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Research

Isolation of Lactic Acid Bacteria (LAB) from *Mimosa pudica* (Semalu) for Production of Bacterial Cellulose

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ABSTRACT

Bacterial cellulose (BC) is a potential eco-friendly biopolymer. BC has higher crystallinity and purity compared to plant cellulose. Scientific studies on the production of BC from lactic acid bacteria (LAB) are minimal compared to other common bacteria such as *Acetobacter xylinum*. LAB was screened and isolated from different tissues of *Mimosa pudica* (medicinal plant) using MRS broth and agar as the selective medium. LAB isolates were subjected to 16S rRNA gene sequencing of all the bacterial isolates. BC was produced from all LAB isolates by incubating at 30 °C for 14 days in herbal tea medium (*Strobilanthes crispus*) and HS medium (control) with 130 rpm agitation. BC produced by two selected bacterial isolates was characterized using FESEM, FTIR, XRD, and TGA. Molecular analysis of the 16S rRNA gene of all the potential LAB isolates shows 99.86 - 100% identity to 16S rRNA sequences of other *Lactobacillus plantarum* strains. Two selected *L. plantarum* strains (LBM001 & LBM004) produce BC in sphere-like particles with a 1.4 to 2.2 µm diameter range of microfiber. FTIR analysis shows that BC produced by LBM001 and LBM004 have four similar cellulose regions identified in cellulose from other sources, which are O-H stretch (3400-330 cm^{-1}), C-H stretch (2970-2800 cm^{-1}), O-H bending (1620cm^{-1}) and C-O-C stretch (1100-1073 cm^{-1}). XRD analysis shows BC produced by the *L. plantarum* strains consists of two different XRD peaks at the 2θ angle of 21.53° and 21.85° instead of a single peak (22.76°) identified in the BC produced by *A. xylinum* and plant cellulose. A similar TG and DTG curved pattern was detected in the BC produced by the *L. plantarum* strains with the BC produced by *A. xylinum* and plant cellulose. The LAB isolates from *M. pudica* have potential in BC production based on the multiple characterization studies.

Key words: Bacterial cellulose, lactic acid bacteria, medicinal plant, microfibril, *Mimosa pudica*

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INTRODUCTION

Bacterial cellulose (BC) is an alternative cellulose material to plant cellulose that has potential in cellulose-related industries (Hashim *et al*., 2021). Alternative cellulose sources, including bacteria, fungi, and algae, will avoid continuous wood pulping and deforestation (Sumardee *et al*., 2021). The high purity of BC produced by *Acetobacter* species in nanosize (less than 100nm) is an efficient source of nanocellulose (Klemm *et al*., 2005; Siró & Plackett, 2010; Andritsou *et al*., 2018). Compared to plant cellulose, the advantages of BC, including high water-holding capacity, biodegradability, and enhanced mechanical strength, make it suitable to be used as nanocellulose fibers in related industries (de France *et al*., 2017). The high cost of purification and conversion of plant cellulose into nanocellulose gave advantages to BC that are naturally synthesized by bacteria as nanofibrils (de Assis *et al*., 2017; de Melo *et al*., 2017). Besides, avoiding deforestation and extensive use of chemicals for plant cellulose purification and the subsequent nanocellulose conversion step is environmentally friendly.

BC-producing microorganisms can produce BC in static and agitated conditions (Sani & Dahman, 2009). BC production in static conditions requires large physical space and thus contributes to the expensive BC production cost

(Shi *et al*., 2014). BC production on an industrial scale requires suitable reactors to improve the yield with the lowest cost. Exploring new fermentation medium for BC production leads to cost-reducing of final products. For example, oil palm frond (OPF) juice containing multiple sugars, including glucose, fructose, and sucrose, makes it a suitable carbon source for BC synthesis (Hashim *et al*., 2021). Besides that, waste materials from fruits in food industries and local markets show their potential as an alternative to Hestrin and Schramm (HS) fermentation medium for BC production (Andritsou *et al*., 2018). Using agroindustrial resources as a fermentation medium on *Acetobacter xylinum* and other *Acetobacter* species shows potential in BC's yield optimization. The recent discovery of several species of LAB in BC production indicates that several LABs open opportunities for exploring their potential as BC producers in cellulose-related industries (Sumardee *et al*., 2020). *Lactococcus lactis* was the first LAB reported to produce BC in 15 days of agitation in the HS medium (Umamaheswaris *et al*., 2017). In contrast, *Lactobacillus hilgardii* produces floated BC pellicles in 16 days of static fermentation (Khan *et al*., 2020). Screening of LAB strains for their potential as BC producers could be helpful in cellulose-related industries.

In Malaysia, medicinal herbs including *Strobilanthes crispus*, *Gynura procumbens*, *Justicia gendarussa*, *Zingiber officinale*, *Plectranthus aromaticus,* and *Plectranthus amboinicus* have been reported as a potential source for LAB isolation (Fang *et al*., 2019). Besides those plants, many medicinal herbs have not been explored for LAB isolation. *Mimosa pudica* (local name: semalu) is one of the medicinal herbs that has not been tested for the presence of LAB on its surface. This well-known herb with the particular characteristic of thigmonastic and seismonastic movements has been reported with beneficial phytochemicals such as phenolic compounds, alkaloids, flavonoids, glycoproteins, quinone, coumarins, tannins, and saponins (Tunna *et al*., 2015). Scientific investigations have shown the potential of this plant's extracts as anticancer, antidiabetic, and antioxidant (Jagetia & Vanlalhruaii, 2020; Baharuddin *et al*., 2021; Lan *et al*., 2021). Based on the vital characteristics of *M*. *pudica*, different parts of the medicinal herb were chosen in this study for LAB isolation. The LAB strains isolated from the surface of strong characters of medicinal herbs are expected to have strong characters and be very consistent in producing BC. The characteristics of BC produced by the isolated LAB strains from different plant tissues were further explored using FESEM, FTIR, XRD, and TGA.

MATERIALS AND METHODS

Isolation of LAB from *M*. *pudica*

Different tissues of *M*. *pudica* such as leaves, fruits, flowers, and roots (Figure 1) were collected at the roadside of Kolej Kediaman 2 in UMP (GPS coordinate: 3°43'57.1"N 103°07'34.1"E). Each tissue was transferred into a 50 mL centrifuge tube containing 20 mL of MRS broth. Static incubation was carried out at 30 °C for 48 h. Each pre-enrichment culture was subjected to 10^{-4} dilution, spread on MRS agar plates, and incubated at 30 °C for 48 h. Three bacterial colonies from each tissue were randomly chosen and streaked on fresh MRS agar plates for single colony isolation. All the plates were incubated at 30 °C for 48 h in similar static conditions.

Preparation of growth media

The growth media used in this study were MRS broth, MRS agar, Hestrin-Schramm (HS) medium, and herbal tea from dried leaves of *S*. *crispus*. MRS broth was prepared by dissolving 5.23 g of premixed MRS broth powder (Merck, Germany) in 100 mL of distilled water, while MRS agar was prepared by dissolving 31 g of premixed MRS agar powder in 500 mL of distilled water. HS medium was prepared by mixing 5 g peptone, 20 g of glucose, 5 g of yeast extract, 1.5 g of citric acid, and 2.7 g of sodium hydrogen phosphate in a total volume of 1000 mL distilled water. Meanwhile, herbal tea medium was prepared by soaking 10 g dried leaves of *S*. *crispus* in 5 L boiled distilled water for 30 min. After cooling down, 100 g of glucose was mixed in the herbal tea before being transferred into a 1 L Schott bottle. All the prepared media were autoclaved at 121 °C for 15 min. Autoclaved MRS agar was poured into petri dish plates in laminar flow after autoclaving.

Molecular identification of the bacterial isolates

Genomic DNA was isolated from six bacterial isolates, as described by Fang *et al*. (2019) and Ismail *et al*. (2019). Similar PCR amplification of 16S rRNA genes was performed using B27F and 1492R primers. All the PCR products were purified and subjected to single-pass sequencing. PCR amplified products were electrophoresed on 1.0% (w/v) agarose gel together with 1 kb Hyperladder™ (Bioline, USA) in the first lane. The electrophoresis was run in 1× TAE buffer at 80 Volts for 40 minutes. The purified products were subjected to single-pass sequencing using B27F and 1492R primers. Sequences of 16S rRNA genes were subjected to BLASTn analysis for species determination of all the bacterial isolates.

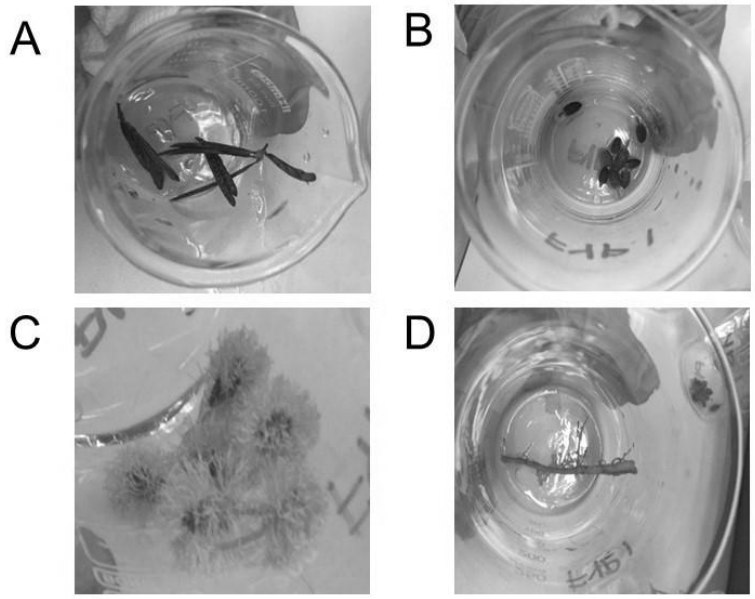

Fig. 1. Different tissues from *M*. *pudica* for LAB isolation. The selected tissues were (A) leaves, (B) fruits, (C) flowers, and (D) roots.

BC production

A single colony from each agar plate was inoculated into a fresh 50ml centrifuge tube containing 5 mL of MRS broth. All the samples were incubated at 30 °C for 48 h in similar static conditions. Centrifugation was carried out at 5,000 r.p.m for 2 minutes for all the samples. The supernatant from each sample was removed and the recovered bacterial pellet was resuspended in a 5 mL herbal tea medium. Then, the bacterial samples were transferred into conical flasks containing a final volume of 100 mL herbal tea medium. All the samples were further incubated at 30 °C for 14 days for BC production, with agitation at 130 r.p.m. After 14 days incubation, BC pellicles were washed overnight in 10% (v/v) NaClO solution, with 50 rpm agitation on a belly dancer. After that, the BC pellicles were rinsed in distilled water three times. The final rinse was performed overnight with 50 rpm agitation on the same belly dancer. All BC pellicles were dried at room temperature for three days.

BC analysis

The dried BC pellicles were subjected to several analyses using multiple instruments. The morphological structure was observed using a field emission scanning electron microscope (FESEM) with 5.0 kV acceleration voltage and a distance of 9.3 mm. The BC image was observed for 100×, 500×, 2,000× and 5,000× magnifications. The functional groups' analysis was performed using FTIR with the ATR platform at the wavenumber range from 4000 cm^{-1} to 700 cm^{-1}. X-ray diffraction patterns of the BC pellicles were determined using an X-Ray Diffractometer (XRD) and thermal stability studies were performed using Thermal Gravimetric Analysis (TGA).

RESULTS AND DISCUSSION

In the present study, potential LAB isolates were isolated from four different tissues of *M*. *pudica* using MRS agar as a selective medium for their growth. Presumptive identification was carried out by observing the morphology of the colony formed on the agar surface (Figure 2). The colonies were smooth, round, creamy-white, and convex, which is similar to the morphology of LAB (Kumar & Kumar, 2014). Screening of twelve potential LAB isolates for BC production (Table 1) showed six isolates with floating pellicles in the 14-day fermented herbal medium. However, no floating pellicles were found in all the bacterial isolates after 14 days of fermentation in the HS medium. This result suggests that the herbal tea medium produced from dried leaves of *S*. *crispus* is suitable for BC production of all the LAB isolates (LBM001-LBM006) compared to the HS medium. HS medium is ideal as the growth medium for *Lactobacillus acidophilus* (Sumardee *et al*., 2020). This contrasting result could be due to the different species of LAB isolates in this study.

Table 1. Screening result of 12 colonies for BC production

Source of bacterial isolation	Colony	Pellicle formation		Isolate
		HS medium	Herbal medium	
Flower	1	-	-	-
	2	-	-	-
	3	-	-	-
Fruit	4	-	+	LBM001
	5	-	+	LBM002
	6	-	+	LBM003
Root	7	-	+	LBM004
	8	-	+	LBM005
	9	-	+	LBM006
Leaf	10	-	-	-
	11	-	-	-
	12	-	-	-

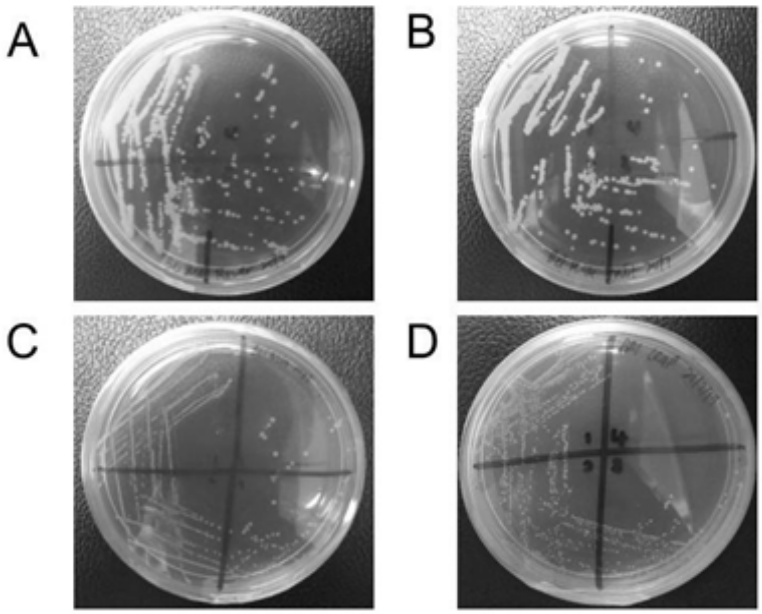

Fig. 2. Single colony isolation of potential LAB from (A) leaves, (B) fruits, (C) flowers, and (D) roots of *M. pudica*.

Purified PCR-amplified product of 16S rRNA genes from all the bacterial isolates showed a good DNA quality with $A_{260/280}$ ratios of more than 1.8 with a concentration range of 120 - 200 ng/µL. However, the samples were diluted to 50-100 ng/µl before single-pass sequencing. The gel photo of the amplified 16S rRNA genes (Figure 3) shows all the products were around 1,500 bp. The size of all the products is almost similar based on the gel photo observation under a UV-gel documentation system. However, DNA sequencing can only determine the exact size of all the PCR-amplified products. Based on the single-pass sequencing result shown in Table 2, all 16S rRNA amplified products of LBM001-LBM006 are 1,450 bp in size after the removal of 27F and 1492R primers' sequences. BLASTn analysis of all the positive isolates (LBM001-LBM006) shows that they belong to *Lactobacillus plantarum* with 99.86 - 100.00 % identity to 16S rRNA genes of other *L. plantarum* sequences that are available in the NCBI GenBank. Table 2 shows all the sequences hit to *L. plantarum* strain 8453 [MT464316.1] with 100% identity, *L. plantarum* strain 7023 [MT464069.1] with 99.93% identity, and *L. plantarum* strain BW14 [MW404313.1] with 99.86% identity. Similar BLASTn results for all LBM001-LBM006 strains were detected in this study due to their 100 % nucleotide similarity, suggesting all the isolates belong to the same strain that naturally spread on fruits and roots of the plant.

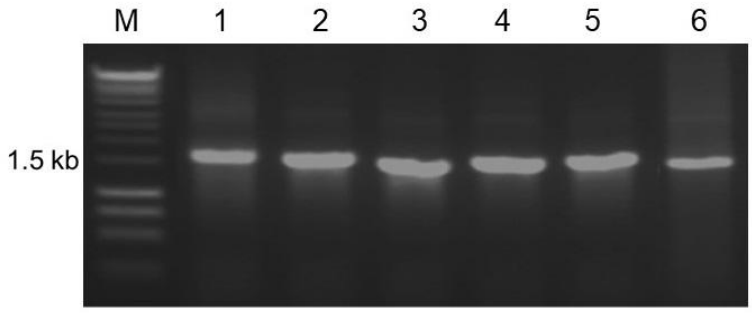

Fig. 3. PCR amplified products 16S rRNA gene of six bacterial isolates with 27F and 1492 primers. Lanes M: 1 kb Hyperladder™ (Bioline, USA), lane 1: LBM001, lane 2: LBM002, lane 3: LBM003, lane 4: LBM004, lane 5: LBM005 and lane 6: LBM006.

Table 2. BLASTn analysis of 16S rRNA gene from bacterial isolates

Isolate	Source of isolation	Size (bp)	Identity (%)	Homologous sequence
LBM001	Fruit	1450	100.00	*L. plantarum* strain 8453 [MT464316.1]
			99.93	*L. plantarum* strain 7023 [MT464069.1]
			99.86	*L. plantarum* strain BW14 [MW404313.1]
LBM002	Fruit	1450	100.00	*L. plantarum* strain 8453 [MT464316.1]
			99.93	*L. plantarum* strain 7023 [MT464069.1]
			99.86	*L. plantarum* strain BW14 [MW404313.1]
LBM003	Fruit	1450	100.00	*L. plantarum* strain 8453 [MT464316.1]
			99.93	*L. plantarum* strain 7023 [MT464069.1]
			99.86	*L. plantarum* strain BW14 [MW404313.1]
LBM004	Root	1450	100.00	*L. plantarum* strain 8453 [MT464316.1]
			99.93	*L. plantarum* strain 7023 [MT464069.1]
			99.86	*L. plantarum* strain BW14 [MW404313.1]
LBM005	Root	1450	100.00	*L. plantarum* strain 8453 [MT464316.1]
			99.93	*L. plantarum* strain 7023 [MT464069.1]
			99.86	*L. plantarum* strain BW14 [MW404313.1]
LBM006	Root	1450	100.00	*L. plantarum* strain 8453 [MT464316.1]
			99.93	*L. plantarum* strain 7023 [MT464069.1]
			99.86	*L. plantarum* strain BW14 [MW404313.1]

A previous study by Fang *et al*. (2019) showed *L*. *plantarum* strains were identified on the leaves of *S*. *crispus*, suggesting the leaves of this medicinal plant are one of the suitable natural hosts for the growth of *L*. *plantarum* strains. Thus, herbal tea produced from *S*. *crispus* was selected in this study as an LAB fermentation medium for BC production to test its suitability as the natural growth medium for LAB. In addition, 20 g/L (w/v) glucose was added into the herbal tea medium as the carbon source for the building of BC by the *L*. *plantarum* strains. For comparison, the HS medium was used as a control in this study. Figure 4 shows the floating BC pellicles in the 14-day fermented herbal tea medium. However, no BC was observed on a cloudy HS medium after the 14-day incubation period. The HS medium with 20% (w/v) glucose is usually used for BC production by *A*. *xylinum*, the well-known BC producer among bacterial species. However, in this study, the cloudy medium after the 14-day fermentation period reflects that the medium is suitable for the growth of the LAB isolates but not ideal for BC production by the isolated *L*. *plantarum*. Even though both HS medium and herbal tea contained 20% (w/v) glucose as the carbon source, the BC production is only successful in herbal tea medium. Thus, the other components, including minerals, active compounds, and other things in the herbal tea that might stimulate BC production by the *L*. *plantarum* should be investigated in the future. Current research focusing on identifying inexpensive medium formulations with high BC yield is actively carried out on *A*. *xylinum* (Chua *et al*., 2020; Hashim *et al*., 2020; Chua *et al*., 2021). A similar effort should be made on *L*. *plantarum* and other LAB strains in the future as alternative potential BC producers that could benefit related industries.

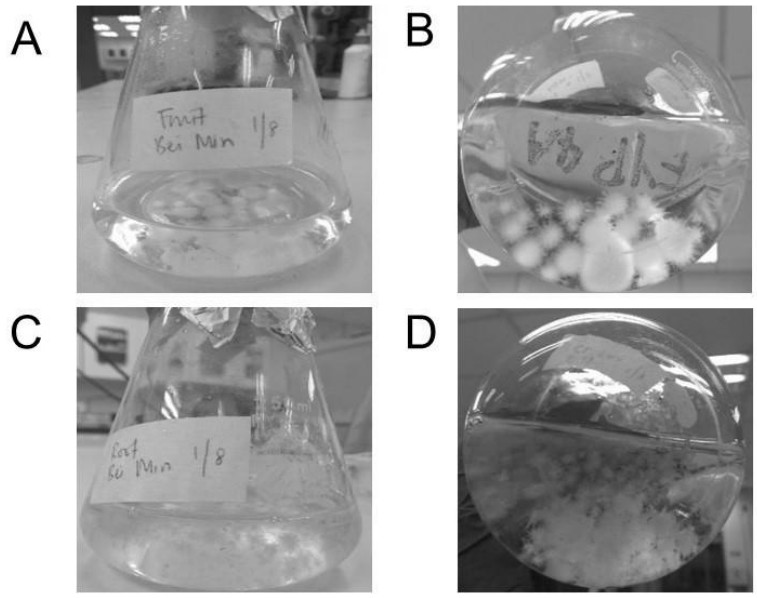

Fig. 4. Recovered BC pellicles from LBM001 and LBM004 in the herbal tea medium after 14 days incubation period. The front (A) and bottom (B) views of BC from LBM001 and similar views for BC from LBM004 are shown in (C) and (D), respectively.

Morphological observation of the BC pellicle produced by LAB isolates was performed using FESEM with different magnifications, as shown in Figure 5. The fiber network can be observed at 100x magnification (Figure 5A). The zoom image with 500x (Figure 5B) and 2000x (Figure 5C) magnified the fiber network. Based on the size measurement in Figure 5D (5000x magnification), the BC fiber produced by LAB isolates is in diameter size ranges of 1.4 µm to 2.2 µm based on the random diameter measurement. Thus, they cannot be categorized as nanoparticles but should be classified as microparticles. Figure 6 shows the diameter size comparison between BC produced from this study (Figure 6A) to another three purified celluloses. BC's fiber network produced by *L*. *acidophilus* (Figure 6B) is in diameter size ranges between 0.8 µm to 1.13 µm. The diameter is smaller than BC produced by *L*. *plantarum* (LBM001-LBM006). BC from *A*. *xylinum* showed a much smaller diameter size of 32.0 nm to 74.4 nm (Figure 6C). This fibril size observed is not so much different compared to the BC produced by *A*. *xylinum* in other media such as oil palm frond juice with a diameter size range of 50 nm to 60 nm (Hashim *et al*., 2021) and the naturally producing BC by *Gluconobacter* with the diameter size range of 50 nm to 100 nm (Tajima *et al*., 2017). Meanwhile, commercialized α-cellulose (Sigma Aldrich, USA) produced from a plant source showed a microfibre with a diameter of 22.1 µm in the labeling position (Figure 6D). Based on the diameter size comparison, it can be concluded that BC produced by LAB including *L*. *plantarum* and *L*. *acidophilus* are 40x to 50x larger than the BC produced by *A*. *xylinum*. Meanwhile, the microcellulose produced by the plant source is 10x to 20x larger than the BC produced by LAB. The BC produced by bacteria is significant for future applications in related industries.

FTIR analysis in Figure 7 highlighted four main functional groups available in the selected cellulose samples for cellulose confirmation. In the first region, the broad peak was observed at 3400 - 3300 $cm^{-1,}$ indicating the O-H stretching and assigned to hydrogen bond and hydroxyl functional groups (Barud *et al*., 2008). The narrow absorption bands in the second region of 2970 to 2800 cm^{-1} correspond to the stretching of C-H (Hashim *et al*., 2021). The bending of -OH is identified in the third region of 1620 cm^{-1}. In the fourth region (1100 to 1073 cm^{-1}), the narrow and intense absorption bands were assigned to C-O-C, C-C, C-H ring and C-OH stretching at the β-(1→4)-glycosidic bond in bacterial cellulose (Sun *et al*., 2008). β-(1→4)-glycosidic bond is a significant bonding in BC. It acts as the connector between carbohydrate monomer and polymer with the C-O-C bonding notation. The chemical bonding (C-O-C stretch, –OH bending, –C-H stretch, –O-H stretch) present in cellulose polymer are significant regions of cellulose. Interestingly, a similar pattern of FTIR spectra was observed for the BC produced by *L*. *plantarum* (LBM001 & LBM004) and BC from *L*. *acidophilus* and *A*. *xylinum*, respectively. This result indicates the cellulose characteristic of BC synthesized by the isolated *L*. *plantarum* strains. The FTIR analysis on plant cellulose (α-cellulose) from Sigma Aldrich, USA also shows a similar FTIR fingerprint. Previous studies reported similar regions representing cellulose functional groups in plant cellulose (Fasoli *et al*., 2016; Hospodarova *et al*., 2018; Javier-Astete *et al*., 2020).

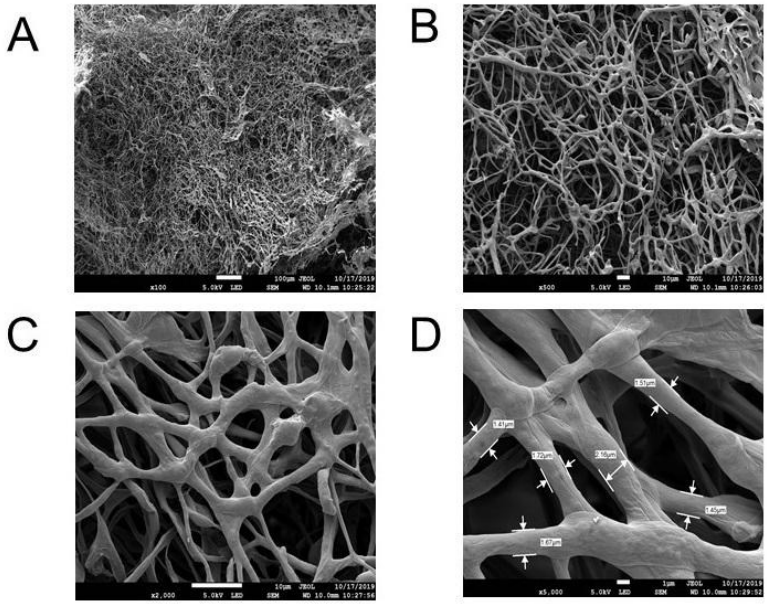

Fig. 5. SEM analysis of BC produced by LBM001 in different magnifications; (A) 100×, (B) 500×, (C) 2000×, and (D) 5000× magnifications.

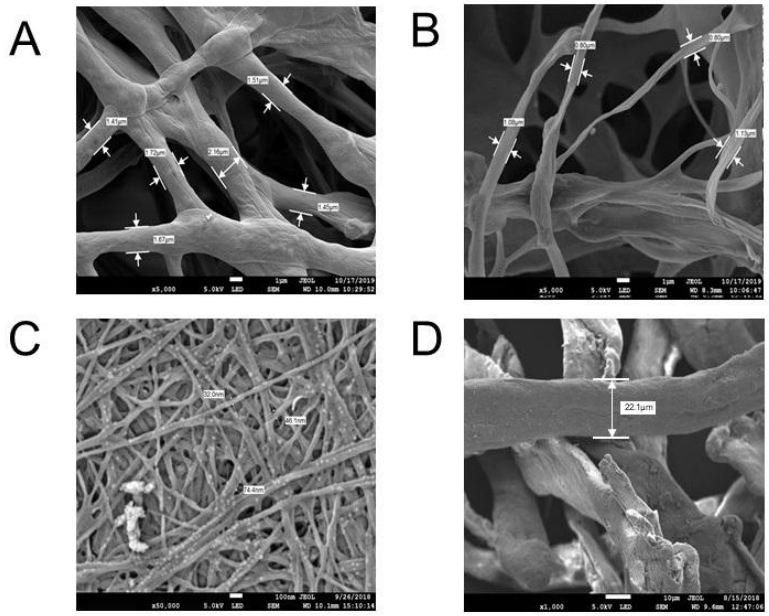

Fig. 6. SEM analysis of (A) BC produced by LBM001 in comparison to (B) BC produced by *L*. *acidophilus*, (C) BC produced by *A*. *xylinum* and (D) plant cellulose.

X-ray diffraction pattern (Figure 8) on the BC produced by LBM001 and LBM004 shows a similar crystalline peak at the 2*θ* angle of 14.55°. The same peak is detected in BC from *A*. *xylinum*, BC from *L*. *acidophilus* and plant cellulose. Unfortunately, the common crystalline peak at 2*θ* angle of 22.76° reported in plant cellulose and BC from *A*. *xylinum* is not detected in the BC produced by LBM001, LBM004, and *L*. a*cidophilus*. However, two separate crystalline peaks are identified at the 2*θ* angle of 21.53° and 21.85°. Interestingly, both peaks are located at the same region of the crystalline peak at the 2*θ* angle of 22.76°. The presence of two separate peaks (disintegrated peaks) in that region is also shown in cellulose type II (Gong *et al*., 2017). Based on their study, the higher the sulfuric acid concentration, the higher the crystallinity observed on the two disintegrated peaks. The increase of

reaction between the crystalline region and sulfuric acid, and complete removal of the amorphous region by the sulfuric acid reaction produces high crystallinity of the two disintegrate peaks. According to de French (2017), some samples of interest might have a mixture of type I and II celluloses. Further calculation needs to be done on intensity versus 2*θ* data for the various polymorphs to obtain theoretical composition patterns for mixtures. Thus, based on Figure 8, the XRD diffraction patterns (2*θ* angle of 21.53° & 21.85°) of the BC produced by *L*. *plantarum* (LBM001 and LBM004) together with BC produced by *L*. *acidophilus* can be assumed as type II cellulose. Meanwhile, a single peak in that region for BC produced by *A*. *xylinum* and α-cellulose (plant cellulose) might be categorized as type I cellulose. However, further investigation is recommended in the future to confirm the formation of type I and II cellulose by bacterial-producing cellulose including *A*. *xylinum*, *L*. *plantarum,* and *L*. *acidophilus,* in different fermentation conditions (static and agitation approaches).

TGA analysis (Figure 9) on the BC produced by LBM001, LBM004 *L*. *acidophilus, and* plant cellulose shows weight loss in three different temperature regions. A minimal change in the weight loss of BC was observed from ambient temperature to 200 °C. Water evaporation from the fibers indicated that the BC from each sample was stable up to 200 °C. Besides that, small molecular fragments such as hydroxyl and methylhydroxyl groups are removed in this phase. As the temperature increased from 200 °C to 500 °C, the BC's weight dropped dramatically due to organic decomposition and cleavage of glycosidic linkages of cellulose. The thermal decomposition of BC, which involved the degradation of the pyran and polymeric chain, was completed at a temperature above 500°C (Manfredi *et al*., 2006). The thermal degradation temperature depends on several parameters such as molecular weight, crystallinity, orientation, nitrogen airflow, and the heating rate (Chang *et al*., 2010). In the DTG curve, the DTG peak appeared at 277.2 °C (decomposition rate of 270.7 µg/min) and 310.1 °C (decomposition rate of 313.54 µg/min) for the BC produced by LBM001 and LBM004, respectively. Meanwhile, BC produced by *L*. *acidophilus* shows the maximum temperature peak at 302.0 °C for a decomposition rate of 512.73 µg/min. A similar TGA/DTG pattern was observed for plant cellulose with a maximum temperature peak at 282.5 °C for a decomposition rate of 434.85 µg/min. The decomposition is due to a cellulose degradation process such as dehydration, depolymerization, and the decomposition of glucosyl groups, followed by the formation of a charred residue (Gea *et al*., 2011).

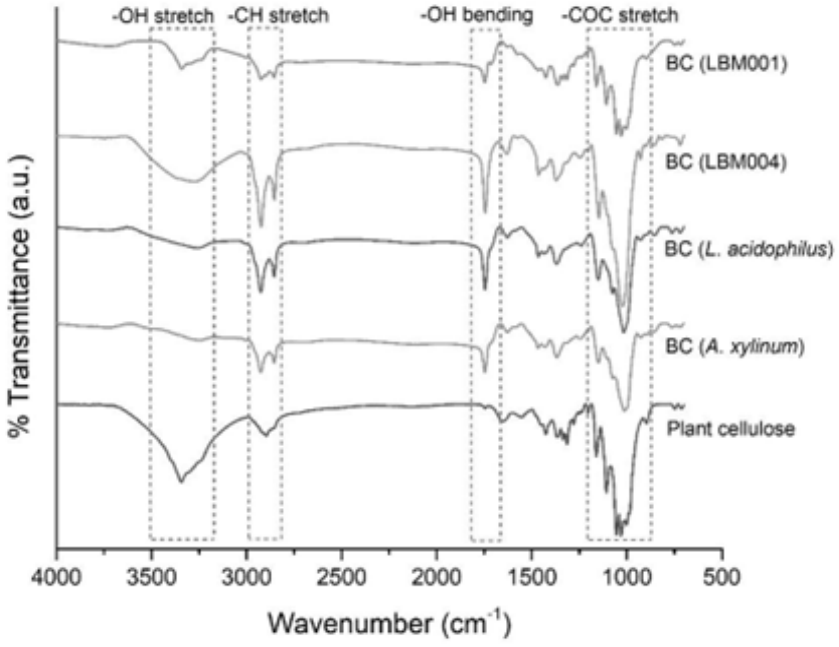

Fig. 7. FTIR spectra of BC produced by LBM001 and LBM004 compared to BC produced by *L*. *acidophilus*, *A*. *xylinum,* and plant cellulose.

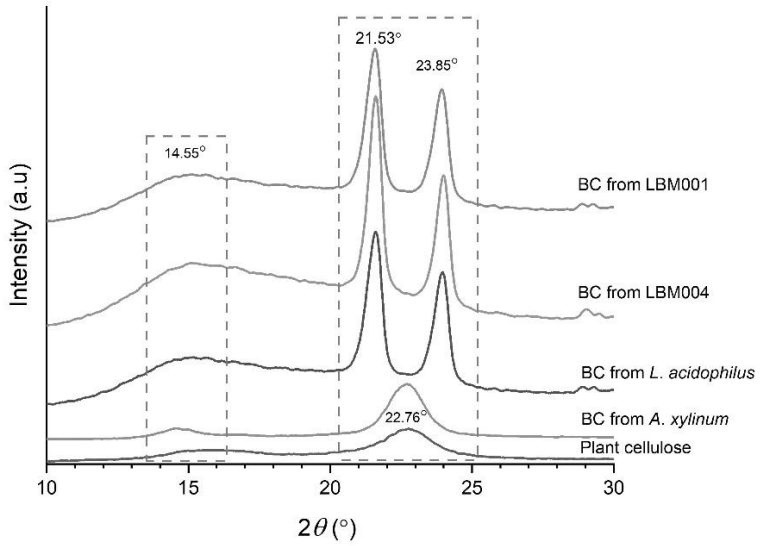

Fig. 8. FTIR spectra of BC produced by LBM001 and LBM004 compared to BC produced by *L*. *acidophilus*, *A*. *xylinum,* and plant cellulose.

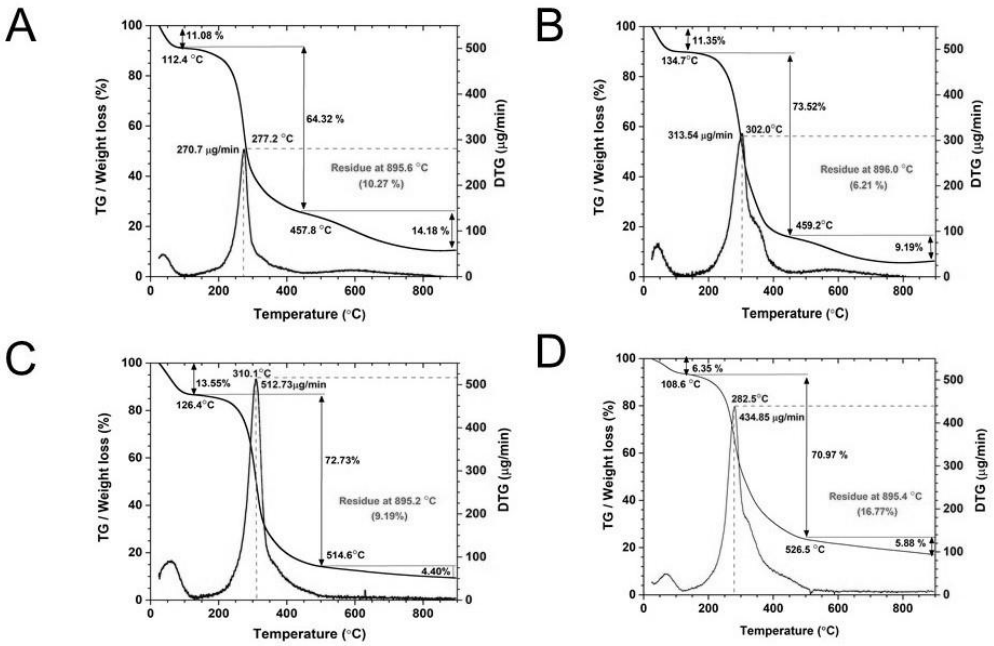

Fig. 9. TG and DTG curves of BC produced by (A) LBM001, (B) LBM004, (C) *A*. *acidophilus* and (D) plant cellulose.

CONCLUSION

This study successfully isolated six LAB strains (LBM001-LBM006) that hit *L. plantarum*. All the bacterial isolates successfully produced BC after 14 days of fermentation in agitation. Herbal tea medium produced from the leaves of *S. crispus* shows potential as a natural fermentation medium for the *L. plantarum* strains in BC production. Multiple characterizations, including FESEM, FTIR, XRD, and TGA, confirm the BC characteristics. In the future, further optimization should be carried out to maximize the BC yield with minimal cost.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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