

Isolation of cellulose-producing bacteria (*Komagataeibacter Saccharivorans*) from rotten sapodilla fruit

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Abstract. Bacterial nanocellulose (BNC) is a nanocellulose produced by bacteria with high purity, crystallinity level, water binding ability, a high degree of polymerization, and excellent mechanical characteristics. The selection of BNC-producing bacteria is one of the critical stages in the production of BNC. This study collected samples from fruit sources and was selected to determine isolates that could produce BNC. Based on the 16s rRNA strain analysis, sapodilla isolate has an identity percentage above 98%, so it can be concluded that it has similarities with the bacteria *Komagataeibacter saccharivorans*. From eleven sources of fruit, two isolates that have the potential to produce cellulose, namely isolate sapodilla, were obtained. The confirmed sapodilla isolate is an acetic acid bacteria, *Komagataeibacter saccharivorans*. The yield of BNC-made isolate sapodilla *Komagataeibacter saccharivorans* (0.432 g/L). Confirmed sapodilla isolates that produce cellulose were mainly determined as cellulose I (adsorption at around 3345, 1430, 1160, and 900 cm⁻¹). Few celluloses II (adsorption at about 1335, 1315, and 1280 cm⁻¹ and a blue-shift of the number of waves from 1430 to around 1425 cm⁻¹) and has a crystallinity index of 52.387 % on HS (Hestrin Scrahm) media with diameter nanofibril about 86.46 nm.

1 Introduction

Bacterial nano-cellulose (BNC) is a nanomaterial synthesized by the bacteria *Komagataeibacter* species cultivated in a medium supplemented with carbon and nitrogen sources. BNC is a high-value material with high purity, high porosity, good mechanical properties, and environmental friendliness [1]. In general, several types of bacteria, including *Komagataeibacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Burkholderia*, *Erwinia chrysanthemi*, *Agrobacteria*, *Rhizobium*, and *Sarcina*, can produce BNC with different levels of BNC productivity. *Komagataeibacter*, formerly known as *Gluconacetobacter* or *Acetobacter*, is the most researched BNC-producing bacteria because it can make BNC massively and efficiently [2]. *Komagataeibacter* is naturally found in several varieties of

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fruits with sour tastes and juices and residues, such as citrus fruits, plums, apples, and grapes [3].

The selection of BNC-producing bacteria is one of the critical stages in the production of BNC. Several studies have stated that cellulose-producing strains can be isolated from several sources, such as rotten fruits, vegetables, and beverages. In the previous research [4], 14 bacterial strains were found in rotten fruits and drinks. The cellulose-producing bacterial strain IITRDKH20 was isolated from rotten apples (*Malus domestica*). Fruits with a sour taste were chosen in this study because most cellulose-producing bacteria like acidic conditions for their growth. Recent studies have focused on the utilization of agro-industrial wastes and other inexpensive substrates as growth media for BNC production, aiming to reduce costs and enhance sustainability [5]. For instance, research has shown that substrates such as sugarcane molasses, fruit peel waste, and even fermented beverages like Kombucha tea can be effective in supporting the growth of *Komagataeibacter* and the subsequent synthesis of BNC [6-8]. These findings open up new avenues for the valorization of waste materials and the development of cost-effective BNC production methods.

The isolation and identification of new BNC-producing strains from diverse sources are crucial for advancing the field. Different strains can exhibit unique characteristics in terms of BNC yield, structural properties, and adaptability to various substrates. In this context, the exploration of fruit and coconut water as sources for isolating new strains of *Komagataeibacter* is particularly promising. These natural sources are rich in nutrients and may harbor bacteria with the potential to produce BNC efficiently. This study aims to isolate and identify BNC-producing bacteria from fruit and coconut water sources and to characterize the BNC produced by these strains. By examining a variety of sources, including Siwalan, apple, pineapple, snakefruit, mango, kecubung, sapodilla, banana, senggani, nam-nam, and coconut water, we aim to identify isolates capable of producing high-quality BNC. The characterization of these isolates will include an assessment of their BNC production yield, structural properties, and potential applications.

2 Material and methods

2.1 Material

Siwalan, Apple, Pineapple, Mango, Snakefruit, Kecubung, Sapodilla, Banana, Senggani, Nam-Nam, and coconut water. Samples used in this research were from several traditional Batu, Malang, and Tuban markets. Selected samples were the fruit that is ripe ideally. Samples collected were stored in a closed container for five days to produce a sour aroma before being isolated. Bactopectone, yeast extract, Na_2PO_4 , acid citrate, bactoagar, and glucose were obtained from Sigma Aldrich. Chemicals used include 98% NaOH, 99% concentrated Acetic Acid (CH_3COOH), and glucose syrup produced by PT Asahimas Chemical. All materials used are analytical grade and used without purification.

2.2 Isolation of bacteria from rotten acidic fruit

Pretreatment for fruit samples was cropped and not peeled, especially formerly Siwalan fruit. After cutting the pieces, the sample was mashed using a hand blender. Mashed samples were weighed as much as 200 g and put in a beaker glass. Sucrose (20 g) was added to the mashed sample. It was dissolved in 1 L of 0.85% saline solution. It was incubated at 30 °C for ten days. Then, a formed pellicle was observed

2.3 Selection of potential bacterial isolates producing BNCs

Isolate bacteria were selected by inoculating bacterial isolates on two media, namely agar HS (Hestrin & Schramm) media and liquid HS media. Sample solution that has been made in stages collection sample diluted 10^{-1} up to 10^{-5} use 0.85% saline solution, then each sample at dilution 10^{-4} and 10^{-5} was inoculated as much as 0.1 mL with spread plate method on HS agar medium duplicate incubated at 30 °C for 24-48 hours. The observation was done after 48 hours on HS medium for comment, a growing colony. Colonies grown on HS media move onto liquid HS medium to keep the isolate that can produce cellulose. Colonies growing on HS media were collected. One ose was then raised on HS media as much as 100 mL and incubated at 30 °C for 24 using a shaker. After that, 10% of the volume of HS is transferred onto new HS media and set under static conditions for 14 days at 30 °C. Then, it was observed whether the isolate produced pellicles. Pellicles were filtered and rinsed with distilled water to remove media residue and other impurities. It was boiled in 10% (w/v) NaOH solution for 20 minutes to remove cell-attached bacteria. Then, it was washed thoroughly with distilled water 2-3 times and dried at 105 °C to determine BNC production (g/L). After comparing BNC production from each potential isolate, isolate bacteria producing the highest number of BNCs were chosen for further steps.

2.4 Analysis of genetic sequences with 16s rRNA method [9]

Selected strains in this research were using 16 s rRNA sequencing. Two oligonucleotide primers, P175 (5'-AGAGTTTGTGATCMTGGCTCAG-3') and P176 (5'-CGGTTACCTTGTTACGACTT-3') were used for amplification in the 16s rRNA gene in a polymerase chain reaction (PCR). The volume of the PCR mixture was estimated to reach 50 L with Composition 1 X PCR buffer, 400 M dNTP, 200 M some primers, 2.5 U Taq polymerase, and 100 ng DNA template. PCR analysis using a PCR machine instrument (ASTEC PC-818, ASTEC, Fukuoka, Japan) with an initial step of 94°C for 10 minutes, continued with 35 cycles of 94, 50, and 72 °C at 30, 30, and 90 seconds, and for final step at 72°C for 10 min to ensure that PCR product extension has complete. 16S rRNA sequences were analyzed for maximum similarity and compared with the culture sequence that has been There is use EzBioCloud server (<http://ezbiocloud.net/>) and NCBI nucleotide database via BLAST search. The degraded data compared with the method of Clustal W multiple aligners. Phylogenetic analysis uses Molecular Evolutionary Genetics Analysis (MEGA) software (version 6).

2.5 Analysis profile growth bacteria

Isolated bacteria from selected fruits were stored in the bottle in an HS medium (Hestrin & Schramm, 1954). For a seed culture, one bottle was added to 100 mL of seed culture medium in condition agitation in the orbital shaker with a speed of 120 rpm for 16 days. Every day, sample seed culture was centrifuged at 9000 rpm for 20 minutes at 7-8 °C pelleted collected and returned suspended in sterile distilled water until set OD using a UV-Vis spectrophotometer at a wavelength of 600 nm).

2.6 Analysis structure

The analysis film structure of BNC film used ATR-FTIR (PerkinElmer 1S8M99LTBM). BNC pieces of 1 cm² (approx.) were used for the recorded spectrum. The spectrum was 450-4000 cm⁻¹ with 16 scans per spectrum.

2.7 XRD analysis

X-ray diffraction (XRD) is a standard method used to determine the crystallinity of bacterial nanocellulose (BNC). In a typical XRD analysis, the BNC sample was placed in an X-ray holder. Scans were performed over a specific range (for example, 10-40° 2θ range) at a particular rate of scan (for example, 2° min⁻¹ or 10°/min). The X-ray diffractometer often employed Ni-filtered Cu-Kα radiation ($\lambda = 1.54 \text{ \AA}$). The crystallinity index of the BNC was then calculated using the Segal method or a similar method. The Segal method involves calculating the ratio of the intensity of the 200 peaks (I₂₀₀) to the intensity of the amorphous peak (I_{am}), often using the formula $CrI = (I_{200} - I_{am}) / I_{200} \times 100\%$.

2.8 FE-SEM analysis

In a typical FE-SEM analysis, the BNC sample was first purified and dried. The sample was then mounted and coated with a thin layer of gold to reduce charge interruptions and enhance the electron signal. The sample was then observed under the FE-SEM at an accelerated voltage (10-15 kV) and various magnifications (for example, 5000x, 20,000x, 32,000x, 40,000x, or 50,000x). The resulting micrographs can provide detailed information about the BNC's fiber diameter, pore size, and overall microstructure. The fiber diameter can be calculated using image processing software, such as ImageJ.

3 Result and discussion

3.1 Isolation, selection, and identification of BNC-producing bacteria

The results of the selection of bacterial isolates from fruits and coconut water were as many as 33 samples, and not all of them could grow on HS agar media. Bacterial isolates were inoculated on HS media so that 24 samples (about 73%) grew colonies. Of the 25 (24 isolates + Kombucha starter control) samples grown on liquid HS media to determine isolates that could produce BNC, there were five isolates (20%) that could produce pellicle, namely Isolate from Apple, Snake fruit, Mango, Sapodilla and SCOBY kombucha starter control. Pellicle thickness from thickest to thinnest was Kombucha starter control > Sapodilla > Snakefruit > Mango.

The yield of BNC produced from the SCOBY kombucha control (3.073 g/L) was more significant than that of mango (0.058 g/L), snake fruit (0.129 g/L), sapodilla isolate (0.432 g/L). Research [1] showed that isolates obtained from soursop fruit showed the highest BC productivity, 0.58 g/L BC. The level of productivity and yield of BNC was influenced by many factors besides the type of bacterial strain, including the type and chemical composition of the growth medium, incubation time and temperature, number of inoculums, and surface area [2]. The BNC results in this study were lower compared to previous studies. Therefore, the selected isolates from this study must be optimized first to obtain the maximum BNC yield.

Isolates from Sapodilla fruit produced the highest yield of BNC compared to isolates from other fruit samples. The sapodilla fruit isolates were identified the strain by the 16s rRNA method. This analysis consists of 5 stages: DNA isolation, PCR, sequencing, processing of sequencing results data, and determining the NCBI Top 10 BLAST and phylogenetic compilation based on the NCBI database. The 16s rRNA sequences of isolated bacteria were compared with several closely related taxa, most from the genus *Komagataeibacter* in the NCBI database. Based on the 16s rRNA strain analysis, sapodilla isolate has an identity percentage above 98%, so it can be concluded that it has similarities with the bacteria *K. saccharivorans*. The phylogenetic tree provides information regarding the evolutionary relationship between new and existing strains (Fig. 1). *K. saccharivorans* have been reported in several studies to produce bacteria cellulose. *K. saccharivorans* strain BC1 was isolated from grapes after going through biochemical and genetic analysis, and the strain was confirmed to be able to synthesize cellulose [3].

Based on Fig. 2, at 0 to 72 h, there was the growth of bacteria from OD 0.0445 at 0 h to OD 0.1647 at 24 h, then OD 0.1379 at 48 h and at 24 h -72 equal to OD 0.1261, which in this period is the lag phase or adaptation of bacteria to grow. The absorbance value obtained in the lag phase is based on the measurements carried out; several points experience an increase and decline. Fluctuating shows the instability of bacteria in development, where the bacteria are still adjusting to the media that is the breeding ground. In the lag phase, the bacteria are still preparing to reproduce.

At 72 to 96 h is the logarithmic phase of the growth of *K. saccharivorans*. It became the highest point at 96 h of the inoculation until before it finally entered the stationary phase. In the log/logarithmic/exponential phase, changes in the growth rate of bacterial cells occurred. Next is the stationary phase; based on the curve in Fig. 2, the stationary phase starts at the 96th hour with an absorbance of 0.22, then continues with measurements at the 120th hour with an absorbance of 0.2273; at the 144th hour, it is 0.2153 and at the 168th is 0.2384. Meanwhile, the *K. saccharivorans* sp *PAPI*, which was isolated from rotten papaya fruit, grew slowly during the first two days of incubation. Then, on the third day, it grew exponentially until the seventh day, after which it entered the stationary phase. The production of cellulose by the bacteria *K. saccharivorans* sp *PAPI* was directly proportional to its cell growth rate [4].

The concentration of sugar, mainly glucose, in the culture medium can significantly affect the yield of Bacterial Nanocellulose (BNC). Glucose is the primary carbon source for BNC-producing bacteria and is used as the building block for cellulose synthesis. According to Fig. 3, the optimum concentration of glucose to produce BNC is about 2%. Glucose concentration is related to the productivity of gluconic acid and can affect the decreasing pH of the medium, inhibiting bacterial growth. Productivity value is closely related to the yield produced after fermentation. In one study, it was observed that the BNC yield increased with the glucose concentration, with higher glucose concentrations supporting higher BNC production. However, the optimal glucose concentration was found to be 20 g/L, and further increases in glucose concentration did not necessarily lead to higher BNC yields [10]. In another study, it was found that BNC yield decreased with an increase in the initial glucose concentration between 40 and 200 g/L, which was attributed to a decrease in pH below 3.0 [9]. The mechanism by which glucose increases gluconic acid production in Bacterial Nanocellulose (BNC) production involves the oxidation of glucose by the enzyme glucose dehydrogenase, which is produced by BNC-producing bacteria such as *Komagataeibacter* species. In this reaction, glucose is converted to gluconic acid, with the concurrent reduction of NAD⁺ to NADH. This reaction is the first step in the metabolic pathway for BNC production, and the gluconic acid produced can be further metabolized to produce the precursors for cellulose synthesis. The rate of this

reaction, and thus the amount of gluconic acid produced, can be influenced by the concentration of glucose in the culture medium. Higher glucose concentrations can lead to higher gluconic acid production up to a certain point. In this study, a glucose concentration of more than 2% can inhibit the growth of the bacteria and thus decrease gluconic acid production.

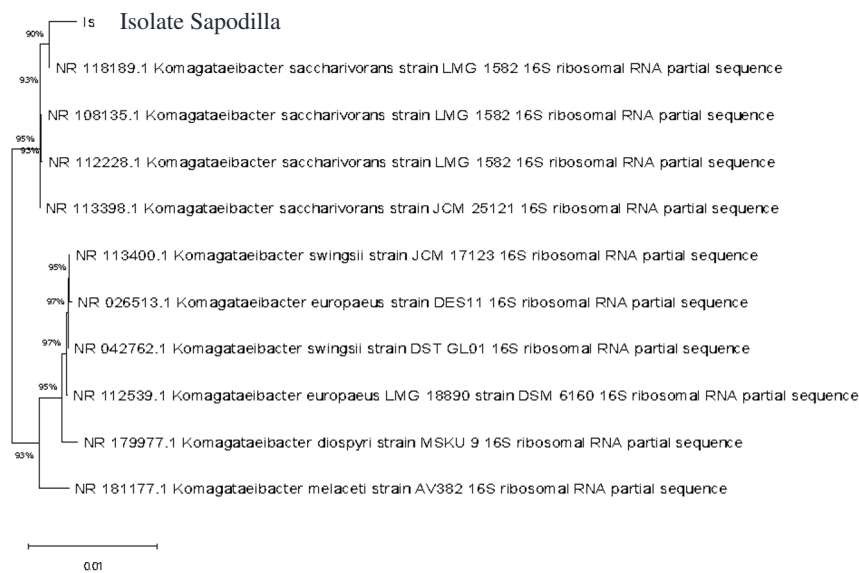


Fig. 1. Phylogenetic analysis.

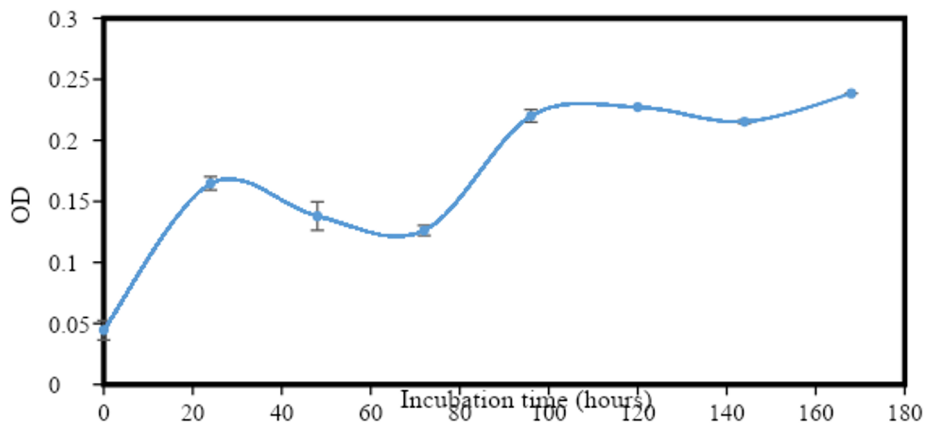


Fig. 2. Growth curve of *K. saccharivorans* sapodilla.

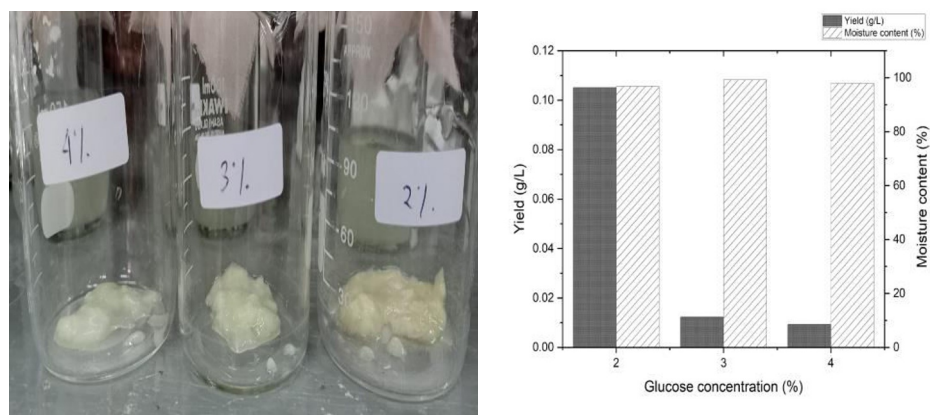


Fig. 3. The effects of glucose concentration on yield of BNC and moisture content.

3.2 FTIR analysis

The FTIR spectra of BNC prepared from different isolates of bacteria are shown in Figure X. All FTIR spectra exhibited several typical vibration bands with little difference (Fig. 4), implying the same chemical structure for the different BNCs prepared from various bacteria isolates. Our study revealed that the BCs produced by isolates bacteria (kombucha, sapodilla, snake fruit, and mango) were mainly determined as cellulose I (adsorptions at around 3345, 1430, 1160, and 900 cm^{-1}) and few celluloses II (adsorption at around 1335, 1315, and 1280 cm^{-1} and a blue-shift of wavenumber from 1430 to around 1425 cm^{-1}).

The vibration of the C-H group at the peaks of the waves 1361 and 2895 cm^{-1} indicates the vibration of the C-H molecule in cellulose. At the peak of wave 1061 cm^{-1} , there is a vibration by the C-O-C molecule produced from the pyranose ring, and the vibration that occurs at the peak of wave 1112 occurs because of the C-C molecule in the cellulose polysaccharide.

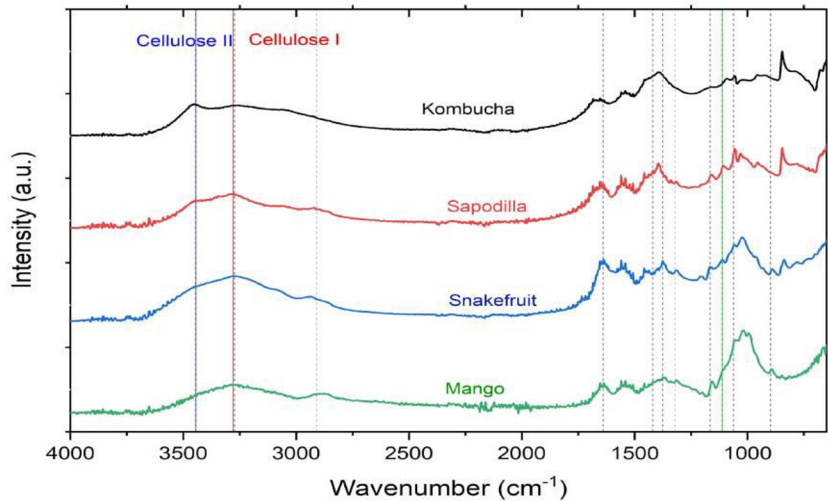


Fig. 4. FTIR analysis.

3.3 XRD analysis

Based on Fig. 6, the diffractogram of the XRD graph peaks at angles of 18° and 22° ; this shows that nanocellulose has the characteristics of cellulose where the peak area is still between $10\text{--}40^\circ$, namely the crystallographic field for cellulose. Crystallinity analysis was seen at the maximum diffraction angle, namely the lattice peak at an angle of 22° and the minimum diffraction amorphous peak at 18° to 19° and obtained a crystallinity index of 52.387%. It means that the crystallinity index of the resulting nanocellulose is 52.387%; this value is smaller than the crystallinity index of nanocellulose produced by bacteria *K. xylinus* in medium HS, usually where the level of good crystallinity is above 80% [11]. Nanocellulose with a lower degree of crystallinity can be more amorphous and flexible, which can be advantageous for specific applications. For example, papermaking and packaging, biomedical applications, and composite materials. However, the suitability of lower crystallinity nano cellulose for these applications can depend on other factors, such as the specific properties of the nitrocellulose and the requirements of the application.

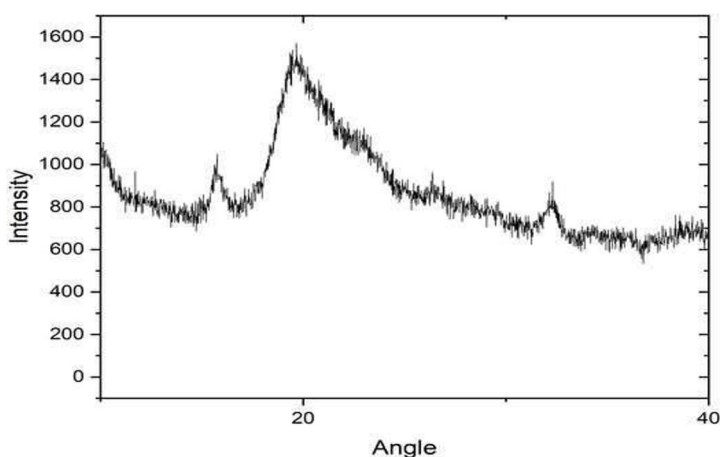


Fig. 6. XRD analysis.

3.4 SEM analysis

Scanning Electron Microscopy (SEM) is a standard method used to analyze the morphology and microstructure of Bacterial Nanocellulose (BNC) produced by *Komagataeibacter*. Based on Scanning Electron Microscopy (SEM) results, the surface of Bacterial Nanocellulose (BNC) produced by the sapodilla isolate (*K. saccharivorans*) appears smooth. The fiber size is relatively excellent, with an average diameter of about 86.46 nm and a fiber size distribution ranging from 20 nm to 150 nm (Fig. 7). Different bacterial strains can produce Bacterial Nanocellulose (BNC) with different morphologies, even when grown in the same medium. The difference in distribution fiber size is essential because the specific characteristics of the bacterial strain, its metabolic pathways, and the enzymes it produces can influence the structure and properties of the BNC it yields.

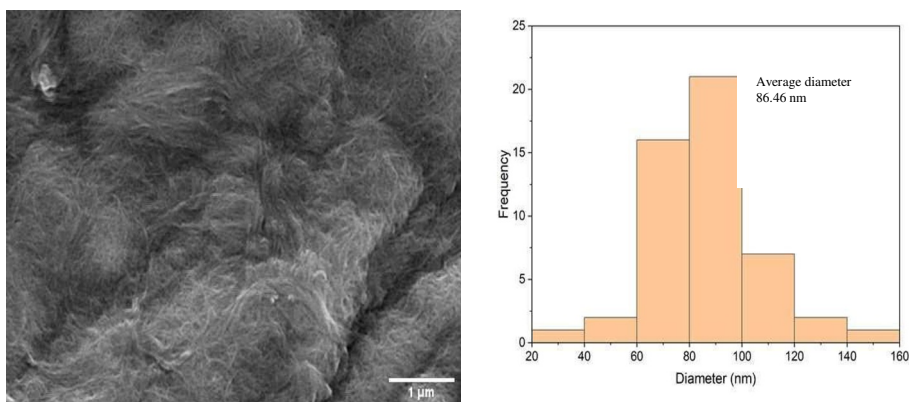


Fig. 7. FE-SEM analysis.

4 Conclusion

The isolation of nanocellulose-producing bacteria from sour-tasting fruits yielded potential nanocellulose-producing bacterial strains, specifically *K. saccharivorans*, from sapodilla fruit isolation. *K. saccharivorans* can produce nanocellulose of type I and II with a yield of 0.105 g/L in liquid HS medium at a glucose concentration of 2% for 14 days of incubation. The resulting nanocellulose has a crystallinity index reaching 52.387% with a diameter nanofibril of about 86.46 nm. Nanocellulose with a lower degree of crystallinity can be more amorphous and flexible, which can be advantageous for specific applications. Further research is needed to enhance productivity and characteristics regarding the suitable growth conditions and medium for nanocellulose production by this bacterial strain.

5 Acknowledgement

The authors would like to acknowledge the financial support from Kementerian Pendidikan, Kebudayaan, Riset dan Teknologi (Center for Higher Education Funding-BPPT) and Lembaga Pengelola Dana Pendidikan (LPDP) for supporting this study.

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