Proceedings of Science and Mathematics
Faculty of science, universiti teknologi malaysia

https://science.utm.my/procscimath/
Volume 27 (2024) 79-85

Isolation and Screening of Nanocellulose Producing Bacteria from Pineapple Wastes

Yamunathevi Mathivanan[a], Shafinaz Shahir[a]*, Zaharah Ibrahim[a], Nik Ahmad Nizam Nik Malek[a,b]
[a]Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia (UTM), 81310, Skudai, Johor, Malaysia.
[b]Centre for Sustainable Nanomaterials (CSNano), Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia.
*Corresponding author: shafinazshahir@utm.my

Abstract
Bacterial nanocellulose (BNC) has many unique properties, including higher purity, porosity, and water retention capacity than plant-derived cellulose. In this study, eleven isolates, most of which were bacteria, were successfully isolated from pineapple wastes. After incubation in Hestrin and Schramm (HS) medium for 10 days at static conditions, all 11 isolates produced BNC. After the alkaline treatment of BNC, it was dried in an oven at 60°C. Four isolates, P1, P2, F1 and J2, recorded the highest BNC dry weight and were chosen as the best BNC producers. The Gram-negative P1, P2 and F1 isolates and J2 yeast cells were identified by 16S rRNA sequencing. According to the results, P1, P2, F1 and J2 showed more than 80% homology to *Klebsiella* YSZ sp., *Acinetobacter* YSZ sp., *Comamonas* YSZ sp. and *Candida* YSZ sp. respectively. In a previous study, *Acinetobacter* sp. produced BNC in Pawpaw juice medium, suggesting that some enzymes for BNC synthesis may be present in *Acinetobacter* YSZ sp. To our knowledge, this study is first to report novel BNC producer, *Klebsiella* YSZ sp., *Acinetobacter* YSZ sp., *Comamonas* YSZ sp., and *Candida* YSZ sp. The findings offer valuable insights into novel BNC-producer from pineapple wastes, paving the way for eco-friendly, large-scale BNC production.
Keywords: Bionanocellulose; Cellulose; Pineapple wastes; *Comamonas* sp.

Introduction

The most abundantly available polymeric material on earth is cellulose, a remarkable and sustainable feedstock. Even though cellulose has been known and studied for several years, nanocellulose has recently become recognized as a noteworthy and superior polymer, according to Trache et al. (2020). This nanotechnology provides alternative bio-based polymers that can be new advanced materials (R et al., 2021). In contrast to plants, certain bacteria can produce cellulose, known as bacterial nanocellulose (BNC). Currently, the demand for cellulose keeps increasing. However, chemical methods for removing polysaccharides, such as hemicellulose and lignin from plant cellulose, are not financially feasible and environmentally friendly. Moreover, this may also change the structure and function of plant-derived cellulose, limiting its application (Abba et al., 2017). Therefore, cellulose synthesis from bacteria could be a fascinating alternative to plant-derived cellulose.

Bacterial strains that undergo the fermentation process frequently produce an extracellular BNC polysaccharide, which has a similar chemical structure as plant-derived cellulose (Aydin & Aksoy, 2010). Many bacterial genera produce BNC, including *Glucanoacetobacter*, *Komagataibacter*, *Acetobacter* and *Achromobacter* (Jamal et al., 2021). The first BNC was produced by *Acetobacter xylinum*, now known as *Gluconabacter xylinus*, discovered by A.J Brown in 1886 (Chawla et al., 2009). BNC is a twisted ribbons-like structure with 20–100 nm diameter (Samyn et al., 2023). According to R et al. (2021), BNC possesses unique characteristics, such as high-water retention capability, crystallinity, flexibility and biodegradability (R et al., 2021). These extraordinary physical features are due to the nanostructure of BNC, with linear β-1,4-glucan chains linked by intra- and inter-molecular hydrogen bonds. Compared to plant cellulose, BNC contains a network of nanofibers that provides a higher capacity to hold water in its native state (Stanisławska, 2016).

Bacterial nanocellulose can be a potential bio-based polymer utilized in various industries, including medical, cosmetics, textiles, environmental and food industries. Bacterial nanocellulose production is a less time-consuming and more environmentally sustainable production method. Since BNC consists of these valuable properties, it might be an alternative to overcome the emerging problem of over-depending on plant-derived cellulose. Therefore, it is crucial to identify potent BNC-producing isolates with higher productivity. Therefore, this study aims to isolate and screen the potential of local BNC producers. The ability to isolate local BNC-producing bacteria is important as it is better adapted with improved productivity for future applications. Acetic acid bacteria that can oxidize ethanol are classified as Gram-negative aerobic bacteria (Klawpiyapamornkun et al., 2015). According to Aydin and Aksoy (2010), various microorganisms, including acetic acid bacteria that produced cellulose, such as *Gluconacetobacter xylinus*, *Gluconacetobacter hansenii*, *Acetobacter aceti*, and *Gluconacetobacter liquefaciens*, have been found and isolated from pineapple wastes. Colony and cell morphologies of the isolated microbes from pineapple wastes were determined, followed by screening for BNC production.

Materials and methods

Chemicals

Chemicals used in this study were glucose (99% purity), citric acid (99.5% purity), disodium hydrogen phosphate (99% purity), and sodium hydroxide (99% purity) from Merck brand, while peptone (99% purity), yeast extract (99% purity), and agar (99% purity) were used from Oxoid brand. All the chemicals used were of analytical grade.

Isolation source

The pineapple waste from 1st wastewater treatment pond (P), fresh pressed pineapple waste juice (J), old pineapple peel waste (W) and fresh peel waste (F) were collected from the producing industry in the Southern part of Malaysia. To avoid further contamination, all the samples were collected separately and stored at 4°C until further studies.

Culture medium

The Hestrin and Schramm (HS) medium was prepared with D-glucose (20 g/L), peptone (5 g/L), yeast extract (5 g/L,) disodium phosphate (2.7 g/L), citric acid (1.15 g/L) and agar (20 g/L) (Aswini et al., 2020). The pH of the medium was adjusted to a pH of 5-6 with 1 M HCl or 1 M NaOH, followed by autoclaving at 121°C, 101.5 kPa for 15 minutes.

Isolation of BNC producer

The isolation of BNC producers was done, as reported by Fernández et al. (2019). BNC-producing bacteria were isolated using the HS medium from the pineapple waste samples. A sample of 2.5 ml (10% v/v) was pipetted separately into 22.5 ml of nutrient broth for the enrichment and cultured for 1-2 days at 30°C, 150 rpm, until the medium turned turbid. The flasks with growth observed were selected and repeatedly streak-plated on HS agar. The HS agar was then incubated for 2-3 days at 30°C to obtain pure colonies. After incubation, distinct colonies were selected from each plate and further screened for potential BNC synthesis.

Screening of BNC producer

The potential of nanocellulose production by each isolate was screened individually. All the isolates were grown overnight in the HS medium until growth was observed. The overnight cultures (10% v/v) were transferred into 90 ml of HS medium, followed by incubation at 30°C for 10 days under static conditions to allow pellicle formation at the air-liquid interface (Fernández et al., 2019). The pellicle formed indicates that the isolate can synthesize nanocellulose. The flasks with pellicle formed were selected for the purification treatment. By comparing the dry weight (g/L) of the BNC produced, the best isolate with a higher yield of BNC was chosen for further analysis.

Purification of BNC

Purifying BNC using alkaline treatment is necessary to eliminate any embedded impurities and bacterial cells (Mathivanan et al., 2024). Treating with sodium hydroxide or potassium hydroxide is the most used treatment method. According to Chawla et al. (2009), the procedure can be applied independently or in combination. The pellicles obtained from the flasks were treated with NaOH (0.1M) for 15 minutes at 80°C to remove the microbial cells and rinsed with distilled water until the pellicle's pH was neutralized

(Aswini et al., 2020). The purified pellicles were then dried in the oven at 60°C until BNC reached constant dry weight. The BNC produced was calculated using the formula developed by Mohammadkazemi et al. (2016), where weight (g) is the produced BNC's dry weight, and volume (L) is the volume of the medium used to synthesize BNC.

$$\text{Dry weight of nanocellulose } (\frac{g}{L}) = \frac{weight\ of\ nanocellulose\ (g)}{volume\ of\ media\ (L)}$$

Characterization of BNC-producing isolate

The morphological and Gram stain reaction of the selected isolate was characterized. The selected isolate's morphological characteristics, such as form, elevation, margin, and colour, were characterized (Jamal et al., 2021). The Gram staining method was used as described by Abba et al. (2017). The stained samples were observed under a light microscope (LEICA DM750).

Results and discussion

Isolation of BNC producer

The nanocellulose-producing microbes were successfully isolated from pineapple wastes. Four samples were collected: first wastewater treatment pond, fresh pressed pineapple waste juice, old pineapple peel waste, and fresh peel waste. A total of 11 isolates were successfully isolated from all the pineapple wastes by dilution streak plate technique. All the plates were incubated at 30°C for 2-3 days to allow for the formation of discrete colonies. Precisely, three isolates were isolated from first wastewater treatment pond (P), three from fresh pressed pineapple waste juice (J), three from old pineapple peel waste (W) and two from fresh peel waste (F). The isolates were further morphologically characterized based on their form, elevation, margin and colour. The morphological characteristics of different isolates obtained are presented in Table 1.

Table 1: Colony morphology of isolates from pineapple wastes.

Source	Isolate	Colony morphology			
		Form	**Elevation**	**Margin**	**Colour**
1st pond (P)	P1	circular	raised	entire	milky-white
	P2	punctiform	raised	entire	white
	P3	irregular	flat	undulate	yellowish
Fresh pressed pineapple waste juice (J)	J1	irregular	umbonate	undulate	milky-white
	J2	circular	raised	entire	milky-white
	J3	circular	raised	entire	milky-white
Old pineapple peel waste (W)	W1	circular	convex	entire	milky-white
	W2	irregular	raised	entire	translucent
	W3	circular	convex	entire	milky-white
Fresh peel waste (F)	F1	circular	flat	entire	translucent
	F2	circular	convex	entire	milky-white

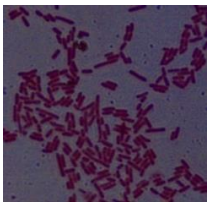
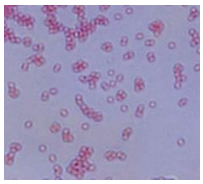
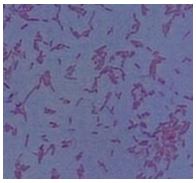
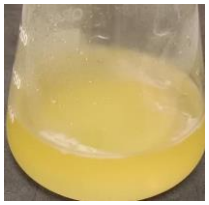
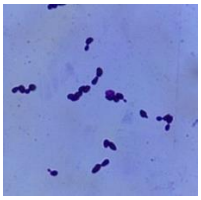
Screening of BNC-producer

All 11 isolates were screened for BNC production under the same conditions. All the isolates showed a potential for BNC production but in different quantities. According to Singhsa et al. (2018), yield and production of BNC were primarily influenced by the type of isolate and fermentation methods used. The screening of nanocellulose production was done using the HS medium, commonly used for BNC

production. The HS medium, which contains both carbon and nitrogen sources, is suitable for use as a medium, and it is known to support better yield of BNC production. Carbon source is essential for BNC production as an energy supply and a precursor molecule (Abba et al., 2017). However, as the concentration of glucose in the HS medium increases, more gluconic acid is produced, which leads to reduced BNC production (Mathivanan et al., 2024; Stanisławska, 2016).

Four of the 11 isolates were chosen for further studies based on the highest yield of BNC produced. The Gram stain reaction of selected isolates was observed using light microscopy under 1000X magnification. Table 2 shows the Gram-staining and BNC production results of the four selected isolates.

Table 2: BNC screening and Gram stain reaction of the selected isolates.

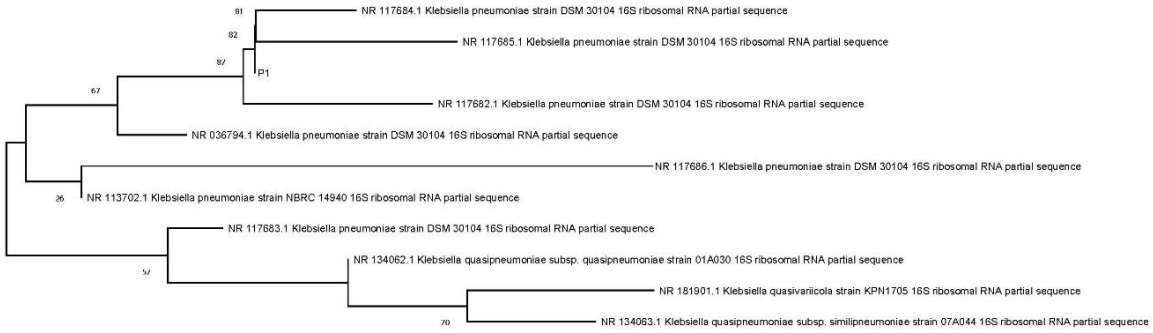
Isolate	BNC screening	BNC dry weight (g/L)	Gram stain reaction
P1		1.67 ± 0.18	 Gram reaction: Negative Shape: Rod
P2		1.65 ± 0.15	 Gram reaction: Negative Shape: Cocci
F1		1.69 ± 0.19	 Gram reaction: Negative Shape: Rod
J2		1.52 ± 0.18	 Yeast cell

Molecular identification of the selected isolate

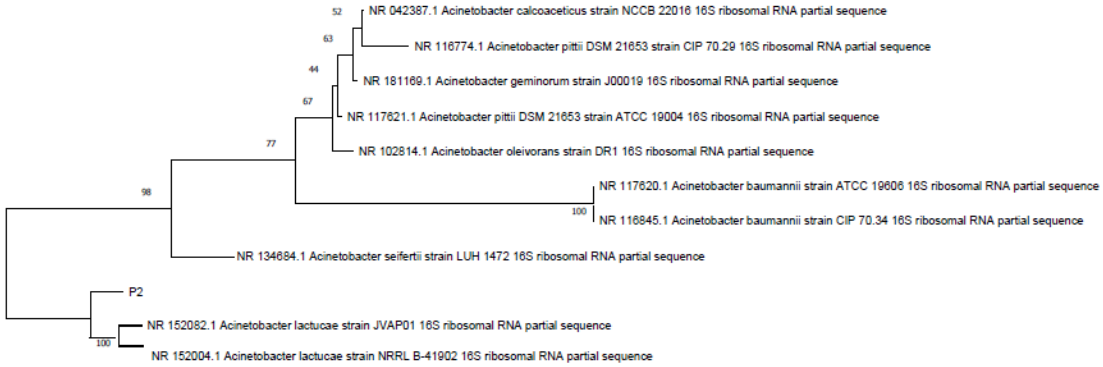
To further confirm the identity of the BNC producer, 16S rRNA sequencing analysis was done at Apical Sdn. Bhd. For the J2 isolate, the ITS technique was employed to determine the genus of the fungi. The polymerase chain reaction (PCR) was carried out by amplifying the highly conserved 16S rRNA gene using a universal primer (27F/1492R). Additionally, the phylogenetic tree was constructed to analyze the evolutionary history of each isolate. Phylogenetic tree aids in the estimation of mode and tempo in which a group of species has evolved. Top hits were acquired in contrast to the 16S gene sequence of isolate P1 (1481 base pairs), P2 (1477 base pairs), F1 (1469 base pairs) and J2 (548 base pairs) after running BLAST-n in the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the phylogenetic

tree was constructed utilizing top hits for each of the isolates. The phylogenetic results for each isolate are shown in Figure 1.

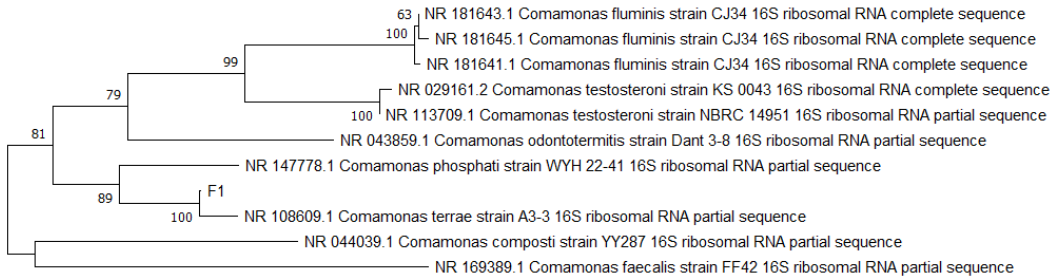
A)



B)



C)



D)

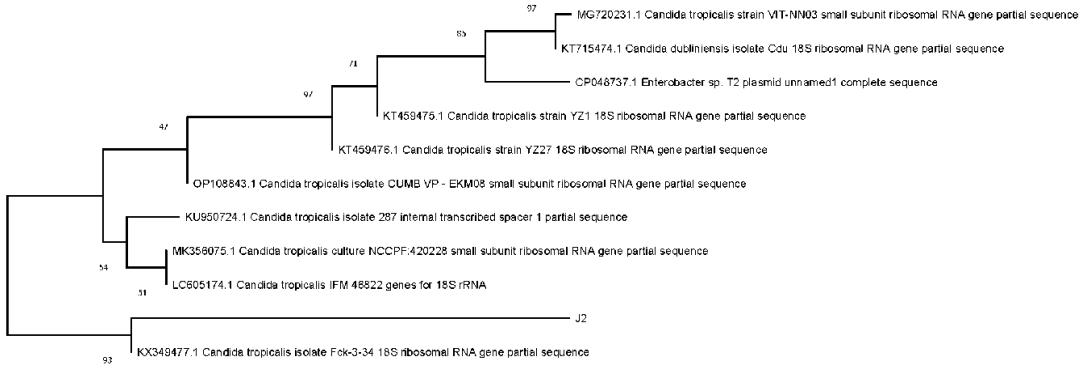


Figure 1 Phylogenetic tree based on 16S rRNA results of a neighbour-joining tree using the bootstrap technique with 1000 resampling: A) isolate P1, B) isolate P2, C) isolate F1 and D) isolate J2

According to Figure 1, the positions of the isolates P1, P2 and F1 indicated that the bacteria were closely related to *Klebsiella* sp., *Acinetobacter* sp. and *Comamonas* sp., respectively, while isolate J2

suggested that the yeast cells were closely related to *Candida* sp. The phylogenetic tree for each isolate suggested that it most likely belongs to another strain of the same species. Each of the BNC-producing isolates obtained is a novel cellulose synthesizing isolate, and their cellulose metabolism has not yet been extensively studied. Therefore, more research on *Klebsiella* sp., *Acinetobacter* sp., *Comamonas* sp., and *Candida* sp. is needed. However, Adebayo-Tayo et al. (2017) revealed that Acinetobacter sp. was able to grow and produced BNC extracellularly on day 15 in the Pawpaw juice medium with a BNC yield of 6.48 mg/L dry weight. The *Acinetobacter* sp. produced the highest BNC yield (6.48 mg/L) in the Pawpaw juice medium as compared to pineapple juice (1.23 mg/L) and watermelon juice medium (3.36 mg/L). Therefore, the need to isolate other bacteria capable of producing BNC will facilitate a more economically viable large-scale production.

Conclusion

The eleven isolates, including bacteria and yeast cells, were successfully isolated from the pineapple wastes and showed potential for BNC production. P1, P2, F1 and J2 isolates were selected based on the highest BNC dry weight produced for further analysis. The 16S rRNA analysis of P1, P2, F1 and J2 showed more than 80% homology to *Klebsiella* YSZ sp., *Acinetobacter* YSZ sp., *Comamonas* YSZ sp. and *Candida* YSZ sp. respectively. The isolation of novel BNC producers from pineapple wastes opens an opportunity for improving cellulose synthesis. BNC production will help minimize the ever-increasing demand imposed on plant-derived nanocellulose. It also contributes to the need for sustainable, large-scale production of BNC for industrial-scale applications. Further studies to optimize the BNC production are recommended.

Acknowledgement

The authors would like to express their appreciation to UTM for the funding via UTMFR Vote No. 21H22 and the Ministry of Higher Education, Malaysia, for providing the scholarship to Yamunathevi Mathivanan under MyBrainSc scholarship.

References

Abba, M., Abdullahi, M., Md Nor, M. H., Chong, C. S., & Ibrahim, Z. (2017). Isolation and characterisation of locally isolated *Gluconacetobacter xylinus* BCZM sp. with nanocellulose producing potentials. *IET Nanobiotechnology, 12*(1), 52–56.

Adebayo-Tayo, B., Akintunde, M., & Sanusi, J. (2017). Effect of different fruit juice media on bacterial cellulose production by *Acinetobacter* sp. BAN1 and *Acetobacter pasteurianus* PW1. *Journal of Advances in Biology & Biotechnology, 14*(3), 1–9.

Aswini, K., Gopal, N. O., & Uthandi, S. (2020). Optimized culture conditions for bacterial cellulose production by *Acetobacter senegalensis* MA1. *BMC Biotechnology, 20*(1), 1–9.

Aydin, Y. A., Aksoy, N. D., & Ao, S.-I. (2010). Utilization of vinegar for isolation of cellulose producing acetic acid bacteria. *AIP Conference Proceedings, 1281*(1), 502–505.

Chawla, P. R., Bajaj, I. B., Survase, S. A., & Singhal, R. S. (2009). Microbial cellulose: Fermentative production and applications. *Food Technology and Biotechnology, 47*(2), 107–124.

Chen, G., Chen, L., Wang, W., Chen, S., Wang, H., Wei, Y., & Hong, F. F. (2019). Improved bacterial nanocellulose production from glucose without the loss of quality by evaluating thirteen agitator configurations at low speed. *Microbial Biotechnology, 12*(6), 1387–1402.

Fernández, J., Morena, A. G., Valenzuela, S. V., Pastor, F. I., Díaz, P., & Martínez, J. (2019). Microbial cellulose from a *Komagataeibacter intermedius* strain isolated from commercial wine vinegar. *Journal of Polymers and the Environment, 27*(5), 956–967.

Jacek, P., Ryngajłło, M., & Bielecki, S. (2019). Structural changes of bacterial nanocellulose pellicles induced by genetic modification of *Komagataeibacter hansenii* ATCC 23769. *Applied Microbiology and Biotechnology, 103*(13), 5339–5353.

Jamal, I. S., Sahari, N. S., Hasmoni, S. H., Ibrahim, Z., Malek, N. A., & Shahir, S. (2021). Characterization of bionanocellulose producing bacteria isolated from tapioca wastewater. In *2021 International Congress of Advanced Technology and Engineering (ICOTEN)* (pp. 1–5). IEEE.

Klawpiyapamornkun, T., Bovonsombut, S., & Bovonsombut, S. (2015). Isolation and characterization of acetic acid bacteria from fruits and fermented fruit juices for vinegar production. *Food and Applied Bioscience Journal, 3*(1), 45–53.

Mathivanan, Y., Shahir, S., Ibrahim, Z., & Malek, N. A. (2024). Isolation and characterization of amorphous nanocellulose producing *Comamonas terrae* YSZ sp. from pineapple wastes. *Polymer Bulletin*.

Meftahi, A., Khajavi, R., Rashidi, A., Rahimi, M. K., & Bahador, A. (2015). Effect of purification on nano microbial cellulose pellicle properties. *Procedia Materials Science, 11*, 206–211.

Mohammadkazemi, F., Faria, M., & Cordeiro, N. (2016). In situ biosynthesis of bacterial nanocellulose-CaCO3 hybrid bionanocomposite: One-step process. *Materials Science and Engineering: C, 65*, 393–399.

Philip, R. R., Thomas, D., Madhavan, A., Sindhu, R., Binod, P., Varjani, S., Awasthi, M. K., & Pandey, A. (2021). Bacterial nanocellulose: Engineering, production, and applications. *Bioengineered, 12*(2), 11463–11483.

Samyn, P., Meftahi, A., Geravand, S. A., Heravi, M. E., Najarzadeh, H., Sabery, M. S., & Barhoum, A. (2023). Opportunities for bacterial nanocellulose in biomedical applications: Review on biosynthesis, modification and challenges. *International Journal of Biological Macromolecules, 231*, 123316.

Singhsa, P., Narain, R., & Manuspiya, H. (2018). Physical structure variations of bacterial cellulose produced by different *Komagataeibacter xylinus* strains and carbon sources in static and agitated conditions. *Cellulose, 25*(3), 1571–1581.

Stanisławska, A. (2016). Bacterial nanocellulose as a microbiological derived nanomaterial. *Advances in Materials Science, 16*(4), 45–57.

Trache, D., Tarchoun, A. F., Derradji, M., Hamidon, T. S., Masruchin, N., Brosse, N., & Hussin, M. H. (2020). Nanocellulose: From fundamentals to advanced applications. *Frontiers in Chemistry, 8*, Article 392.