International Journal on Advanced Science Engineering Information Technology

Vol.8 (2018) No. 4-2
ISSN: 2088-5334

Isolation and Identification of Bacteria-producing Cellulose from Tropical Fruit for Halal Capsule Application

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Abstract*—Bacterial cellulose (BC) is pure cellulose synthesized by various species of bacteria. Raising demands on bacterial cellulose is due to its pure and simpler structure. It has plenty of applications in various industries such as food, medical and cosmetics, rendering it a choice in halal industry application as a substitute for non-halal gelatine. However, challenges arise during the BC production such as high production cost and low volumetric yield. In this study, BC is studied to overcome the barriers to BC production. Isolation and identification of cellulose producing bacteria were carried out on eight different tropical fruit sources using Hestrin-Schramm media in static culture condition. Morphological and molecular identification by microscopic observation, gram staining, and 16S rRNA analysis were conducted to identify the characteristics and strain of the new isolates. Next, the selected colonies were challenged to grow in agitating condition using modified HS media. The effects of carbon concentration and agitation speed on the production of cellulose were investigated using on central composite design (CCD). Three new cellulose producing bacteria were successfully isolated and identified to be similar to *Enterobacter sp*. SJZ-5, *Bacterium sp* NLAE-zl-H356, and *Bulkhoderia sp.* RD_DACAR_02 through morphological and molecular analysis. The most potent strain which is similar to *Enterobacter sp*. SJZ-5 (named as *Enterobacter sp*.M003) has been chosen for BC optimization study for high BC production using modified HS media. Optimization of bacterial cellulose production using response surface methodology (RSM) with 13 runs indicated that the optimal production parameters were 17.5 g/L for carbon concentration at 277 rpm for agitation speed gave 1.7g/L cellulose. It is expected that the newly isolated bacteria will be able to provide an alternative to gelatine for halal capsule production, thus minimizing and replacing non-halal gelatine usage.

***Keywords*—bacterial cellulose; *Enterobacter sp*.M003; gelatine; halal.**

I. INTRODUCTION

Cellulose with a molecular formula of $(C_6H_{10}O_5)n$ is synthesized by many types of plants, algae, some species of bacteria and several types of animals such as tunicates [1]. However, cellulose is normally derived from two types of sources, which are plants and bacteria. This present study focused on bacterial cellulose due to its unique properties such as high purity, crystallinity, water-holding capacity, degree of polymerization and mechanical strength [2]. The complex structure and process of plant-based cellulose production make it less favorable in the organic chemical industry. Researchers nowadays are more attracted in isolating the bacterial cellulose instead of plant cellulose. The bacteria-producing cellulose have been isolated from different potential sources such as fruits, vegetables, flowers, vinegar and fermented drinks [3]. In particular, tropical fruits have a big potential to be further developed and commercialized for production of bacteria cellulose due to their availability and low cost. There are various potential applications of bacterial cellulose in different fields such as food additives, production of paper, biomaterials in medical fields and electrical instrument [4]. Latest finding by Ullah and co-workers showed that bacterial cellulose (BC), having both immediate and sustained drug release properties; is a promising material for alternative gelatin capsules [5]. According to Gelatin Manufacturers of Europe [6], gelatin is made mostly from pigskin where about 80% gelatin in the market is extracted from pigskin, 15% from cattle hide split, and the remaining 5% comes from pig and cattle bones,

poultry and fish. Gelatine is an important and preferred raw material in the capsulation of medicine or supplement due to its cheap production cost, toughness and low breaking rates. Observation on the demands for gelatine in pharmaceutical industry alarms the Muslim and vegetarian on the need to identify an alternative source of material. Bacterial cellulose that is sustainable, from halal sources [7] and cheap would be the option.

The sources of bacterial cellulose are from various species of bacteria belonging to genera Gluconacetobacter (formerly Acetobacter), Aerobacter, Agrobacterium, Azotobacter, Rhizobium, Sarcina, Pseudomonas, Salmonella, Rhodococcus and Achromobacter [6]. This study involves isolation, identification, and production of BC from newly isolated bacteria from tropical fruits in Malaysia. Following static cultivation, agitated culture with modified Hestrin-Schramm medium was used as alternative to enhance yield of BC.

II. Material and Method

A. Culture Media

Hestrin-Schramm (HS) medium (consisted of 2.0 % glucose as carbon source and 0.5% yeast extract, 0.5% peptone, 0.12% citric acid, 0.27% disodium hydrogen phosphate and 1.5% agar) at pH 6.0 was used for this study. Fructose was used to replace glucose in modified HS-medium to observe the suitability of fruit juice as a component in the growth media.

B. Isolation of Cellulose Producing Bacteria

The juices from tropical fruits such as pineapple, papaya, banana, mango, guava, watermelon, and mangosteen were separated from residues and filtered, then diluted to 10^{-1} to 10^{-5}. Samples were labelled with ID as mentioned in Table 1. Next, 100 µl of each sample was inoculated on standard HS medium agar pH 6 and incubated at 37˚C for 2 days. The colonies formed were isolated and purified by repeated streaking onto new agar plates. Then, the single colony formed was inoculated in a standard HS fermentation broth grown at 30 ˚C for 7 days without agitation. The positive isolate was observed for a white pellicle ring on the surface of the liquid medium.

Next, positive samples producing white pellicle ring isolates were challenged with agitated culture condition by incubating in HS fermentation broth an agitated condition gradually from 50 rpm until 150 rpm at 30 ℃ for 3 days. The samples that failed to produce white pellicles were directly discarded.

Finally, the sample that produced the similar white pellicle ring was chosen for further identification and stored in 50% glycerol at -80◦C. The flask with thick pellicle denominated 003 was further analyzed for growth optimization process.

C. Morphology and Molecular Identification of BC Producing Bacterial Strains

The morphology of isolates was identified by performing gram staining, colony morphology, and microscopic observation.

Genomic DNA was isolated using innuPREP DNA Micro Kit (Analytikjena, AG) according to the manufacturer's instruction. The 16S rRNA was amplified using universal primers. Forward primer 27F: 5' AGAGTTTGATCMTGGCTCAG 3' and reverse primer 1492R: 5' TACGGTTACCTTGTTACGACTT 3'. Polymerase Chain Reaction (PCR) was performed in 50 µl reaction volumes containing 0.2 µM forward and reverse primers, 2 µL of extracted DNA samples, 0.5 X PCR master mix, and sterile distilled water. PCR was performed using gradient master cycler (Eppendorf, Germany) under the following conditions: 94℃ for 30 seconds, (94℃ for 20 seconds, 53℃ for 1 minute and 68℃ for 1 minute) for 35 cycles, and finally extension reaction 68℃ for 5 minutes.

The amplified DNA was sent to 1st Base Laboratories (Sri Kembangan, Selangor) for sequencing analysis. Sequences were analysed by searching the similarity in Gene bank via BLAST algorithm. The phylogenetic tree was constructed using the neighbour-joining method.

D. Optimization of Bacteria Cellulose Production

The positive isolate was the one that was successfully isolated, grown in modified HS media, and agitated This strain was grown in modified HS media by replacing glucose with fructose as the carbon source.

In this experiment, the constant variables were period of incubation time (3 days) and the carbon source (fructose). Response Surface Methodology (RSM) was chosen to study the effect of fructose concentration and aeration rate on bacterial cellulose production. Central Composite Design (CCD) with five levels was generated using the statistical analysis software; Minitab 18 (State College, Pennsylvania). The samples were incubated for three days, and the yield of BC (g/L) was measured. All data were analysed with analysis of variance (ANOVA).

E. Purification of BC

The pellicle was separated from residual medium and other contaminants. After that, they were boiled with 2.0% NaOH solution for 30 minutes at 80◦C and washed thoroughly with distilled water. Next, the pellicles were neutralized with 4% acetic acid, followed by repeated washing with distilled water. Finally, the pellicles were dried at room temperature, and then the dried yield was measured as follows:

$$The\ yield\ of\ dried\ BC = \frac{Total\ weigth\ of\ sample\ and\ filter\ (g) - Weight\ of\ filter\ (g)}{volume\ used\ (mL)} \quad (1)$$

Dried white pellicle from isolate number 003 was later characterized for cellulose by Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM).

F. FTIR

The dried pellicle was sent to laboratories for FTIR analysis. FTIR spectrum of BC pellicles was recorded in the spectral range of 4000 – 400 cm^{-1} at a resolution of 4 cm^{-1}.

G. Scanning Electron Microscopy (SEM)

The structure of BC was observed by SEM operating at 15.0 kV. The dried pellicle was dried and gold coated for 90 s. The micrographs were acquired at a magnification of 10,000 X.

III. RESULTS AND DISCUSSION

All fruit samples showed positive results based on the formation of white colonies after spreading and streaking on Hestrin-Schramm agar (Fig. 1). The white colonies were further tested. Those that were able to form white pellicle using agitate culture method and on modified HS media were also tested in optimization experiment. After a thorough screening, all bacteria colonies isolated from all the fruit samples tested can produce cellulose as a large ring adhering to the wall of the flask but sank inside the modified HS media in the static culture except for sample with ID number 003. The isolate number 003 showed a thick white pellicle floating on the culture medium (Fig. 2). It is chosen for the further experiment because floating pellicle shall minimize the steps in isolating cellulose in the manufacturing plant. In modified HS media, fructose (fruit sugar) was used to replace glucose as a carbon source in line with by Kurosumi and co-workers [7] who reported the possibility of bacterial cellulose production from fruit wastes.

A. Identification of BC Producing Bacterial Strains

Through sequencing and BLAST analysis, the isolate 003 has high similarity with *Enterobacter sp.* SJZ-5, therefore it was named *Enterobacter sp.* M003.The summary of findings for morphology, colony and molecular identification are tabulated in Table 1. The isolates from guava, watermelon, and papaya were similar to Bacterium NLAE-zl-H356, and *Burkholderia sp*., respectively (Table 2).

Further, a phylogenetic tree was constructed using the neighbor-joining method to identify the relatedness of strain with the species thus supported the results from BLAST analysis (Fig.3*). Burkholderia sp*.; isolated from the watermelon and papaya were not chosen for further experiments due to it being pathogenic. Burkholderia is a genus of proteobacteria that refers to a group of gram-negative, obligate aerobic and rod-shaped bacteria [8]. Several members of this genus are prominent opportunistic pathogens where *B. mallei and B. pseudomallei. B. cepacia, B. mallei, B. pseudomallei, B. cenocepacia,* and *B. pseudomallei* can cause cystic fibrosis and are very challenging to treat [8]. Therefore, the subsequent study only focuses on isolate with ID number 003 in which isolate number 003 from mangosteen was identified as *Enterobacter sp.* M003 is a facultative anaerobic and gram-negative bacillus. Enterobacter is a genus of Enterobacteriaceae, and several strains of these bacteria are pathogenic and can cause opportunistic infections to a host with a weakened immune system [9]. However, this genus is less dangerous compared to the Burkholderia genus.

Furthermore, based on our observation *Enterobacter sp*. M003 produced the highest amount of cellulose compared to other isolates.

Meanwhile isolates 007 and 008 were identified as multidrug-resistant bacteria; therefore they were not chosen for the further experiments.

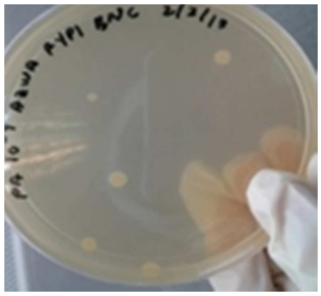

Fig. 1 White colonies of cellulose producing bacteria

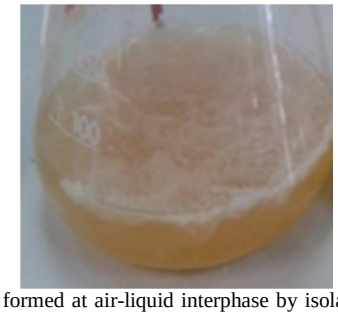

Fig. 2 The pellicle formed at air-liquid interphase by isolate *Enterobacter sp*. SJZ-5

TABLE I
PHYSIOLOGICAL IDENTIFICATION OF BACTERIA PRODUCING CELLULOSE

Isolate	**Pellicle in static culture**	**Pellicle in agitate culture**	**Structure of bacteria**	**Gram**	**Structure of colony**
001 (Apple vinegar-as control)	√	√	Coccus	-ve	Round, white
002 (Pineapple)	√	√	Coccus	-ve	Round, white, smaller
003 (Mangosteen)	√	√	Bacillus	-ve	Round, white
004 (Mango)	√	√	Bacillus	-ve	Round, white
005 (Banana)	√	√	Coccus	-ve	Round, white
006 (Guava)	√	√	Bacillus	-ve	Round, white
007 (Watermelon)	√	√	Bacillus	-ve	Round, white
008 (Papaya)	√	√	Bacillus	-ve	Round, white, smaller

TABLE II
FINAL SUMMARY OF THE BACTERIA IDENTIFICATION ANALYSIS BY MOLECULAR IDENTIFICATION

Sample	Sources	Closest relatives	% ID	E value	Query Cover (%)	Accession Number
003	Mangosteen	*Enterobacter sp. SJZ-5*	81	0.0	31	LC014954.10
006	Guava	*Bacterium NLAE-zl-H356*	97	0.0	92	JX006570.1
007	Watermelon	*Burkholderia sp.*	97	0.0	93	KU597495.1
008	Papaya	*Burkholderia sp.*	97	0.0	99	KU597495.1

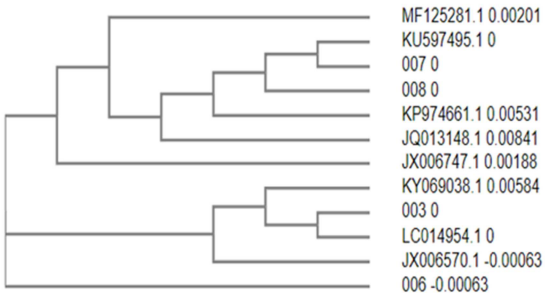

Fig. 3 Phylogenetic tree of isolate 003,006,007,008 by the neighbour-joining method, inferred from 16S rRNA gene sequence analysis

B. Optimization of Bacterial Cellulose Production

Table 3 shows the experimental design and result from the optimization experiment. Based on the results it is shown that 10 g/L fructose and agitation speed at 250 rpm gave the highest cellulose yield.

TABLE III
A SET OF 13 RUN EXPERIMENTS USING CCD

Run	Carbon concentration (g/L)	Agitation speed (rpm)	Yield (g/L)
1	20	150	1.12
2	20	150	1.22
3	10	50	0.48
4	20	9	0.0
5	20	150	1.23
6	30	250	1.12
7	20	150	1.55
8	6	150	0.55
9	34	150	1.18
10	20	290	1.68
11	30	50	0.16
12	10	250	1.75
13	20	150	1.52

Analysis of variance (ANOVA) was employed to find the significant parameters and estimate conditions of production of bacterial cellulose. F-value of 7.91 in ANOVA represents the accuracy of the model and p-value indicated the interaction between the significant model terms. A significant p-value must be below than 0.05 while p-value with greater than 0.10 is considered as an insignificant model. Also, the coefficient of regression (R2), adjusted R2 and predicted R2 indicate the quality fit of the polynomial model equation.

Table 4 summarised ANOVA result of a quadratic model where yield of BC is the response being measured. Based on Table 4, the F-value of this model is 7.91 and the p-value is 0.008 (<0.05), which implies a significant model. Meanwhile, the non-significant value of 0.073 for lack of fit showed that the quadratic model fitted and is valid for the present study. R^2 and adjusted R^2 were 0.8496 and 0.7423, respectively. This suggests that the model could explain 84.96 % variability in response and this model is good enough as R^2 above 0.6 is considered worthwhile. This value must not exceed one and not less than one in the determination of a successful experiment. The total variability that was not explained by regression in this model is 15.04 %. Fig. 3 visualized the relationship between the responses and experimental levels of each parameter. The highest yield obtained was 1.7 g/L. The optimal parameters are 17.57 g/L for carbon concentration in 277 rpm for the aeration rate.

Optimization results showed that a high amount of carbon sources did not increase the amount of cellulose. This observation may be due to the excess of carbon sources which is metabolized to other substances via gluconic acid pathway [10]. Result also showed that high agitation speed led to high cellulose production. *Enterobacter* sp is a facultative anaerobic bacterium as such they do not use oxygen directly in the synthesis of cellulose production. However, energy is required to activate the intermediate metabolites and synthesize the activator, c-di-GMP, which is the activator of BC biosynthesis [11]. Therefore, a high agitation rate shall produce more cellulose from this strain.

TABLE IV
ANALYSIS OF THE VARIANCE OF A QUADRATIC MODEL FOR A YIELD OF BC

Source	Sum of squares	F-Value	P-Value
Model	3.33691	7.91	0.008
A-carbon concentration	0.00060	0.01	0.935
B- aeration rate	2.66329	31.57	0.001
A^2	0.34964	4.14	0.081
B^2	0.38561	4.57	0.070
AB	0.02250	0.27	0.621
Lack-of-Fit	0.43252	3.65	0.122
R^2 : 0.8496 Adjusted R^2: 0.7423 Predicted R^2: 0.1540	Standard Deviation : 0.29		

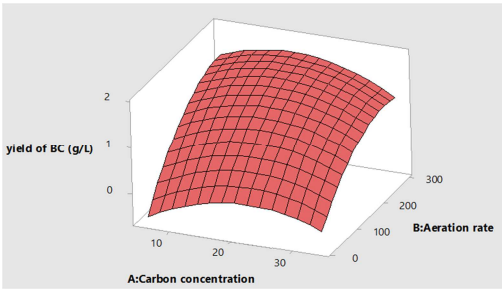

Fig. 4 The 3-Dimensional surface plot showing the interaction between carbon sources concentration and aeration rate leading to yield of BC.

C. Characterization of Bacterial Cellulose

Fig.5 shows the compact structure of cellulose obtained following air-drying method used in this study. It is expected that freeze drying could retain the structure, and the grain appearance of BC whereby moisture in gelatinous membrane would turn into ice and then transform into gas in vacuous circumstances [12].

Also, TEM is preferable to observe bacterial cellulose structure because it can show the nano whiskers structure of cellulose. TEM has up to 50 million magnification level while SEM only up to 2 million level of magnification. Moreover, TEM seeks to observe inside the surface [13].

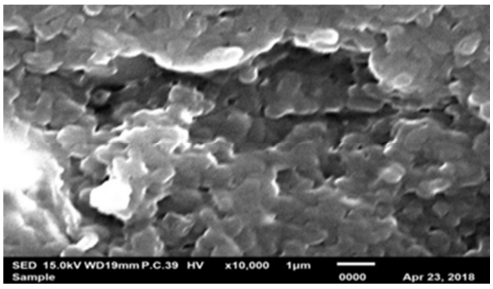

Fig. 5 SEM of cellulose pellicle from *Enterobacter sp*. M003

Comparison of FTIR results between bacterial cellulose (BC) and microcrystalline cellulose (MCC) was carried out to validate the structure of BC. Figures 6 and 7 visualized the absorbance peak at 3277.757 cm^{-1} which is due to OH-stretching vibrations. The spectra also showed absorbance peaks at 2924.03 cm^{-1} and 2358.92 cm^{-1} corresponding to the C-H stretching vibration. Besides, the bands between 1500-1235 cm^{-1} represent bacterial cellulose due to the in-plane vibration of CH_2, CH, OH groups [14]. This statement supported the result in Fig. 6 and Fig. 7 which show that several absorbance peaks between 1500 and 1235 cm^{-1}. MCC has a sharp peak of 1058.06 cm^{-1} and moves towards a long wave that represents the CO-H stretching vibrations due to the looser structure and longer distance between cellulose macromolecules for MCC.

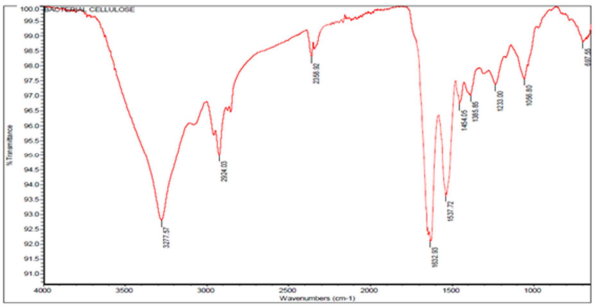

Fig. 6 FTIR spectra for bacterial cellulose (BC CM^{-1})

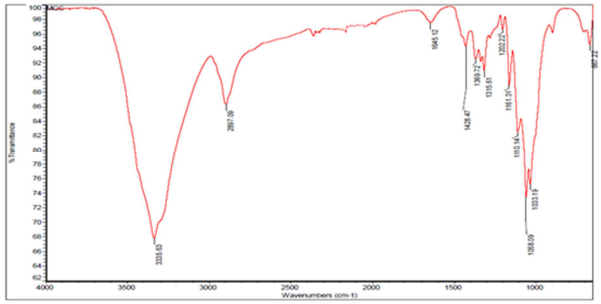

Fig. 7 FTIR spectra for microcrystalline cellulose as the positive control (MCC CM^{-1})

In contrast, BC did not show a sharp peak because of its compacted structure. The results showed FTIR spectra, and absorbance peaks between the two samples were similar except for some parts. This result occurred due to the breaking of some chemical bond thus decreasing the absorbance peak and shifting the absorbance peak to lower or higher value [15].

Hence, according to the results obtained, it was proven that the bacterial cellulose from *Enterobacter sp*. M003 is true bacterial cellulose. The comparison of absorbance peaks from FTIR analysis for both samples was tabulated in Table 5.

TABLE V
COMPARISON OF ABSORBANCE PEAKS FOR BC AND MCC

Absorbance peaks (cm^{-1})	**Bacterial Cellulose (BC)**	**Microcrystalline Cellulose (MCC)**
	3277.57	3335.63
	2924.03 2358.92	2897.09
	1632.93 1537.72 1454.05 1385.85 1233.00 1056.80	1645.12 1428.47 1361.72 1315.61 1202.22 1161.31 1110.14 1058.09 1033.19
	697.53	667.22

IV. CONCLUSIONS

The present study reported the isolation, identification and production process optimization of BC from selected tropical fruits. The isolates were identified as *Enterobacter sp*. SJZ-5, *Bacterium* NLAE-zl-H356, and *Burkholderia sp*. The highest yield obtained was 1.7 g/L. The optimal parameters when *Enterobacter sp*. M003 was used to investigate the optimum production process were 17.57 g/L for carbon source concentration at 277 rpm aeration rate. The result suggested that *Enterobacter sp*. M003 can produce BC, under agitation conditions, and fructose is a suitable carbon source to replace glucose for the BC synthesis. Therefore, *Enterobacter sp*. M003 is a suitable strain for high BC production scale in the bioreactor.

ACKNOWLEDGMENT

We would like to thank the Kulliyyah of Engineering, International Islamic University Malaysia for providing the laboratory facilities for this project.

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