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Isolation of Bacterial Cellulose Nanofibers Producing Acetic Acid Bacteria from Fermented juices

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ABSTRACT

Bacterial cellulose (BC) are high purity, ultra-fine network of nanofibrils structure, and non-toxic. The nanofibrils bacterial cellulose used for many applications in the food, medical and cosmetics industries. The objectives of this study aim to isolate and identify of nanofibrils bacterial cellulose producing acetic acid bacteria (AAB). We studied, isolation of cellulose producing AAB from 26 samples of Kombucha culture and different kinds of fermented fruit, and herb juices on Histrin Schramm. One hundred and seventeen bacterial isolates were obtained. The isolates were tested for their biochemical characteristics and bacteria cellulose nanofibrils production. Four isolates: RC1, RC3, RJ1, and RG2 were found to produce highest of bacteria cellulose (approximately 9-11 mm) from 1% (v/v) concentration of acetic acid in HS broth. The morphology and nanofibers were investigated by SEM and XRD, the bacterial cellulose nanofibrils size was 4.10, 4.95, 4.38, and 4.91 nm, respectively. However, isolates RJ1, RC3, and RC1 were identified to *Komagataeibacter hansenii* and then, isolates RG2 was identified to *K. maltaceti*.

Keywords: Bacterial cellulose, Bacterial cellulose producing acetic acid bacteria, Nanofibrils

INTRODUCTION

Bacterial cellulose (BC) is an exopolysaccharide ((C₆H₁₀O₅)_n), which is mainly produced by the genus *Komagataeibacter* (Dufresne and Farnworth, 2000; Hornung et al., 2006). The synthesis of bacterial cellulose in *Komagataeibacter* occurs between the outer membrane and cytoplasmic membrane by a cellulose synthesizing complex, which is in association with pores at the surface of bacteria (Bielecki et al., 2005). Bacterial cellulose synthase is the most important enzyme in this process. The bacterial cellulose leaves the pores as fibrils, a ribbon of crystalline bacterial cellulose. The polymer bacterial cellulose is connected by inter-and intramolecular hydrogen bonding network. The bacterial cellulose is of high purity and highly crystalline, with ultra fine network structure of diameter 20-100 nm. It is non-toxic and has high water absorption capacity and high biocompatibility (Petersen and Gatenholm, 2011; Jozala et al., 2016; Ullah et al., 2016).

The structure of BC has different properties from plant cellulose. Plant cellulose make up the cell walls, is a tough, mash-like bulkwork, water retention 60% and has hemicellulose and lignin, used chemical treat such as chlorine gas, carbon disulfide, carbon monoxide. Plant cellulose is a nonpurity to use in application. However, the BC is a Rapid growth period of the cells, Moist skin, Mesh-like ribbons, Non-hemicellulose and lignin, Water retention value of 100% and without toxic or allergic side effects. The BC used for many applications in the food, medical and cosmetics industries. The aim of this study to isolate and identify of nanofibrils bacterial cellulose producing acetic acid bacteria.

MATERIAL AND METHODS

Materials

Collection of samples

Total of 26 samples obtained from different region of Thailand (Chiang Mai, Chumphon, and Ranong) were collected. These were included in fermented fruit juices (star fruit, cultivated banana, santol, lichee, mix of fruits, nipa palm, mangosteen, sapodilla, langsat, guava,), fermented herb juices (lime, indian gooseberry, olive, myrabolan wood, turmeric, black galangal, butterfly pea, bilimbi, coconut peel, rosella, betel nut peel, marigold, and banana peel) and commercially-produced Kombucha tea culture. The samples were kept as its original until used.

Isolation of bacteria cellulose producing acetic acid bacteria (AAB)

Each sample 10 ml was inoculated into the 90 ml of Histrin Schramm medium (HS, glucose 20 g/l, peptone 5 g/l, yeast extract 5 g/l, Na₂HPO₄ 2.70 g/l, and citric acid 1.15 g/l) supplemented with 50 ppm cycloheximide to inhibit yeasts and mould. After incubation at 30°C for 3 days, samples produced bacteria cellulose layer on the top of HS were selected and serially diluted spread on HS agar, incubated at 30°C for 3 days. Bacterial strains that produce rough colonies or likely bacteria cellulose on HS agar were selected and re-streaked on HS agar. The pure cultures were tested for their basic biochemical (catalase, oxidase test), and stored at 4°C (Kerstens et al., 2006; Carreira et al., 2011).

Determination of bacteria cellulose production

The single colony of bacterial strains were inoculated into 10 ml HS medium, incubated at 30°C for 7 days. After incubation, bacteria strains produced BC as layer on top of HS were selected for the next experiments (Yim et al., 2017).

Identification of bacteria cellulose producing acetic acid bacteria by DNA analysis

Genomic DNA of the collected isolates were extracted according to Sambrook and Russell's protocol (Sambrook and Russell, 2001). The 16S rRNA gene amplified using polymerase chain reaction (PCR) according to Kumdhithahutsawakul's protocol (Kumdhithahutsawakul et al., 2019). The forward primer 27F (5'-AGAGTTTGATCCTGGCTGAG-3') and reverse primer 1492R (5'-TACCTTGTTACGACTT-3') used in PCR amplification (Chen et al., 2017).

Effect of acetic acid on bacterial cellulose production

A single colony of isolates were inoculated to 10 ml of HS medium and adjusted with the different concentration at 1-10% (v/v) of acetic acid, incubated at 30°C for 7 days. The data of surface colors, surface roughness and the thickness of bacterial cellulose were recorded. The total number of each strains were counted by plating on HS agar. Finally, the morphology and structure of BC were investigated using scanning electron microscopy (SEM), and X-ray diffraction (XRD) (Yim et al., 2017).

RESULTS

Isolation of bacteria cellulose producing acetic acid bacteria

The samples were measured pH between 2.28-3.26. The bacteria cellulose producing AAB were isolated from fermented fruit juices, fermented herb juices, and commercially produced Kombucha tea culture. Total of 117 bacterial strains were isolated and determined of the cellulose producing acetic acid bacteria. All the bacterial strain had some biochemical similarities to the reference strain such as Gram negative, rod shaped, oxidase negative, catalase positive, and glucose fermentation positive. However, bacteria cellulose producing AAB were found to be high in Indian gooseberry and Mix of fruits samples, respectively (Table 1 and Figure 1).

Table 1. Characterization of samples used for bacterial cellulose nanofibers AAB isolation

Sources/Samples	pH	Fermentation time of sample (months)	BC production	Total isolates
1. Chiang Mai				
Indian gooseberry	2.77	13	+	8
Star fruit	2.47	13	+	5
Cultivated banana	2.83	14	+	2
Lime	2.97	9	+	6
Olive	2.94	60	+	5
Santol	3.55	36	+	3
Lichee	3.42	36	+	2
Kombucha	2.28	1	+	2
2. Chumphon				
Myrabolan wood	2.81	13	+	1
Mix of fruits	3.04	24	+	12
Tumeric	4.19	13	+	4
Black galingal	3.11	9	+	2
Butterfly Pea	2.98	13	+	1
3. Ranong				
Nipa palm	3.26	28	+	5
Mangosteen	2.76	9	+	9
Indian gooseberry	2.57	7	+	8
Spodilla	3.04	13	+	4
Myrabolan wood	2.56	15	+	2
Langsat	2.45	1	+	1
Guava	3.00	1	+	4
Bilimbi	2.43	29	+	6
Coconut Peel	3.25	1	+	6
Rosella	2.96	1	+	8

Table 1. Characterization of samples used for bacterial cellulose nanofibers AAB isolation (Cont.)

Sources/Samples	pH	Fermentation time of sample (months)	BC production	Total isolates
Betal nut peel	2.87	1	+	1
Marigold	3.12	1	+	5
Banana Peel	2.20	28	+	5

Determination of bacteria cellulose production

Fifty-seven isolates were produced bacteria cellulose to the surface of HS medium. However, bacteria cellulose producing AAB (Figure 1.) were found to be high in Mixed of fruits sample 10 isolates of SX1, SX2, SX3, SX4, SX5, SX6, SX7, SX8, SX9, and SX10, Rosella sample 5 isolates of RJ1, RJ2, RJ3, RJ4, and RJ5, and Coconut Peel sample 5 isolates of RC1, RC2, RC3, RC4, and RC5.



Figure 1. Colony morphology of bacterial cellulose producing AAB, (a); colonies on HS agar, (b); surface of colonies under stereo microscope, and (c); Gram strain of bacterial cellulose producing AAB.

Effect of acetic acid on bacterial cellulose production

To evaluate the effect of acetic acid concentration on bacterial cellulose production. Among 39 isolates were grown and produced bacteria cellulose at 1-2% acetic acid concentration, at least 4 isolates: RC1, RC3, RJ1, and RG2 could grow and produce high thickness of bacteria cellulose of 9-11 mm at 1% (v/v) of acetic acid. The total bacteria number showed 6.02, 7.27, 7.20, and 7.48 log CFU/ml, respectively. The surface colors of BC of all isolates were yellow. Moreover, at 2% (v/v) of acetic acid, isolates RC3 and CB1 were produced bacteria cellulose with 6 and 9 mm of thickness and total bacteria counts were 6.45 and 5.64 log CFU/ml, respectively (Table 2). The morphology of BC showed that BC fibril networks were dense and contained

large amount of aggregated of bacterial cell between nanofibrils (Figure 2). According to the size of crystallite of isolates RC1, RC3, RJ1, and RG2 were observed at peak around $2\theta = 14.8^\circ$, 16.4° , and 22.58° and calculated size as 4.10, 4.95, 4.38, and 4.91 nm, respectively (Figure 3).

Table 2. Effect of acetic acid on bacterial cellulose nanofibrils production

Isolate	Acetic acid concentration					
	0%		1%		2%	
	Thickness (mm)	Total bacteria (log CFU/ml)	Thickness (mm)	Total bacteria (log CFU/ml)	Thickness (mm)	Total bacteria (log CFU/ml)
C1	4	6.67	11	6.02	-	-
RC3	3	6.27	11	7.27	6	6.45
RJ1	3	6.22	11	7.20	-	-
RG2	3	6.15	11	7.48	-	-
CB1	5	5.66	9	5.85	9	5.64

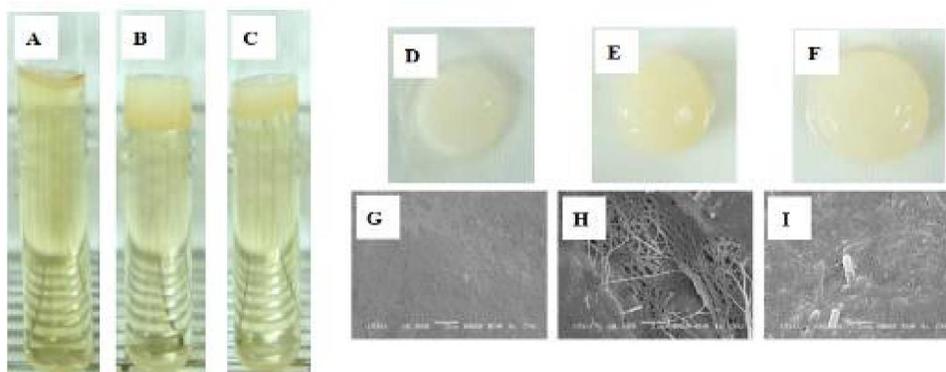


Figure 2. Bacterial cellulose production of the isolate RC3. A: BC production at 0% acetic acid; B: BC production at 1% (v/v) acetic acid; C: BC production at 2% acetic acid. D, E, F: surface and surface color of BC. G, H, and I: BC nanofibrils structure from SEM.

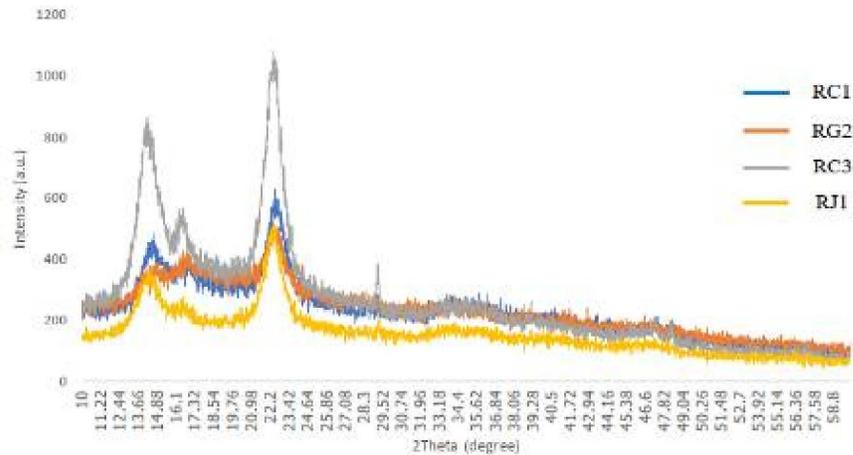


Figure 3. X-ray diffraction analysis of bacterial cellulose Series1 Series2 Series3 Series4 produced by isolates RC1, RC3, RG2, and RJ1.

Identification of cellulose producing AAB by DNA analysis

The 16S rRNA sequences of the isolates RC1, RC3, RG2, and RJ1 had length of 1028, 1316, 1326, and 1252 bp, respectively. The phylogenetic tree was constructed using 16S rRNA gene sequence of the isolates RJ1, RC3, and RC1 suggesting that RJ1, RC3, RC1 were *Komagataeibacter hansenii* NBRC14820 with 99.92%, 99.77%, and 99.71% similarity, respectively. And isolate RG2 was closely related to *Komagataeibacter maltaceti* LGM1529 with 99.68% similarity. (Figure 4).

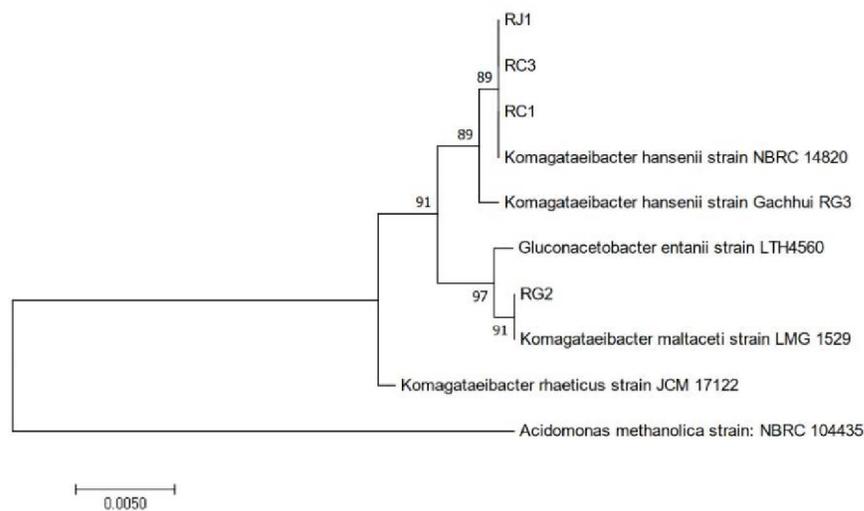


Figure 4. Phylogenetic tree constructed based on 16S rRNA sequences.

DISCUSSION

In the present study, we investigated isolation of bacterial cellulose producing AAB of the strains *Komagataeibacter hansenii* and *K. Maltaceti* from fermented fruit juices, fermented herb juices, and commercially produced Kombucha tea culture. The family of *Acetobacteraceae* and the genus *Komagataeibacter*, which were formerly known as *Gluconacetobacter* (Yamada et al., 2012) was produced the highest of bacteria cellulose as bacterial cellulose producers. Different strains of acetic acid bacteria can synthesize bacterial cellulose. The reported strains of *K. Xylinus* (Soemphel et al., 2018; Volova et al., 2018) *G. Intermedius* (Tyagi and Suresh, 2016) *K. Rhaeticus* and *K. Hansenii* were isolated from Kombucha tea and *K. Maltaceti* was isolated from vinegar (Rozenberga et al., 2016; Machado et al., 2018). Genus of *Komagataeibacter* is dominant genus in bacterial cellulose production.

The supplementation of 1% (v/v) acetic acid to the HS medium increased of bacterial cellulose production. As Toda et al. (1997) found that the bacterial cellulose production increases 4 times in Glucose Yeast Peptone medium with 2% (v/v) concentration of acetic acid. However, 1% (v/v) concentration of acetic acid in HS medium, increases 1.5 times of bacterial cellulose production (Rani and Appaiah, 2013).

The intensity of X-ray diffraction was related to the size of crystallite and the position of the diffraction peak was related to the crystal form. Bacterial cellulose was showed three signature peaks at the $2\theta= 14.7^\circ$, 16.2° , and 22.4° (Gea et al., 2011; Yim et al., 2017).

CONCLUSION

In this study, bacterial cellulose producing AAB were isolated and identified from fermented fruit juices, fermented herb juices, and commercially produced Kombucha tea culture. It showed that 1% (v/v) of acetic acid in HS medium was the best for producing bacterial cellulose. The morphology and nanofibrils were investigated by SEM and XRD, The BC fibrils were dense and show a large amount of aggregated of bacterial cell between nanofibrils. However, crystallite size obtained was calculated to indicated bacterial cellulose nanofiber size as 4.56 nm. According to the nucleotide sequences of 16S rRNA, isolates RC1, RC3 and RJ1 were identified to *K. Hansenii*. And isolate RG2 was identified to *K. Maltaceti*. Finally, the family of

Acetobacteraceae is suggesting for bacterial cellulose nanofibers production and will be used in many industries applications.

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