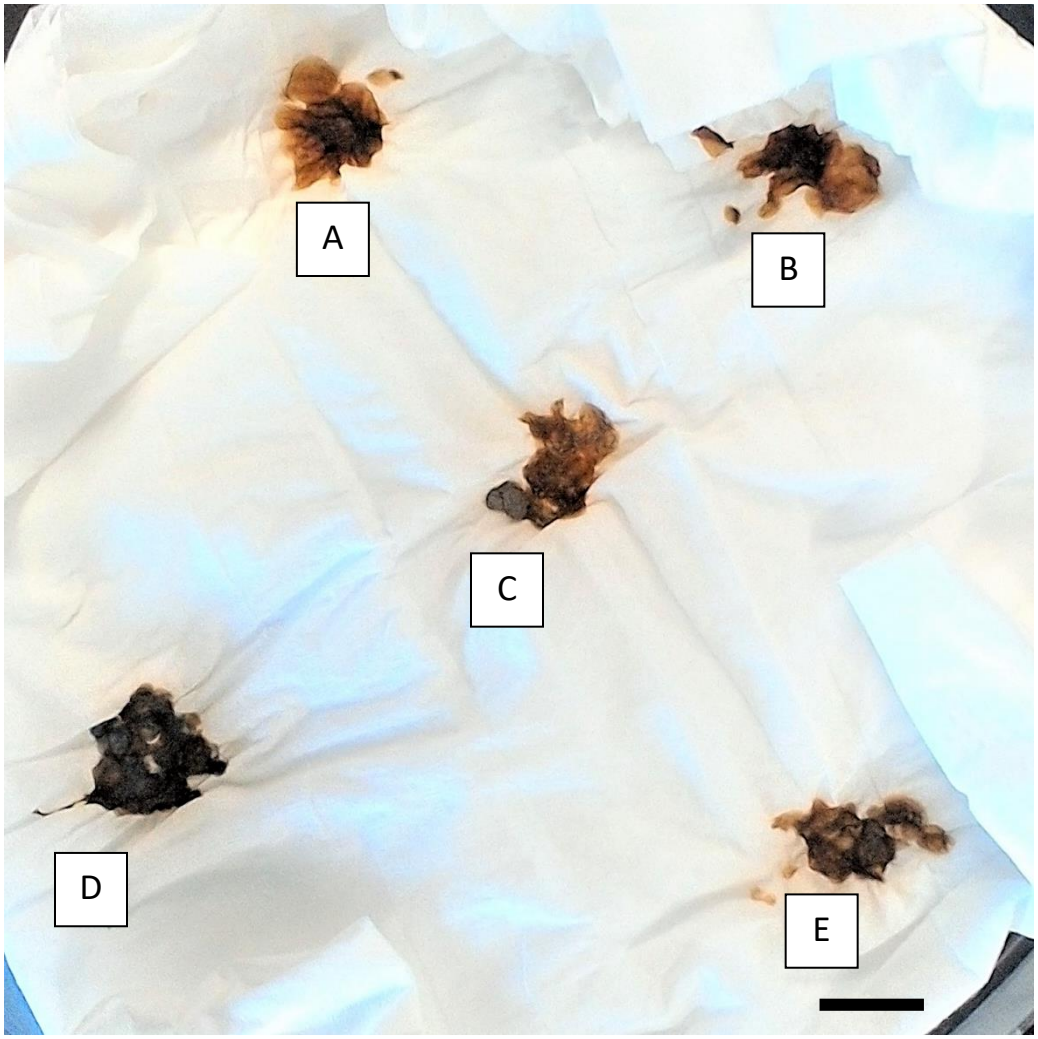


Figure 7

Click here to access/download;Figure;Microbial Cellulose Spheres JoVE Figure 7.pdf



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
100 mL graduated cylinder			
1000 mL beaker			
25 mL graduated cylinder			
250 mL Erlenmeyer baffled flask	Chemglass	CLS-2040-02	
500 mL beaker			
Balance			
Biochar			Ponderosa pine heat treated under argon gas, heated at 15 °C per minute to 800 °C
Black tea			
Deionized water			
Distilled white vinegar			
Elastic band			
Microbial starter culture	Cultures for Health		
Mine waste			Collected from Butte, MT: 46.001978,-112.582465. Mine waste contains soil and metals originating from past copper mining. Mn, Si, Ca, Al, and Fe were the five most prevalent elements measured in the mine waste through x-ray diffraction.
Mortar and pestle			
Orbital shaker			Used various brands
Paper towel			
Polystyrene microbeads	Polybead	17138	3 micron diameter
Stir rod			
Sucrose			
Tea kettle			
TGA	TA Instruments	TA Q500	400 °C/min to 800 °C, 100 mL/min N2
Thermometer			
XRF Analyzer	ThermoFisher Scientific	10131166	

Response to Reviewer Comments for
Microbial Cellulose Spheres Encapsulate Solid Materials

By Bitterman, et al.
Submitted to Journal of Visualized Experiments

We would like to thank the editor and reviewers for their comments and corrections to this manuscript. We have thoroughly reviewed the comments and incorporated them into the revised version of our document.

Editor:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The authors have proof-read and fixed all spelling and grammatical errors to the best of our abilities.

2. Please define all abbreviations before use. E.g. don't use "BC" before defining it "Bacterial Cellulose (BC)" (Lines 32, 48, etc.), Line 225: TGA, etc.

We believe that we had defined bacterial cellulose as BC in the summary and thermal gravimetric analysis in the abstract. After further consideration, we agreed that BC should also be defined in the abstract and the following change was made (lines 31-32):

"Bacterial cellulose (BC) spheres have been increasingly researched since the popularization of BC as a novel material."

The authors also decided to define "rpm" as rotations per minute in step 2.5 of the protocol:

"set the speed to 125 rotations per minute (rpm)."

3. In line 51-52, use "Symbiotic Culture Of Bacteria and Yeast…SCOBY".

Thank you for this suggestion. The authors agree that capitalizing those letters make the acronym easier to understand. The same edit was made in step 1.1 of the protocol.

4. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

The authors thank the editor for this reminder and have highlighted the most essential steps of the protocol.

5. Please avoid the use of personal pronouns (we, our) in the protocol. E.g. Line 111.

Verbiage updated to avoid personal pronouns. For example, step 1.1:

> "One can be purchased from Cultures for Health."

For example, step 3.2:

> "Appropriate solids include biochar (260 ± 140 µm), mine waste (350 ± 140 µm), and polystyrene microbeads (3 µm) and data for these materials are outlined in the Representative Results section."

6. Line 110: What quantity of SCOBY is used?

The authors thank the reviewer for their interest and agree that information is pertinent for the protocol. Typically, SCOBY starter cultures come as a dehydrated cylindrical mat about 1 cm thick. The amount used is usually around 50 g. The text was updated in step 1.1 to clarify:

> "Obtain a starter culture of bacterial cellulose, approximately 50 g, in the form of a Symbiotic Culture Of Bacteria and Yeast (SCOBY)."

7. Line 112: Covered with?

Various materials may be used to cover the SCOBY containing beaker. However, we have updated that sentence in step 1.1 and included the material we use, which is a paper towel.

8. Line 131: What strainer is used? Mesh size?

To separate the liquid from the SCOBY mats, it is acceptable to use gloved hands. A strainer with a particular mesh size is not used. The sentence has been revised to make the directions clearer in step 1.6.1:

> "Using gloved hands to hold back the SCOBY mats, drain the liquid from the hotel…"

9. Line 162: What do you mean by "acceptable temperature"?

The authors thank the editor for pointing out the ambiguity in this phrase. An acceptable temperature is room temperature, which can be roughly anywhere from 18 – 27 °C. The wording has been updated to reduce confusion and a more specific temperature range is included in step 2.4:

"Once the liquid temperature is at room temperature (20 – 25 °C), add…"

10. Line 171: How should the spheres be removed? How should they be stored (dry/in liquid media etc.)?

The authors have not experimented with the best methods for sphere storage. We believe that was not within the scope of our project. Spheres are isolated by gently decanting the liquid. Spheres can then be gently poured into the container of choice. We have reworded those steps in the protocol to reduce confusion. For example, step 2.6:

"Once the spheres have formed, they can be gently poured from the flask and analyzed by the researcher, disposed of, or used in a way not outlined in this paper."

For example, step 3.3:

"…particles for analysis, disposal, or a different use."

11. Line 179: What do you mean by "mine waste"? Please provide more details.

The authors thank the editor for pointing out our oversight in not clearly describing mine waste. A brief description of the mine waste is included in the materials table. This explains where the mine waste was sampled from and the most common elements in the sample. We have also included a reference to the materials table for those interested in knowing the specifics of all solid materials used for the encapsulation study. Step 3.2 has been modified to say:

"Please see the attached materials table for further descriptions of biochar, mine waste, and microbeads."

Below is the relevant entry in the Materials Table:

Mine waste			Collected from Butte, MT: 46.001978,-112.582465. Mine waste contains soil and metals originating from past copper mining. Mn, Si, Ca, Al, and Fe were the five most prevalent elements measured in the mine waste through x-ray diffraction.

12. Line 187-192: Please move this to the figure legends section.

This figure description was moved to the appropriate section.

13. Figure 1: Consider using the same terms in the description and the labels E.g. "hotel" is used in the protocol (line 127), but "stock culture" is used in the figure label. Do they both refer to the same thing?

Verbiage was updated in step 1.5 of the protocol to avoid confusion:
"This vessel is commonly referred to as stock culture or a hotel."

14. Please provide the relevant details for TGA in the materials table.

The authors thank the editor for pointing out this oversight. The materials table has been updated with the ramp rate, maximum temperature, and gas flowrate of the thermal gravimetric analyses.

Reviewer 1

Comments:

The manuscript describes a motivation and protocol for producing spheres of bacterial cellulose in a mixed culture. The spheres are developed and tested as an encapsulant for dispersed particles like biochar. The work is clear, well-motivated, and adds to the active area of BC production with a new, practical technique. I recommend the work be published after minor revisions to address some of the below questions.

The authors thank the reviewer for their summary of the manuscript and detailed comments. We appreciate your interest in this research. We have addressed each of your questions to the best of our ability.

1. The particles, like biochar, are added after 3 days of sphere growth. Does this mean that if you were to cut the spheres in half the particles would only be present a certain radial distance into the sphere? Is this a specification that can be designed into the process?

It is very likely that the reviewer is correct and particles may only be distributed towards the outer perimeter of the sphere. Our work in this area is ongoing, and we decided not to include a method for sphere visualization in this manuscript.

2. The procedure for removal of stray BC mass from the walls of the vial could be clearer. Is there a criterion for differentiating between spheres of certain sizes and random masses?

The authors thank the reviewer for this insight and agreed to change the protocol to better describe the method of removing irregular BC shapes. There is no quantitative criterion for deciding which masses of BC to remove; rather it is up to the experimenter

to qualitatively choose which non-spherical shapes they do not want. The note after step 2.5 of the protocol has been edited to give researchers an idea of which BC masses to remove (lines 184-187):

> "NOTE: If irregular shapes form in the flask contents or if cellulose clumps stick to the walls of the flask, they should be removed to prevent further irregular BC masses from forming. Tweezers are a good instrument for removing unwanted BC masses, including thin strings, rings, tubular shapes, and other clearly non-spherical shapes."

3. The protocol calls for careful removal of the product spheres at the end of the process. This needs more elaboration. Are the spheres vulnerable to destruction or damage due to fragility or is there another aspect that requires care? Thinking in terms of scale-up, would filtration or other flow process cause problems?

The authors thank the reviewer for their interest and advice on sphere removal. As mentioned in response to editorial comment #10 above, the removal and storage of spheres was not a focus of our research, but we agree that is pertinent information for future research and potential commercialization. BC spheres are somewhat fragile and their structures are affected when in contact with dry materials, such as kim-wipes. Quantifying structural properties of the spheres is a part of our future research.

Reviewer 2

Comments:

The paper can be considered to review again after carefully concerning my comments as follows.

The authors thank the reviewer for the comments and have implemented most of the suggested changes. We are grateful for the interesting references and have incorporated some into the introduction. The formatting of the document was left unchanged, as the current order is prescribed by the journal to focus on the method described by the protocol.

1. The English should be revised by an native English.

Thank you for this comment. The manuscript has been revised by English speakers.

2. The order of manuscript is also not following the standard order. The good order should be: abstract, introduction, experimental, results and discussion. Please revised it.

The authors thank the reviewer for this observation and understand that the journal format is not standard. Please note that we have followed the prescribed JoVE template.

3. In Creation and Maintenance of Bacterial Cellulose Starter Culture do you think the yeast can be died by hot water?

To clarify, creating the bacterial cellulose starter culture does not involve yeast being directly added to hot water. Rather, yeast and bacteria are present in the SCOBY mat and exposing those organisms to hot water could potentially kill them. The authors think that yes, the yeast can be killed by hot water.

4. The introduction section should be enriched by citing some refs:
https://doi.org/10.1016/j.polymertesting.2017.05.013
https://doi.org/10.1007/s00289-017-2162-4
https://doi.org/10.1080/09276440.2019.1680205
https://doi.org/10.3390/ma12091407

The authors would like to thank the reviewer for sharing additional interesting research on bacterial cellulose. Our research was not focused on the addition of BC to other materials, but we have added two of the noted references to the introduction to portray the growing interest in the field of biomaterials and BC's advantages (lines 76-80):

> "Studies have also explored the beneficial effects of using BC to produce improved biocomposites. Using epoxy-resin as a base, researchers have showed that the addition of BC improves material characteristics like fatigue life, fracture toughness, and tensile and flexural strength[9, 10]. As shown by past and current research, many are interested in commercializing BC use."

5. The conclusion should be added.

The authors thank the reviewer for their insight. Please note that the journal does not request a conclusion section.

6. The chemical structure and crystal phase of BC should be studied by FTIR, NMR, AND XRD

The authors thank the reviewer for this comment and agree that the chemical structure and crystal phase of BC is relevant information. Structural characteristics of spheres will be a part of or future research. One group has studied the structure of BC membranes and a reference to that characterization has been added to the introduction (lines 73-76):

> "Holland et al. have studied the crystallinity and chemical structure of BC using X-ray diffraction and Fourier transform infrared spectroscopy[8]. We assume BC capsules will exhibit similar characteristics and will investigate structural properties with future research.

Reviewer 3

Comments:

Dear authors, the manuscript entitled "Microbial Cellulose Spheres Encapsulate Solid Materials" present simple study on well known method of BC spheres production in shaking cultures of symbiotic culture of bacteria and yeast. The research can by published in JoVE however before should be improved.

The authors thank the reviewer for the taking the time to review our work so carefully. We sincerely appreciate your comments and suggestions, most of which we gladly incorporated into the paper. You will find our detailed response below.

Major concerns:

1*. Line 53-54. The sentence seems to be an aim of study that should be placed on the end of introduction. Authors should avoid inserting this kind of sentence in the middle region of introduction.*

The authors thank the reviewer for this observation and agree that the sentences in that section needed editing to make more sense in that region of the introduction (lines 52-57):

"Brewing kombucha relies on a Symbiotic Culture Of Bacteria and Yeast, more commonly known as a SCOBY. Using this symbiotic culture of organisms, a similar technique is employed to create BC spheres. This biomaterial may be employed to help…"

2. Line 62; Bacterial cellulose offers an advantage over synthetic cellulose because…….. What exactly authors means in terms of synthetic cellulose? Probably authors means about plant cellulose which purification process is much more complicated than purification of BC. Please correct it.

The authors would like to express much gratitude to the reviewer for pointing out the confusion in this sentence. We believe this information is not relevant for the scope of this study and this sentence has been removed.

3. Line 85-86; …BC sphere CRF's could limit eutrophication,… Could Authors more clearly write how BC spheres can be applied in the process of water eutrophication reduction? The cost of this technology could be a first limitation of proposed idea.

The authors made this claim with reference to using the BC spheres as a method of encapsulating fertilizers to, potentially, lower their release rate. A lower release rate would allow crops sufficient time to uptake the fertilizer and prevent excess runoff into bodies of water. We agree that this technology might be difficult to scale up, but similar slow release fertilizers have been prepared and piloted using polymer coatings. The BC

coating would have the advantage of also adding carbon to the soil. Parts of this explanation has been inserted in the introduction of the text to better explain to readers how BC spheres can limit eutrophication (lines 95-99):

> "Adapting BC into a fertilizer coating may further improve CRF technologies. By lowering fertilizer release rate, crops will have sufficient time to uptake the fertilizer and prevent excess runoff into bodies of water, thereby reducing eutrophication and unoxygenated zones. Similar slow-release fertilizers have been prepared and piloted using polymer coatings[20]."

4. Line 72; Other publications….. could be replaced by Many researchers

Verbiage has been updated.

5. Line 74; Much of this research could be replaced by; Many or a lot of research

The authors thank the reviewer for their input on the grammatical structure of this sentence. After some discussion, we have agreed that the current wording is clear and concise as is.

6. Line 80-82. The sentence is not clear. Please simplify it.

The authors agree with the reviewer that this sentence needed to be simplified. The sentence in the introduction has been changed it for clarification (lines 89-93):

> "Bacterial cellulose may work as a favorable encapsulating material for CRF's. Fertilizers may leach out of BC membranes or discharge as BC biodegrades[15, 16]. BC's high water swelling capacity can also act as a beneficial soil amendment[17–19] because both fertilizer nutrients and moisture may release into the ground through application of BC spheres."

7. Line 197; The standard deviation tends to increase with time… The standard deviation is not a factor but is used for expression of population diversity. This sentence should be removed and replaced by more precise finding that with the age of the SCOBY culture, the variation in the amount and morphology of obtained BC spheres increased.

The authors thank the reviewer for their comment and agree that this interpretation needed to be removed. After a closer look at Figure 2 showing the growth of sphere diameters, we have decided the variations in mean diameter were not significant enough to claim that they increased uniformly with time.

8. Line 212; were plain BC.., better use; non-modified

The authors thank the reviewer for pointing out the poor wording here. After some discussion have agreed that writing “BC” implies it was not modified. “Plain” has been removed.

9. Line 276; If the organisms are exposed to high heat….. replace by; If the organisms are exposed to high temperatures.

Verbiage has been updated.

10. Line 277. Authors wrote "The temperature of the room in which the flask is shaking also affects sphere growth. Shaking at room temperatures over 30 °C causes irregular BC shapes to form (Fig.4A)". The room temperature in scientific reports it is a range between 20-25°C. Moreover Authors wrote that the "temperatures ranging from 22-24°C". Could Authors indicate exact conditions under which the culture was carried out in terms of temperature?

The authors agree that the differences in temperature ranges introduce ambiguity into the text. With the means available to us, it was not possible to control the temperature range more precisely than what is shown in the data or continually record the temperature. The sentence in line 304: “Shaking at room temperatures over 30 °C causes irregular BC shapes to form (Fig. 4A).” was included because of the journal’s request to discuss what could go wrong if certain protocol steps are not followed. In general, we perform all experiments at room temperature. For clarity, we changed all the temperature ranges to 20 – 25 °C.

11. Line from 269 to 290 is not a discussion. This section needs more references and comparison of obtained results with discoveries earlier reported by other researchers.

The authors thank the reviewer for their input and agree that the discussion needed more references. The JoVE manuscript template requests discussion of the following items: “critical steps in the protocol, modifications and troubleshooting of the method, limitations of the method, the significance of the method with respect to existing/alternative methods, and future applications or directions of the method”. Since this paragraph discusses critical steps in the protocol, we have decided to keep it in the manuscript, but with more references added.
For example, lines 299-300:

> “The temperature of the room in which the flask is shaking also affects sphere growth[3, 28, 29].”

For example, lines 304-305:

> “Different culture conditions affect the success of BC sphere production, as also shown by Hu and Catchmark[4]”

For example, lines 305-306:

> "The presence of baffles accelerated sphere development compared to smooth-walled flasks[6]."

For example, lines 313-315:

> "Speeds of 125, 140, and 150 rpm produce spheres but have variance in sphere size, number, and shape, as reported previously[6, 29]."

For example, lines 316-317:

> "As a BC formation process, agitated culture is preferable to static culture, as previously stated[2]."

For example, line 325:

> "This is beneficial because of BC's environmentally friendly material properties[29, 31]."

For examples, lines 326-327:

> "Although BC is an interesting and potentially valuable biomaterial, there are still challenges for its widespread use as previous studies indicate[18, 32].

For example, lines 328-329:

> "Tubular and strand-like structures sometimes form in the media[2, 18, 32s]."

Minor Concerns:
Please consult the style of manuscript with colleagues that have experience in scientific reports writing.

The authors thank the reviewer for their observation. We understand that the journal format is unique and emphasizes focus on methods of the research. Our document, as written, attempts to follow the unique format of this publication.