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Physicochemical characterization of high-quality bacterial cellulose produced by *Komagataeibacter* sp. strain W1 and identification of the associated genes in bacterial cellulose production†

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In the last few decades, bacteria capable of bacterial cellulose (BC) synthesis and the characterization of BC have been well-documented. In this study, a new BC-producing bacterial strain was isolated from fermented vinegar. The BC morphology, composition and diameter distribution, and the genes associated with BC production were analyzed. The results showed that one out of five isolates belonging to *Komagataeibacter* was a BC-producer, which mostly produced the typical cellulose I consisting of nanofibrils and had several functional groups similar to typing paper (*i.e.*, plant cellulose). Several known genes such as *glk*, *pgm* and *UPG2* in glucose metabolisms, *bcsA*, *bcsB*, *bcsC* and *bcsD* in BC synthesis and *cmcax*, *ccpAx*, *bglxA* and other genes in BC synthesis regulation or c-di-GMP metabolisms have also been found in the strain W1 based on genome sequencing and gene annotations. The functions of BcsX and BcxY might also be important for BC synthesis in *Komagataeibacter* sp. W1. Our study provided a new BC-producing bacterial strain that could be used to prepare high-quality BC and to study BC synthesis mechanisms.

1. Introduction

Cellulose is an organic compound that acts as a basic structural material of most plants and often combines with hemicellulose, lignin and pectin.[1] Although plant-derived cellulose has been used in several areas such as paper-making, all-cellulose composite preparation and dietary fiber intake,[2] it needs to be purified by enzymatic and/or mechanical treatments before further use.[1,3] In addition to the high cost, these processes may also change the cellulose performance and limit its applications in several areas.[1] Microorganisms including algae (*e.g.*, *Cladophora* and *Vallonia*), fungi (*e.g.*, *Dictyostelium* and *Saprolegnia*) and bacteria (*e.g.*, *Acetobacter*, *Achromobacter*, *Acidomonas*, *Aerobacter*, *Agrobacterium*, *Alcaligenes*, *Ameyamaea*, *Asaia*, *Gluconobacter*, *Gluconacetobacter*, *Granulibacter*, *Komagataeibacter*, *Kozakia*, *Neoasaia*, *Neokomagataea*, *Pseudomonas*, *Rhizobium*, *Saccharibacter*, *Sarcina*, *Swaminathania*, *Tanticharoenia* and *Zoogloea*)[4–6] also produce cellulose and have attracted people's attention due to its higher purity, crystallinity and water-holding capacity, lower production cost, thinner fiber diameter (*i.e.*, ~30–50 nm) and better biocompatibility as compared to plant cellulose.[7,8] Now bacterial cellulose (BC) has been widely used as food material, paper-making material, acoustic or filter membrane, cosmetic and clothing material, medical material and electronic paper substrate.

It is hypothesized that cellulose production in bacteria helps them to move to the oxygen-rich medium surface, protects them from ultraviolet light and retains moisture, and establishes close contact with a preferred host to facilitate efficient host–bacteria interactions.[9,10] Based on molecular evidence, the synthesis of BC is a multistep process including two main mechanisms: the synthesis of uridine diphosphoglucose (UDPGlc), followed by the polymerization of glucose into long and unbranched chains, *i.e.*, β-1,4-D-glucan, associated with hydrogen bonding.[1,7] While the former mechanism is well known, the latter still needs exploring.

As the most two important members of BC producers, both *Gluconacetobacter* and *Komagataeibacter* are formerly grouped into *Acetobacter* and now regrouped into independent genera based on 16S rRNA gene sequences and phenotypic, ecologic

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and chemotaxonomic characteristics.[11] Studies have shown that *Gluconacetobacter* is the most-well known genus due to its highest BC production ability among the reported BC-producers,[1,7] while *Komagataeibacter* contributes mostly to the rapid pH decrease and BC production during vinegar fermentation.[12,13] Although more than 14 species of *Komagataeibacter* have been regrouped from *Gluconacetobacter*,[14,15] their BC production characteristics warrant further investigations. For example, most studied BC-encoding genes are *bcsA*, *bcsB*, *bcsC* and *bcsD*, but the functions of other genes such as *bcsX* and *bcsY* are little known.[16] A recent study showed that *gqqA* encoded a novel protein involving in bacterial quorum quenching and cellulose formation, indicating more attention should be paid to BC synthesis mechanisms in bacteria.[17]

Since BC production is naturally occurring, most BC-producing bacteria are often isolated from the carbohydrate-containing substances such as rotten fruits, kombucha and vinegar.[16,18,19] In the present study, a bacterium belonging to the genus *Komagataeibater* was firstly isolated from a famous vinegar factory located in Yongchun, Quanzhou, China. By subjecting the strain to Hestrin and Schramm (HS) medium, a typical BC membrane was found and characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD) and Fourier transform infrared (FTIR) spectroscopy. Although the genomes of several species of *Komagataeibacter* have been sequenced, the gene information associated with BC synthesis and regulation is little known except for *K. nataicola*.[6] To identify the genes involving in glucose utilization and BC synthesis, draft genome sequencing and associated annotations (*e.g.*, COG functional prediction, NR annotation and Swissprot annotation) were also conducted in this study. The fully aim of this study was to give more precise work on understanding of BC synthesis mechanisms in bacteria and its importance in current and future applications.

2. Materials and methods

2.1. Sampling and bacteria isolation

To isolate BC-producing bacteria, both the vinegar and BC membranes were collected from Yongchun Laocu Industry Co., Ltd and stored in sterile plastic bottles (Corning, USA) in ice bath for taking back to lab. Before use, the BC membranes were washed carefully by sterile Milli-Q water to remove the surface bacteria, followed by soaking in 20 mL PBS containing 0.85% NaCl[20] and shaking at 200 rpm for 30 min. After that, aliquots of vinegar and washing buffer were streaked onto HS agar medium consisting of 2% glucose, 0.5% peptone, 0.5% yeast extract, 0.68% $Na_2HPO_4 \cdot 12H_2O$, 0.051% $MgSO_4 \cdot 7H_2O$, 0.115% $C_6H_8O_7 \cdot H_2O$ and 1.8% agar (pH was adjusted to 6.0 by 1 M NaOH and HCl).[21] All isolates were purified by re-streaking onto HS agar medium three times and subjected to HS liquid medium following 7 d incubation to pick out the bacteria capable of BC production. The obtained BC was pretreated as previously described and the BC-producing bacteria in the supernatant were mixed with 30% glycerol by 1 : 1 (v/v) and stored at −80 °C or re-streaked onto HS agar medium following 7 d incubation and stored at 4 °C for further use.

2.2. Bacterial identification

All isolates that could grew on HS agar medium were collected and re-cultivated in HS liquid medium for 7 d, followed by centrifugation at 8000 × *g* for 5 min. The collected bacterial biomass was used to extract total DNA by using a FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) according to the manufacturer's instructions. The 16S rRNA gene was amplified by the universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (ACGGCTACCTTGTTACGACTT) on a T100 Thermocycler (BioRad, USA).[20] The polymerase chain reaction (PCR) mixtures consisted of 12.5 μL of 2 × Mix (Yifeixue Biotechnology, Nanjing, China), 1.5 μL of primer pair (10 μM), 10 μL of PCR degrade water and 1 μL of DNA template. The PCR programs for 16S rRNA amplification were: 5 min at 94 °C for pre-denaturation, 35 s at 94 °C for denaturation, 30 s at 55 °C for annealing and 1.5 min at 72 °C for extension (35 cycles), and 10 min at 72 °C for a final extension.[20] The PCR products were purified and sequenced by GenScript Co., Ltd. (Nanjing, China). Based on the sequence analysis by BLAST similarity search in the NCBI database, the phylogenetic tree was constructed by using the neighbor-joining algorithm in the MEGA 5.0 program.

To have an observation of bacterial morphology, the biomass was pretreated by a sequential dehydration procedure and freeze-dried for 24 h (FreeZone 6 plus, Labconco, USA) according to Wang *et al.*[20] After a spray-gold treatment, the morphology was observed by SEM (Quanta™ 250 FEG, FEI, USA).

2.3. Bacterial cellulose characterization by SEM, XRD and FTIR

To observe the porous structure and fibril distribution on BC, one colony of the strain W1 was inoculated in HS liquid medium for 7 d, followed by washing and shaking treatments as shown above. After centrifugation at 8000 × *g* for 5 min, the bacterial biomass was re-suspended in 10 mL sterile Milli-Q water, aliquots of which were transferred to a new medium to a final OD_{600} at 0.01. After 7 d of incubation, the BC membranes were collected and washed by Milli-Q water, followed by boiling at 100 °C for 2 h by 0.1 M NaOH and another 2 h by Milli-Q water. The pre-treated BC was soaked in sterile Milli-Q water overnight at room temperature and then oven-dried at 50 °C. The dried BC was treated by spray-gold and observed by SEM. To evaluate the diameter distribution of BC fibrils, the SEM image of BC was analyzed on a NanoMeasurer 1.2 by calculating 100 nanofibrils randomly. In this study, the difference of each group of fibrils was 10 nm, and the results were presented as % of the total fibril numbers.

Similar to SEM, all samples used for further characterization by XRD (Bruker D8 ADVANCE, Germany) and FTIR (Thermo Scientific Nicolet iS5, USA) were pre-treated as described. Of which, XRD pattern was obtained using nickel filtered copper K_α radiation, with 0.1° step, from 4° to 70° (2θ, angle), while FTIR analysis was conducted in the attenuated total reflection (ATR) mode with 32 scans per measurement between 400 and 4000 cm^{-1}.

2.4. Draft genome sequencing and key functional genes annotation

To have an overview of BC synthesis mechanisms in the strain W1, the draft genome sequence and key functional genes were analyzed by Majorbio, Shanghai, China. Before sequencing, the total DNA was extracted as previously described and the DNA quality was evaluated by Nanodrop® ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) based on DNA concentration and OD_{260}/OD_{280}. The established sample was fragmented to 400–500 bp by using M220 Focused-ultrasonicator™ (Covaris, Woburn, MA, USA), followed by library construction by using TruSeq™ DNA Sample Prep Kit (Illumina, Inc., USA) and sequencing on an Illumina Miseq (PE) platform by MiSeq Reagent KiT v3 (Illumina, Inc., USA). After data transformation by Base Calling, the raw sequences were saved as FASTQ and deposited in the NCBI-Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/Traces/sra) with the accession numbers PRJNA388252 (BioProject number), SAMN07173612 (BioSample number) and SRP108180 (SRA Study number).

Before genome assembly, the low-quality data in the raw sequences were removed based on the following criteria: (1) the adapter sequences, (2) the sequences include A, G, C or T base in the 5′ terminal, (3) the terminal sequences with low quality values (<20), (4) the reads include ≥10% N and (5) the small sequences (<25 bp) after removing the adapter and low-quality data. The genome assembly was performed on a SOAP denovo platform (v2.04, http://soap.genomics.org.cn/) by multi-optimization of K-mer parameters, followed by a further base-proofreading by GapCloser (v1.12).[22] The genome prediction was conducted by Glimmer (v3.02, http://www.cbcb.umd.edu/software/glimmer/). For functional gene annotation, all protein sequences in correspond to the predicted genes were analyzed by blastp research (BLAST 2.2.28+) against the known sequences in Nr, String, Genes and GO databases. Of which, the String annotation can obtain COG function analysis information, *i.e.*, the protein function, classification and evolution status. Moreover, the blast results from Genes database can assign a certain gene to a KO number in KEEG pathway.

3. Results and discussion

3.1. Taxonomic characterization of BC-producing bacterial isolate

By using the typical HS agar medium for isolation of BC-producing bacteria, a total of five strains were obtained. However, only one (*i.e.*, strain W1) produced BC in HS liquid medium. As shown in Fig. 1A, limited colonies of the strain W1 were found on HS agar medium, which might be due to the fact that BC-producing bacteria often use ~50% energy to produce BC as compared to normal bacteria that use most energy to reproduce offspring.[23] In addition, the strain W1 formed unregular colonies that might contain BC as the colonies were tough and smooth on agar medium (Fig. 1A). SEM observation found that the cells of strain W1 were short rod-shaped without flagellum (Fig. 1B). The size of each cell was 1.58 ± 0.11 μm long by 0.91 ± 0.09 μm wide (Fig. 1B).

To further identify the strain W1, its 16S rRNA sequence (GenBank accession number MF187480) was aligned with the known sequences retrieved from the NCBI database. All three groups that were often confused with each other previously were listed. The results showed that although the 16S rRNA sequence of W1 shared high similarity with most reference sequences, it belonged to the Group I, *i.e.*, the genus *Komagataeibacter* (Fig. 2). However, the strain hadn't been exactly classified at species level, thereby being named as *Komagataeibacter* sp. W1 (Fig. 2). Since *Komagataeibacter* is regrouped from the genus *Gluconacetobacter*, a typical and widely-studied BC-producing family, the strain isolated in this study could be a good candidate for BC preparation.[14,15] Studies also showed that *Komagataeibacter* contributed mostly to pH decrease in vinegar production,[12,13] indicating that the strain W1 was an important member in vinegar fermentation and BC production in the sampling site.

3.2. Morphological characteristics of cellulose produced by *Komagataeibacter* sp. strain W1

As shown above, the strain W1 produced BC membranes, which were pale yellow and transparent before and after treatment in 0.1 M NaOH bath, respectively (Fig. 3A–D). To have an *in situ* observation of the BC with or without NaOH treatment, all

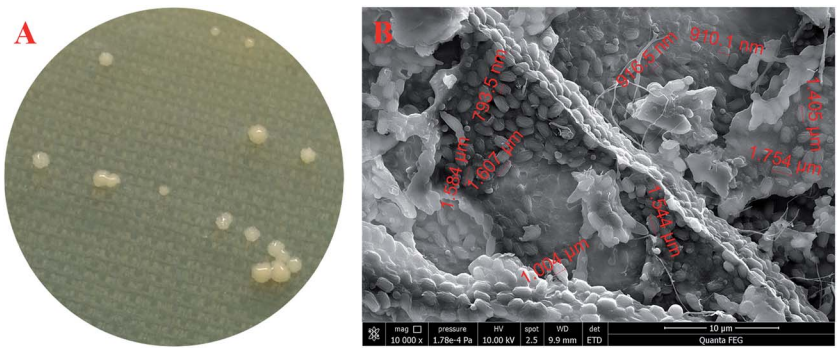

Fig. 1 Colony morphology (A) and SEM observation ((B), 10 000×) of *Komagataeibacter* sp. W1 isolated from spiced vinegar fermentation tank in Yongchun, Quanzhou, Fujian, China.

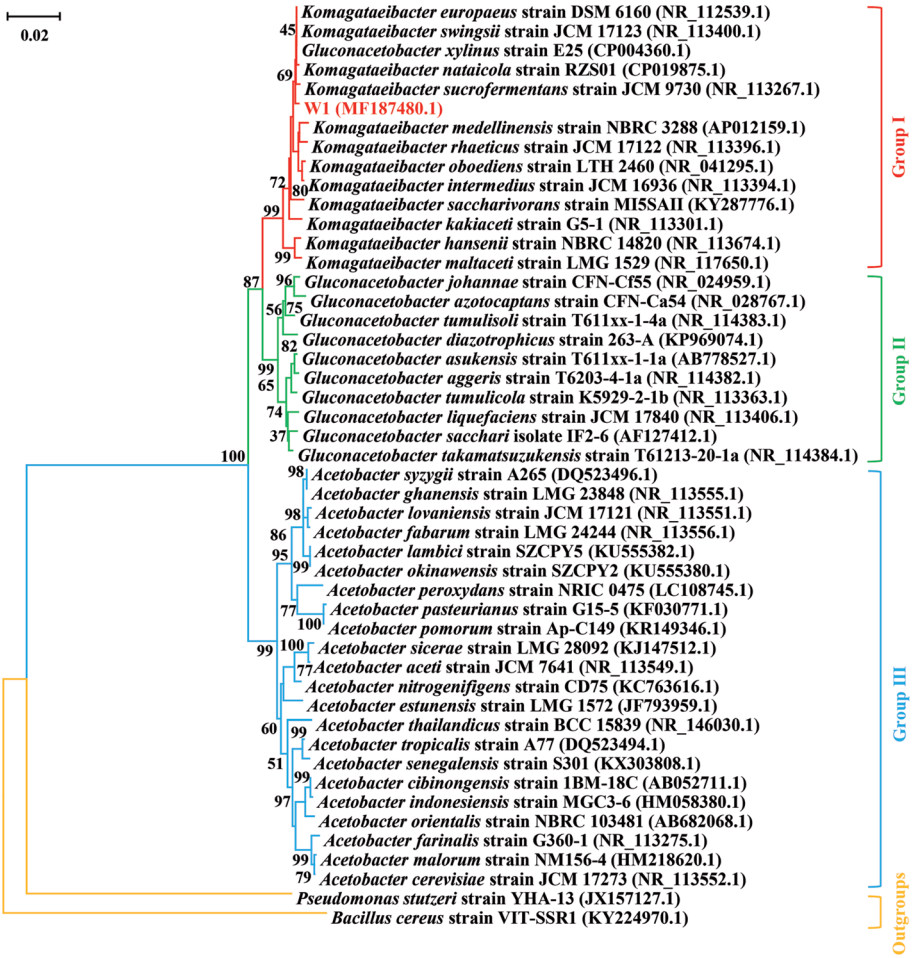

Fig. 2 Neighbor-joining phylogenetic trees of bacterial 16S rRNA sequence of *Komagataeibacter* sp. W1 retrieved from the fermentation tank of spiced vinegar in Yongchun, Quanzhou, Fujian, China. Reference sequences were retrieved from NCBI database. The tree root was constructed with bootstrap values calculated from 1000 resamplings. The numbers at each node indicate the percentage of bootstrap supporting. Scale bars indicate 20 mutations per 1000 bases. Group I, II and III were often confused with each other previously and have been classified into three independent groups recently.

samples were oven-dried and BC images were taken by SEM. Results showed that the BC membranes had a three-dimensional structure and the bacteria distributed both on the surface of and inside BC membranes (Fig. 3E–L). It was also apparent that the bacteria produced fibrils on the cell surface (Fig. 3H), where several terminal complexes consist of BC synthase catalytic subunits, by a three-way branching pattern.[23,24] In this case, a single cell can produce a ribbon of BC with 10–100 microfibrils.[23] As a result of cell mitosis, the fibril assembly occurs, forming the glucan chain sheets by van der Waals bonding followed by stacking of the sheets by H-bonding to form the crystalline structure,[25,26] which were supported by our data as shown in Fig. 3L. We hypothesized that the strain W1 produced a typical BC with high purity (Fig. 3I–L).

To test the diameter distribution of the BC fibrils produced by *Komagataeibacter* sp. W1, 100 fibrils on SEM image were selected randomly and calculated by NanoMeasurer 1.2. As expected, 98% of the fibrils had the diameter <100 nm, 77% of which were <50 nm (Fig. 4), indicating that the BC obtained in this study consisted of nanofibrils. This was in line with previous studies that the visible BC fibrils consist of 40–60 nm cellulose ribbons, which are assembled by microfibrils consisting of 3–4 nm subelementary fibrils.[27,28]

3.3. XRD and FTIR analyses of cellulose produced by *Komagataeibacter* sp. strain W1

Based on a SEM observation, the BC produced by *Komagataeibacter* sp. strain W1 had some typical characteristics of the known BC, *e.g.*, including nanofibrils and pellicles (Fig. 3). To further verify the BC compositions and purity, XRD and FTIR analyses were also performed.

Fig. 5 shows XRD pattern of the BC produced by the strain W1. Similar to previous studies, three typical diffraction peaks were observed at 2θ 14.5°, 16.6° and 22.7° (Fig. 5), corresponding to $(1\bar{1}0)$, (110), and (200) planes, respectively.[29,30] Studies have shown that the broad peaks at 2θ 14.5° and 22.7° are associated with the presence of cellulose I_α and cellulose I_β phases, *i.e.*, $100_{1\alpha}$, $110_{1\beta}$ and $010_{1\beta}$ planes at 14.5° and $110_{1\alpha}$ and $200_{1\beta}$ at 22.7°.[31,32] Thus, we concluded that W1-produced

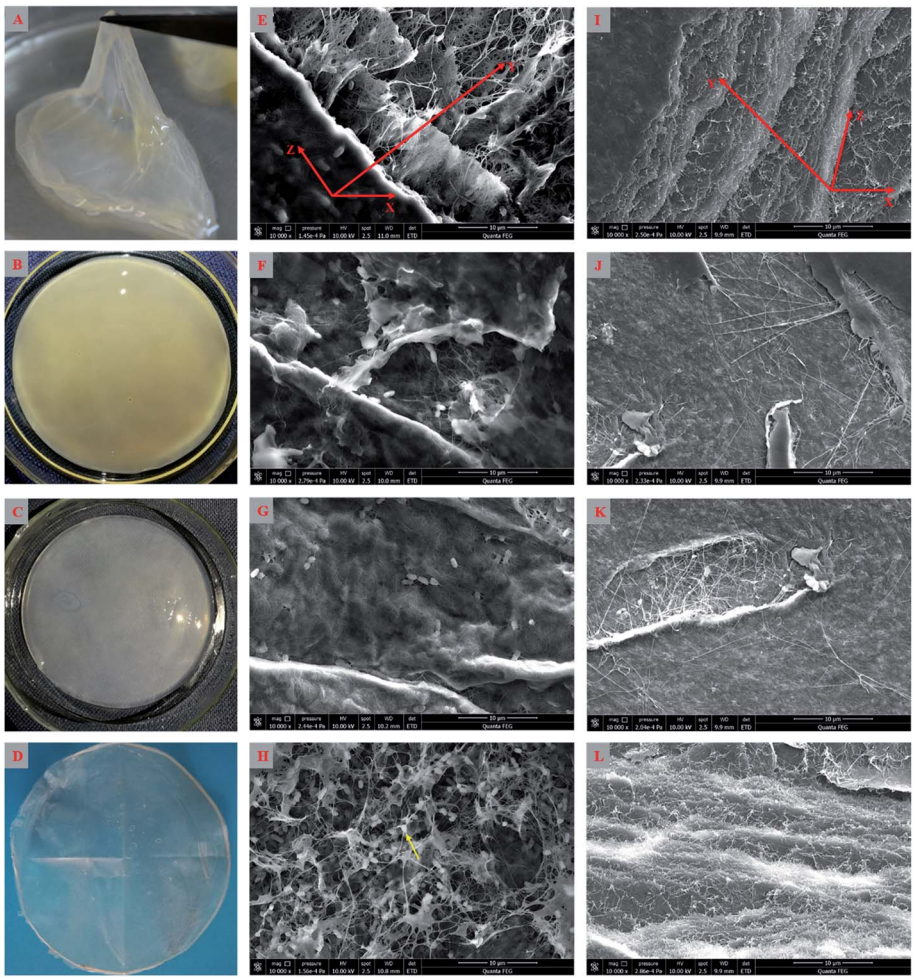

Fig. 3 The appearance (A–D) and SEM images (E–L) of the bacterial cellulose membranes produced by *Komagataeibacter* sp. W1. (A and B) indicate the samples without the treatment in 0.1 M NaOH bath for 2 h; (C and D) are the samples with the treatment in 0.1 M NaOH bath for 2 h before and after oven-drying at 50 °C, respectively; (E–H) indicate the bacterial distribution on the surface of and inside bacterial cellulose membranes, *i.e.*, (E) full observation, (F) surface bacterial membranes, (G) bacterial distribution on the horizontal scale and (H) three-dimensional structure of the bacterial cellulose containing bacteria, of which the yellow arrow indicates the bacterial cellulose production and secretion site locating on the cell surface; (I–L) indicate the horizontal and cross-sectional observations of the bacterial cellulose with additional treatment in 0.1 M NaOH bath for 2 h, *i.e.*, (I) full observation, (J) horizontal surface textile structure of bacterial cellulose, (K) inter-horizontal textile structure of bacterial cellulose and (L) three-dimensional-cross-sectional structure of the bacterial cellulose. Red arrows indicate three-dimension structure of the bacterial cellulose.

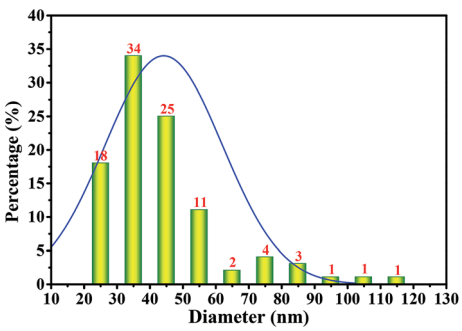

Fig. 4 Diameter distribution of the bacterial cellulose fibrils produced by *Komagataeibacter* sp. W1. The diameter calculation was performed on Nano Measurer 1.2 by calculating 100 nanofibrils randomly on SEM image of the bacterial cellulose membranes (50 000×).

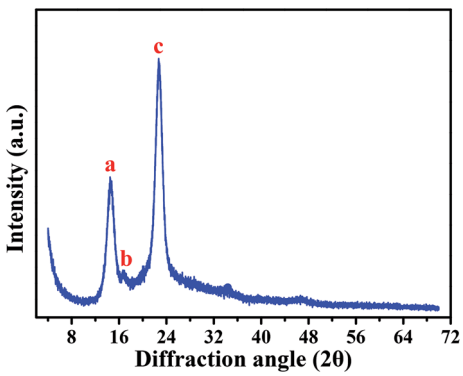

Fig. 5 XRD analysis of pure bacterial cellulose produced by *Komagataeibacter* sp. W1.

BC was pure. However, similar to other studies, the broad diffraction peaks with sharpness observed in this study indicated that the products included semi-crystalline BC (Fig. 5).[29,30]

In addition to XRD characterization, FTIR analysis is also an important alternative to identify the types and purity of BC.[24,33] To have an comparative view on functional groups in cellulose, the FTIR spectrums of BC and typing paper (*i.e.*, plant cellulose) were analyzed. Since the BC was difficult to be powdered, the ATR mode with 32 scans per measurement between 400 and 4000 cm^{-1} was used in this study according to previous studies.[34,35] As shown in Table 1 and Fig. 6A, the W1-produced BC contained 17 functional groups, most of which were similar to those that in typing paper (Fig. 6B) and the BC produced by other bacteria.[36,37] For example, the typical wavenumbers of BC produced by *Komagataeibacter* sp. W1 (*i.e.*, 1426, 1335, 1314, 1160, 1108, 1054 and 1030 cm^{-1}) were also found in *A. xylinum* ATCC 10245 (*i.e.*, 1426, 1314, 1160 and 1053 cm^{-1}), *G. xylinus* BCRC12335 (*i.e.*, 1335, 1163, 1111, 1060 and 1035 cm^{-1}) and *K. saccharivorans* PE5 (*i.e.*, 1425, 1319, 1157 and 1023 cm^{-1}).[38,39]

Specifically, the typical absorption at around 3300 cm^{-1} (peak 1) corresponds to stretching vibration of intra and inter O–H in cellulose I.[34,40,41] While the weak peak at around 2900 cm^{-1} (peaks 2 and 3) corresponds to C–H stretching of CH_2 and CH_3 groups or CH_2 asymmetric stretching (Fig. 6), the latter is often found in plant cellulose (Fig. 6B).[2,34] For both BC and typing paper, a peak at around 1650 cm^{-1} (peak 4) is observed, corresponding to H–O–H bending of absorbed water.[2,40] Although it is thought that the peak at around 1430 cm^{-1} (peak 5) may originate from CH_2 scissoring,[42] most studies attribute it to CH_2 symmetric bending or O–H in plane bending.[2,43] The peak at around 1360 cm^{-1} corresponds to C–H bending, which also results in a typical absorption at 1280 and 1204 cm^{-1} (Fig. 6A).[9,29,43] While the peak at 1335 cm^{-1} (peak 7) may correspond to C–H deformation or O–H in-plane bending,[2,9] the absorption at around 1315 cm^{-1} (peak 8) is assigned to out-of-plane wagging of the CH_2 groups.[42] As a typical indicator of the presence of C–O–C antisymmetric bridge stretching of 1, 4-β-D-glucoside in BC, the absorption at around 1160 cm^{-1} is also observed.[2,36] It has shown that the peaks at 1000–1100 cm^{-1} can be assigned to C–O stretching vibration in primary alcohol and C–O–C skeletal vibration.[2,36,43] However, these hypotheses are controversial. For example, some studies attribute the absorption at 1030 and 1054 cm^{-1} to the bending of C–O–H bond of carbohydrates[44–46] or C–O–C

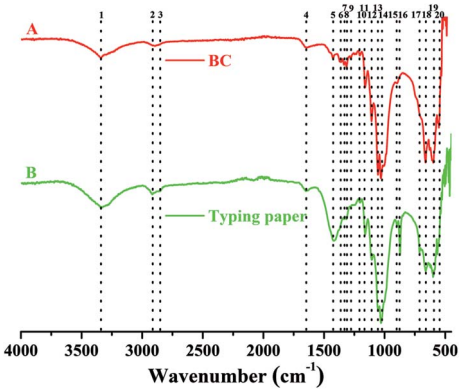

Fig. 6 FTIR analysis of W1-produced bacterial cellulose on a Nicolet iS5 in the ATR mode with 32 scans per measurement between 400 and 4000 cm^{-1}.

Table 1 Comparative analysis of functional groups on BC and typing paper by FTIR

Peak number	Wavenumber (cm^{-1}) BC	Typing paper	Functional groups[a]
1	3338	3332	O–H stretching vibration
2	2895	2917	C–H stretching of CH_2 and CH_3 groups
3	—[b]	2865	CH_2 asymmetric stretching
4	1642	1636	H–O–H bending of absorbed water
5	1426	1420	CH_2 symmetric bending or O–H in plane bending
6	1361	—	C–H bending
7	1335	1335	C–H deformation or O–H in-plane bending
8	1314	1316	Out-of-plane wagging of the CH_2 groups
9	1280	—	C–H bending
10	1204	1204	
11	1160	1160	C–O–C antisymmetric bridge stretching of 1,4-β-D-glucoside
12	1108	1105	C–C bonds of the monomer units of polysaccharide or C–O bending vibration
13	1054	1052	The bending of C–O–H bond of carbohydrates or C–O–C pyranose ring skeletal vibration
14	1030	1029	
15	899	897	Antisymmetric out-of-phase ring stretching of β-glucosidic linkages between the glucose units
16	—	872	UK[c]
17	—	712	The monoclinic I_β form of cellulose
18	663	661	O–H out-of-phase bending vibration
19	598	600	
20	552	558	

[a] More information on the relationships between wavenumber and assignment can be found in Gea *et al.*[66] and Moharram *et al.*[41] [b] Not detected. [c] Unknown.

pyranose ring skeletal vibration.[1,40,46] Moreover, Gao *et al.*[47] showed that the peak at 1030 cm^{-1} might also be associated with the presence of OCH_3, while the peak at 1108 cm^{-1} indicated C–C bonds of the monomer units of polysaccharide[45] or C–O bending vibration,[9] *i.e.*, skeletal vibration. Furthermore, the peaks at 1162, 1107 and 1055 cm^{-1} can be assigned to C–C stretching vibration, skeletal vibration and ring vibration, respectively.[48] Besides, we also found four peaks at 400–1000 cm^{-1} (Fig. 6A). Among of which, the peak at around 900 cm^{-1} corresponds to antisymmetric out-of-phase ring stretching of β-glucosidic linkages between the glucose units,[2,43,49] which is designated as an "amorphous" absorption band, mainly contributing to the increase of BC intensity.[45,50] For the peak at 712 cm^{-1}, it is assigned to the monoclinic I_β form of plant cellulose,[9] but which is absent in BC (Fig. 6). Unlike the peak 17, the peaks 18–20 are present in both BC and typing paper, corresponding to O–H out-of-phase bending vibration.[2]

Since two weak peaks occur at around 1430 and 900 cm^{-1} (Fig. 6A), we hypothesized that the BC produced by *Komagataeibacter* sp. W1 mainly consisted of pure cellulose I,[50–52] which was in accordance with XRD data (Fig. 5). Other peaks corresponding to cellulose I are also found in this study, *e.g.*, 3338, 1160 and 899 cm^{-1}, which have been well studied previously.[53,54] However, another three weak peaks at 1335, 1314 and 1280 cm^{-1} and the blue-shift of wavenumber from 1430 to 1426 cm^{-1} give the evidence of the presence of cellulose II.[53,54]

3.4. Genome sequence identification in BC-producing bacterial isolate for cellulose synthesis and regulation

It is well-known that the BC production in bacteria is associated with several enzymes, including glucokinase (EC 2.7.1.2), phosphoglucomutase (EC 5.4.2.2), UTP-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) and cellulose synthases (EC 2.4.1.12).[23] However, these enzymes vary in different genera of BC-producing bacteria.[55] Moreover, the complete genomes of *Komagataeibacter* have only been sequenced in four species such as *K. nataicola* RZS01, *K. xylinus* E25, *K. hansenii* ATCC 23769 and *K. medellinensis* NBRC3288, while draft genomes are available for *K. europaeus* LMG18494, *K. intermedius* AF2, *K. rhaeticus* AF1, *K. kakiaceti* JCM 25156 and *K. oboediens* 174Bp2.[6,16,56,57] To have an overview of the relevant enzymes in *Komagataeibacter* sp. W1, we also sequenced the draft genome and analyzed the functional genes and signaling pathways associated with BC synthesis.

After removing the low-quality sequences, the total bases of clean data were about 1.32 Gb (Table S1†). The genome assembly showed that there were 168 scaffolds and 285 contigs in the draft genome (Table S2†). Moreover, a total of 3705 genes (*i.e.*, open reading frames, orfs) were found based on sequence alignment with the known information in the KEEG database (Tables S3 and S4†). However, only 1783 and 1732 out of 3705 genes were annotated to COG functional categories and certain KEEG pathways, respectively (Fig. 7 and Table S5†). As shown in Fig. 7, there were 113 genes that corresponded to carbohydrate transport and metabolisms, of which 3 orfs were responsible for encoding glucokinase, phosphoglucomutase and UTP-glucose-

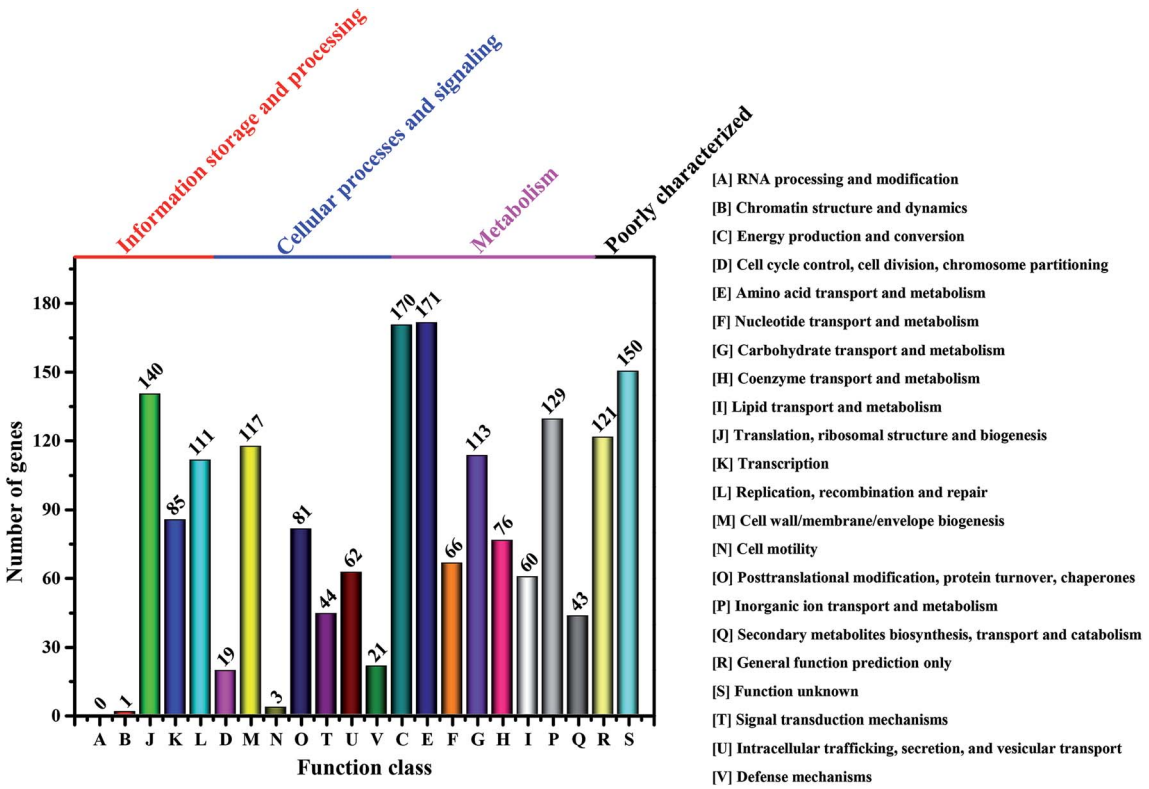

Fig. 7 Number of genes associated with general COG functional categories based on the total number of protein coding genes in the genome.

Table 2 Open reading frames and corresponding proteins information based on NR and Swissprot annotations

Open reading frames number	Amino acid length (aa)	NR top hit	Similarity (%)	Swissprot top hit	Similarity (%)	Protein names
Glucose transformation						
orf1235[a]	322	WP_019084814.1	100	P21908.2	81	Glucokinase [*Komagataeibacter europaeus*]; glucokinase = glucose kinase [*Zymomonas mobilis* subsp. *mobilis* ZM4 = ATCC 31821]
orf1086[a]	555	WP_019091484.1	100	P38569.1	100	Phosphoglucomutase [*Komagataeibacter europaeus*]; phosphoglucomutase = PGM = glucose phosphomutase [*Komagataeibacter xylinus*]
orf1122[a]	280	WP_019084882.1	100	P27897.1	97	UTP-glucose-1-phosphate uridylyltransferase [*Komagataeibacter europaeus*]; UTP-glucose-1-phosphate uridylyltransferase = alpha-D-glucosyl-1-phosphate uridylyltransferase = UDP-glucose pyrophosphorylas
Cellulose synthesis						
orf0140[a]	1530	—	—	Q9WX75.1	79	Cellulose synthase catalytic subunit [UDP-forming]
orf0492[b]	1328	WP_026019532.1	100	Q9WX71.1	88	Cellulose synthase [*Komagataeibacter europaeus*]; cellulose synthase 2 operon protein C = flags: precursor [*Komagataeibacter xylinus*]
orf0493[b]	386	WP_019084639.1	100	Q9WX70.1	94	Acyltransferase [*Komagataeibacter europaeus*]; Putative membrane-bound transacylase BcsY [*Komagataeibacter xylinus*]
orf0494[b]	223	WP_019090487.1	100	Q9WX69.1	99	BcsX [*Komagataeibacter europaeus*]; protein BcsX [*Komagataeibacter xylinus*]
orf0495[a,b]	1558	—	—	Q9RBJ2.1	92	Putative cellulose synthase 2 = cellulose synthase catalytic subunit [UDP-forming]
orf1576	321	WP_019085254.1	92	Q76KK0.1	60	Hypothetical protein [*Komagataeibacter europaeus*]; cellulose-complementing protein [*Komagataeibacter xylinus*]
orf1578[a,b]	691	WP_026018529.1	100	Q9WX61.1	96	Cellulose synthase [*Komagataeibacter europaeus*]; cellulose synthase 1 catalytic subunit [*Komagataeibacter xylinus*]
orf1579[a,b]	802	—	—	Q9WX62.1	97	Cyclic di-GMP-binding protein = CDGBP = cellulose synthase regulatory subunit = cellulose synthase protein B/flags: precursor
orf1580[b]	1293	WP_026019647.1	100	Q9WX63.1	96	Cellulose synthase 1 operon protein C/flags: precursor [*Komagataeibacter xylinus*]; cellulose synthase [*Komagataeibacter europaeus*]
orf1581[b]	156	WP_010507046.1	100	Q9WX64.1	100	Cellulose synthase operon protein D [*Komagataeibacter xylinus*]; cellulose synthase [*Komagataeibacter europaeus*]
Cellulose synthesis regulation						
orf1575	354	WP_019085253.1	100	P37696.1	86	Endoglucanase [*Komagataeibacter europaeus*]; probable endoglucanase = cellulase = endo-1,4-beta-glucanase = flags: precursor [*Komagataeibacter hansenii*]
orf1582	734	WP_010507044.1	100	Q5BFG8.1	53	Beta-glucosidase [*Komagataeibacter europaeus*]; beta-glucosidase B = beta-D-glucoside glucohydrolase B = cellobiase B = gentiobiase B
orf1669	325	WP_019086736.1	100	P58599.1	60	Endoglucanase [*Komagataeibacter europaeus*]; endoglucanase = cellulase = endo-1,4-beta-glucanase = flags: precursor [*Ralstonia solanacearum* GMI1000]
orf2722	698	WP_010508536.1	100	Q9WX70.1	51	Acyltransferase [*Komagataeibacter europaeus*]; putative membrane-bound transacylase BcsY [*Komagataeibacter xylinus*]

[a] The orfs that can be annotated to known pathways in KEEG pathway database as shown in Table 3. [b] The full sets of cellulose synthase genes in the *bcs2* and *bcs1* operons, respectively.

1-phosphate uridylyltransferase, respectively, and 14 orfs were responsible for encoding cellulose synthases and associated co-enzymes (Table 2).

By KEEG analysis, the enzymes encoded by orfs 1235, 1086 and 1122 were involved in glucose transformation in starch and sucrose metabolism pathway (Table 3 and Fig. S1†). All these enzymes co-produced the precursor (*i.e.*, UDP-glucose) of BC (Fig. 8A), which was in accordance with the documented studies.[23,27] Once UDP-glucose produced, the domain A encoded by *bcsA* and attached inner cell membrane is thought to catalyze the UDP-glucose to cellulose.[27] This process also needs another core enzyme, *i.e.*, BcsB, to accelerate the BC synthesis by combining to c-di-GMP.[27,58,59] After that, the newly synthesized BC is crystallized with the aid of BcsD and then extruded by BcsC (Fig. 8B and C).[60] As expected, all the genes associated with above proteins encoding were detected in the strain W1 and the corresponding amino acid sequences shared high similarity with *K. europaeus* and *K. xylinus* (Table 2). However, both BcsC and BcsD could not be annotated to certain KEEG pathways (Table 3). As stated by previous studies, these genes are located in *bcs* operon1 (*bcs1*).[6,27] Similar to Zhang *et al.*,[6] we also found two upstream genes *cmcax* (orf1575) and *ccpAx* (orf1576), and one downstream gene *bglxA* (orf1582) in *bcs1* (Table 2 and Fig. 8A). While *ccpAx* encodes a cellulose-complementing protein,[61,62] *cmcax* and *bglxA* encode endo-β-1,4-glucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21), respectively, both of which assist cellulose biosynthesis by hydrolysing tangled glucan chains when a failure in chain arrangement occurs

Table 3 Open reading frames and corresponding proteins information based on KEEG pathway annotation

Open reading frames ID	Enzyme ID	Enzyme names	Ko ID (gene ID)	Ko names (gene names)	KEEG ID
orf1235	2.7.1.2	Glucokinase	K12407; K00845	*GCK*; *glk*	ko00010; ko00052; ko00500; ko00520; ko00521; ko00524
orf1086	5.4.2.2	Phosphoglucomutase	K01835	*pgm*	ko00500; ko00520
orf1122	2.7.7.9	UTP-glucose-1-phosphate uridylyltransferase	K00963	*UGP2*; *galU*; *galF*	ko00500
orf0140, orf0495, orf1578, orf1579	2.4.1.12	Cellulose synthase (UDP-forming)	K00694	*bcsA/B*	ko00500
orf1575, orf1669	3.2.1.4	Endoglucanase	K01179	*cmcax*	ko00500
orf1582	3.2.1.21	Beta-glucosidase	K01188	*bglxA*	ko00500

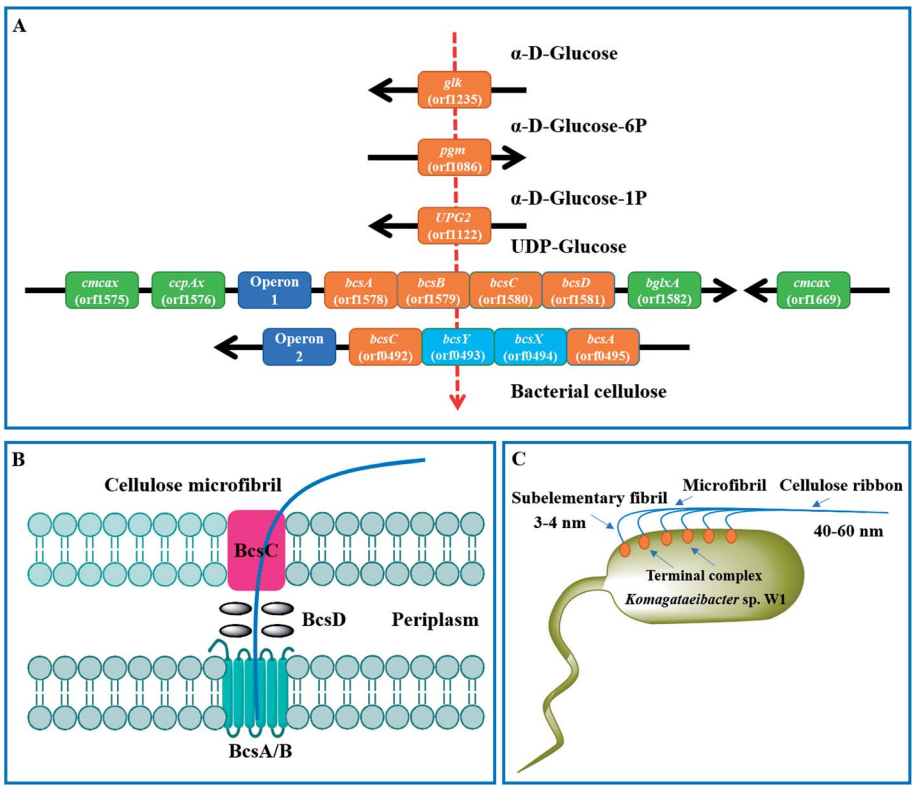

Fig. 8 Mechanisms of BC synthesis in *Komagataeibacter* sp. W1. (A) genes associated with glucose transformation and BC synthesis; (B) proposed BC synthesis and excretion pathways by the regulation of *bcs* operon1;[60] (C) BC assembly programs.[27]

(Fig. S2†).[6,63] It was interesting to note that another *cmcax* gene (orf 1669) was also found in *Komagataeibacter* sp. W1 (Table 2), which was different from *K. nataicola*[6] but similar to *K. europaeus* (Table 2) and its functions in BC synthesis regulation warranted further studies. As shown in Table 2 and Fig. 8A, another *bcs* operon (*bcs2*) for BC synthesis was also found in this study. The *bcs2* was composed of *bcsA* (orf0495), *bcsX* (orf0494), *bcsY* (orf0493) and *bcsC* (orf0492), of which *bcsX* and *bcsY* were located in the middle of *bcsA* and *bcsC* as previous report,[6] but their potential roles in BC synthesis remained unknown. Morgan *et al.*[64] showed that the protein encoded by *bcsA* contained the catalytically active subunit with a PilZ domain, which was responsive to c-di-GMP. Similar to Zhang *et al.*,[6] we also found another *bcsA* (orf0140), which might be located on the reverse strand and its encoded protein did not contain a PilZ domain. Except for above genes, another *bcsY* (orf2722) was also located in the genome of *Komagataeibacter* sp. W1, which might involve in the expression of acyltransferase (Table 2).

As described, the PilZ domain is in response to the second messenger c-di-GMP. c-di-GMP is produced from 2 molecules of GTP by diguanylate cyclases (DGCs) and is broken down into 5′-phosphoguanylyl-(3′-5′)-guanosine by specific phosphodiesterases (PDEs).[6] The c-di-GMP level can stimulate BC synthesis, leading to a 100-fold of BC increase.[65] Similar to Zhang *et al.*,[6] we found three *cdg* operons containing a c-di-GMP PDE gene (orf2263, orf2943 and orf3022) followed by a DGC gene (orf2264, orf2944 and orf3023). Besides, four standalone c-di-GMP PDE genes (orf1651, orf1917 and orf1918), one standalone cAMP DPE gene (orf2811) and three standalone DGC genes (orf0413, orf1556 and orf 2256) were also located in the genome of *Komagataeibacter* sp. W1 (Table S4†). Given the presence of two *bcs* operons in BC synthesis and three *cdg* operons in c-di-GMP regulation, it was possible that all these operons were attributed to the great ability of BC biosynthesis in *Komagataeibacter* sp. W1.

4. Conclusions

In this study, a BC-producing bacterium namely *Komagataeibacter* sp. W1 was isolated from a fermented vinegar. The SEM, XRD and FTIR analyses revealed that the pure BC produced by W1 was mostly cellulose I and composed of nanofibrils. By a draft genome sequencing, all typical genes associated with BC synthesis and regulation were found. However, the functional verification of some genes that might involve in BC synthesis co-regulation warranted future research.

Conflicts of interest

There are no conflicts to declare.

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