CHARACTERIZATION AND EXPLORATION OF APPLICATIONS OF NANOCELLULOSE DERIVED FROM *SARGASSUM MUTICUM*

By Cody O’Brien

Cody O’Brien, from Jacksonville, FL, is a senior Civil/Environmental Engineering student at Florida State University. In his second year of university, he pursued the Undergraduate Research Opportunity Program (UROP) with colleagues to research the use of algae, including the invasive species *Sargassum muticum*, for creating stronger air-dry clay bricks. Seeking to continue research in utilizing invasive organisms, he was awarded the IDEA Grant to study nanocellulose isolated from *S. muticum* and their real-world applications. This project resulted in the first documented extraction and characterization of cellulose nanocrystals (CNCs) from *S. muticum.* He hopes to pursue a career in sustainable engineering and better incorporate the built world with the natural world. Outside of school and research, Cody works as a tutor and enjoys the outdoors. He would like to thank Robert and Mary Frappier for their support for this project through the IDEA Grant program and Dr. Qian Zhang, his advisor. He also commends Dr. Joel Smith, Tracey Bell, Dr. Nebojsa Bogdanovic, and everyone else who provided their time and expertise through the undertaking.

Abstract

Sargassum muticum is an invasive species of marine algae affecting Florida and other areas along the Atlantic Ocean. There is urgency to find real-world applications of this species or its products, such as its cellulose nanofibers (CNFs) and nanocrystals (CNCs), collectively known as nanocellulose. The objective of this project was to attempt the first documented extraction of CNFs from *S. muticum*, perform tests and imaging on them, and explore their future applications in medicine, concrete reinforcement, insulation, and other fields. Nanocellulose acts as a more sustainable alternative to the standard materials used for those purposes, offering improved human and environmental health outcomes. The algae was dried, grinded, and bleached, and then refluxed in a sodium hydroxide solution to remove the proteins. Then, a chloroform-methanol-water solution was used to remove the algae's lipids and pigments. Finally, a hydrochloric acid solution was used to further refine the remaining carbohydrate-based cellulose. The sample was centrifuged between each refluxation. The final product was characterized using Fourier-transform infrared spectroscopy (FT-IR), X-ray diffractometry (XRD), and transmission electron microscopy (TEM), which confirmed the successful extraction of CNCs, contrary to original expectations. These CNCs, measuring between 70 and 700 nm in length and below 10 nm in width, compared favorably with CNCs from other sources, including other species of algae.

Background

Algae are plant-like protist organisms that primarily consist of proteins, lipids, and carbohydrates. These carbohydrates, like glucose (monosaccharides), are generally arranged into larger chains (polysaccharides) called cellulose, which makes up the cell walls in plants and some other organisms.

Cellulose can then be broken into cellulose nanofibers (CNFs) and cellulose nanocrystals (CNCs), collectively known as nanocellulose. CNFs and CNCs are essentially thin (usually <20 nm wide) chains of carbohydrates, with CNFs being longer (usually >1,000 nm long) and more curved and interwoven than the rod-like CNCs (usually <1,000nm long). A significant overlap can be found in the utilization of both CNFs and CNCs.

S. muticum was chosen for this study as it is an invasive species in need of sustainable applications for society, and limited literature exists on its nanocellulose components. The original goal of this research project was to extract CNFs from this species and, time and resources permitting, test their effectiveness in permeable concrete reinforcement, building upon a prior experiment that utilized full algae in air-dried clay bricks. Cengiz (2017) found that addition of CNFs extracted from *Cladophora* freshwater algae to concrete improved the concrete's flexural strength by a maximum factor of 2.7 (p.1). On the other hand, adding standard commercial CNFs (derived from cotton) weakened the concrete (p. 6). The researchers attributed the improved strength to the more intricate and fibrous structure of the freshwater algal CNFs. Another paper, Zhang (2022), reported that added cotton-derived CNFs substantially improved many properties of pervious concrete, including its compressive and flexural strength and freeze damage resistance. Given the findings of previous studies, it could be extrapolated that algal CNFs could build on these improvements.

Outside the realm of concrete, nanocellulose purposes have been studied in fields like medicine, insulation, and food packaging. Shaghaleh (2018) explored the usage of CNFs and CNCs as sustainable base materials for creating biocomposites and polymeric materials. One of these described processes entails breaking nanocellulose down into glucose and other monomers and creating glycopolymers, which in turn can be utilized in antiviral and antibacterial medicines (Shaghaleh, 2018, p. 5). Berglund (2021) covered a process of weaving alginate-CNFs into "fabrics" that have strong insulative properties and reduced flammability compared to other common insulators. Zhai (2018) found that CNFs help reduce surface tension and stabilize pickering emulsions between oil and water. Wang (2024) described a variety of ways nanocellulose can create sustainable food packaging, such as thin films and coatings, that are healthier for human consumption-related uses than currently utilized materials like plastics.

Several CNF extraction methods were studied. Cengiz (2018) covered refluxing freshwater algae through multiple solutions to remove proteins, lipids, and pigments, before using hydrochloric acid (HCl) to disintegrate the remaining cellulose into CNFs. Zhai (2018) also covered using hydrochloric acid to disintegrate bacterial cellulose into nanofibers. Meanwhile, in Lee (2018), researchers extracted CNFs from cellulose fibers (after conventional protein and lipid removal) using the more recently discovered TEMPO-mediated oxidation method. Sirvio (2018) described using a choline chloride and imidazole-based (CCIMI) solvent to swell up cellulose fibers and cause them to disintegrate into cellulose nanofibers. Due to the lack of resources available to conduct TEMPO-mediated oxidation or fiber swelling, the Cengiz method was pursued for this project.

Due to limitations of time, resources, and unexpected turns in the extraction process, this research project did not progress into the second planned stage of testing the nanocellulose's effectiveness in strengthening permeable concrete. However, detailed observations of the product, CNCs instead of the anticipated CNFs, were made.

Methodology

S. muticum samples were procured from the Gulf Specimen Marine Laboratory (Panacea, Florida, USA) in February 2024. The algae samples were air-dried for a week, oven-dried for 5 days at 40 °C, and partially ground with a mortar and pestle. 100 g of the algae was added to 500 mL of a 4% v/v solution of aqueous sodium hypochlorite (NaOCl). These components were combined in a 2 L round-bottom flask, equipped with a magnetic stirrer and refluxing apparatus, with the flask submerged in an oil bath set to 100 °C. 12 g of successfully bleached algae was filtered, dried, and ground into a finer powder.

The bleached algae was refluxed in 120 mL of 2 M sodium hydroxide (NaOH) solution for 2 hours, with the oil bath set to 130 °C, to remove the proteins from the algae structure. The solution was then neutralized using a 3 M HCl solution and centrifuged at 12,660 g of force for 10 minutes to isolate the deproteinized algae from the solution, since filtration was not successful.

For the removal of oils and pigments, the deproteinized algae was refluxed in 120 mL of a 4:2:1 by volume chloroform-methanol-distilled water solution for 20 minutes. The oil bath was set to 150 °C. The sample was then centrifuged and dried again.

Finally, the remaining product was refluxed in 120 mL of 2 M HCl solution for about 18 hours, while the oil bath was set to 110 °C. Ideally, this final step would break down the remaining cellulose into nanocellulose (in this objective, CNFs) in a process called acid hydrolysis. The remaining solution was centrifuged to extract the sample, which was dried for three weeks at 40 °C and pulverized into a finer dust with an electric grinder in preparation for testing.

Characterization, Analysis, and Discussion

X-ray diffractometry was performed on the prepared samples using a Rigaku Powder XRD machine with the voltage and current settings set to 5 kV and 40 mA respectively. The intensity of the X-rays scattered by the sample was measured over a $2\theta = 5°$ to $2\theta = 40°$ beam angle interval and plotted on the chart below. The profile closely resembles those from prior studies on both cellulose and nanocellulose products, revealing that the sample has similar arrangements of molecules to them. The two abnormal peaks found between $2\theta = 20°$ and $2\theta = 30°$ were likely caused by cross-contamination with an inorganic material in the testing system.

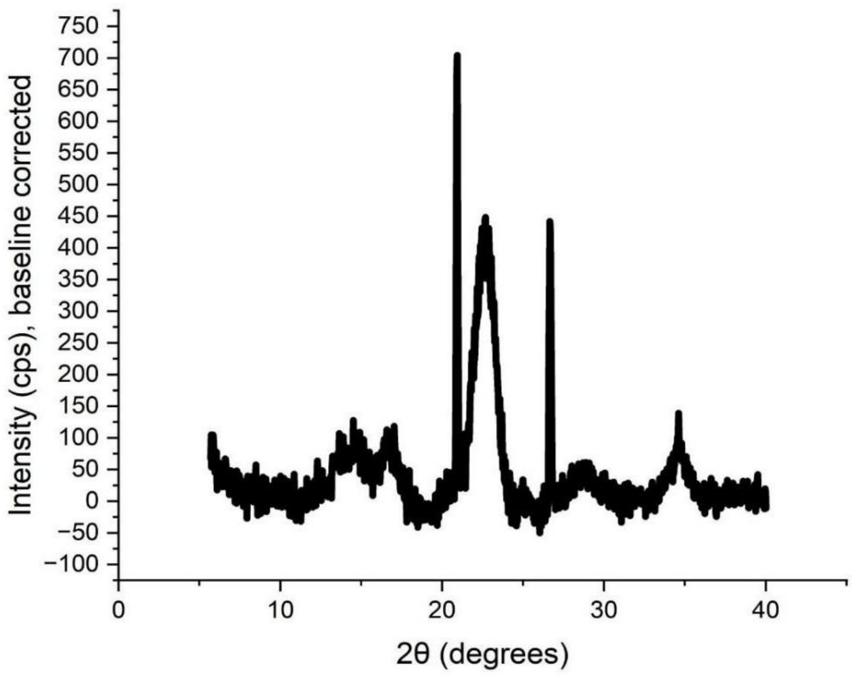

Fig. 1: XRD spectra of *Sargassum muticum* nanocellulose

The samples then underwent Fourier-transform infrared spectroscopy (FT-IR) testing with the Thermo-Fisher Nicolet iS50 FT-IR spectrometer. The transmittance of infrared radiation through the material over different wavelengths was plotted on the chart below. The profile resembles those from previous studies of nanocellulose, indicating that similar molecular structures and organic compounds are present.

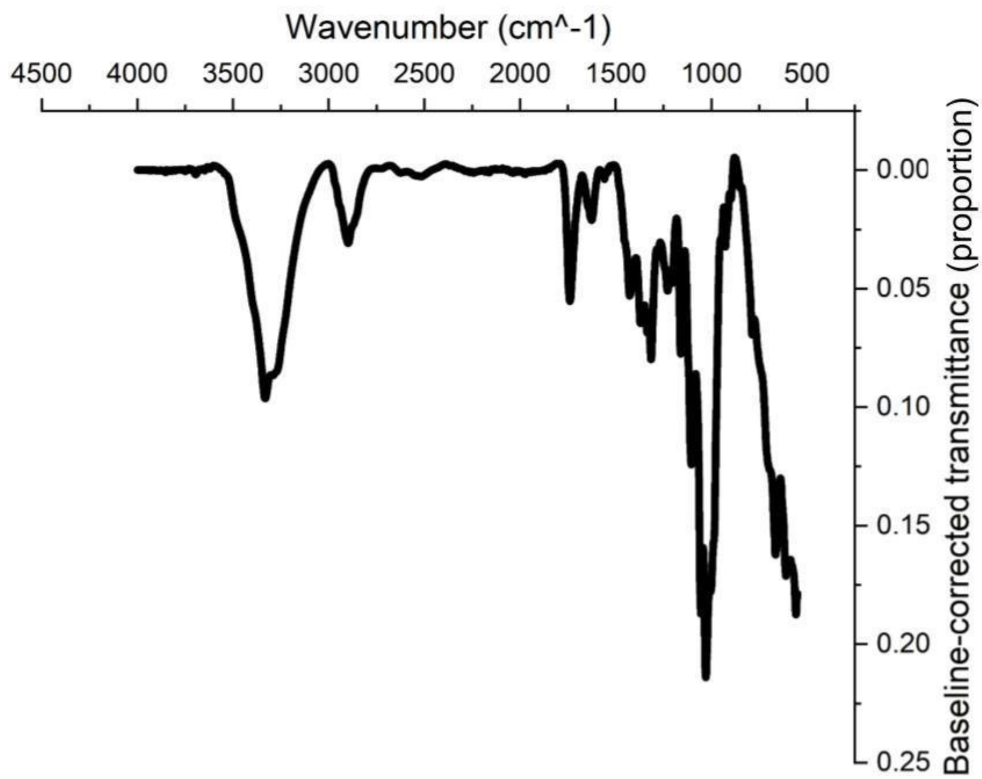

Fig. 2: FT-IR spectra of *Sargassum muticum* nanocellulose

Finally, the samples underwent transmission electron microscopy (TEM) imagery. In preparation for imagery, the sample was partly dissolved in 40 °C distilled water with sonication, although the concentration of the aqueous solution could not be determined. Then, the solution was stained onto copper grids with uranyl acetate dye for improved image contrast. These grids were placed in the Hitachi 7800 electron microscope; the voltage difference was set to 100.00 kV and the filament voltage and current were set to 10 V and 10 mA respectively. The following images of cellulose nanocrystals (CNCs) were taken; they were identified apart from CNFs through comparison to imagery from prior studies, due to their more rod-like composition. The spots were caused by fluid evaporation from the grids. The scale bar sizes are given below each image.

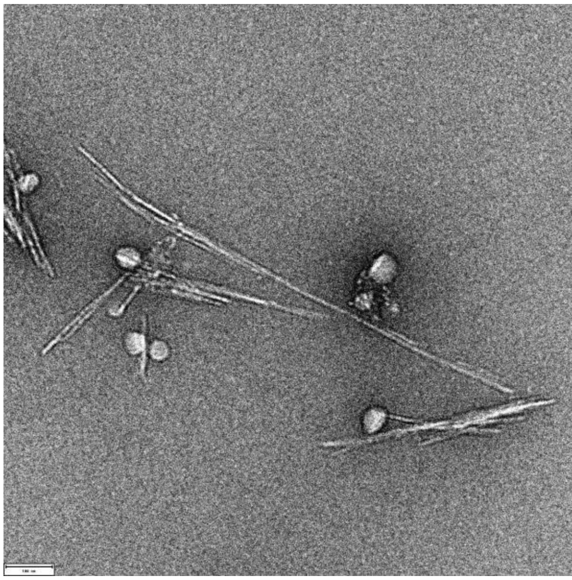

Fig. 3: 100 nm

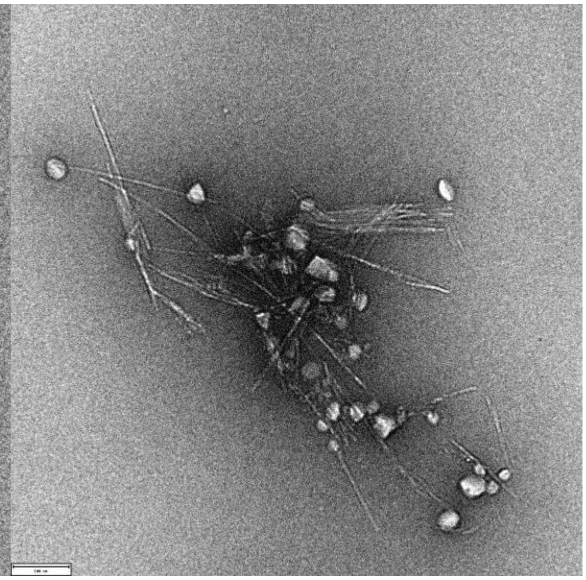

Fig. 4: 200 nm

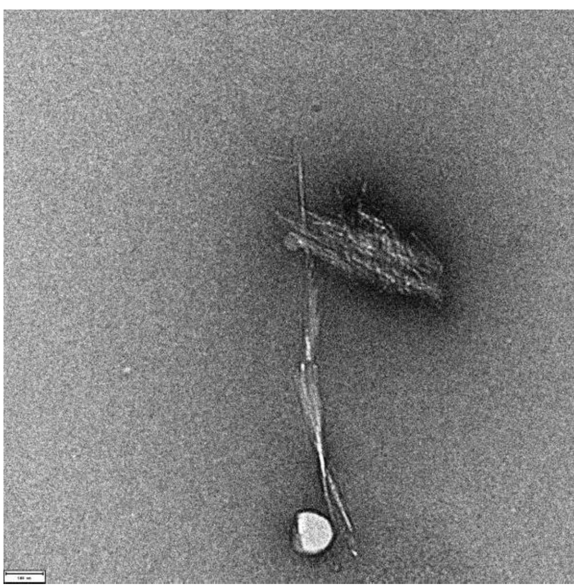

Fig. 5: 100 nm

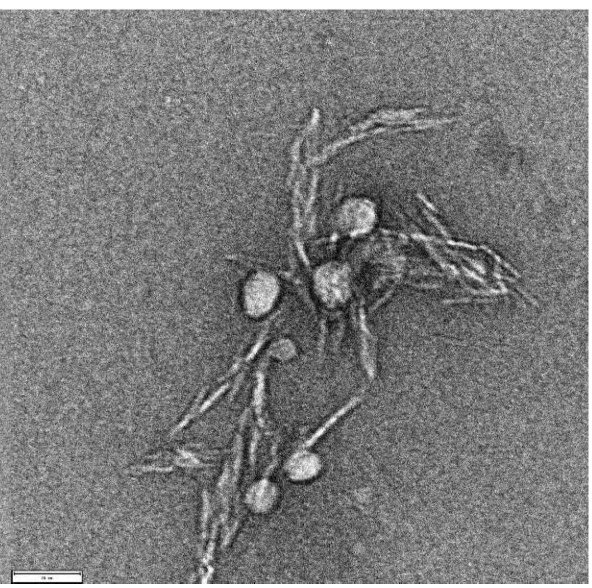

Fig. 6: 70 nm

The CNCs observed in this study predominantly ranged between 200 and 500 nm in length, although those found in some "clusters" (such as those seen in Fig. 6) averaged only around

70 nm in length. Some CNCs, particularly those found in Fig. 3, reached approximately 700 nm in length. Virtually all CNCs fell below 10 nm in width.

In comparison, CNCs extracted from *Sargassum fluitans* (the most similar algae to *S. muticum* with existing CNC data) in prior research averaged only 43.72 nm in length, with a standard deviation of 7.44 nm (Doh, 2020, p. 4). However, the study had several deviations in extraction methods from this one, including a different order of refluxations and different chemicals at each of these stages. Additionally, it is possible the study observed only one size grouping of CNCs while conducting TEM imagery. On the other hand, Trache (2017) reported that CNCs extracted from red marine algae primarily measured between 400 and 600 nm in length. The extraction method was not specified.

Non-algal CNCs were also considered. Foster (2018) found that CNCs from rice, soy, and mengkuang leaves mostly fell between 200 and 300 nm in length (p. 36). The same study reported that CNCs isolated from tunicate (a marine invertebrate commonly known as the "sea squirt") consistently reached or exceeded 1,000 nm in length. Additionally, bacteria-sourced CNCs primarily ranged between 830 and 1,230 nm in length, with outliers in each direction (Arserim-Uçar, 2021, p. 5).

CNFs, the original main subject of the study, were not clearly identified within the samples. The patterns observed in the following image may indicate cellulose nanofiber (CNF) presence, particularly in the narrower artery-like shapes, although they are more likely a staining abnormality.

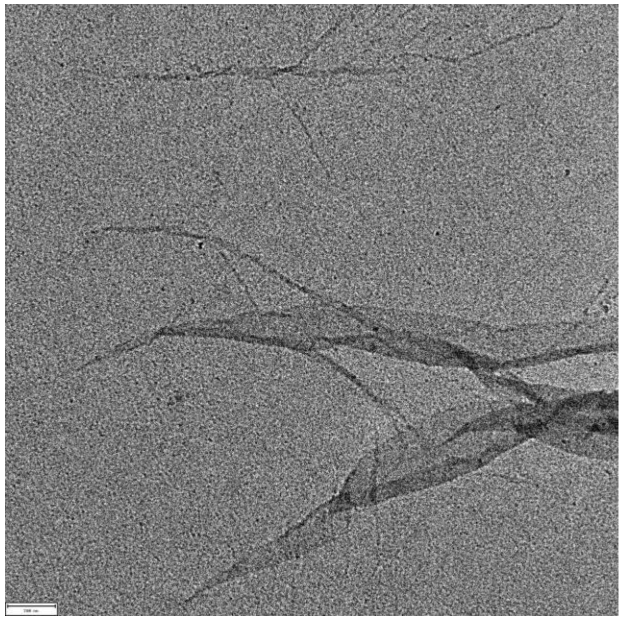

Fig. 7: 200 nm

Conclusion

The most notable takeaway from the project was the unpredicted successful extraction of CNCs instead of CNFs with the chosen method. This different outcome may have occurred due to the unusually long time the cellulose sample spent in hydrochloric acid during acid hydrolysis: 18 hours, as compared to ten hours in Cengiz (2018) and only one hour in Zhai (2018). On the other hand, Doh (2020) reported only 30 minutes of acid hydrolysis in sulfuric acid (H_2SO_4) in their process, indicating another step in our process may have played a greater role. It is also possible that the CNCs more easily dissolved from the powdered sample into the aqueous solution used in TEM than any possibly present CNFs.

During the extraction process, several procedural flaws in Cengiz (2018) were discovered, such as testing of the pH of a solid substance. It also did not detail a method of extracting the algae (or later, cellulose) from the solution after each refluxing step; centrifugation was chosen after traditional filtration/straining methods failed in the steps following bleaching. The Cengiz study further did not outline the risk of a foaming reaction seen during the bleaching process in this experiment, although it worked with freshwater algae instead of marine algae.

Future goals of research in this field include the design of a more easily scalable CNC extraction process for *S. muticum* that includes preservation of its protein and lipid extracts, as well as the more direct application of *S. muticum*-derived nanocellulose in real-world applications. Additionally, the large variance in recorded CNC lengths in this study may also indicate a need for a size sorting system or an extraction method that allows CNC length to be specified.

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