Project Context: Hello! If you're reading this, I assume you are a part of the Ultra Fabrics scholarship committee or company. This is a summary of what this project is, the purpose, concept, and how I plan to use this information moving forward. I interviewed several synthetic biologists and researchers with similar research during the summer before this and decided to make this experiment a school project for my biology class. My high schools' resources were minimal but now that I'm attending college there are more opportunities for me to continue more advanced research and in this area.

Concept: Use waste cooking oil from restaurants and residential areas in populated areas as a feedstock for bacterial cellulose. Because a lot of waste cooking oil is produced in densely populated areas and bacterial cellulose can be grown in a warehouse anywhere it could be a cheap way to get feedstock and produce a useful product for nearby consumers.

Pros: This experiment offered me some interesting insight into cellulose producing bacterial strains. I will most likely complete a literature review on bacterial cellulose in sustainable textile fiber production during my undergraduate and use this experience as a start to investigate sustainable cost-effective feedstocks. This was my first time attempting research like this so it was a great opportunity despite being imperfect. I plan on doing more research on specific carbon sources that bacterial cellulose grows effectively on and finding an easily available sustainable feedstock.

Cons: This experiment didn't produce conclusive results, but I doubt that waste cooking oil is a viable carbon source for bacterial cellulose. A lack of resources left a lot to be desired. The results were wildly different from oil-based industrial waste feedstocks. Cooking oil can likely be recycled in other ways that may be more efficiently than as a feedstock for bacterial cellulose textiles. There are other residential and commercial wastes that might be easily sourced from urban or suburban areas such as organic food waste that is likely a better alternative.

Investigation of the growth bacteria cellulose of refined soybean oil and used refined soybean oil as the sole carbon source by the bacteria ***Komagataeibacter xylinus***

Article I. <u>Introduction</u>

This study investigates the use of refined soybean oils and used refined soybean oils commonly used in restaurants and home kitchens as carbon source for *Komagataeibacter xylinus*. Bacterial cellulose is a valuable biopolymer which has potential uses in medicine, food, textiles and agriculture (Zh.t). Bacterial cellulose is commonly produced by acetic acid bacteria such as *Komagataeibacter xylinus, Komagataeibacter rhaeticu*s and *Komagataeibacter hansenii*. Several methods of decreasing the cost of bacterial cellulose production using different low-cost carbon sources. Various agricultural, industrial, and municipal wastes have been considered as possible low-cost and sustainable feedstocks. This includes pecan citrus pulp water, industrial hardwood, coconut water, waste beer yeast, soybean oil effluent and kitchen wastes. (Anna Żywicka, 2018) (Nan Qiao, 2019) (Hamada El-Gendi, 2022)

I chose this project because I had spent a very long time searching for easier ways to make fibers for yarn in a search to find a way to create an infinite supply of yarn for myself. Sadly, I am not capable of growing an infinite supply of yarn for my crochet addiction. However, it's not realistic for me to grow a flax field or keep a flock of sheep in my backyard so bacterial cellulose seemed like a pretty good option. It can likely be grown much more easily in suburban and urban areas without needing as much space to grow. However, I still can't do that because there simply isn't enough research on it for me to get staple spun yarns like I want. So of course, the only reasonable solution in my mind was to center my future plans and scientific career on sustainable textile fibers and bacterial cellulose. Which motivated me to do this experiment. This study seeks to explore the use of kitchen wastes, specifically refined cooking oils and waste refined cooking as a carbon source for cellulose-producing acetic acid and bacterial cellulose.

Article II. <u>Hypothesis</u>

Experimental group	Carbon source	Media	Expected outcome
1	glucose	2% glucose (20 g/L)	This is a control that should yield a standard which the other two outcomes can be compared to. This is likely to be the most productive culture because it contains an easily metabolized carbon source
2	Soybean oil (pure)	2% soybean oil 20 (g/l)	This refined oil may be less suitable for production of bacterial cellulose due to a lack of free fatty acids in the solution making the carbon source less readily available.
3	Soybean oil (waste)	2% soybean oil (20 g/l)	There will likely be more growth on this plate than the unused waste oil however it may not display as much growth as standard medium due to an increased amount of free fatty acids

Article III. <u>Background</u>

K.xylinus and other cellulose producing acetic acid bacteria have previously demonstrated an ability to metabolize fatty acids and lipids as a carbon source from industrial wastes. As mentioned previously, one study used soybean oil effluent retrieved from has demonstrated that fatty acid lipids are a viable carbon source for *k.xylinus* . (Nan Qiao, 2019) Another study used rapeseed oil as a supplement to increase the bacterial cellulose yield by 500%. (Anna Żywicka, 2018) This demonstrates that *k.xylinus* can grow on various oils as carbon sources. Another acetic acid bacteria *K.rhaeticus* demonstrated the ability to metablolize kitchen wastes with refined cooking oils with little or no changes in bacterial cellulose production (Zhi-Yu Li, 2021). There may be different effects on growth may be due to the different composition of refined kitchen oils in comparison to agricultural or industrial waste oil. Rapeseed oil is an unrefined form of canola oil that contains high amounts of erucic acid, and the soybean oil effluent was stated to contain high amounts of free fatty acids. While refined soybean oil and canola oil (the refined form of rapeseed oil), contain mostly fatty acids in the form of triglyceride esters. Canola oil contains less than 2% erucic acid as mandated by the Food and Drug administration (Food and Drug Adminstration, 2023). Similarly, refined soybean oil also contains less than 0.03% of fatty carboxylic acids (Food and Drug Adminstration, 2023), Considering the findings of the previous experiments involving used kitchen waste and the outcomes of using various crude oils containing free fatty acids, this may suggest that *k.xylinus* and possibly other cellulose producing acetic acid bacteria are more easily able to use free fatty carboxylic acids as a carbon source.

When refined oils are used for frying or cooking the triglyceride esters undergo a hydrolysis reaction freeing the esters from their bonds and turning them into FFAs (Frank T.

Orthoefer, 2007). This leads to used cooking oil having a lower smoke point and being unhealthier for people as high FFAs are said to increase obesity and may cause diabetes.

Article IV. Methodology

Section 4.01 Variables and risk assessment

This experiment heavily references the previously mentioned studies on various bacterial cellulose to better compare bacterial cellulose production in this experiment with other similar studies on different carbon sources.

(a) Independent variables: The carbon source used in each group

Glucose is the standard carbon source used in Hestrin Schramm medium, which is the control medium for many experiments.

Soybean oil and Waste soybean oil are used as the oils referenced in this study. Not only has soybean oil effluent been used as a carbon source for *k.xylinus* in another experiment but soybean oil is the most used cooking oil being 30% vegetable oil consumption (U.s Environemental Protection Agency, 2001). Making this seem like a better choice when keeping the potential use of waste cooking oils in mind. The waste soybean oil refers to oil that was used to fry food in a home kitchen.

(b) Dependent variables: Growth of bacterial cellulose pellicle

(c) Control variables: room temperature at 26º Celsius, amount of medium, petri dish size, bacteria, time allowed for growth

(d) Risk assessment/important safety procedures:

The bacteria used is a common acetic acid bacteria used as a basis for bacterial cellulose experiments obtained from the American Type Culture Collection *Komagataeibacter xylinus*

(American Type Culture Collection, 1997) . All metal and glass materials that come in constant with the bacteria and used to prepare and inoculate culture will be maintained in bacterial cellulose. The plates were cultured at room temperature 26º Celsius which is safe for *k.xylinus* as stated on its product sheet but also is not likely to culture dangerous bacteria and pathogens. Surfaces were wiped with a 10% bleach solution after use. Goggles and gloves should be worn during the experiment and hair should be pinned back throughout the experiment.

Preparing the experiment and Culture

(e) Materials

- Peptone (5.0 grams)
- Citric acid (1.5 grams)
- Yeast extract (5.0 grams)
- Glucose (2.0 grams)
- Sodium diphosphate/ Na2HPO4 (2.7 grams)
- Distilled water
- Scale (measured in grams)
- Soybean oil (2 grams)
- Waste soybean oil (2.0 grams)
- 15 petri dishes
- Gloves
- Acetic Acid Bacteria *K.xylinus*
- Scale
- Stir Bar
- 1 Liter flask
- Inoculation loops
- Label maker
- Autoclave sterilizer.
- 10% bleach solution
- Goggles
- 3 Erlenmeyer flasks
- Ph probe

(f) <u>Preparation and sterilization of media and materials.</u>

1. Label 15 petri dishes. 5 as HS crotrol medium. 5 as 2% soybean oil medium. 5 as 2% waste soybean oil medium.
2. Autoclave glassware, stir bar, and tweezer at 121º Celsius for 20 minutes.
3. A medium containing was made in added to a 1 Liter flask:

- Peptone 5.0 g
- Yeast extract. 5.0 g
- $Na2HPO4$ 2.7 g
- Citric acid.. 1.5 g
- Distilled water.. 1.0 L

4. Then 101.42 liters of premade solution (additional grams account for added ingredients) are placed into 3 Erlenmeyer flasks.
5. 2 grams of a different carbon source, glucose, soybean, and waste cooking soybean oil.
6. After the carbon source is added the whole solution is autoclaved at 121 degrees Celsius and cooled for 20 minutes. The unused oil leaves a thin layer on the top of the medium when left to sit. The used oil formed thicker clusters of oil with small spots of debris left over from its use. The control medium had no film on the top.
7. For each medium add 15 milliliters of medium into the appropriately labeled petri dishes. 25 milliliters of each solution should be left over left over.
8. Take the freeze-dried sample of *k.xylinus* obtained from the ATCC. Remove the pellet from the glass vial carefully using the autoclaved tweezers was and rehydrate in 15 ml in standard glucose HS medium.
9. Using an inoculation loop streak each of the 15 plates with the bacteria using the rehydrated solution.

10. To prevent contamination of the medium the Ph of medium was taken from a sample of the mediums that was separated from the one that was going to be used for the cultures. Around 20 milliliters of each.

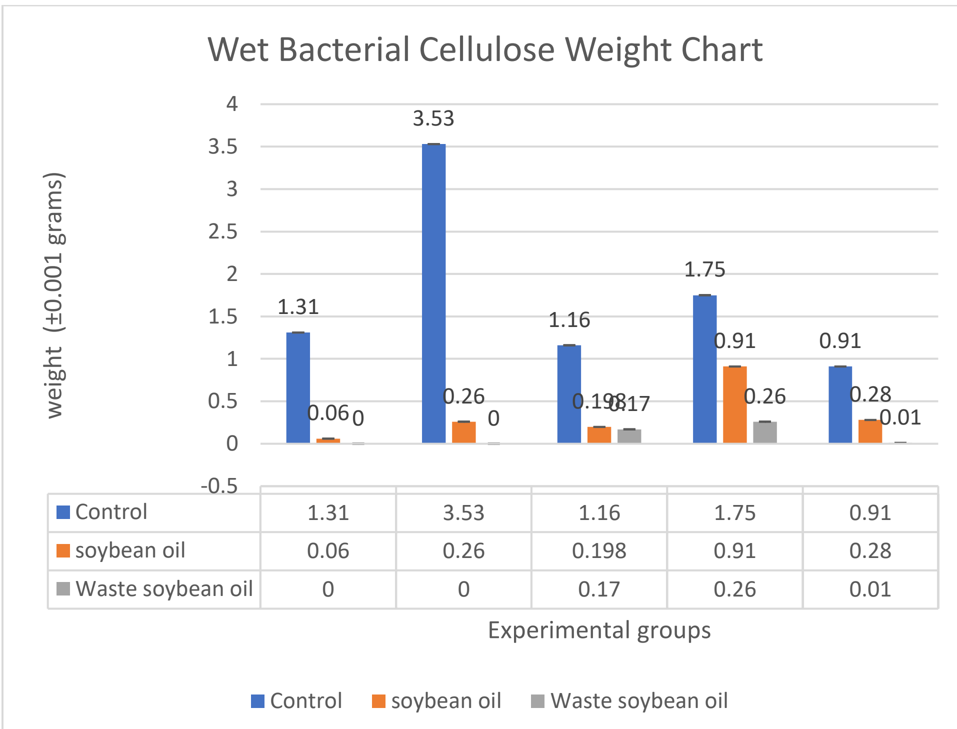
Table 1: Ph of different mediums

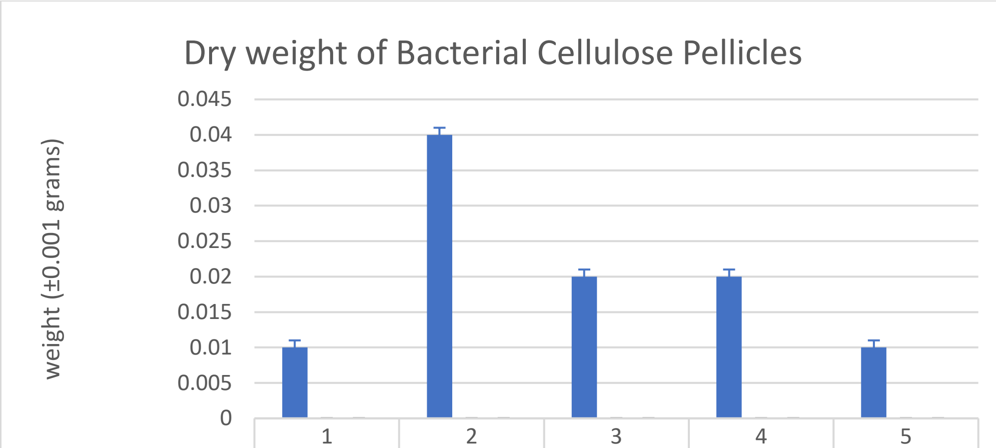
Control	Soybean oil	Waste soybean oil
6.23	6.61	6.48

11. The bacteria were then cultured for at room temperature for 12 days at 26º Celsius. Even after 12 days of growth the plates using oil as a substrate did not form solid pellicles, instead forming small bubbles of bacterial cellulose that did not come together or floated to the bottom. Autoclave the tweezers again.
12. The pellicles were removed carefully with the autoclaved pair of tweezers and weighed in grams on a scale.
13. The pellicles were placed onto parchment paper and allowed to dry for one day. After they were dry the pellicle would be weighed on the scale again.
14. Once the experiment concluded the petri dishes and bacterial products were disposed of through Stericycle, the school labs bio-waste company, and surfaces were wiped with 10% bleach solution.

Article V. <u>Results</u>

On the first day of culture there were no significant changes aside from the condensation clearing but I could see some debris on the bottom of the plate. On day 2, I was able to take a picture of newly formed condensation on the lids of the petri that had formed on all the plates. This was most likely indicating that the *k.xylinus* was going through fermentation reactions and releasing CO_2, which meant that growth could be seen on all of the plates. On day 7, there was an attempt to remove one of the pellicles of the control but after attempting to pull the edge of the pellicle with a pair of tweezers it appeared that the pellicles could not be removed easily including in the control. On day 12 the pellicles of the control were strong enough to picked up as a solid mass with a pair of tweezers. The oil groups still had most of their pellicles floating in clumps at the top or sinking to the bottom of the petri dish.





	1	2	3	4	5
Control	0.01	0.04	0.02	0.02	0.01
Soybean oil	0	0	0	0	0
waste soybean oil	0	0	0	0	0

Article VI. Strengths, Weaknesses and Limitations

Section 6.01 Strengths

The ability of bacterial cellulose to be grown on refined soybean oil used for commercial cooking and consumption is clear. This experiment offers a direct side by side comparison of each carbon source. The 5 trials leave less room for error in the results. This study uses soybean oil which is the most used oil in households and commercially for consumption.

Section 6.02 Weaknesses

The yield in the cultures with oil as a carbon source was so low that it returned as weighing nothing on the scale. The fragility of the pellicle made it impossible to lyse the pellicles using NaOH as most experiments measuring bacterial cellulose growth do before drying the pellicle meaning oil content and cells would have also been measured in the weight. The small yield produced from the experiment severely limits the experiments results and what information can be gathered. The culture was left at room temperature at 26º Celsius however since an incubator wasn't used some fluctuations in temperature may have occurred. The culture's growth time was initially going to be 7 days which is in line with other experiments however the pellicles of all the plates seemed too weak to remove easily, so it was left to grow for 5 more days until day 12. Making this study less comparable and that several pellicles had been disturbed before the culture was complete.

Section 6.03 Limitations

Given that this study has a particular interest in observing how different oils affect the growth of bacterial cellulose the ability to compare with soybean oil effluent or unrefined soybean oil as an experimental group likely would have been beneficial to understanding the results. No analysis was done on the characteristics of the bacterial cellulose such as tensile strength. The available equipment in the lab meant it wasn't possible to test composition of the waste cooking oil is severely lacking and no tests were done to check what compounds or chemicals were in it. Overall due to constraints within available lab equipment and tools this experiment left a lot of room for further data gathering and analysis.

Article VII. Conclusion and Discussion

The acetic acid bacteria can produce bacterial cellulose using any of the three experimental groups as a carbon source. When comparing wet weight the glucose-based medium had the best results, generating the largest amount of bacterial cellulose in both the dry and wet states. The soybean oil-based medium that was not demonstrated larger amounts of growth while the waste soybean oil demonstrated the worst growth overall. All the pellicles lost a significant amount of mass once they were dried. When comparing dry weight, the glucose medium again demonstrated the most growth with both the oil mediums reading as zero on the scale in weight. Ph seemed to have little to no effect on growth.

Regarding the initial hypothesis in relation to the effect of free fatty acids in waste cooking oil as opposed to triglycerides esters in oil. This experiment may suggest that there is either a week or negative correlation between free fatty acids and bacterial cellulose growth on the surface. However, due to the lack of analysis on the compounds within there is a lot of room for speculation as to what the cause of the difference in growth could have been. These results do not align with previous literature research for bacterial cellulose growth using oil as a carbon source. For now, it may be best to consider that aspect of this experiment inconclusive,

Despite the results of this experiment being somewhat lackluster there is still some good insight to be found within these results. Further research including unrefined oils in direct comparison to refined oil. Deeper analysis on the chemical composition of different oils being used as carbon sources and the different effects of various compounds and fats on bacterial cellulose growth is certainly worth further study. Understanding what a good low-cost medium for bacterial cellulose may be and why might open the door for the creation of more sustainable textiles and medical products in the future.

For me I think this experiment serves as strong basis for a research avenue which I can pursue in university. I believe there is quite a lot of room for growth and experimental study in this area.

Article VIII. <u>Bibliography</u>

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