Characterization of Bionanocellulose Producing Bacteria Isolated from Tapioca Wastewater

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***Abstract*—This study was conducted to explore the potential of isolating bionanocellulose (BNC) producing bacteria from tapioca wastewater. A total of ten bacteria were successfully isolated and only one isolate named BPNC 3 produced white gelatinous materials in Hestrin and Schramm (HS) medium believed to be the BNC. According to 16S rRNA analysis, bacterium BPNC 3 was identified as *Asaia* sp. The BNC produced by *Asaia* sp. BPNC3 was characterized via Fourier transformed-infrared spectroscopy (FTIR) and Field-emission scanning electron microscope (FESEM). The FTIR spectrum showed the presence of two signature peaks at 3276.69 cm^{-1} and 2923.99 cm^{-1} indicative of nanocellulosic material. The FESEM micrograph showed characteristics of network fibrils typically present in nanocellulose structure.**

Keywords*— *Bionanocellulose; Acetic Acid Bacteria; Tapioca Wastewater; Asaia sp.

I. INTRODUCTION

Nanocellulose is a promising material that have multifunctional application in broad range of industries such as medical, cosmetics, pharmaceutical, bioremediation and biotechnology. This emerging and developing nanotechnology could provide interesting natural polymers that could become a novel and advanced material especially in nano- form [1]. There are three categories of nanocellulose which includes cellulose nanocrystal (CNC), cellulose nanofibrils (CNF) and bacterial nanocellulose (BNC). Bacterial nanocellulose (BNC) that are synthesized by the acetic acid bacteria (AAB) will be the focus of this study due to its appealing characteristics such as high degree of polymerization, high purity, good biocompatibility, biodegradability, high crystallinity and excellent mechanical properties [2] in broad-range of industrial application.

Acetic acid bacteria (AAB) are usually involved in the fermentation process where the BNC is one of the specific products produced from their metabolic activities. There are various bacteria that have been identified for their ability to produce BNC such as *Acetobacter, Gluconobacter, Komagataeibacter, Enterobacter, Rhodoccus* and *Sarcina* that are frequently found in fermented and food-based product such as alcohol, vinegar, fruit juices and Nata de Coco [3]. Moreover, BNC production is eco-friendly. The production also less time-consuming process since bacteria occur naturally in a pure form thus no extensive process is needed in order to remove impurities or contaminants such as lignin, pectin and hemicellulose like plant-derived cellulose [4].

The application of BNC serves a broad-range of industrial applications including medical, pharmaceutical, cosmetics, textiles and foods industries. The example of application in the food industry including sauces and gravies; frostings and icings; sour cream and cultured dairy products; whipped toppings and aerated desserts, and frozen dairy products. For medical application, BNC have been studied extensively as a wound care dressing where BNC have been found able to heal skin wounds. Based on the following application, the BNC have become fascinating advanced materials to be used in industries as they can produce good nanocellulose in high-scale of production [5]. This study explored the possibility of isolating new BNC-producing bacterial strains from samples containing high content of starch and fibres such as tapioca wastewater.

II. MATERIALS AND METHODS

A. Culture Media

Hestrin-Schramm (HS) medium was used in this study which consists of 20g/L glucose, 5g/L peptone, 5g/L yeast extract, 2.7g/L disodium hydrogen phosphate and 1.15g/L citric acid. The pH of the media was adjusted to 6.0-7.0 with 1M sodium hydroxide (NaOH).

B. Isolation and Screening of BNC Producing Bacteria

The wastewater samples were collected from nearby tapioca chip manufacturer. The samples were serially diluted to 10^{-1} to 10^{-9} with saline solution. 100µl of each diluted

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sample were spread onto HS agar and incubated at 30°C for 3-5 days. After the incubation period, the colonies formed were restreaked onto fresh HS agar to obtain single pure colonies. The single pure colonies were inoculated into HS broth for 3-5 days at 30°C under static condition for the BNC screening. Isolates producing white gelatinous materials on the surface of the liquid media were considered as positive samples for BNC production.

C. Characterization and Identification of BNC Producing Bacteria

The successfully screened BNC producing bacteria were characterized to determine the genus and species of bacteria. Gram staining and biochemical test such as oxidase ad catalase test were done to observe for their taxanomic characteristics. Molecular characterization via 16s rRNA was done for identification of the bacteria.

D. Purification of BNC

Bionanocellulose produced was by the selected bacteria were separated from the culture medium by centrifugation at 3300xg for 15 min and washed with distilled water. The pellicle and suspended bacterial cells were treated with 0.1 M NaOH at 90°C for 3h in order to discard the attached cells followed by repeated washings with distilled water until pH became neutral. The purified BNC was filtered using Whatman filter paper and was oven dried at 60°C until constant weight. The dried BNC was later characterized via Fourier-Transform Infrared (FTIR) and Field Emission Scanning Microscopy (FESEM).

E. FTIR

The dried BNC was analysed using FTIR. Absorption spectra were collected at wave numbers ranging from 600-4000 cm^{-1} at resolution of $4cm^{-1}$ with an average 32 scans.

F. FESEM

The BNC sample was placed on copper stubs and sputter-coated with a platinum layer. The morphology of coated BNC sample was viewed using FESEM under 30,000 and 60,000 magnification.

III. Results and Discussion

A. Isolation and Sreening of BNC Producing Bacteria

A total of 10 colonies were isolated from mix culture (i.e. spread plate) after each colony was individually streaked onto fresh HS agar to obtain pure culture. A total of 6 isolates were successfully isolated from tapioca wastewater, 2 from the cloudy greyish sludge and 2 from yellowish sludge sample. The 10 isolated bacteria were characterized according to their colony morphologies. The Gram staining procedure was also done to determine their cellular shape and Gram staining reaction using light microscopy under 1000x magnification. Table 1 summarises the colony morphologies of ten isolates obtained and the Gram staining reactions. The 10 isolated pure colonies obtained were further screened for nanocellulose production using HS medium and the production of BNC by were shown in Table 2. Only bacterium BPNC 3 produced bacteria nanocellulose (BNC) under static condition after 3-5 days at 30°C (Figure 1).

Table 1 Colony morphology and Gram staining reaction of the isolates.

Isolates	Gram Stain Reaction	Colony morphology			
BPNC 1	+ve oval	Shiny Milky White	Circular	Raised	Entire
BPNC 2	+ve oval	Opaque Milky White	Circular	Raised	Entire
BPNC 3	-ve Rods	Brown	Circular	Raised	Entire
BPNC 4	+ve oval	Shiny Milky White	Irregular	Flat	Undulate
BPNC 5	+ve rods	Brown	Circular	Flat	Entire
BPNC 6	+ve cocci	Brownish	Circular	Flat	Entire
BPNC 7	-ve rods	Opaque Milky White	Circular	Raised	Entire
BPNC8	+ve rods	White	Root-like feature	Flat	Undulate
BPNC 9	-ve cocci	Transparent	Circular	Flat	Entire
BPNC 10	-ve cods	Transparent	Circular	Flat	Entire

Table 2 The production of BNC by each of the ten isolates

Isolates	BNC Prodution
BPNC 1	Absent
BPNC 2	Absent
BPNC 3	Present
BPNC 4	Absent
BPNC 5	Absent
BPNC 6	Absent
BPNC 7	Absent
BPNC 8	Absent
BPNC 9	Absent
BPNC 10	Absent

Figure 1 The BNC produced by BPNC 3 under static condition at 30°C after 3-5 days.

B. Morphological Characterization of Bacterium BPNC 3

The colony morphology of the selected bacterium BPNC 3 was determined based on its colour, size, texture, surface, edge and elevation. The bacterium is Gram negative rod shaped (Figure 2). Interestingly, bacterium BPNC 3 also revealed other unique characteristics whereby the colonies changed colour from brown to milky white and to pink after being stored at 4^{O}C as shown in Figure 3. Pigments can be produced by some bacteria at lower temperature to act as a mechanism to survive ecological stresses. Since cellular metabolic pathways such as glycolysis, tricarboxylic acid cycle, electron transport chain and pentose phosphate pathway are down regulated, alternative secondary pathways such as production of pigments can be used as an energy source [15].

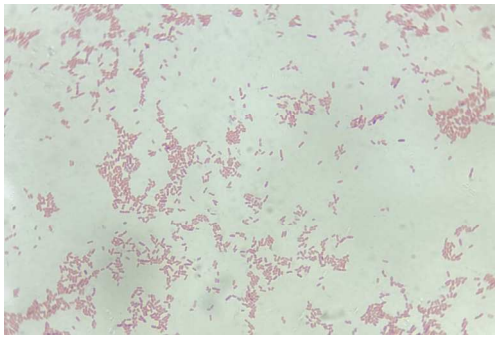

Figure 2 Gram stain of bacterium BPNC 3 showing Gram negative rods under light microscope at 1000x magnification

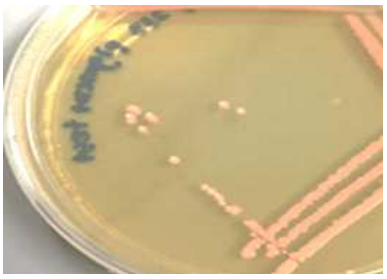

Figure 3 The colour change of bacterium BPNC 3 after being stored for 6 days at 4°C

C. Biochemical Characterization of Bacterium BPNC 3

Two biochemical tests were carried out on bacterium BPNC 3 which were oxidase and catalase test. BPNC 3 gave positive results for both tests. The findings indicated similar results/reaction as obtained in previous studies mainly on the screening and characterization of BNC producing bacteria [6, 7].

D. Molecular Identification of Selected BNC Producing Bacteria

Sequencing of the 16s rRNA gene and phylogenetic trees construction were carried out in order to identified selected bacterium BPNC 3. According to the phylogenetic analysis of 16S rRNA gene sequences (Figure 4), bacterium BPNC 3 was allocated to the cluster of genus *Asaia* and in broad family of *Acetobacteraceae* [8]. From the top ten Blast result against 16S ribosomal RNA sequences (Bacterium and Achaea) database, bacterium BPNC 3 obtained the highest similar percentages identity to *Asaia prunellae* with 99.86%, followed by *Asaia bogorensis and Asaia spathodeae* with 99.79%, *Asaia platycodi* and *Asaia siamensis* with 99.72%, Asaia *krungthepensis* with 99.65 and *Asaia lannensis* with 99.50% (Figure 4).

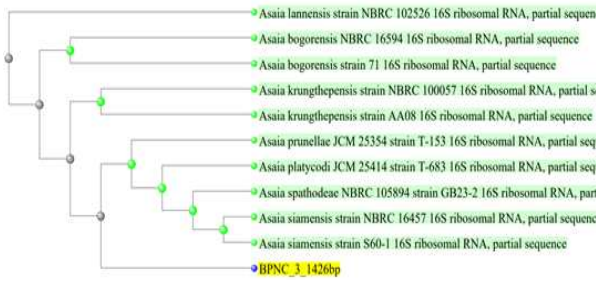

Figure 4 The phylogenetic tree-neighbour joining (unrooted tree) by NCBI Blast tree method.

E. Characterization of BNC produced by Asaia sp. BPNC 3

Figure 5 shows the BNC produced by isolate BPNC 3 showed distinctive and clear characteristics of the native cellulose due to the presence of fibrous network which consist of pores and tunnel. The presence of pores and tunnels structure explained the hydrophilicity properties of BNC membranes which mainly involved the presence of hydrogen and Van der Waals forces that causes the filaments to interact with each other to be kept separately by the adsorbed water layers [13]. This consequently resulted in the formation of cellulose network.

The peaks obtained from the absorption spectra of the BNC samples analysed with FTIR (Figure 6, Table 3) have disclosed the conformational characteristics of the pure form of cellulose, merely the BNC vibration peaks of cellulose type I with strong absorption peaks at 3276.69 cm^{-1} which assigned to the stretching of hydroxyl (OH) group [9]. In addition, this -OH stretching of cellulose I is associated with crystalline structure of native BNC produced by bacterial species [10]. Strong peaks were also acquired at 2923.99 cm^{-1} and 2854.62 cm^{-1} indicating stretching of C-H of methyl group present in the cellulose [11, 12]. These two strong peaks are considered among the typical bands found in pure native cellulose.

Furthermore, the peaks obtained at 1451.40 cm^{-1} and 1151.06 cm^{-1} represents the vibrational peaks corresponding to the CH_2 and stretching of C-O-C group of glycosidic bonds. Meanwhile, peaks at 1040.69 cm^{-1} and 1021.65 cm^{-1} indicates the bonds that exist in sugar ring that act as bridge between the sugar rings. Therefore, almost all bands in FTIR spectrum of the bacterial nanocellulose correlate with the bonding or functional groups in the chemical structure of the cellulose.

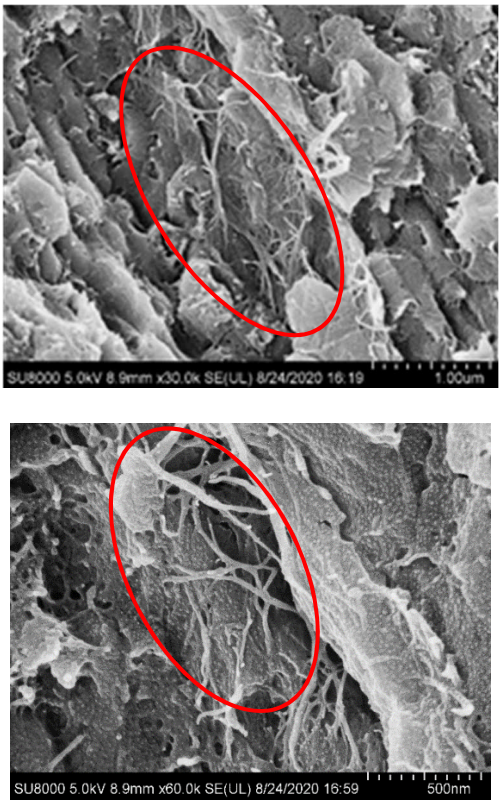

Figure 5 The FESEM micrograph of the fibrils structure of BNC at (top) 30000x (bottom) 60000 x magnification

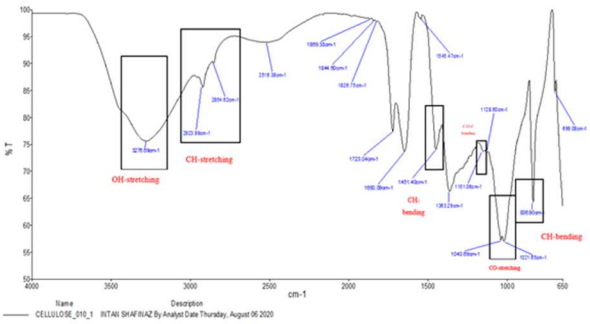

Figure 6 The FTIR spectrum of BNC produced by *Asaia sp.* BPNC 3

Table 3 Summary of FTIR bands assignment

Wavenumber peaks (cm-1)	Bonding/Functional group	Notes
3276.69	O-H stretching	▪ Broad and strong ▪ May come from cellulose or water molecules
2923.99 and 2854.62	C-H stretching	▪ Symmetric and asymmetric stretching of C-H for alkane (-C-H) ▪ Sugar ring
1723.04 and 1650.09	C=O	▪ Sharp and strong ▪ No C=O for cellulose
1451.40	CH_2 bending	
1363.29	CH bending	▪ May overlap with other bonding
1151.06	C-O-C stretching	
1040.69 and 1021.65	C-O stretching	▪ In sugar ring and bridging between the sugar ring
836.90	C-H bending	

IV. CONCLUSION

In conclusion, ten colonies of bacteria were successfully isolated from the tapioca wastewater. Among these ten isolates, only bacterium BPNC 3 was able to produce BNC after further screening using HS medium. The bacterium BPNC 3 has been identified as a species *Asaia* and in broad family of *Acetobacteraceae*. The FTIR analysis of BNC showed the presence of two signature peaks at 3276.69 cm^{-1} and 2923.99 cm^{-1} indicative of nanocellulosic material while the FESEM micrograph showed the existence of network fibrils within the BNC structures revealed distinctive characteristics of the native cellulose. Further work is in progress to optimize BNC production and study its potential use in bioremediation.

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