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In Vivo Curdlan/Cellulose Bionanocomposite Synthesis by Genetically Modified *Gluconacetobacter xylinus*

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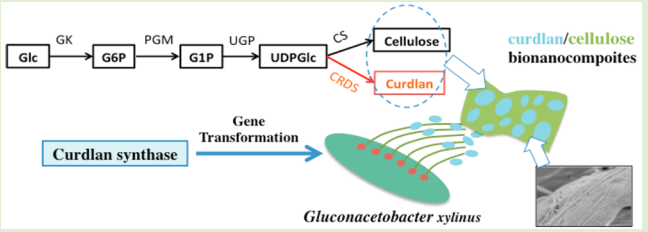
ABSTRACT: Bacterial cellulose pellicle produced by *Gluconacetobacter xylinus* (*G. xylinus*) is one of the best biobased materials having a unique supernetwork structure with remarkable physiochemical properties for a wide range of medical and tissue-engineering applications. It is still necessary to modify them to obtain materials suitable for biomedical use with satisfactory mechanical strength, biodegradability, and bioactivity. The aim of this research was to develop a gene-transformation route for the production of bacterial cellulose/Curdlan (β-1,3-glucan) nanocomposites by separate but simultaneous in vivo synthesis of cellulose and Curdlan. Modification of the cellulose-nanofiber-producing system of *G. xylinus* enabled Curdlan to be synthesized simultaneously with cellulose nanofibers in vivo, resulting in biopreparation of nanocomposites. The obtained Curdlan/cellulose composites were characterized, and their properties were compared with those of normal bacterial cellulose pellicles, indicating that Curdlan mixed with the cellulose nanofibers at the nanoscale without disruption of the nanofiber network structure in the pellicle.



INTRODUCTION

Exopolysaccharides (EPS) are natural polysaccharides generated by microorganisms, including yeast, fungi, and bacteria.[1] Because of their diverse physical and chemical properties, EPS play an essential role in areas such as food additives, biofilm synthesis, pathogen persistence, and medical uses.[1−5] The biosynthesis of EPS involves three stages: substrate processing, intracellular polymerization of oligosaccharides to polysaccharides, and extrusion and exudation of products to the extracellular region. Microbial cellulose nanofibers secreted by the Gram-negative bacterium *Gluconacetobacter xylinus* have attracted widespread attention because of the unique cellulose-nanofiber-producing mechanism and the hierarchical three-dimensional nanofiber structure. *G. xylinus* has cellulose-synthesizing terminal complexes (TCs); these are a group of proteins that aggregate in a specific manner, each of which is not only responsible for molecular biosynthesis, but also for the self-assembly of the products to nanofibers before excretion.[6−8] In this way, the TC structure enables *G. xylinus* to act as a cellulose-synthesizing nanomachine. The glucan chain of a cellulose molecule is first polymerized intracellularly, using uridine diphosphate glucose (UDP-glucose) as a substrate, by cellulose synthase, and then extruded through the cell membrane through pores associated with TCs to induce the self-assembly of a nanofiber on the surface of the bacterial cell. Finally, a cellulose membrane (continuous pellicle) with an ultrafine network structure of the cellulose nanofibers and unusual physiochemical properties is constructed at the gas−liquid interface between the culture medium and the surrounding atmosphere.

The resulting pellicle exhibits excellent versatile properties, such as biocompatibility,[9] high water absorption capacity, high crystallinity, and high mechanical strength.[10] Further investigations for the unique three-dimensional network structure constructed with cellulose nanofibers have also been performed to open wider application fields of tissue engineering,[11,12] electronic device,[13] emulsifiers,[14] nanocomposite,[15] and so on.[16,17] Recently, in particular, a variety of research concerning cellulose nanocomposites having nanofibrillar cellulose networks have been extensively conducted for various cellulose sources in addition to the pellicle; that is, nanopapers that are often composites containing at least little bits of hemicellulose,[18,19] cellulose nanofibril composites,[20,21] or electrospun fiber networks with cellulose or its derivatives.[22,23]

Although their superior properties make the microbial cellulose nanofibers and pellicle excellent materials for the above applications, because of the high production cost, research has focused on their use as high-value-added materials.[24] Because of its high porosity, water absorbance, mechanical properties, formability, and biocompatibility, the pellicle is considered to be a potential material for biomedical applications.[25−28] However, the in vivo degradation of cellulose

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Table 1. Bacterial Strains, Plasmids, and Primers Used in This Study[a]

bacterial strain, plasmids, and primers	relevant characteristics	source
Bacterial Strains		
Gluconacetobacter xylinus AY201	derivative of *G. xylinus* ATCC23769	laboratory stock
Agrobacterium sp. ATCC31749	wild type (wt)	laboratory stock
Vectors		
pTI99 vector	Amp^r, pTrc99A derivative	Sunagawa et al.[41]
pTI99-*crdS* vector	Amp^r, pTI99 containing the *crdS* gene	this study
Primers		
forward primer (FP)	5′- CGGAGCTCATGTATTTCAGTGCTGAAGG-3′	this study
afterward primer (AP)	5′-CCGGTACCTCACCCGAATGCCCGTGC-3′	this study

[a]Amp^r: ampicillin resistance.

is limited because of the absence of β-1,4-glucosidic linkage hydrolase in animal and human bodies.[29] Furthermore, although a microbial cellulose pellicle is a stable material and can stimulate cell growth in tissue-engineering applications, cell adhesion is not sufficient for such applications.[30]

In recent years, many approaches have been used to obtain the desired properties for biomedical applications. One possibility is to modify the pellicle during culturing by adding host polymers to the culture medium to tailor the crystallinity and network morphology.[31,32] Disruption of the higher-ordered structure of the pellicle improves the biodegradability and processability, but the mechanical strength decreases. Another possibility is to modify the formed pellicle membrane to enhance the hydrophilicity or bioactivity, but this is an expensive and complex procedure.[33,34]

In this study, we developed a method for synthesizing a novel bioactive composite membrane by directly modifying the cellulose-synthesizing metabolic pathway of *G. xylinus* to secrete biocomposites of cellulose and Curdlan (β-1,3-glucan). Curdlan is an extracellular polysaccharide with a linear structure containing β-1,3-glucosidic linkages, and is produced by yeast, fungi, and bacteria.[35] Since Curdlan was first detected in *Agrobacterium* sp., it has become an essential material for biomedical applications because of its nonionic gelation properties and low toxicity in vitro and in vivo.[36,37] The molecular genetics of Curdlan synthesis in *Agrobacterium* sp. ATCC31749 has been studied in detail.[38,39] Similar to *G. xylinus*, *Agrobacterium* sp. can use UDP-glucose as a precursor for EPS production. Transformation of a Curdlan synthase gene from *Agrobacterium* sp. ATCC31749 can therefore be used to introduce an enthetic Curdlan biosynthesis system into *G. xylinus*. Cooperation of the two independent polysaccharide-biosynthesizing systems results in bioblending of the cellulose nanofiber pellicle and Curdlan in vivo. Here, it should be noted that in the case of *Agrobacterium* sp., another two supporting proteins in addition to the Curdlan-molecular synthase gene *crdS* introduced in this study are, in general, necessary for Curdlan secretion into an extracellular environment by creating passage ways for the β-1,3-glucan chains to pass through the cell wall, which were not transformed to *G. xylinus* in this study. Thus, the aim of the present study is also to propose a novel potential biosynthetic strategy only by introducing one Curdlan synthase gene *crdS* without the other supporting genes to allow the extracellular secretion, in order to provide Curdlan/bacterial cellulose nanocomposites directly from a bacterium, *G. xylinus*. Then, novel composites with promising physical properties and bioactivities could be obtained using this modified nanomachine, without any further processing, using *G. xylinus* as the host.

MATERIALS AND METHODS

Materials. Glucose, Bacto peptone, yeast extract, disodium hydrogen phosphate, critic acid, and water-soluble aniline blue were purchased from Wako Pure Chemical Industries Ltd. (Japan). Nutrient broth (NB) medium was purchased from BD Ltd. (Japan). Aniline blue fluorochrome was purchased from Biosupplies Pty Ltd. (Australia). Ampicillin sodium salt and Curdlan (for control experiments) were purchased from the Sigma-Aldrich Co., LLC (Japan). Cellulase was purchased from the Yakult Co., Ltd. (Japan). All chemicals were used without further purification.

The bacterial strains and plasmids used in this study are listed in Table 1. *G. xylinus* was cultured statically at 30 °C in Hestrin−Schramn (HS) medium.[40] Ampicillin (100 μg/L) and cellulase (0.1%, wt/vol) were added to the medium as necessary. Curdlan was detected using HS medium containing 2% agar and 0.005% (wt/vol) aniline blue fluorochrome. *Agrobacterium* sp. was incubated in NB medium at 28 °C.

Plasmid Construction and Transformation. *Agrobacterium* sp. ATCC31749 genome DNA was extracted using an Isoplant II DNA extraction kit (Nippon Gene Co., Ltd., Japan). The gene encoding Curdlan synthase (*crdS*) was amplified using *Agrobacterium* sp. ATCC31749 as a template, and the primers FP and AP were amplified by the polymerase chain reaction (PCR) using a PCR kit (Takara LA Taq DNA polymerase, Takara Bio Co., Ltd., Japan). The PCR was performed using an S1000 Thermal Cycler system (Bio-Rad Laboratories Inc., U.S.A.) with preincubation at 94 °C for 1 min, and 30 cycles at 98 °C for 10 s and 68 °C for 2 min. The amplified DNA fragments (approximately 2.8 kb) were recycled from agarose gel and purified, followed by cloning into the *Eco*RI and *Hind*III restriction sites of the pTI99 vector using a ligation kit (Ligation High Ver. 2, Toyobo Biotech. Co., Ltd., Japan) to construct the expression vector pTI99-*crdS*, which was then sequenced by the Takara Bio Co., Ltd., Japan. *G. xylinus* AY201 was transformed with pTI99-*crdS* by electroporation, according to the method used in previous studies.[42] Gene amplification and plasmid construction were confirmed by gel electrophoresis. Gene-transformed *G. xylinus* cells were incubated in HS medium at 30 °C, with shaking, in the presence of 0.1% (v/v) cellulose for 2.5 days, until the culture reached the mid log phase. The cells were harvested by centrifugation, washed with 10% (v/v) glycerol, concentrated 100-fold in 10% (v/v) glycerol, and stored at −80 °C. Frozen cells (100 μL) were thawed on ice, and then pTI99-*crdS* was mixed with the cells in an ice-cold 0.1 cm long electroporation cuvette (Bio-Rad Laboratories Inc.). The electroporation was performed using an electroporation machine (Bio-Rad Laboratories Inc.) under the conditions 2.5 kV pulse, 200 Ω, and 25 mF. After electroporation, precooled HS medium (1 mL) was added immediately. The cells were incubated at 30 °C for 3 h, diluted, and transferred to HS/agar plates containing ampicillin for screening of the recombinants.

Cell Growth, Composite Production, and CrdS Protein Expression by Gene-Transformed *G. xylinus*. For composite production, gene-transformed *G. xylinus* was cultured in HS medium containing 100 μg/L ampicillin in a static environment. The pellicles were collected and purified by washing with 4% sodium dodecyl sulfate

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(SDS) solution at 70 °C for 2 h and then 0.1% NaOH for 4 h to remove the entrapped bacteria, followed by immersion in deionized water overnight until the pH became neutral. The resulting composite mats were freeze-dried and weighed. The mass of the product was calculated based on the culture volume. In order to determine the amount of secreted Curdlan, the obtained composites films were treated with 1 M NaOH solution for 6 h to dissolve the Curdlan. The alkali-treated films were washed in deionized water overnight and then freeze-dried and weighed. For control experiments, pure Curdlan film was prepared by the casting method. Curdlan powder was dissolved in 1 M NaOH solution to ensure a final concentration of 1%. The Curdlan solution was poured on glass plates and kept undisturbed at room temperature for 3 days, then dried in an oven at 55 °C overnight.

For protein analysis, gene-transformed *G. xylinus* was cultured at 30 °C, with shaking, for 72 h in HS medium containing 100 μg/L ampicillin and 1 mg/mL cellulase. The cells were harvested and disrupted by ultrasonication (50 W, 20 min, with 1 min intervals) and centrifuged to recycle the lysates. The lysates were ultracentrifuged at 132000*g* for 90 min to precipitate the membrane protein fraction. The entire process was conducted at 4 °C. The precipitate was treated with (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)/SDS buffer, before SDS polyacrylamide gel electrophoresis (PAGE).

Detection of Synthesized Curdlan on Agar Medium. Gene-transformed *G. xylinus* was cultured on HS/agar medium containing the β-1,3-glucan-specific dye aniline blue (0.01%, w/v). After incubation at 30 °C for 7 days, the gene-transformed *G. xylinus* colonies had been stained blue, whereas the color of the control colonies on agar did not change.[43]

Field-Emission Scanning Electron Microscopy (FE-SEM). FE-SEM was performed to study the nano/microscale structures of the composites. Purified samples were fixed on an SEM grid, coated with platinum, and examined using an ultrahigh-resolution scanning electron microscope (SU8000, Hitachi, Japan) at an accelerating voltage of 1 kV and a working distance of 1.5 mm.

Fluorescence Microscopy. The purified samples were cut into 1 × 1 cm pieces and fixed on a glass slide. Aniline blue fluorochrome (0.01%, wt/vol, 30 μL) was added to the samples. The stained samples were observed with a fluorescence microscope, using an Hg lamp with UV emission.

Wide-Angle X-ray Diffraction. The crystalline structures of the secreted microbial cellulose nanofibers in the composited pellicles were studied by WAXD using a Rigaku RINT2000 V/PC XRD system (Rigaku Co., Ltd., Japan). The WAXD patterns were obtained using graphite-monochromated Cu Kα radiation (λ = 1.54056 Å) at 40 kV and 20 mA.

Water Contact Angle Measurements. Dried samples were fixed on a glass slide, and the water contact angles were measured using a drop shape analysis system equipped with a charge-coupled device camera (DropMaster DM300, Kyowa Interface Science Co., Ltd., Japan). The images were captured immediately at 10 and 20 s after pure water was dripped onto the sample surface. The angle for each sample was measured three times in parallel.

RESULTS AND DISCUSSION

Gene Transformation of *G. xylinus* and Screening of Curdlan/Cellulose Composite Production. *Agrobacterium* sp. ATCC 31749 produces Curdlan extracellularly in large amounts, because of its efficient mechanism for the use and regeneration of UDP-glucose as the major precursor for Curdlan synthesis. In previous studies, four genes at two separate loci (locus 1 and locus 2) were found to be the essential genes for Curdlan production and regulation. A three-gene-cluster at locus 1 contains the putative Curdlan synthase gene *crdS* and two supporting genes, *crdA* and *crdC*. The *crdS* gene (1623 bp) encodes a transmembrane protein CrdS, which is responsible for catalysis of β-1,3-linkage formation.[38] CrdS has substrate-binding and catalytic motifs, and shares significant homologies with β-glycan synthases in the GT-2 family, such as BcsA, which is the cellulose synthase protein in *G. xylinus*.[39] Transformation of the *crdS* gene from *Agrobacterium* sp. into *G. xylinus* would therefore enable a metabolic pathway for Curdlan/cellulose synthesis to be developed (as shown in Figure 1).

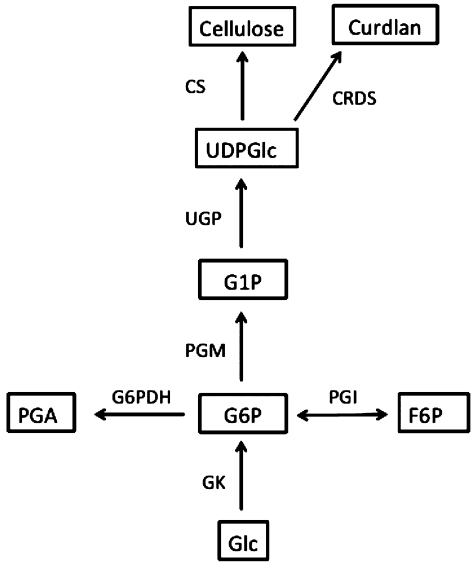

Figure 1. Putative metabolic pathway of genetically modified *G. xylinus*. Glc: glucose; G6P: glucose-6-phosphate; G1P: glucose-1-phosphate; UDPGlc: uridine diphosphoglucose; PGA: phosphogluconic acid; F6P: fructose-6-phosphate; GK: glucokinase; G6PDH: glucose-6-phosphate dehydrogenase; PGI: phosphoglucoisomerase; PGM: phosphoglucomutase; UGP: pyrophosphorylase uridine diphosphoglucose; CS: cellulose synthase; CRDS: Curdlan synthase.

The pTI99 vector (5359 bp), a shuttle vector derived from ptrc99A, was chosen for *crdS* transformation. The successful construction of the pTI99-*crdS* vector (~7000 bp) and synthesis of the CrdS protein (~60 kDa) in the cell membrane were confirmed using gel electrophoresis (Figure 2) and SDS-PAGE (Figure 3), respectively. After gene transformation,

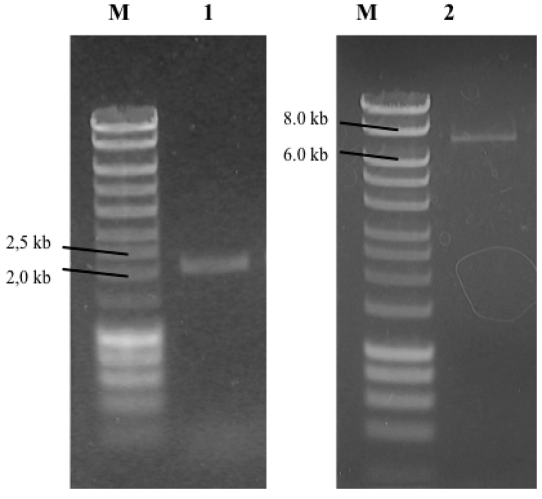

Figure 2. PCR products of *crdS* gene and pTI99-*crdS* vector. M: marker; Lane 1: *crdS* amplified from genomic DNA of *Agrobacterium* sp. ATCC 31749; Lane 2: confirmation of construction of pTI99-*crdS* vector.

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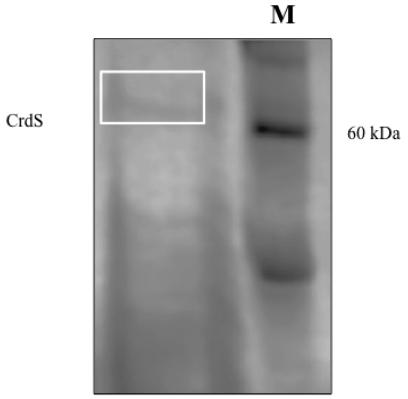

Figure 3. SDS-PAGE analysis of proteins in total membrane fractions prepared from gene-transformed *G. xylinus*. M: marker.

Curdlan production was screened using the aniline blue staining method. Aniline blue dye is widely used for β-1,3-glucan detection because it can selectively bind the β-1,3-glucan linkage and thereby stain Curdlan-producing colonies blue. The gene-transformed *G. xylinus* colonies were compared with wild type *G. xylinus* (Figure 4). After culture for 1 week, only the gene-transformed colonies were blue, indicating that Curdlan was secreted extracellularly.

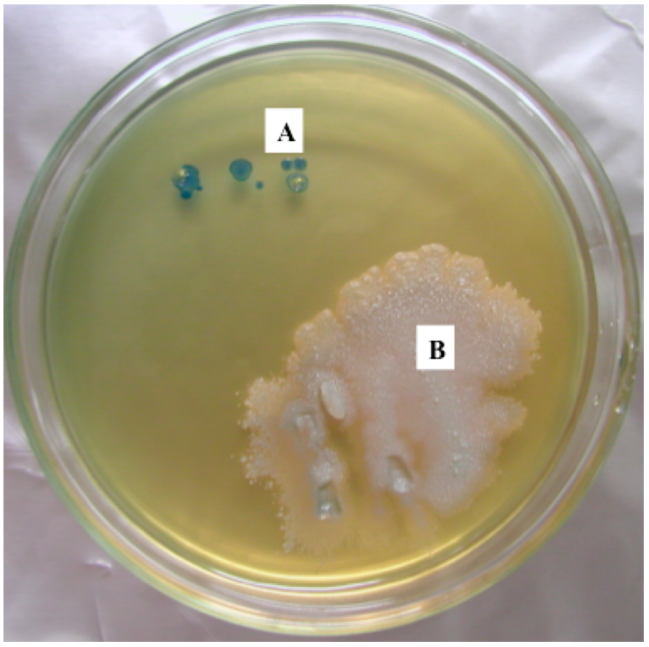

Figure 4. Screening of Curdlan production by aniline blue staining. A: colonies of gene-transformed *G. xylinus*; B: colonies of *G. xylinus* AY201 (control).

The gene-transformed colonies were much smaller than the control (Figure 4), and this greatly affects the productivity of gene-transformed *G. xylinus*. Curdlan synthesis was further confirmed by observation of the composite pellicle using a fluorescence microscope in the presence of aniline blue fluorochrome (Figure 5). Aniline blue fluorochrome is adsorbed and forms a complex with β-1,3-glucan, with high specificity, to give light-blue fluorescence under UV light.[44] The biosynthesized Curdlan/cellulose composite emitted a specific light-blue fluorescence when a UV filter was used, indicating the presence of a Curdlan/cellulose complex in the pellicle network matrix.

The yields of the products from gene-transformed *G. xylinus* were lower than those from the control (Figure 6). After treatment with 1 M NaOH solution, the weight of the composite films reduced by 12.2 ± 5.6%, whereas the weight of the control cellulosic pellicle only dropped by 4.3 ± 2.7%. The comparative study indicates that Curdlan was synthesized in a small proportion after genetic modification. Because the secreted Curdlan is strongly complexed with cellulose microfibrils at the nanoscale, it is still difficult to directly and accurately calculate the amount of secreted cellulose and Curdlan. Namely, only a part of Curdlan fraction could be dissolved in the alkali, and thus separated from the composite pellicle. Therefore, a more suitable method is required to determine the ratio of Curdlan and cellulose. The slow growth rate of genetically modified colonies and the low yield of the composites might result from the competition between the cellulose and Curdlan biosynthetic systems. Both CrdS and BscA transfer UDP-glucose to the growing glucan chain. The two metabolic pathways are therefore competitive with each other, which may decrease the production of Curdlan.

It has been reported that the mutant Curdlan-production-supporting genes *crdA* and *crdC* of *Agrobacterium* strains failed to synthesize Curdlan at a normal level. The CrdA and CrdC protein functions are still unknown, but they are believed to assist the transport of Curdlan polymeric chains across the membrane of the cell envelope.[35] In this study, Curdlan was successfully synthesized and secreted extracellularly simply by transforming the *crdS* gene to *G. xylinus*.

Here, why did the Curdlan extrusion occur without another two supporting proteins presumably working for the extracellular Curdlan secretion? One possible reason is that the TCs of *G. xylinus* in the cell membrane provide passageways for Curdlan chains to pass through the cell membrane when cellulose is being secreted. Namely, the Curdlan secretion might be also using the same pore-like structure[45] on the cell wall for cellulose chains extrusion. Therefore, although the supporting proteins for Curdlan secretion were absent, Curdlan was still able to be secreted through TCs. Similar to the cellulose-synthesizing process, Curdlan is thought to be first synthesized and accumulated in vivo. In this case, when the intracellular Curdlan concentration reaches a certain level of turgor pressure, the Curdlan is forced to be extruded through the pores associated with TCs. In this way, Curdlan is not only synthesized in vivo, but is also secreted along with synthesized cellulose nanofibers. This unique in vivo bioblending process produces a novel Curdlan/cellulose nanocomposite.

Curdlan/Cellulose Bionanocomposite Morphology. The morphology of the secreted Curdlan/cellulose composite was examined using field-emission scanning electron microscopy (FE-SEM); the images are shown in Figure 7. The fiber morphology of the secreted composite, shown in Figure 6C, differs greatly from that of the control cellulose pellicle and Curdlan film (Figure 7A,B). A gel-like Curdlan substance covers the surface of the cellulose fiber network, resulting in elimination of the pore structure. A high-magnification image (Figure 7D) shows the nanoscale composite structure. Curdlan covers the surface of the secreted cellulose nanofibers and fills the pores between cellulose nanofiber networks, indicating formation of a reinforced-concrete-like structure. The FE-SEM images suggest that although Curdlan and cellulose nanofibers were both produced by gene-transformed *G. xylinus*, cellulose

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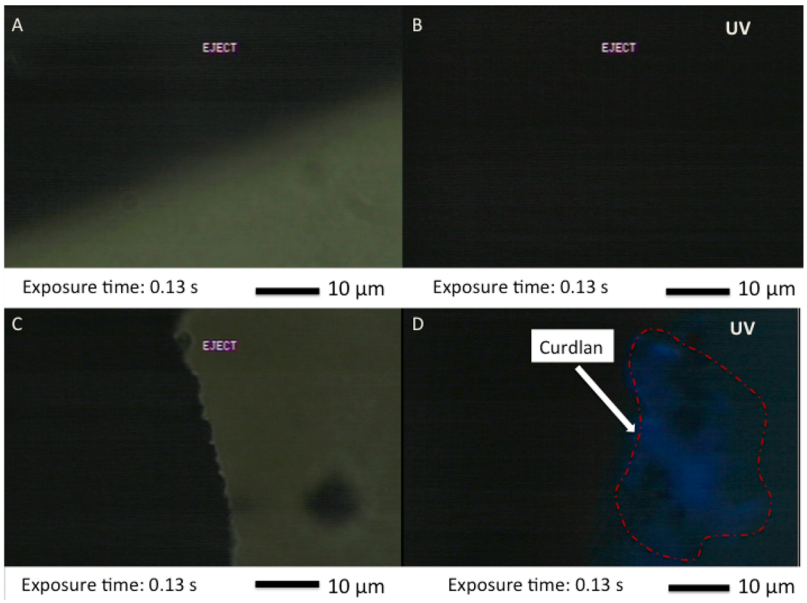

Figure 5. Staining with aniline blue fluorochrome: (A) cellulose pellicle observed without UV light; (B) cellulose pellicle observed with UV light; (C) Curdlan/cellulose composites stained by aniline blue fluorochrome, observed without UV light; and (D) Curdlan/cellulose composites stained by aniline blue fluorochrome, observed with UV light.

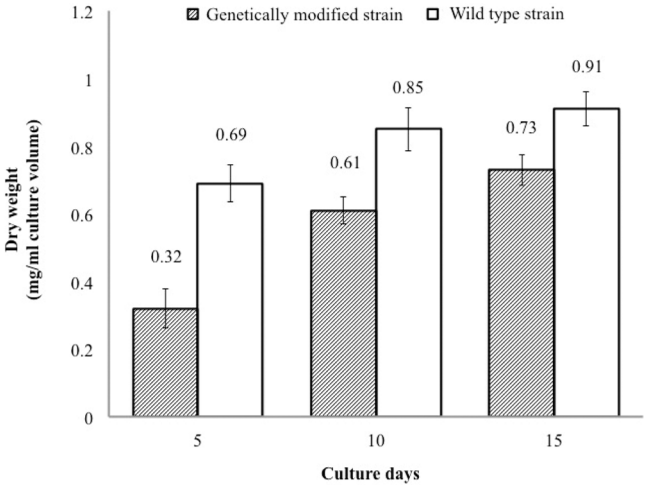

Figure 6. Differences between production yields of gene-transformed *G. xylinus* and *G. xylinus* AY201.

nanofibers were synthesized and secreted preferentially. When Curdlan was synthesized and accumulated to an intracellular concentration with an increase in turgor pressure high enough to induce outward secretion, the cellulose nanofiber networks were well constructed. Curdlan secretion therefore did not disrupt the cellulose network structures, but only wrapped the cellulose nanofibers.

Crystalline Properties of Curdlan/Cellulose Bionanocomposite. The crystalline properties of the resulting composite pellicle were examined using wide-angle X-ray diffraction (WAXD). Figure 8 shows the WAXD patterns of the Curdlan/cellulose bionanocomposite and cellulose pellicle. Curdlan is a low crystalline polysaccharide (crystallinity is about 30%).[46] Both patterns show the diffraction peaks corresponding to cellulose I (2θ = 14.3°, 17.0°, and 22.8°), indicating that Curdlan secretion had almost no influence on the higher-ordered cellulose crystalline structure.[47]

It is assumed that cellulose molecules were polymerized and secreted in preference to Curdlan. Preferential cellulose synthesis resulted in a low Curdlan yield and also led to a lag in Curdlan secretion. It is thought as described above that Curdlan is secreted extracellularly through the TCs on the cell wall after secretion and crystallization of the cellulose polymeric chains. The secreted Curdlan therefore causes little damage to the tightly packed β-1,4-glucan chains, resulting in little change in the crystalline structure of the bionanocomposite. The WAXD results show that there were no detectable differences between the crystalline properties of cellulose nanofibers in the Curdlan/cellulose composite and the control pellicle; this supports the hypothesis that Curdlan is synthesized in low amounts and the secretion of Curdlan did not disturb the cellulosic pellicle formation in the culture medium. In addition, if Curdlan is produced separately and it follows secretion of cellulose nanofibers, a close interaction of the secreted Curdlan with the already produced cellulose crystalline nanofibers possibly inhibits crystallization of the Curdlan, as indicated in Figure 8.

Surface Properties of Curdlan/Cellulose Bionanocomposite. The time-lapse contact angle measurements are shown in Figure 9. The water contact angle of Curdlan film was maintained at 107.3 ± 5.5° after 20 s when water was dropped on the film surface, revealing the great hydrophobicity of Curdlan film. The Curdlan/cellulose bionanocomposite and control pellicle were both hydrophilic. However, the presence of Curdlan increased the surface hydrophobicity of the composite. When water was dropped on the surface of the films, the water contact angle decreased slightly from 46.1 ± 5.1° to 43.0 ± 4.2° after 20 s, whereas the water contact angle of the control pellicle film dropped significantly from 25.7 ± 2.6° to 10.9 ± 3.1°. This indicated that the Curdlan substance not only slightly increases the hydrophobicity of the bionanocomposites, but also prevents the water from permeating the surface of the cellulose microfibril network. These data corroborate the FE-SEM results, suggesting that the decrease in the surface porosity improved the water resistance

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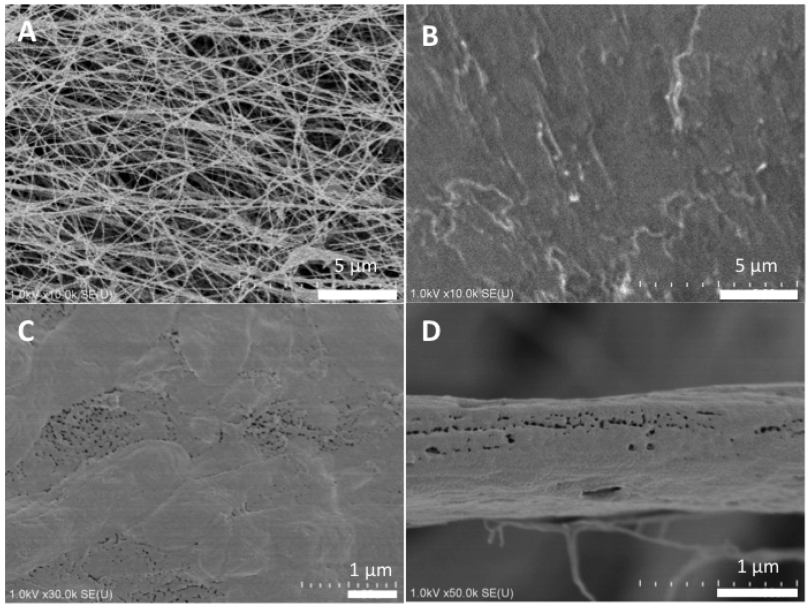

Figure 7. FE-SEM images of (A) cellulose pellicle secreted by *G. xylinus* AY201; (B) Curdlan film prepared by coating method; (C) Curdlan/cellulose composites secreted by gene-transformed *G. xylinus*; and (D) higher magnification of C.

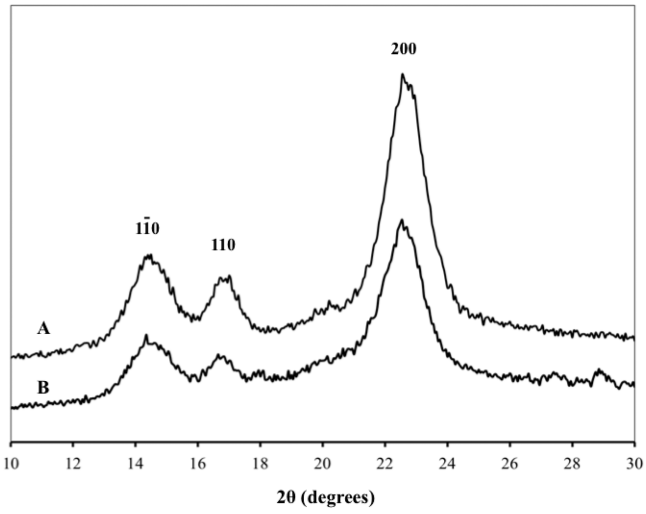

Figure 8. WAXD patterns of Curdlan/cellulose bionanocomposite (A) and control cellulose pellicle (B).

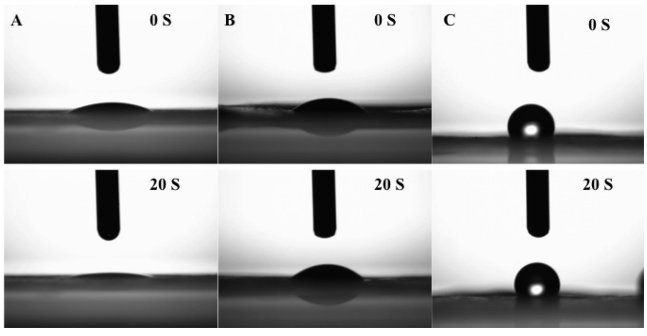

Figure 9. Water contact angle measurements of cellulose pellicle from wide type (A), Curdlan/cellulose bionanocomposite (B), and Curdlan film (C) at 0 and 20 s after water was dropped on sample surface.

of the bionanocomposite. The Curdlan covering the surface of the cellulose microfibrils and nanofibers prevented permeation of water into the composite substrate, and therefore, the composite has a wide range of potential applications as biomedical materials.

CONCLUSIONS

In this study, we developed a feasible, environmentally friendly approach to Curdlan/cellulose bionanocomposite synthesis. *G. xylinus*, which was used as a natural nanocellulose-producing machine, was modified simply by introducing a Curdlan synthase gene, *crdS*, for the purpose of combining two separate cellulose and Curdlan metabolic pathways. Curdlan extrusion successfully occurred without another two supporting proteins working for the extracellular Curdlan secretion, accompanied by secretion of crystalline cellulose nanofibers, and therefore, this gene transformation enabled production of a Curdlan/cellulose bionanocomposite pellicle. Curdlan secretion significantly changed the surface morphology of the composite film, but had little influence on the crystalline structure, suggesting that Curdlan secretion may be of lower priority than cellulose nanofiber production. The water permeability of the bionanocomposites was low, because the pore structures on the composite surface were covered with Curdlan. Further investigations will focus on optimizing the metabolic modification strategy to control the Curdlan/cellulose proportion to regulate the surface properties. This work is expected to expand the use of cellulose-based composites in biomedical and other material application.

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Notes

The authors declare no competing financial interest.

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