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Isolation and characteristics analysis of a novel high bacterial cellulose producing strain *Gluconacetobacter intermedius* CIs26

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ABSTRACT

A strain producing bacterial cellulose (BC) screened from rotten mandarin fruit was identified as *Gluconacetobacter intermedius* CIs26 by the examination of general taxonomical characteristics and 16S rDNA sequence analysis. Furthermore, Fourier transform infrared (FT-IR) spectrum showed that pellicle produced by strain CIs26 was composed of glucan, and had the same functional group as a typical BC. X-ray diffractometry (XRD) analysis indicated that the BC was type I in structure with crystallinity index of 75%. BC yields of strain CIs26 in Hestrin–Schramn (HS), citrus waste modified HS (CMHS) and citrus waste solution (CWS) mediums were 2.1 g/L, 5.7 g/L, and 7.2 g/L, respectively. It was shown that citrus waste could stimulate BC production of strain CIs26 efficiently. Based on the ability of utilization of citrus waste, this strain appeared to have potential in BC manufacture on an industrial scale.

1. Introduction

Cellulose is the most abundant biopolymer in the nature, and as well as one of the most important natural resources widely used in industry because of its availability, degradability and renewability (Vandamme, De Baets, & Steinbüchel, 2002). Although cellulose from plant is the most available source at present, cellulose from bacteria (named bacterial cellulose, BC) is expected to exhibit superior features over plant cellulose, such as extreme purity, high crystallinity, Young's modulus and water holding capacity, as well as excellent biological affinity and biodegradability (Shoda & Sugano, 2005). Unlike plant cellulose, BC is a relatively high purity biopolymer free of hemicellulose, lignin and other impurities, composed of β-D-glucoses through β-1,4-glycosidic linkages. BC produced by bacteria is initially extruded from the cell surface as microfibers and entangle together to form ribbons. Such a ribbon is very thin, with a width of only one-hundredth that of plant cellulose (Ross, Mayer, & Benzimann, 1991; Shoda & Sugano, 2005).

Many bacteria had been reported to produce extracellular cellulose, such as *Gluconacetobacter* (formerly *Acetobacter*), *Agrobacterium*, *Sarcina*, *Aerobacter* and *Achromobacter* (Ross et al., 1991). Among these, *Gluconacetobacter* is the most important genus, and *Gluconacetobacter xylinus* was investigated as the most efficient strain, with the high BC yield reaching to around 7–8 g/L in industrial scale (Hessea & Kondob, 2005; Keshk & Abu Haija, 2011; Toda, Asakura, Fukaya, Entani, & Kawmura, 1997; Tokoh, Takabe, Fujita, & Saiki, 1998; Uraki et al., 2007). Other reported *Gluconacetobacter* strains included *Gluconacetobacter hansenii* from rotten apple with BC production of 1.33 g/L in shaking condition (Jung, Park, & Chang, 2005; Park, Park, & Jung, 2003), *Acetobacter* sp. V6 producing 4.98 g/L BC in shaking flask (Jung et al., 2010), *Gluconacetobacter swingsii* from home made vinegar with BC yield of 2.8 g/L (Castro et al., 2011), and *Gluconacetobacter sacchari* from kombucha tea with BC yield of 2.7 g/L (Trovatti, Serafim, Freire, Silvestr, & Neto, 2011). It is interesting to note that *Gluconacetobacter kombuchae* sp. nov., a nitrogen-fixing bacterial strain that exhibited cellulose-producing ability even in nitrogen-free broth (Dutta & Gachhui, 2007). Though BC yields of previously reported strains were varied with culture media and conditions, most of them were lower than 5 g/L.

Citrus is one of the most important fruits in the world, and mandarin is still the main variety in China. About 30% peels and dregs waste are left in citrus juice industry at present, and most of them were used as feed ingredient or discarded immediately, which caused huge waste of natural resources. Orange juice had been proved to stimulate the efficiency of BC production (Basavaraj, Hungund, & Gupta, 2010; Kurosumi, Sasaki, Yamashita, & Nakamura, 2009). Mandarin dregs contain similar nutritional

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components with orange juice and could be served as the source substitute of BC. In this work, a cellulose producing bacterial strain from rotten mandarin fruit was isolated, and its morphological, physiological, biochemical and genic characteristics were investigated. The influence of citrus waste on BC yield and the corresponding cells and cellulose configuration was compared in Hestrin–Schramn (HS) and citrus waste solution (CWS) mediums.

2. Materials and methods

2.1. Materials

2.1.1. Rotten fruits and citrus waste solution preparation

Over ripen mandarin fruits were collected from citrus orchard in Wenzhou, China (main producing area of mandarin orange), and stored at 25 °C. Those rotten fruits with the odor of ethanol and acetic acid were selected for bacterial screening.

After the mandarin fruits were peeled and squeezed, the remained dregs waste was mixed with water at the ratio of 1:8 (w/w), including 150 U/ml pectinase and 50 U/ml cellulase (Imperial Jade Bio-technology Co., Ltd., China), hydrolyzed at 45 °C for 2 h. The samples were then filtered through a filter cloth (74 μm). The resulting filtrate was collected as citrus waste solution.

2.1.2. Culture medium

The screening medium contained 5 g yeast extract, 10 g glucose, 5 g $CaCO_3$, 17 g agar in 1 l citrus waste solution. The medium was mixed with 5% ethanol (v/v) before poured into petri dishes.

The Hestrin–Schramn (HS) medium was prepared according to Hestrin and Schramm (1954). Citrus waste modified HS (CMHS) medium was prepared as same as standard HS medium except that all the components were dissolved in citrus waste solution instead of distilled water.

The citrus waste solution (CWS) medium consisted of 5 g yeast extract, 5 g glucose, 30 g sucrose, 3 g $(NH_4)_2SO_4$, 2 g Na_2HPO_4 and 1 ml lactic acid in 1 l citrus waste solution, and adjusted pH to 5.5.

2.2. Methods

2.2.1. Microorganism isolation

Rotten fruits were cut into small pieces and 25 g mixed pieces were chose to put into conical flasks containing 225 ml sterile saline with glass beads. The flasks were shaken at 28 °C for 30 min. The flask samples were diluted stepwise with sterile saline. Then 0.1 ml appropriate dilution was spread onto the screening medium, cultured at 28 °C for 4 days. Those acid producing isolates were picked out and inoculated into CWS medium at 28 °C for 10 days. Finally, one strain producing thick cellulose-like pellicle was picked out for further analysis.

2.2.2. Identification of BC producing isolate

Morphological, physical and biochemical analyses were carried out according to Bergey's Manual of Systematic Bacteriology (Brenner, Krieg, Staley, & Garrity, 2004), The Prokaryotes: A Handbook on the Biology of Bacteria (Dworkin, Falkow, Rosenberg, Schleifer, & Stackebrandt, 2006) and other reports (Cleenwerk & De Vos, 2008; Yamada & Yukphan, 2008). The colony configuration, pigment formation, and cell morphology were evaluated through a microscope (Olympus ZX31, Olympus Corporation, Japan) and a transmission electron microscope (TEM, H-7650, Hitachi, Japan).

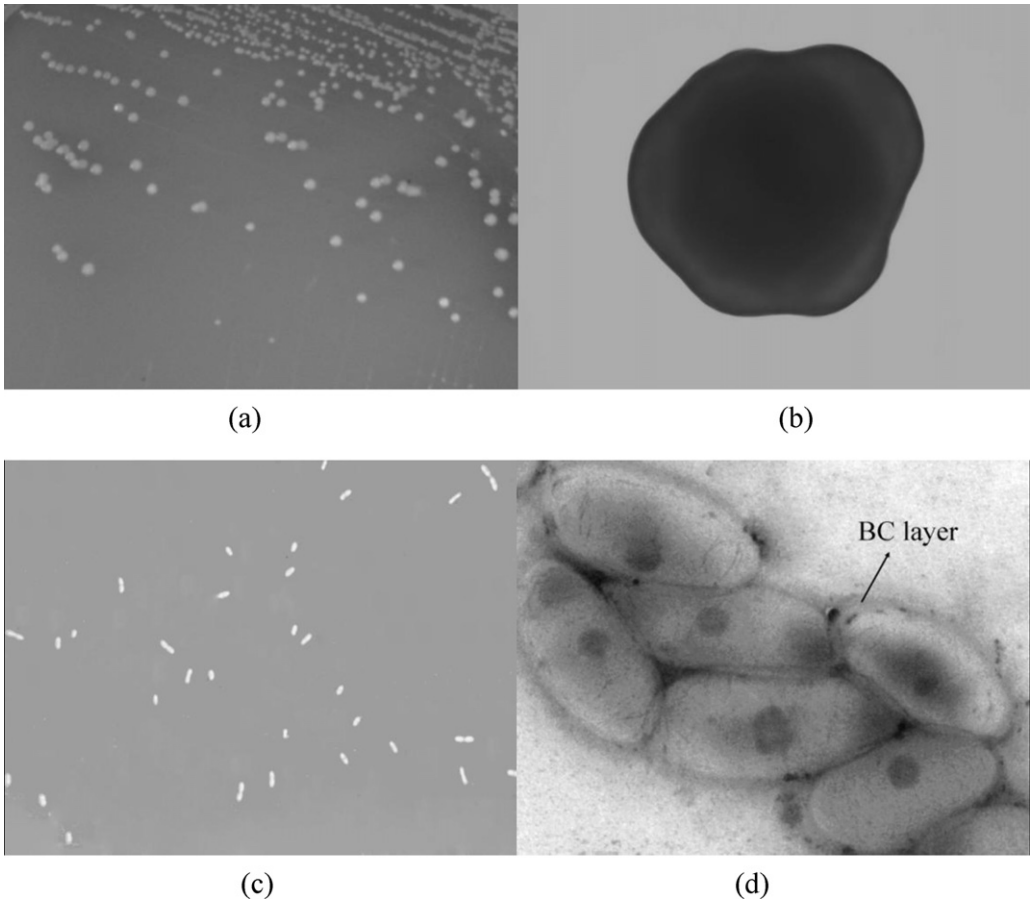

Fig. 1. Morphology observation of strain CIs26. (a) Colonies without magnification, (b) colony of 100 times magnification, (c) cells of 1000 times magnification (negative stain), and (d) cells through transmission electron micrograph.

Physiological and biochemical characteristics such as acetic acid production, oxidation reaction of acetate and lactate, growth conditions on 3% ethanol in the presence of 5% acetic acid (v/v), growth in 30% (w/v) glucose, were also investigated.

16S rDNA sequence analysis was carried out through the procedures of DNA extraction, PCR amplification, cloning and sequencing of 16S rDNA (3730 DNA Analyzer, Applied Biosystems Inc., USA) in Invitrogen Corporation (Shanghai, China).

2.2.3. Cultivation and harvest of BC pellicles

The bacterial strain was incubated in CWS medium at 28 °C for 8 days. The pellicles were harvested and washed with distilled water thoroughly to remove residual medium and other impurities. Then the pellicles were rinsed in 2 wt.% NaOH solution at 90 °C for 60 min to eliminate microorganism cells. Finally, the samples were washed with distilled water repeatedly until the pH was less than 7.0. The wet cellulose pellicles were dried at 60 °C to obtain constant weight. These pellicles were further used for FT-IR and XRD analysis.

2.2.4. Fourier transform infrared (FT-IR) spectroscopy

Thin BC pellicles obtained were evaluated through Fourier transform infrared spectra (Nicolet 6700 spectrophotometer, Thermo Scientific Inc., USA). The scan was done from 4000 cm^{-1} to 1000 cm^{-1} with resolution of 0.5 cm^{-1} for each measurement.

2.2.5. X-ray diffractometry (XRD)

XRD pattern of cellulose film was characterized through thin-film X-ray diffractometry (XRD, X'Pert PRO, PNAlytical Company, Holland). The radiation was Cu Kα of 1.54 Å, generated at a voltage of 40 kV and current of 40 mA. The diffraction angle ranged from 8.0° to 80° (2θ) with step size of 0.033°.

Crystallinity index (CrI) could be calculated by following formula (1) (Segal, Creely, Martion, & Conrad, 1959):

$$\mathrm{CrI} = \frac{I_{(200)} - I_{(\mathrm{am})}}{I_{(200)}} \quad (1)$$

where $I_{(200)}$ is the intensity at (2 0 0) peak and $I_{(\mathrm{am})}$ is the minimum intensity between (1 $\bar{1}$ 0) and (2 0 0) peaks.

2.2.6. Scanning electron microscope (SEM)

The BC pellicle was fixed with 2.5% glutaraldehyde solution for more than 4 h, then washed 3 times in the phosphate buffer. The sample was fixed with 1% OsO_4 solution for 1 h and washed 3 times again. The fixed pellicle was dehydrated by a stepwise ethanol (50%, 70%, 80%, 90%, 95% and 100%) for 20 min, then transferred to the mixture of alcohol and iso-amyl acetate solution (v:v = 1:1) for 30 min, subsequently transferred to pure iso-amyl acetate for 1 h. At last, the pellicle was dehydrated in Hitachi Model HCP-2 critical point dryer with liquid CO_2, then coated with gold–palladium and observed under SEM (H-7650, Hitachi, Japan).

3. Results and discussion

3.1. Microorganism isolation and identification

After screening of strains, one BC producing strain CIs26 was isolated from rotten mandarin fruit. By cultivation of strain CIs26, the pellicle was formed at air–liquid interphase with good hardness.

The morphological, molecular, physical and biochemical analyses were carried out to identify the strain. Colonies configurations were shown in Fig. 1(a) and (b) and cells morphology were shown in (c) and (d). As shown in Fig. 1(a), the colonies were round with irregular edge and raised elevation in center. The surfaces of colonies were smooth and their colors were cream white, without pigment diffusion into the medium. It is interesting to note that the

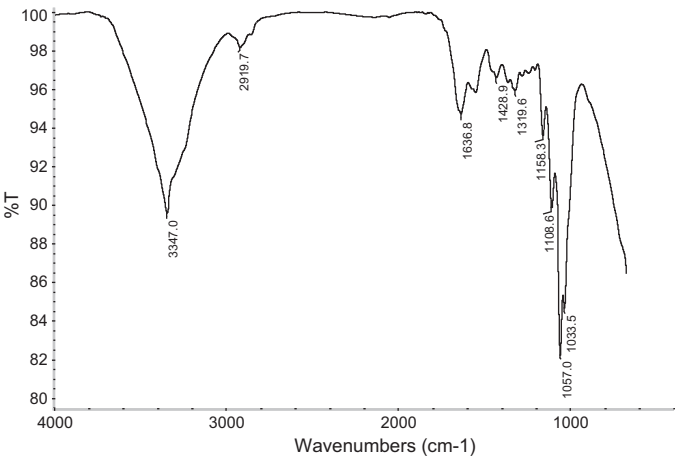

Fig. 2. FT-IR spectrum of BC membrane from strain CIs26.

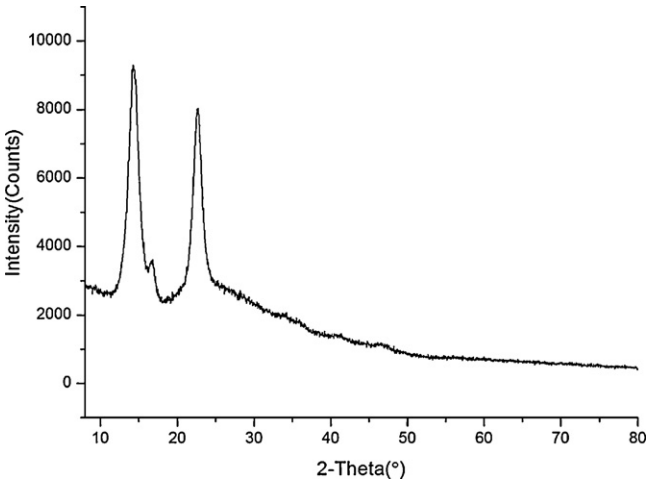

Fig. 3. XRD pattern of BC membrane from strain CIs26.

colonies were mucoid, covered by sticky substances (in Fig. 1(b)). Furthermore, most of the cells were of short rod shape, with little ellipse shaped cells. The cells occurred singly or in pairs with the size about 0.2–0.5 μm in width and 0.5–2.0 μm in length (Fig. 1(c)). It was obvious to find in Fig. 1(d), the surface of CIs26 cells were covered with a translucent BC layer, which was the same observation as the cellulose producing cells of *G. hansenii* PJK (Jung et al., 2005).

16S rDNA sequence of strain CIs26 was 1490 bp, having a homology of 99% with *Gluconacetobacter intermedius* strain AB099297.1. Hence, the stain was identified to belong to the group

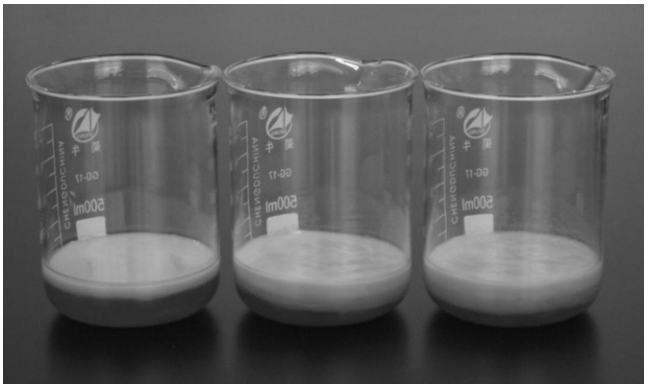

Fig. 4. Growth and BC production of strain CIs26 in different media (HS, CMHS and CWS, respectively, from left to right).

Table 1
Physiological and biochemical characteristics of strain CIs26 comparing with description in Bergey's Manual of Systematic Bacteriology.

Test	Results of CIs26	Description in Bergey's manual
Gram stain	−	−
Production of acetic acid from ethanol	+	+
Production of catalase	+	+
Production of water soluble pigment	−	−
Oxidation of acetate to CO_2 and H_2O	+	+
Oxidation of lactate to CO_2 and H_2O	+	+
Growth in the presence of 0.35% acetic acid (pH 3.5)	+	+
Growth on 3% (v/v) ethanol in the presence of 5% acetic acid	−	+
Requirement of acetic acid for growth	−	−
Growth only in the presence of acetic acid and ethanol and glucose	−	−
Growth on the medium of Carr and Passmore	(+)	(+)
Growth on carbon source ethanol	+	+
Growth in the presence of 30% (w/v) glucose	+	+
Production of cellulose	+	+
Ubiquinone type	Q10	Q10

Symbols: +, positive; −, negative; (+), weak positive.

of *Gluconacetobacter*. However, the physiological and biochemical characteristics of this strain were not strictly the same as *G. intermedius* described in Bergey's Manual of Systematic Bacteriology (Table 1). According to Bergey's manual, strains of *G. intermedius* could grow in the medium containing 3% ethanol and 5% acetic acid, but CIs26 could not survive. Therefore, CIs26 might be a new isolate and we named it as *G. intermedius* CIs26.

3.2. Fourier transform infrared (FT-IR) spectroscopy

The structure of BC pellicle produced by CIs26 in static conditions was investigated by FT-IR spectroscopy (Fig. 2). The spectrum had good compatibility with IR spectra of glucan in database of Nicolet software. A characteristic absorption band appeared at 3347 cm^{-1} was assigned to the stretching vibration of O—H bond. The absorption band at 2919 cm^{-1} was represented the

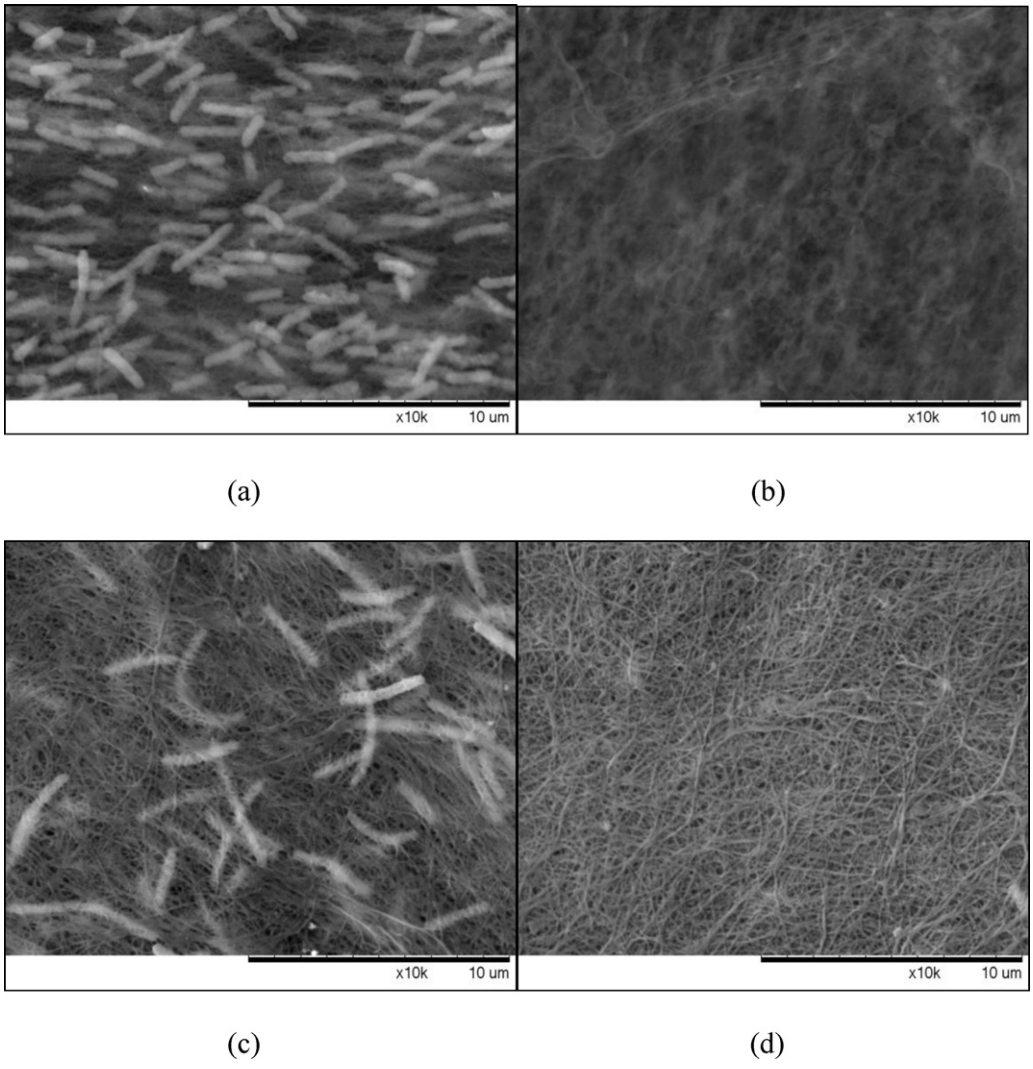

Fig. 5. SEM pictures of cells and BC in HS and CWS media. (a) Cells and BC in HS medium, (b) BC in HS medium, (c) cells and BC in CWS medium, and (d) BC in CWS medium.

C—H stretching vibration (Sheykhnazari, Tabarsa, Ashori, Shakeri, & Golalipour, 2011). Absorption signal at 1636.8 cm^{-1} indicated the stretching vibration of carboxyl group (C=O) (Trovatti et al., 2011). Furthermore, absorption band at 1428 cm^{-1} and 1158 cm^{-1} were revealed the existences of CH_2 and C_1OC_4, respectively. The absorption signals observed at 1108 cm^{-1}, 1057 cm^{-1} and 1033 cm^{-1} indicated stretching vibration of C_2O_2, C_3O_3 and C_6O_6, respectively. These results were similar with the reports of Yan, Chen, Wang, Wang, and Jiang (2008) and Shah, Ha, and Park (2010).

3.3. X-ray diffractometry (XRD)

Fig. 3 shows the XRD pattern of BC pellicle produced from strain CIs26. Three distinct peaks appeared at 2θ equaling to 14.4°, 16.5°and 22.6°, which were assigned to reflexion planes of (1 1 0), (1 $\bar{1}$ 0) and (2 0 0), respectively. The XRD pattern showed a typical crystalline form of cellulose I, and was in agreement with relative reports (Cai & Kim, 2010; Keshk & Sameshima, 2006). The crystallinity index was calculated to be 75% according to formula (1).

3.4. Effect of citrus waste on BC production

As seen in Fig. 4, CIs26 produced much thicker cellulose pellicle in CMHS medium and CWS medium than in HS medium. The yields of BC in CWS, CMHS and HS media were 7.2 g/L, 5.7 g/L and 2.1 g/L, respectively. The results indicated that citrus waste was a good natural resource for BC production. The nutrition components such as sugar, amino acid, vitamin, metal ions and other microelements in the waste might stimulate BC production by strain CIs26.

3.5. Scanning electron microscope (SEM) observation

Fig. 5 shows the cellular growth and BC production by CIs26 under static culture conditions in HS and CWS media. The fibers and cells assembled randomly to form a crossed network structure. The fibers in CWS medium were much thicker than those in HS medium. BC network structure was close-knitted in CWS medium, but fine and loose-knitted structure was observed in HS medium. Moreover, cells grew much more in numbers and shorter in length in HS than those in CWS medium. It was indicated that strain CIs26 preferred BC synthesization than cells propagation in CWS medium, while HS medium became preferential during cells multiplication. This phenomenon indicated the simulative effect of citrus waste on BC production by strain CIs26.

4. Conclusion

G. intermedius CIs26 was screened from rotten mandarin fruit, and identified as a new isolate of *G. intermedius* group. This strain had been collected in China General Microbiological Culture Collection Centre (CGMCC) with collection number of CGMCC No. 4663. Strain CIs26 produced semitransparent BC pellicle with good quality. The cellulose was type I in structure and its crystallinity index was 75%. Citrus waste was found to be an effective nutritional resource for the growth of CIs26. The BC yield reached to 7.2 g/L in CWS medium, which was much higher than those of other reported *Gluconacetobacter* strains. It demonstrated that citrus waste could be utilized in BC industry, which created a new perspective for BC production.

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