Applied Microbiology and Biotechnology (2018) 102:1155–1165
https://doi.org/10.1007/s00253-017-8680-z

BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Enhanced bacterial cellulose production by *Gluconacetobacter xylinus* via expression of *Vitreoscilla* hemoglobin and oxygen tension regulation

Miao Liu[1] · Siqi Li[1] · Yongzhen Xie[1] · Shiru Jia[1] · Ying Hou[1] · Yang Zou[2] · Cheng Zhong[1]

Received: 31 October 2017 / Accepted: 23 November 2017 / Published online: 4 December 2017

Abstract
Oxygen plays a key role during bacterial cellulose (BC) biosynthesis by *Gluconacetobacter xylinus*. In this study, the *Vitreoscilla* hemoglobin (VHb)-encoding gene *vgb*, which has been widely applied to improve cell survival during hypoxia, was heterologously expressed in *G. xylinus* via the pBla-VHb-122 plasmid. *G. xylinus* and *G. xylinus-vgb*$^+$ were statically cultured under hypoxic (10 and 15% oxygen tension in the gaseous phase), atmospheric (21%), and oxygen-enriched conditions (40 and 80%) to investigate the effect of oxygen on cell growth and BC production. Irrespective of *vgb* expression, we found that cell density increased with oxygen tension (10–80%) during the exponential growth phase but plateaued to the same value in the stationary phase. In contrast, BC production was found to significantly increase at lower oxygen tensions. In addition, we found that BC production at oxygen tensions of 10 and 15% was 26.5 and 58.6% higher, respectively, in *G. xylinus-vgb*$^+$ than that in *G. xylinus*. The maximum BC yield and glucose conversion rate, of 4.3 g/L and 184.7 mg/g, respectively, were observed in *G. xylinus-vgb*$^+$ at an oxygen tension of 15%. Finally, BC characterization suggested that hypoxic conditions enhance BC's mass density, Young's modulus, and thermostability, with *G. xylinus-vgb*$^+$ synthesizing softer BC than *G. xylinus* under hypoxia as a result of a decreased Young's modulus. These results will facilitate the use of static culture for the production of BC.

Keywords Bacterial cellulose · *Gluconacetobacter xylinus* · Oxygen tension · *Vitreoscilla* hemoglobin · Young's modulus

Introduction

Cellulose, the most abundant biopolymer on Earth, is synthesized by both plants and microorganisms (Divne et al. 1994). Bacterial cellulose (BC) produced by bacteria has the same chemical structure as plant cellulose but is devoid of hemicellulose and lignin (Ross et al. 1991). *Gluconacetobacter xylinus* is the most widely studied BC-producing microorganism due to its relatively high production yield and the appealing characteristics of BC (Fang and Catchmark 2014; Kubiak et al. 2014; Li et al. 2012). *G. xylinus* is an obligate aerobe that produces, in static culture, a BC pellicle at the air-liquid interface with its cells embedded in it in order to access oxygen (Hestrin and Schramm 1954). BC produced in static culture has a finer three-dimensional network structure and a higher mechanical strength than the one produced in agitated culture (Czaja et al. 2004). It has been successfully used in many fields, including the paper industry (Shah and Brown 2005), food packaging (Fabra et al. 2016), fuel cells (Evans et al. 2003), cell culture (Yin et al. 2014), and medical materials (Czaja et al. 2006; Maneerung et al. 2008). However, the low yield of BC production limits its commercial application. Researchers have endeavored to improve BC production by reducing culture time and cost. The BC membrane has a layered structure with a much denser zone at the upper film/air interface (Tang et al. 2010). This thin upper surface layer (of approximately 1 mm in thickness) has been found to be the only strictly aerobic zone for the growth of *G. xylinus*, and only the cells in this aerobic zone are able to produce BC. These active cells constitute approximately a 10% of the total bacterial cells of the culture and this number remains

✉ Cheng Zhong
chzhong.tju@gmail.com

[1] Key Laboratory of Industrial Fermentation Microbiology, Ministry of Education, Tianjin University of Science and Technology, Tianjin 300457, People's Republic of China

[2] Tianjin Jialihe Livestock Group Co., Ltd, Jin Wei Road, Beichen District, Tianjin 300402, People's Republic of China

constant (Hornung et al. 2006). Thus, it is suggested that oxygen is one of the most important factors for BC pellicle production.

In agitated culture, most cells are distributed around the surface of BC spheres, and only a few of them are randomly scattered inside. Such a particular arrangement seems to suggest that cellulose is only synthesized at the interface, where oxygen and substrate are found in sufficient amounts (Czaja et al. 2004). We had previously shown that *G. xylinus* cell growth is enhanced in agitated culture compared to static culture, which could be explained by an increased dissolved oxygen concentration (Liu et al. 2015). However, the conversion rate of glucose to BC is lower in agitated culture because of the hydrodynamic stress that induces the accumulation of self-protection metabolites (Liu et al. 2015). To eliminate the hydrodynamic stress of the culture media and the shear stress of the stirring paddles, an airlift reactor has been used to culture *Acetobacter xylinum* for BC production (Valla et al. 1989). In addition, the supply of oxygen-enriched air instead of air has been shown to increase the rate and yield of BC production in agitated culture by 57.6 and 63.6%, respectively (Chao et al. 2001). In contrast, in static culture, BC yield significantly increases under hypoxic conditions compared to atmospheric and oxygen-enriched air conditions, even though changes in the oxygen tension in the gaseous phase do not influence cell density or gluconic acid contents. These results have been confirmed in four additional BC-producing strains, showing that an oxygen tension of 10% is optimal for BC production in static culture (Watanabe and Yamanaka 1995).

Vitreoscilla hemoglobin (VHb), a homodimeric oxygen-binding protein, was first studied by Webster and Hachett in the 1960s (Webster and Hackett 1966). The *vgb* gene, coding for VHb, has been successfully expressed in various bacteria to optimize cell density and metabolite synthesis for industrial applications (Dogan et al. 2006; Stark et al. 2015; Suen et al. 2014; Zhang et al. 2007). VHb has been shown to help alleviate the effects of oxygen limitation in a variety of microorganisms (Frey and Kallio 2003; Xiong et al. 2007). VHb increases the level of dissolved oxygen in the cell by enhancing oxygen delivery to the respiratory apparatus under hypoxia (Frey and Kallio 2003). In this study, *G. xylinus* CGMCC 2955 was genetically modified to express VHb using plasmid pBla-VHb-122. Both *G. xylinus* and *G. xylinus*-*vgb*$^+$ were statically cultured under various oxygen tensions in the gaseous phase to investigate the effects of oxygen tension and of the expression of *vgb* on cell growth and BC production. Glucose consumption, BC conversion rate, and gluconic acid production were also analyzed. The characteristics of BC and the possible relationship between oxygen utilization and BC synthesis are discussed.

Materials and methods

Microorganisms and culture medium

G. xylinus CGMCC 2955 was used for BC production in this study. This strain was isolated in our laboratory and was deposited in the China General Microbiological Center Collection with the registered number 2955. *Escherichia coli* DH5α was used as cloning host.

The culture medium for *G. xylinus* CGMCC 2955 was composed of 25 g/L glucose, 10 g/L peptone, 7.5 g/L yeast extract, and 10 g/L disodium phosphate, and the initial pH was adjusted to 6.0.

Culture and BC production

For oxygen-limited cultures, unventilated rubber plugs were used to prevent air from entering into culture flasks. For cultures grown under aerobic conditions, flasks were covered with gauze. To investigate the effect of oxygen tension on cell growth and BC production, gas with the required oxygen tension was continuously supplied into the static culture. Cells were inoculated into 150 mL of fresh media in 500-mL flasks at an initial OD_{600} of 0.02 and were cultured statically at 30 °C for 15 days.

At the end of the culture, BC was harvested from the air-liquid interface, rinsed with distilled water, and placed in a NaOH (0.1 mol/L) boiling bath for 20 min to remove the attached bacterial cells and media. BC was further purified using distilled water until the pH of BC became neutral. Finally, BC was dried at 80 °C for 10 h until a stable weight was obtained and the dry weight of each pellicle at room temperature was recorded.

Construction of plasmid pBBR-122

VHb was expressed in *G. xylinus* from plasmid pBla-VHb-122, a generous gift from Cheng-Kang Lee. pBla-VHb-122 contains the *vgb* gene driven by a constitutive *bla* promoter (Chien et al. 2006). pBBR-122 was constructed by digesting pBla-VHb-122 with *Eco*R I to delete the *vgb* and *bla* genes. The resulting DNA fragment, of around 4000 bp in length, was purified using the Tiangen DNA purification kit and was recircularized at 16 °C overnight using a DNA ligase. The resulting plasmid DNA was transformed into *E. coli* DH5α and transformants were selected on Luria Bertani (LB) medium plates containing 50 μg/mL of kanamycin. Polymerase chain reaction (PCR) using the forward and reverse primers 5′-gacggcatgatgaacctgaat-3′ and 5′-tcttgcccgcctgatgaatg-3′, respectively, was used to screen the transformants. Positive transformants were further confirmed by restriction enzyme digestion.

Electroporation

G. xylinus cells grown to exponential phase ($OD_{600} = 0.5$) were harvested by centrifugation at 4 °C, washed twice with cold 1 mM HEPES, and resuspended in 1/300 volumes of 15% glycerol. The cells were distributed into aliquots and frozen at − 80 °C until electroporation was conducted. For electroporation, a Gene Pulser apparatus (Eppendorf Inc.) set to 2.0 kV was used. Plasmid DNA (2 μL) was added to 100 μL of competent *G. xylinus* cells in a cold 0.2-cm electroporation cuvette and a pulse was applied. Pulsed cells were transferred to 1 mL of culture medium containing 4% (*v*/*v*) cellulase and were incubated at 30 °C and 180 rpm overnight. The culture was then diluted and plated on culture agar plates with kanamycin (25 μg/mL). *G. xylinus* transformants were screened for the presence of pBla-VHb-122 by PCR with the forward and reverse primers 5′-acatgcatgcatgtgaattcgacgaaagggcctcgtgatacgcct-3′ and 5′-ccgctcgagttaagcgtagtctggaacgtcgtatgggtattcaaccgcttgagcgtac-aaatctgc-3′, respectively. Selected transformants were further confirmed by restriction enzyme digestion with *Eco*R I. The sequence of *vgb* in the *G. xylinus* transformants (named *G. xylinus*-*vgb*$^+$) was confirmed by DNA sequencing.

Western blot

The expression of VHb in *G. xylinus* was analyzed by Western blot. Control and recombinant strains were cultured in 150 mL of culture media with 4% cellulase under oxygen-limited conditions at 30 °C and 180 rpm for 3 days. Cells were harvested by centrifugation at 5000 rpm for 5 min, washed twice with 5 mL of phosphate buffer solution (PBS), and resuspended in 500 μL of PBS. Cells were sonicated for 9 min (KQ-500E, KunShan, China) and centrifuged at 12,000 rpm for 10 min. The supernatant was defined as the soluble fraction. The pellet, defined as the insoluble fraction, was washed with PBS, centrifuged at 12,000 rpm for 1 min, and resuspended in 300 μL of 8 mol/L urea. The proteins of the crude extractions (soluble or insoluble fractions) were separated by SDS-PAGE and transferred onto a nitrocellulose (NC) filter membrane (BioTrace, Mexico) by electroblotting (Beijing LIUYI Biological Technology Co., LTD, China). The NC membrane was then blocked with a skim milk solution (5% *w*/*v* PBS) for 1–2 h, washed with PBS to remove any residual milk, and incubated at 4 °C overnight with an anti-6×His antibody (1/5000 in 5% skim milk, Abclonal), which recognizes the N-terminal 6×His tag of VHb expressed from pBla-VHb-122. After washing thrice with PBS, the NC membrane was incubated with IRDye 800CW Goat anti-Mouse IgG (H + L) (1/5000, Li-COR) for 1–2 h and was washed again thrice with PBS. Finally, the NC membrane was scanned with Odyssey imaging systems (Li-COR, USA).

Real-time PCR

Cells cultured for 3 days were harvested by centrifugation (4 °C, 5000 rpm, 5 min), washed with PBS thrice, and stored in liquid nitrogen until RNA was extracted. For RNA extraction, cells were ground into a powder and RNA was purified using a RNA kit (Omega, USA). RNA concentration and quality were determined with the BioSpectrometer® basic (Eppendorf, Germany). Total RNA was used for cDNA synthesis with the oligo(dT)18-primer and the RevertAid First Strand cDNA Synthesis Kit, according to the manufacturer's instructions. RT-PCR was performed on a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA) with the DyNAmo Color Flash SYBR Green qPCR Kit (Thermo Fisher Scientific, Waltham, MA) and primers F 5′-ATCAAAGCCACTGTTCCTG-3′ and R 5′-TTAG GCTGCTCCAAAGATTC-3′. All the reactions were performed in triplicate. No-template controls were included for each PCR. The following thermal profile was used for all the PCR: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplicon dissociation curves were obtained after cycle 40 by heating the samples from 60 to 95 °C with a ramp speed of 0.3 °C/s. Gene expression levels in wild-type *G. xylinus* and *G. xylinus*-pBBR-122 were used as controls. Expression of the DNA gyrase subunit B gene (*gyrB*) was used as an internal standard to normalize all the quantifications (Tsuge et al. 2006). The relative expression level of *vgb* was calculated using the $2^{-\Delta\Delta CT}$ method.

BC characterization

To determine tensile strength, hot-dried BC samples were cut into small pieces (10 cm in length, 1 cm in width) and were measured with a tensile tester (6P-TS2007S). Thickness was measured with a thickness gauge (970243, Lorentzen & Wettre, Sweden). Thermogravimetric (TG) analysis was carried out with a Shimadzu DTG-60 TGA. The samples were heated from 25 to 800 °C at a rate of 10 °C/min under nitrogen.

Results

Heterologous expression of VHb in *G. xylinus*

Plasmid pBla-VHb-122 was introduced into *G. xylinus* by electroporation. Randomly selected transformants were checked for the presence of the *vgb* gene by PCR. Positive colonies were further confirmed by restriction enzyme digestion with *Eco*R I. The expected *vgb* gene fragment was observed in *G. xylinus*-*vgb*$^+$ and its DNA sequence was identical to that of the original pBla-VHb-122.

Western blot analysis of protein extracts from *G. xylinus* and *G. xylinus-vgb*$^+$ is shown in Fig. 1a, b. VHb was successfully expressed in *G. xylinus-vgb*$^+$ as shown by the prominent protein bands (lanes 3 and 6) with the expected molecular weight of ~ 16 kDa (Hart et al. 1990). VHb was detected in both the soluble (Fig. 1a) and insoluble fractions (Fig. 1b). RT-PCR was used to quantify expression of *vgb* in *G. xylinus-vgb*$^+$. As shown in Fig. 2c, the $2^{-\Delta\Delta CT}$ value for *vgb* in *G. xylinus-vgb*$^+$ was 1046.8 times higher than that in *G. xylinus* and *G. xylinus*-pBBR-122. Thus, *G. xylinus-vgb*$^+$ was confirmed to be a positive transformant.

Effect of VHb expression on BC synthesis under oxygen-limited and aerobic conditions

A preliminary experiment to study the effect of VHb expression on BC production was performed. Previous studies had demonstrated that intracellular accumulation of VHb often results in variations in engineered product formation, cell density, and oxidative metabolism in various heterologous hosts under oxygen-limited conditions (Chien et al. 2006; Frey and Kallio 2003; Ramandeep et al. 2001; Zhang et al. 2007). Therefore, *G. xylinus* and *G. xylinus-vgb*$^+$ were cultured under both oxygen-limited and aerobic conditions. BC membranes were obtained after 15 days of static culture. Under oxygen-limited conditions, BC membranes proved to be too tender and fragile to be sampled. Therefore, BC yield could not be calculated under these conditions. Under aerobic culture conditions, the BC yield of *G. xylinus* and *G. xylinus-vgb*$^+$ increased with culture time until it plateaued on days 8 and 10, respectively (Fig. 2a). The maximum BC yield of *G. xylinus-vgb*$^+$ was 24.5% higher than that of *G. xylinus*. The pH value and glucose consumption of *G. xylinus-vgb*$^+$ were virtually the same as those of *G. xylinus* (Fig. 2b, c). Still, the glucose to BC conversion rate of *G. xylinus-vgb*$^+$ was 24.5% higher than that of *G. xylinus* on day 15 (Fig. 2d). Together, these results suggest that VHb expression promotes BC production in the stationary phase under aerobic conditions. However, VHb remains in its physiologically active reduced form under hypoxic conditions and turns into inactive oxy-VHb in oxygen-enriched environments (Park et al. 2002). In addition, VHb is known to bind oxygen at low extracellular oxygen concentrations, allowing bacteria to survive in hypoxic environments (Webster 1988). Therefore, the effect of VHb expression on BC production under hypoxic conditions is expected to be more significant than that under aerobic conditions.

Effect of oxygen tension and VHb expression on BC production

To investigate the effect of oxygen tension and VHb expression on BC production, *G. xylinus* and *G. xylinus-vgb*$^+$ were cultured under different oxygen tensions. BC is synthesized at the air/cellulose pellicle interface, rather than at the medium/cellulose interface, in static cultures (Jonas and Farah 1998). Therefore, the oxygen tension in the gaseous phase, rather than that of the liquid phase, was regulated. Specifically, *G. xylinus* and *G. xylinus-vgb*$^+$ were cultured under atmospheric (21%, control), oxygen-enriched (40 and 80%), and hypoxic culture conditions (10 and 15%). The oxygen tension was expressed as the percent of oxygen volume in the gaseous phase at 1 atm.

The effect of oxygen tension on cell density, glucose consumption, pH, and gluconic acid concentration in *G. xylinus* and *G. xylinus-vgb*$^+$ cultures is shown in Fig. 3. *G. xylinus* and *G. xylinus-vgb*$^+$ cells were collected on days 5 and 15, which corresponds to the exponential growth and stationary phases, respectively (Fig. 2a). As shown in Fig. 3a, on day 5, the cell densities of *G. xylinus* and *G. xylinus-vgb*$^+$ were higher at higher oxygen tensions (10–80%). On day 15, cell densities at different oxygen tensions were almost the same, except for that at 80% oxygen tension (Fig. 3b). The glucose concentration in the culture broths of *G. xylinus* and *G. xylinus-vgb*$^+$ was the same and was exhausted on day 15 (Fig. 3c, d). This indicates that neither the oxygen tension nor VHb expression influences glucose utilization in *G. xylinus*, which is consistent with the results of the preliminary experiment (Fig. 2c). The pH level was higher under oxygen-enriched conditions than that under atmospheric or hypoxic conditions on day 5 (Fig. 3e), but was similar in all three conditions on day 15 (Fig. 3f). This variation in the pH value could be attributed to variations in the gluconic acid concentration (Liu et al. 2015). On day 5, at oxygen tensions of 10 to 21%, almost 90% (*w*/*w*) of the glucose was converted to gluconic acid both in *G. xylinus* and *G. xylinus-vgb*$^+$ (Fig. 3g). In contrast, the glucose to gluconic acid conversion rate was of 56 and 22.8% at oxygen tensions of 40 and 80%, respectively (Fig. 3g). Expression of *vgb* slightly reduced this conversion rate under hypoxia. On day 15, the gluconic acid concentration dropped to similar levels in all the oxygen tensions tested (Fig. 3h). Therefore, variations in gluconic acid concentration are consistent with pH variations.

The effect of oxygen tension on BC production and on glucose to BC conversion rate in *G. xylinus* and *G. xylinus-vgb*$^+$ is shown in Fig. 4. In *G. xylinus*, higher BC yields and conversion rates were observed under hypoxia compared to atmospheric conditions. Specifically, a 71.5 and 46.3% increase in BC yield was observed at oxygen tensions of 10 and 15%. This increase is higher than the 25% increase previously reported by others (Watanabe and Yamanaka 1995). In contrast, under oxygen-enriched conditions, BC production was inhibited. Similarly, in *G. xylinus-vgb*$^+$, BC yield and glucose to BC conversion rate increased significantly under hypoxic conditions. As oxygen tension decreased from 21 to 10% and 15%, BC yield increased by 77.3 and 89.8%, respectively (Fig. 4a), and glucose to BC conversion rate increased

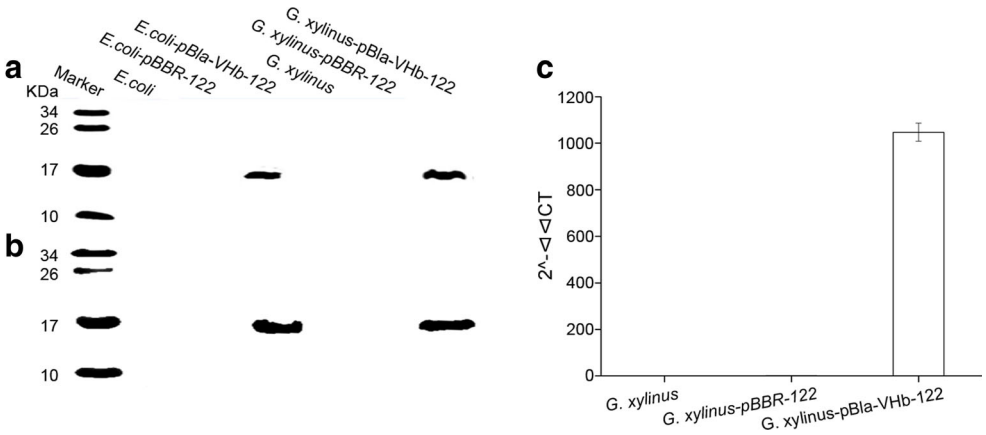

Fig. 1 Western blot of protein extracts from *G. xylinus* and *G. xylinus-vgb*$^+$. **a** Insoluble samples. **b** Soluble samples. **c** Relative expression levels of *vgb* in *G. xylinus*, *G. xylinus*-pBBR-122, and *G. xylinus-vgb*$^+$ using *gyr*B as the reference gene

by 78.2 and 90.8%, respectively (Fig. 4b). Notably, the BC yield and conversion rate at 10% oxygen tension were slightly lower than those at 15%. In this strain, an inhibition of BC production and conversion rate was observed under oxygen-enriched conditions. In particular, as oxygen tension increased to 40 and 80%, BC yield and glucose to BC conversion decreased by 48.7 and 76.5%, respectively (Fig. 4).

Finally, the effect of VHb expression on BC production and BC conversion rate at different oxygen tensions was analyzed (Fig. 4). Under atmospheric conditions, the BC yield and conversion rate of *G. xylinus-vgb*$^+$ were 22.3 and 21.7% higher than those of *G. xylinus*. At oxygen tensions of 10 and 15%, the BC yield of *G. xylinus-vgb*$^+$ was 26.5 and 58.6% higher than that of *G. xylinus*, respectively (Fig. 4a), and the conversion rate of *G. xylinus-vgb*$^+$ was 26.5 and 59.5% higher than that of *G. xylinus*, respectively (Fig. 4b). Under oxygen-enriched conditions, the BC yield and conversion rate of *G. xylinus-vgb*$^+$ were almost the same as those of *G. xylinus*. These results indicate that *vgb* expression significantly increases glucose to BC conversion and promotes BC production under hypoxia, supporting our hypothesis that the effect of VHb expression would be more significant under hypoxia than under aerobic culture.

BC production using gluconic acid as carbon source

Next, we wanted to study whether *G. xylinus* can use gluconic acid as a secondary carbon source for BC production when

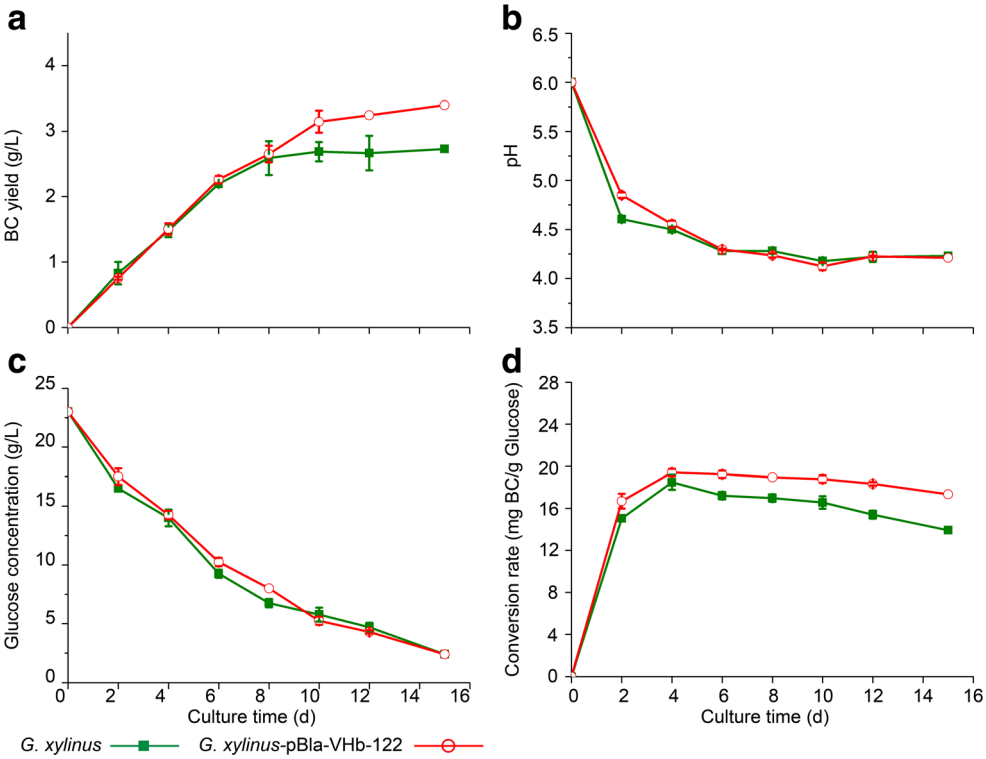

Fig. 2 BC yield (**a**), pH (**b**), glucose concentration (**c**), and glucose to BC conversion rate (**d**) of *G. xylinus* and *G. xylinus-vgb*$^+$

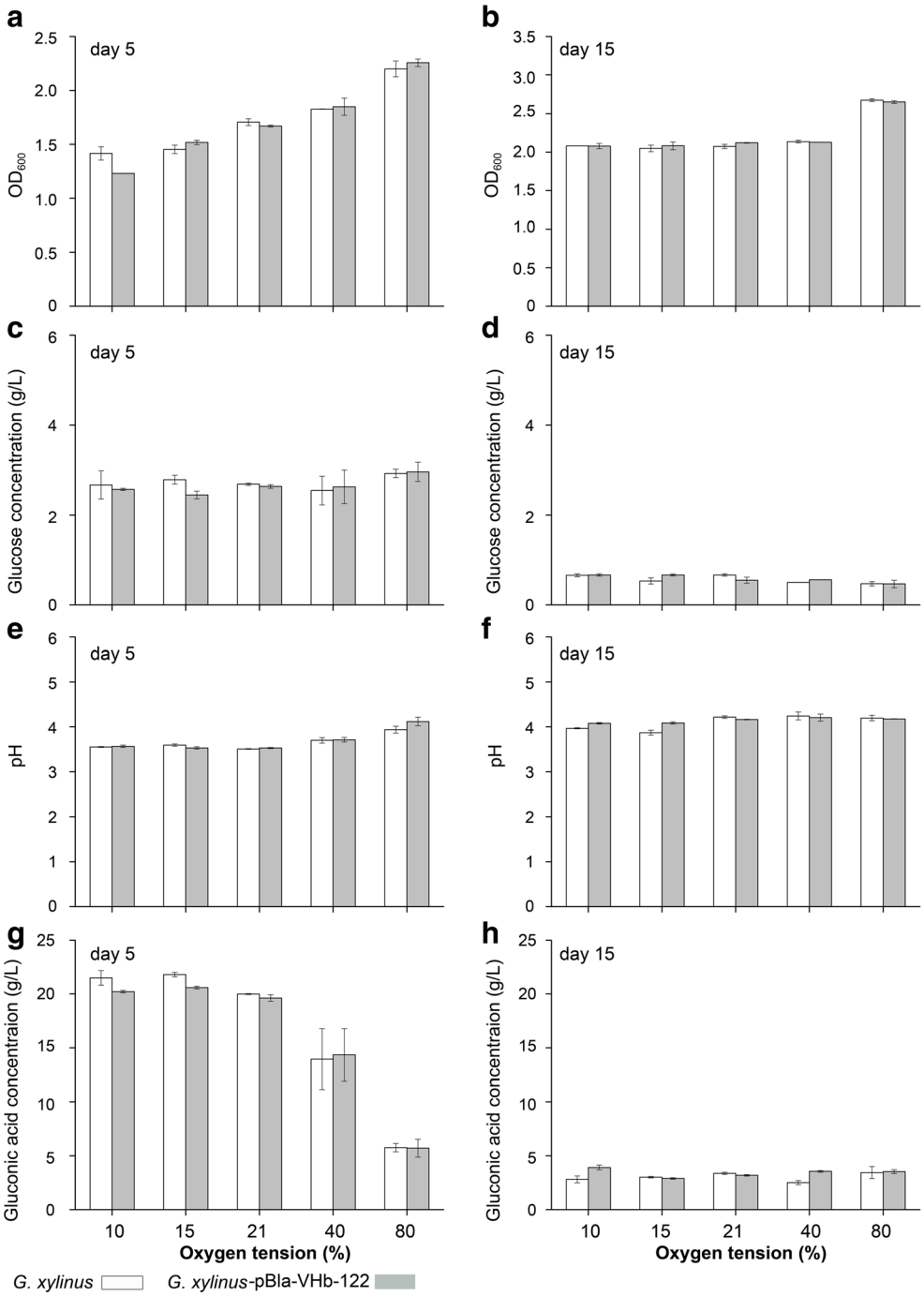

Fig. 3 Effect of oxygen tension on cell growth, pH, and gluconic acid production in *G. xylinus* and *G. xylinus-vgb*$^{+}$. OD_{600} (**a**, **b**), glucose concentration (**c**, **d**), pH value (**e**, **f**), and gluconic acid concentration (**g**, **h**) on days 5 and 15, respectively

glucose is exhausted. Glucose and gluconic acid at a concentration of 0.85 mol C/L were used as sole carbon sources for BC production. A negative control with no carbon source added was used. BC yield and dry cell weight after 7 days of static culture are shown in Table 1. Compared to the control, the dry cell weight and BC yield from gluconic acid were increased by 2.55 folds and 14.17 folds, respectively. Compared to glucose, the use of gluconic acid resulted in a 32.05% higher cell weight but a 6.96% lower BC yield.

Characterization of BC produced under various oxygen tensions

BC produced by *G. xylinus* and *G. xylinus-vgb*$^{+}$ under different oxygen tensions was characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD), tensile strength, and thermogravimetric analysis. The results showed that the thickness of the BC membrane decreased as oxygen tension increased from 10 to 80%. There was no apparent difference

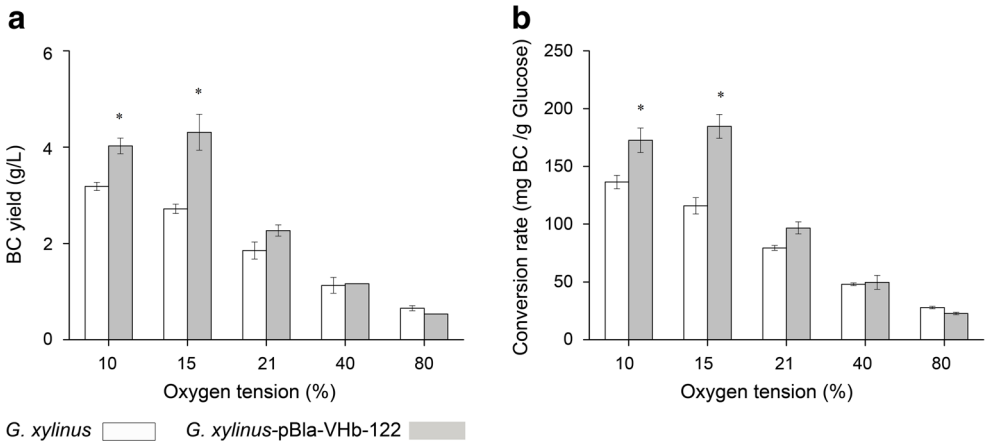

Fig. 4 Effect of oxygen tension on BC yields (**a**) and BC conversion rate from glucose (**b**) by *G. xylinus* and *G. xylinus-vgb*$^+$

in fibril width, network structure, and crystallinity among BC membranes produced at different oxygen tensions (SEM and XRD data not shown). As oxygen tensions decreased from 80 to 10%, the mass density of BC increased (Fig. 5a). In addition, VHb expression increased the mass density of BC at oxygen tensions of 10 and 15%.

As shown in Fig. 5b, the Young's modulus of BC produced by either *G. xylinus* or *G. xylinus-vgb*$^+$ under hypoxic conditions was higher than that of BC produced under atmospheric or oxygen-enriched conditions. Surprisingly, the Young's modulus of the BC produced by *G. xylinus* under oxygen-enriched conditions was nearly the same as that of BC produced under atmospheric conditions. Furthermore, VHb expression tended to decrease the Young's modulus of BC irrespective of the oxygen tension at which it was produced. Table 2 shows the thermal performance of BC produced under various oxygen tensions. An initial weight loss occurred at 100 °C, which could be attributed to the evaporation of absorbed moisture. A decrease of 50 °C in the decomposition temperature of the second weight loss stage and an increase in the total weight loss were observed when oxygen tensions raised from 10 to 80%. Compared to the BC of *G. xylinus*, the BC of *G. xylinus-vgb*$^+$ had a slightly smaller weight loss.

Discussion

In this study, the effect of oxygen on cell growth, BC synthesis, and BC conversion rate in *G. xylinus* was investigated. Oxygen availability was modified at two levels: intracellularly, by expressing VHb that affects intracellular oxygen utilization; and extracellularly, by statically culturing both *G. xylinus* and *G. xylinus-vgb*$^+$ under different oxygen tensions. *G. xylinus-vgb*$^+$ showed higher BC yields and glucose to BC conversion rates than *G. xylinus* under hypoxic and atmospheric conditions (Fig. 4a). Increasing oxygen tensions from hypoxia to oxygen-enriched conditions resulted in higher cell density (Fig. 3a) but lower BC yields and conversion rates (Fig. 4a) both in *G. xylinus* and *G. xylinus-vgb*$^+$. This would indicate that both VHb expression and oxygen tension can affect BC productivity.

Table 1 Dry cell weight and BC yield with glucose or gluconic acid as carbon sources

Carbon source	Control	Glucose	Gluconic acid
Dry cell weight (g/L)	0.29 ± 0.02	0.78 ± 0.15	1.03 ± 0.05
BC yield (g/L)	0.18 ± 0.01	2.92 ± 0.27	2.73 ± 0.19

In most studies, VHb has been expressed from its own native oxygen-regulated promoter (Pvgb). This promoter has been widely applied to direct high-level expression of several genes (Liu et al. 2008; Liu et al. 2005; Tsai et al. 1996). However, Pvgb is only induced under oxygen-limited conditions (Frey and Kallio 2003; Tsai et al. 1996). In this study, the *bla* promoter was used to express VHb, which does not need the addition of an inducer. As shown in the preliminary experiment, the BC yield of *G. xylinus-vgb*$^+$ in static culture was approximately 24.5% higher than that of *G. xylinus* (Fig. 2a). This suggests that VHb is expressed by the *bla* promoter in *G. xylinus-vgb*$^+$ and that it promotes BC production in static culture. VHb is a homodimeric heme protein, which can be concurrently accumulated both in its soluble and insoluble forms when expressed in *E. coli* (Hart et al. 1990). In this study, VHb was detected both in soluble and insoluble protein samples (Fig. 1a, b). This differs with previously published results in *A. xylinum* where VHb was only detected in soluble samples (Chien et al. 2006). This could be explained by differences in strain and culture conditions.

The oxygen tension in the extracellular environment was then altered and the effect on cell growth and BC production in *G. xylinus* and *G. xylinus-vgb*$^+$ was studied. Unlike previous reports, (Watanabe and Yamanaka 1995), in this study, we found that *G. xylinus* and *G. xylinus-vgb*$^+$ cell growth was promoted at higher oxygen tensions (10–80%) during the exponential growth phase (Fig. 3a). However, in the stationary

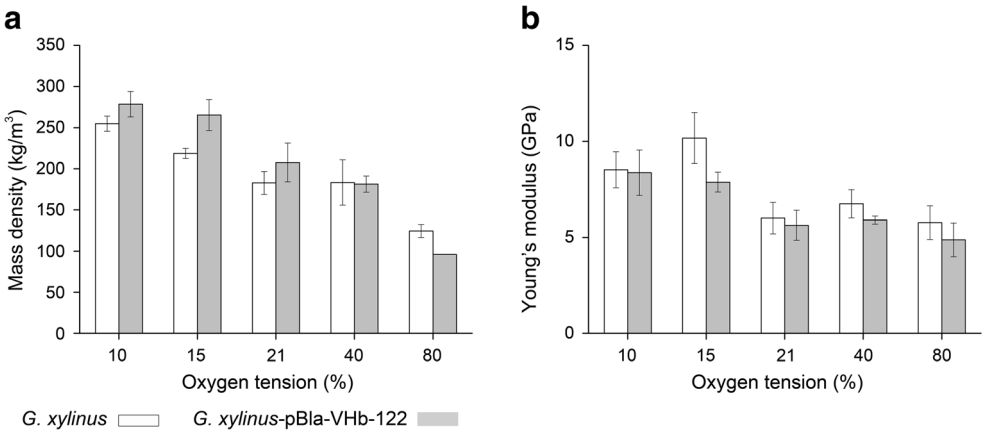

Fig. 5 Mass density (**a**) and Young's modulus (**b**) of BC produced at various oxygen tensions

phase, the final cell density reached almost the same at oxygen tensions of 10 to 40% (Fig. 3b), which is consistent with the results of Watanabe et al. (Watanabe and Yamanaka 1995). The initial glucose concentration in the media was the same for all the cultures and conditions tested. However, under hypoxia, more glucose is converted to gluconic acid during the exponential growth phase, which would explain the lower cell densities. The conversion rate from glucose to gluconic acid was dependent on the culture condition. Like it was reported previously, agitated culture would promote the conversion as compared to static culture (Liu et al. 2015). Nevertheless, gluconic acid is used for cell growth once glucose is exhausted, which would explain the similar final cell densities achieved under the different culture conditions. This inference is supported by the results shown in Fig. 3 and Table 1, which prove that gluconic acid can be utilized as carbon source for cell growth and BC production, which is in agreement with our previous work (Liu et al. 2015). We and others had previously suggested that an excessive accumulation of gluconic acid could be one of the main reasons for a low BC productivity (Chawla et al. 2009; Liu et al. 2015). However, here, we show that BC yield is higher under hypoxic conditions, when gluconic acid production is higher, than that under oxygen-enriched conditions. Therefore, gluconic acid production on its own does not explain the effect of oxygen tension on BC production. Gluconic acid is also considered to be closely related to pH value (Liu et al. 2015). Metabolic flux analysis has shown that *G. xylinus* converts up to 40% of the glucose to gluconic acid, its main byproduct, and pH level decreases as gluconic acid accumulates and increases as it is consumed (Liu et al. 2015; Zhong et al. 2013). In the present work, the pH value increased with oxygen tension (Fig. 3e), which is also consistent with the variations in gluconic acid concentration observed (Fig. 3g).

The BC yield of both *G. xylinus* and *G. xylinus-vgb*$^+$ increased at lower oxygen tensions. In addition, the BC yield increased as VHb was expressed under both atmospheric and hypoxic conditions (Fig. 4a). However, the BC yield of *G. xylinus-vgb*$^+$ was slightly lower at 10% oxygen tension than that at 15%, which was not observed with *G. xylinus* (Fig. 4a). This suggests that VHb expression only increases BC production within a certain oxygen tension range, with 15% being the

Table 2 Thermal performance of BC determined from the TG/DTG spectra

Samples		Second weight loss		Third weight loss		Total weight loss (%)	Max weight loss rate (% min^{-1})
		Temperature (°C)	Weight loss (%)	Temperature (°C)	Weight loss (%)		
10%	*G. xylinus*	287.00	68.56	442.00	14.74	83.30	1.20
	GX-*vgb*$^+$	286.99	65.07	442.01	15.26	80.33	1.27
15%	*G. xylinus*	287.01	67.79	442.05	16.16	83.95	1.24
	GX-*vgb*$^+$	286.89	67.15	441.91	15.92	83.07	1.23
21%	*G. xylinus*	275.07	76.07	441.94	8.98	85.05	1.25
	GX-*vgb*$^+$	275.10	75.14	441.14	9.16	84.30	1.28
40%	*G. xylinus*	260.08	72.03	441.91	13.31	85.34	1.28
	GX-*vgb*$^+$	260.05	73.31	442.01	12.96	86.27	1.27
80%	*G. xylinus*	234.97	69.63	442.01	15.14	84.77	1.10
	GX-*vgb*$^+$	234.92	73.29	441.93	14.25	87.54	1.19

GX-vgb$^+$ *G. xylinus-vgb*$^+$

optimal oxygen tension. On the other hand, under oxygen-enriched conditions, cell growth, rather than BC synthesis, is benefited (Fig. 3a, b; Fig. 4a). Therefore, we propose a two-step culture strategy with an initial phase of cell growth under oxygen-enriched conditions until the exponential growth phase is reached, followed by a second hypoxic phase for BC synthesis.

As shown from the BC biosynthesis pathway (Imai et al. 2014), oxygen is not directly involved in BC synthesis. Then, how come both VHb protein and oxygen tension affect BC yield? As an oxygen carrier, VHb has a relatively normal association rate constant (k_{on}) for oxygen binding, but its rate constant for oxygen dissociation (k_{off}) is unusually high, thus facilitating oxygen diffusion (Orii and Webster 1986). These characteristics are consistent with its putative role in sequestering oxygen from the environment and feeding it to the respiratory chain (Ramandeep et al. 2001). In both native and heterologous hosts, VHb stimulated the electron transport chain through its catalytic function, raising the activity of the terminal oxidase cytochrome *o* (Tsai et al. 1996). A study that measured NAD(P)H fluorescence in *E. coli* has shown that VHb expression affects NAD(P)H generation and likely maintains cells grown under low oxygen densities in a more oxidized state (Zhang et al. 2007). In addition, flux distribution analysis has revealed that in VHb-expressing cells, the ATP synthesis rate from substrate-level phosphorylation is lower, under oxygen-limited conditions, but the overall ATP production rate is higher (Tsai et al. 1996). In the context of our results, these observations would suggest that VHb expression might affect BC synthesis by manipulating the energy metabolism. Additional reports support this inference. For instance, BC synthesis by *Enterobacter* sp. FY-07 is subjected to energy production under aerobic and anaerobic conditions, with glucose catabolism and anaerobic nitrate respiration identified, respectively, as the effective energy production modes for BC biosynthesis (Ji et al. 2016). In *A. xylinum*, ethanol cannot be used as a substrate for BC production, but it can function as an energy source for ATP generation promoting BC production (Naritomi et al. 1998). In addition, energy is required to activate intermediate metabolites and to synthesize the activator of BC synthase, c-di-GMP (Ross et al. 1986). All together, these data suggest that VHb expression and oxygen tension indirectly affect BC synthesis by manipulating energy metabolism. However, we observed that an increase in oxygen tension from 10 to 21% resulted in a decrease in the BC yield, while VHb expression resulted in a higher BC yield. Therefore, the regulatory mechanisms of energy metabolism by VHb and oxygen tension, and their influence on BC production seem to be different and deserve further investigation.

BC characterization analysis indicated that the BC produced under atmospheric and oxygen-enriched conditions had similar Young's modulus values (Fig. 5b), suggesting that under oxygen-enriched conditions, the BC produced is tough and could be used to produce paper (Cheng et al. 2011; Henriksson et al. 2008), blood vessels (Bäckdahl et al. 2006), and other high-strength composites (Nakagaito et al. 2005). On the other hand, under hypoxic conditions, *G. xylinus-vgb*$^+$ synthesized softer BC than *G. xylinus* as shown by a lower Young's modulus (Fig. 5b). Furthermore, a higher weight loss temperature and a lower total weight loss indicated an improved thermostability of the BC produced under hypoxia (Table 2). These characteristics could fulfill its application to ion exchange (Choi et al. 2010), biomedical (Cai and Kim 2010), and electronic devices (Feng et al. 2012), among others (Shah et al. 2013). X-ray diffraction suggested that the crystallinity index of BC was not significantly affected by oxygen tension or VHb expression (data not shown). This would suggest that the assembly process of BC fibers, rather than the crystalline process, is influenced by oxygen utilization in *G. xylinus*.

In conclusion, the heterogeneous expression of VHb in *G. xylinus* and the regulation of oxygen tension influence BC production and cell growth. Increasing oxygen tension is beneficial for cell growth but not for BC production. On the other hand, reducing oxygen tension together with VHb expression significantly facilitates BC production. Therefore, we suggest a two-step culture strategy for BC production by *G. xylinus*, in which oxygen-enriched air would be first supplied to favor cell growth before the exponential growth phase, followed by a hypoxic phase to increase BC production during the stationary phase. Although the mechanisms by which VHb and oxygen tension affect BC synthesis manipulated are different, they are both related to energy metabolism. The results presented here on the effect of oxygen tension and VHb heterogeneous expression on BC production are predicted to be useful for the rational design of a culture strategy specifically valuable for the production of highly demanded BC for various applications.

Acknowledgements The authors would like to express their appreciation to Professor Lee Cheng-Kang from the National Taiwan University of Science and Technology for the generous gift of plasmid pBla-VHb-122.

Funding information This work was funded by the National Natural Science Foundation of China (no. 21576212 and no. 31470610), the Natural Science Foundation of Tianjin (15JCZDJC32600), and the Innovation Foundation for Doctor Dissertation of Tianjin University of Science and Technology (2016001).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

References

Bäckdahl H, Helenius G, Bodin A, Nannmark U, Johansson BR, Bo R, Gatenholm P (2006) Mechanical properties of bacterial cellulose and interactions with smooth muscle cells. Biomaterials 27(9): 2141–2149. https://doi.org/10.1016/j.biomaterials.2005.10.026

Cai Z, Kim J (2010) Bacterial cellulose/poly(ethylene glycol) composite: characterization and first evaluation of biocompatibility. Cellulose 17(1):83–91. https://doi.org/10.1007/s10570-009-9362-5

Chao Y, Sugano Y, Shoda M (2001) Bacterial cellulose production under oxygen-enriched air at different fructose concentrations in a 50-liter, internal-loop airlift reactor. Appl Microbiol Biotechnol 55(6):673–679. https://doi.org/10.1007/s002530000503

Chawla PR, Bajaj IB, Survase SA, Singhal RS (2009) Microbial cellulose: fermentative production and applications. Food Technol Biotechnol 47(2):107–124

Cheng KC, Catchmark JM, Demirci A (2011) Effects of CMC addition on bacterial cellulose production in a biofilm reactor and its paper sheets analysis. Biomacromolecules 12(3):730–736. https://doi.org/10.1021/bm101363t

Chien LJ, Chen HT, Yang PF, Lee CK (2006) Enhancement of cellulose pellicle production by constitutively expressing Vitreoscilla hemoglobin in *Acetobacter xylinum*. Biotechnol Prog 22(6):1598–1603. https://doi.org/10.1021/bp060157g

Choi YJ, Ahn Y, Kang MS, Jun HK, Kim IS, Moon SH (2010) Preparation and characterization of acrylic acid-treated bacterial cellulose cation-exchange membrane. J Chem Technol Biotechnol 79(1):79–84

Czaja W, Krystynowicz A, Bielecki S, Jr BR (2006) Microbial cellulose—the natural power to heal wounds. Biomaterials 27(2):145–151. https://doi.org/10.1016/j.biomaterials.2005.07.035

Czaja W, Romanovicz D, Brown RM (2004) Structural investigations of microbial cellulose produced in stationary and agitated culture. Cellulose 11(3):403–411. https://doi.org/10.1023/B:CELL.0000046412.11983.61

Divne C, Stahlberg J, Reinikainen T, Ruohonen L, Pettersson G, Knowles JK, Teeri TT, Jones TA (1994) The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei*. Science 265(5171):524–528. https://doi.org/10.1126/science.8036495

Dogan I, Pagilla KR, Webster DA, Stark BC (2006) Expression of *Vitreoscilla* hemoglobin in *Gordonia amarae* enhances biosurfactant production. J Ind Microbiol Biotechnol 33(8):693–700. https://doi.org/10.1007/s10295-006-0097-0

Evans BR, O'Neill HM, Malyvanh VP, Lee I, Woodward J (2003) Palladium-bacterial cellulose membranes for fuel cells. Biosens Bioelectron 18(7):917–923. https://doi.org/10.1016/S0956-5663(02)00212-9

Fabra MJ, López-Rubio A, Ambrosio-Martín J, Lagaron JM (2016) Improving the barrier properties of thermoplastic corn starch-based films containing bacterial cellulose nanowhiskers by means of PHA electrospun coatings of interest in food packaging. Food Hydrocoll 61:261–268. https://doi.org/10.1016/j.foodhyd.2016.05.025

Fang L, Catchmark JM (2014) Characterization of water-soluble exopolysaccharides from *Gluconacetobacter xylinus* and their impacts on bacterial cellulose crystallization and ribbon assembly. Cellulose 21(6):3965–3978. https://doi.org/10.1007/s10570-014-0443-8

Feng Y, Zhang X, Shen Y, Yoshino K, Feng W (2012) A mechanically strong, flexible and conductive film based on bacterial cellulose/graphene nanocomposite. Carbohydr Polym 87(1):644–649. https://doi.org/10.1016/j.carbpol.2011.08.039

Frey AD, Kallio PT (2003) Bacterial hemoglobins and flavohemoglobins: versatile proteins and their impact on microbiology and biotechnology. FEMS Microbiol Rev 27(4): 525–545. https://doi.org/10.1016/S0168-6445(03)00056-1

Hart RA, Rinas U, Bailey JE (1990) Protein composition of *Vitreoscilla* hemoglobin inclusion bodies produced in *Escherichia coli*. J Biol Chem 265(21):12728–12733. https://doi.org/10.1128/MMBR.00036-05

Henriksson M, Berglund LA, Isaksson P, Lindström T, Nishino T (2008) Cellulose nanopaper structures of high toughness. Biomacromolecules 9(6):1579–1585. https://doi.org/10.1021/bm800038n

Hestrin S, Schramm M (1954) Synthesis of cellulose by *Acetobacter xylinum*. II. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. Biochem J 58(2):345–352

Hornung M, Ludwig M, Gerrard AM, Schmauder HP (2006) Optimizing the production of bacterial cellulose in surface culture: evaluation of product movement influences on the bioreaction (Part 1). Eng Life Sci 6(6):537–545. https://doi.org/10.1002/elsc.200620162

Imai T, Sun S, Horikawa Y, Wada M, Sugiyama J (2014) Functional reconstitution of cellulose synthase in *Escherichia coli*. Biomacromolecules 15(11):4206–4213. https://doi.org/10.1021/bm501217g

Ji K, Wang W, Zeng B, Chen S, Zhao Q, Chen Y, Li G, Ma T (2016) Bacterial cellulose synthesis mechanism of facultative anaerobe *Enterobacter* sp. FY-07. Sci Rep-UK 6:21863. https://doi.org/10.1038/srep21863

Jonas R, Farah LF (1998) Production and application of microbial cellulose. Polym Degrad Stab 59(1–3):101–106. https://doi.org/10.1016/S0141-3910(97)00197-3

Kubiak K, Kurzawa M, Jędrzejczak-Krzepkowska M, Ludwicka K, Krawczyk M, Migdalski A, Kacprzak MM, Loska D, Krystynowicz A, Bielecki S (2014) Complete genome sequence of *Gluconacetobacter xylinus* E25 strain—valuable and effective producer of bacterial nanocellulose. J Biotechnol 176(1):18–19. https://doi.org/10.1016/j.jbiotec.2014.02.006

Li Y, Tian C, Tian H, Zhang J, He X, Ping W, Lei H (2012) Improvement of bacterial cellulose production by manipulating the metabolic pathways in which ethanol and sodium citrate involved. Appl Microbiol Biotechnol 96(6):1479–1487. https://doi.org/10.1007/s00253-012-4242-6

Liu M, Zhong C, Wu XY, Wei YQ, Bo T, Han PP, Jia SR (2015) Metabolomic profiling coupled with metabolic network reveals differences in *Gluconacetobacter xylinus* from static and agitated cultures. Biochem Eng J 101:85–98. https://doi.org/10.1016/j.bej.2015.05.002

Liu Q, Zhang J, Wei XX, Ouyang SP, Wu Q, Chen GQ (2008) Microbial production of l -glutamate and l -glutamine by recombinant *Corynebacterium glutamicum* harboring *Vitreoscilla* hemoglobin gene *vgb*. Appl Microbiol Biotechnol 77(6):1297–1304. https://doi.org/10.1007/s00253-007-1254-8

Liu T, Chen JY, Zheng Z, Wang TH, Chen GQ (2005) Construction of highly efficient *E. coli* expression systems containing low oxygen induced promoter and partition region. Appl Microbiol Biotechnol 68(3):346–354. https://doi.org/10.1007/s00253-005-1913-6

Maneerung T, Tokura S, Rujiravanit R (2008) Impregnation of silver nanoparticles into bacterial cellulose for antimicrobial wound dressing. Carbohydr Polym 72(1):43–51. https://doi.org/10.1016/j.carbpol.2007.07.025

Nakagaito AN, Iwamoto S, Yano H (2005) Bacterial cellulose: the ultimate nano-scalar cellulose morphology for the production of high-strength composites. Appl Phys A 80(1):93–97. https://doi.org/10.1007/s00339-004-2932-3

Naritomi T, Kouda T, Yano H, Yoshinaga F (1998) Effect of ethanol on bacterial cellulose production from fructose in continuous culture. J Ferment Bioeng 85(6):598–603. https://doi.org/10.1016/S0922-338X(98)80012-3

Orii Y, Webster DA (1986) Photodissociation of oxygenated cytochrome o(s) (*Vitreoscilla*) and kinetic studies of reassociation. J Biol Chem 261(8):3544–3547

Park KW, Kim KJ, Howard AJ, Stark BC, Webster DA (2002) *Vitreoscilla* hemoglobin binds to subunit I of cytochrome bo ubiquinol oxidases. J Biol Chem 277(36):33334–33337. https://doi.org/10.1074/jbc.M203820200

Ramandeep, Hwang KW, Raje M, Kim KJ, Stark BC, Dikshit KL, Webster DA (2001) Vitreoscilla hemoglobin. Intracellular localization and binding to membranes. J Biol Chem 276(27):24781–24789. https://doi.org/10.1074/jbc.M009808200

Ross P, Aloni Y, Weinhouse H, Michaeli D, Weinberger-Ohana P, Mayer R, Benziman M (1986) Control of cellulose synthesis *Acetobacter xylinum*. A unique guanyl oligonucleotide is the immediate activator of the cellulose synthase. Carbohydr Res 149(1):101–117. https://doi.org/10.1016/S0008-6215(00)90372-0

Ross P, Mayer R, Benziman M (1991) Cellulose biosynthesis and function in bacteria. Microbiol Rev 55(1):35–58

Shah J, Brown RM Jr (2005) Towards electronic paper displays made from microbial cellulose. Appl Microbiol Biotechnol 66(4):352–355. https://doi.org/10.1007/s00253-004-1756-6

Shah N, Ul-Islam M, Khattak WA, Park JK (2013) Overview of bacterial cellulose composites: a multipurpose advanced material. Carbohydr Polym 98(2):1585–1598. https://doi.org/10.1016/j.carbpol.2013.08.018

Stark BC, Pagilla KR, Dikshit KL (2015) Recent applications of *Vitreoscilla* hemoglobin technology in bioproduct synthesis and bioremediation. Appl Microbiol Biotechnol 99(4):1627–1636. https://doi.org/10.1007/s00253-014-6350-y

Suen YL, Tang H, Huang J, Chen F (2014) Enhanced production of fatty acids and astaxanthin in *Aurantiochytrium* sp. by the expression of *Vitreoscilla* hemoglobin. J Agric Food Chem 62(51):12392–12398. https://doi.org/10.1021/jf5048578

Tang W, Jia S, Jia Y, Yang H (2010) The influence of fermentation conditions and post-treatment methods on porosity of bacterial cellulose membrane. World J Microbiol Biotechnol 26(1):125–131. https://doi.org/10.1007/s11274-009-0151-y

Tsai PS, Hatzimanikatis V, Bailey JE (1996) Effect of *Vitreoscilla* hemoglobin dosage on microaerobic *Escherichia coli* carbon and energy metabolism. Biotechnol Bioeng 49(2):139–150. https://doi.org/10.1002/(SICI)1097-0290(19960120)49:2<139::AID-BIT3>3.0.CO;2-R

Tsuge S, Nakayama T, Terashima S, Ochiai H, Furutani A, Oku T, Tsuno K, Kubo Y, Kaku H (2006) Gene involved in transcriptional activation of the *hrp* regulatory gene *hrpG* in *Xanthomonas oryzae* pv. oryzae. J Bacteriol 188(11):4158–4162. https://doi.org/10.1128/JB.00006-06

Valla S, Coucheron DH, Fjaervik E, Kjosbakken J, Weinhouse H, Ross P, Amikam D, Benziman M (1989) Cloning of a gene involved in cellulose biosynthesis in *Acetobacter xylinum*: complementation of cellulose-negative mutants by the UDPG pyrophosphorylase structural gene. Mol Gen Genet 217(1):26–30. https://doi.org/10.1007/bf00330938

Watanabe K, Yamanaka S (1995) Effects of oxygen tension in the gaseous phase on production and physical properties of bacterial cellulose formed under static culture conditions. Biosci Biotechnol Biochem 59(1):65–68. https://doi.org/10.1271/bbb.59.65

Webster DA (1988) Structure and function of bacterial hemoglobin and related proteins. Adv Inorg Biochem 7:245–265

Webster DA, Hackett DP (1966) The purification and properties of cytochrome o from *Vitreoscilla*. J Biol Chem 241(14):3308–3315

Xiong X, Xing J, Li X, Bai X, Li W, Li Y, Liu H (2007) Enhancement of biodesulfurization in two-liquid systems by heterogeneous expression of *Vitreoscilla* hemoglobin. Appl Environ Microbiol 73(7):2394–2397. https://doi.org/10.1128/AEM.02372-06

Yin N, Santos TM, Auer GK, Crooks JA, Oliver PM, Weibel DB (2014) Bacterial cellulose as a substrate for microbial cell culture. Appl Environ Microbiol 80(6):1926–1932. https://doi.org/10.1128/AEM.03452-13

Zhang L, Li Y, Wang Z, Xia Y, Chen W, Tang K (2007) Recent developments and future prospects of *Vitreoscilla* hemoglobin application in metabolic engineering. Biotechnol Adv 25(2):123–136. https://doi.org/10.1016/j.biotechadv.2006.11.001

Zhong C, Zhang GC, Liu M, Zheng XT, Han PP, Jia SR (2013) Metabolic flux analysis of *Gluconacetobacter xylinus* for bacterial cellulose production. Appl Microbiol Biotechnol 97(14):6189–6199. https://doi.org/10.1007/s00253-013-4908-8