ISSN 1330-9862
(FTB-2058)

review

Microbial Cellulose: Fermentative Production and Applications

*Prashant R. Chawla, Ishwar B. Bajaj, Shrikant A. Survase and Rekha S. Singhal**

Food Engineering and Technology Department, Institute of Chemical Technology, University of Mumbai, Matunga, IN-400019 Mumbai, India

Received: February 19, 2008
Accepted: December 3, 2008

Summary

Bacterial cellulose, an exopolysaccharide produced by some bacteria, has unique structural and mechanical properties and is highly pure as compared to plant cellulose. This article presents a critical review of the available information on the bacterial cellulose with special emphasis on its fermentative production and applications. Information on the biosynthetic pathway of bacterial cellulose, enzymes and precursors involved in bacterial cellulose synthesis has been specified. Characteristics of bacterial cellulose with respect to its structure and physicochemical properties are discussed. Current and potential applications of bacterial cellulose in food, pharmaceutical and other industries are also presented.

Key words: microbial cellulose, homopolymer, *Acetobacter xylinum*, *Acetobacter hansenii*, fermentation

Introduction

Polysaccharides are a structurally diverse group of biological macromolecules of widespread occurrence in nature. They can be divided according to their morphological localization as: intracellular polysaccharides located inside, or as part of the cytoplasmic membrane; cell-wall polysaccharides forming a structural part of the cell wall; and extracellular polysaccharides located outside the cell wall. Extracellular polysaccharides occur in two forms: loose slime, which is non-adherent to the cell and imparts a sticky consistency to bacterial growth on a solid medium or an increased viscosity in a liquid medium; and microcapsules or capsules, which adhere to the cell wall. They have a definite form and boundary, being only slowly extracted in the water or salt solutions. It is therefore possible to separate capsules and microcapsules from loose slime by centrifugation (*1*).

Exopolysaccharides are long chain polysaccharides consisting of branched, repeating units of sugars or sugar derivatives, mainly glucose, galactose and rhamnose in different ratios. They are classified into two groups: homopolysaccharides (cellulose, dextran, mutan, pullulan, curdlan), and heteropolysaccharides (gellan, xanthan) (*2*). Homopolysaccharides consist of repeating units of only one type of monosaccharides (D-glucose or D-fructose) joined by either a single linkage type (*e.g.* 1→2 or 1→4) or by a combination of a limited number of linkage types (*e.g.* 1→2 and 1→4). Heteropolysaccharides consist of multiple copies of oligosaccharides, containing three to eight residues, produced by a variety of microorganisms. Exopolysaccharides find wide industrial applications in food, pharmaceutical and other industries like textile, paper, cosmetics, gelling agents and medicines for wound dressing (*3*).

Microbial cellulose is an exopolysaccharide produced by various species of bacteria, such as those of the genera *Gluconacetobacter* (formerly *Acetobacter*), *Agrobacterium*, *Aerobacter*, *Achromobacter*, *Azotobacter*, *Rhizobium*, *Sarcina*, and *Salmonella* (*4*). Production of cellulose from *Acetobacter xylinum* was first reported in 1886 by A.J. Brown (*5*). He observed that the resting cells of *Acetobacter* produced cellulose in the presence of oxygen and glucose.

*Corresponding author; Phone: ++91 22 24 145 616; Fax: ++91 22 24 145 614; E-mail: rekha@udct.org

The molecular formula of bacterial cellulose $(C_6H_{10}O_5)_n$ is the same as that of plant cellulose, but their physical and chemical features are different (*6*). Bacterial cellulose is preferred over the plant cellulose as it can be obtained in higher purity and exhibits a higher degree of polymerization and crystallinity index. It also has higher tensile strength and water holding capacity than that of plant cellulose, making it more suitable raw material for producing high fidelity acoustic speakers, high quality paper and dessert foods (*4*). Fibrils of bacterial cellulose are about 100 times thinner than that of plant cellulose, making it a highly porous material, which allows transfer of antibiotics or other medicines into the wound while at the same time serving as an efficient physical barrier against any external infection. It is therefore used extensively in wound healing (*7*).

Microbial cellulose exists as a basic structure known as microfibrils, which are composed of glucan chains interlocked by hydrogen bonds so that a crystalline domain is produced. This microfibrillar structure of bacterial cellulose was first described by Mühlethaler in 1949 (*8*). Electron microscopic observations showed that the cellulose produced by *Acetobacter xylinum* occurs in the form of fibres. The bacteria first secreted a structurally homogeneous slimy substance within which, after a short time, the cellulose fibers were formed.

Acetobacter xylinum produces two forms of cellulose: (*i*) cellulose I, the ribbon-like polymer, and (*ii*) cellulose II, the thermodynamically more stable amorphous polymer (*9*). The differences in the assembly of cellulose I and cellulose II outside the cytoplasmic membrane are described in Fig. 1. Microfibrillar structure of bacterial cellulose is responsible for most of its properties such as high tensile strength, higher degree of polymerization and crystallinity index. Bacterial cellulose is used as a diet food and to produce new materials for high performance speaker diaphragms, medical pads (*10*) and artificial skin (*11*). Relatively high cost of the production of cellulose may limit its application to high value-added products as well as speciality chemicals (*12*). Significant cost reductions are possible with improvements in fermentation efficiency and economics of scale, the lower limit of the cost of microbial cellulose being determined by the price of the raw material substrates. Consequently, *Acetobacter* cellulose may always be more expensive to produce than conventional sources of cellulose (*4*). For this reason, successful commercialization of *Acetobacter* cellulose will depend on careful selection of applications where its superior performance can justify its higher cost.

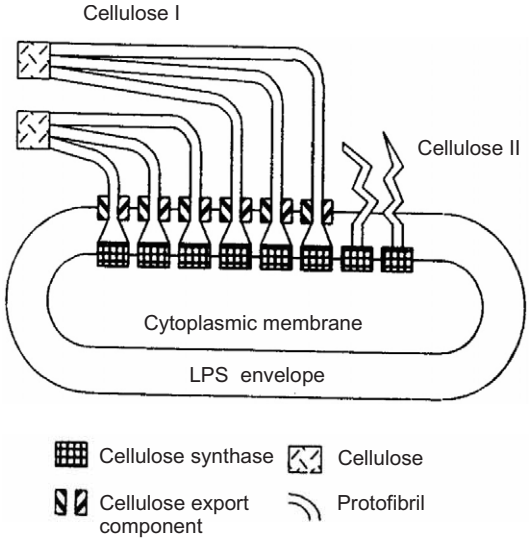

Fig. 1. Assembly of cellulose microfibrils by *A. xylinum* (*36*)

Strains Producing Cellulose

Cellulose is found in groups of microorganisms like fungi, bacteria, and algae. In green algae, cellulose, xylan, or mannan may serve as structural cell wall polysaccharides. Cellulose is found, although in small quantities, in all of the brown algae (Phaeophyta), most of the red algae (Rhodophyta), and most of the golden algae (Chrysophyta (Chrysophytes)) (*13*). It was also reported to be present in some fungi, forming inner cell wall layer, usually in association with β-1→3/β-1→6--linked D-glucan. Chitin is completely replaced by cellulose in Oomycetes, accounting for about 15 % of the wall dry mass (*14*). Gram-negative species like *Acetobacter*, *Agrobacterium*, *Achromobacter*, *Aerobacter*, *Sarcina*, *Azotobacter*, *Rhizobium*, *Pseudomonas*, *Salmonella* and *Alcaligenes* produce cellulose. Cellulose is also synthesized by the Gram-positive bacterium *Sarcina ventriculi*, accounting for about 15 % of the total dry cell mass (*15*). The most effective producers of cellulose are *A. xylinum* (*16–19*), *A. hansenii* (*20,21*) and *A. pasteurianus* (*22*).

A. xylinum has been applied as model microorganism for basic and applied studies on cellulose. It is most commonly studied source of bacterial cellulose because of its ability to produce relatively high levels of polymer from a wide range of carbon and nitrogen sources (*23*). It is a rod-shaped, aerobic, Gram-negative bacterium that produces cellulose in the form of interwoven extracellular ribbons as part of primary metabolite. This bacterium grows and produces cellulose from a wide variety of substrates and is devoid of cellulase activity (*24*). Various strains producing cellulose and their yields are depicted systematically in Table 1.

It is important to preserve the chosen bacterial strain to guarantee reproducibility of the work as well as to shorten the preparation time. Various techniques like freezing in a suspension using glycerol, dimethyl sulphoxide (DMSO) or skimmed milk as protective agents and drying in gelatin drops have been studied for the preservation of the strain. A useful preservation method should provide high survival rates of *A. xylinum*, and should have no influence on the cellulose formation. The use of glycerol and skimmed milk as protective agents for freezing is not recommended, since they alter the structure of cellulose produced by *A. xylinum* and influence the bacterial metabolism. Freezing in a suspension with DMSO has proven to be more efficient with high survival rates and no determinable influence on the structure of the formed bacterial cellulose. Drying the bacterial cells in gelatin drops had no effect on the morphological structure and kinetic parameters, but showed very low survival rate (*25*).

Table 1. Different strains producing microbial cellulose

Microorganism	Carbon source	Supplement	Culture time	Yield/(g/L)	Reference
A. xylinum BRC 5	glucose	ethanol, oxygen	50 h	15.30	(*75*)
G. hansenii PJK (KCTC 10505 BP)	glucose	oxygen	48 h	1.72	(*20*)
G. hansenii PJK (KCTC 10505 BP)	glucose	ethanol	72 h	2.50	(*21*)
Acetobacter sp. V6	glucose	ethanol	8 day	4.16	(*44*)
Acetobacter sp. A9	glucose	ethanol	8 day	15.20	(*47*)
A. xylinum BPR2001	molasses	none	72 h	7.820	(*52*)
A. xylinum BPR2001	fructose	agar oxygen	72 h	14.10	(*64*)
A. xylinum BPR2001	fructose	agar	56 h	12.00	(*64*)
Acetobacter xylinum ssp. *sucrofermentans* BPR2001	fructose	oxygen	52 h	10.40	(*68*)
Acetobacter xylinum ssp. *sucrofermentans* BPR2001	fructose	agar oxygen	44 h	8.70	(*68*)
Acetobacter xylinum E25	glucose	no	7 day	3.50	(*78*)
G. xylinus strain (K3)	mannitol	green tea	7 day	3.34	(*46*)
Gluconacetobacter xylinus IFO 13773	glucose	lignosulphonate	7 day	10.10	(*48*)
Acetobacter xylinum NUST4.1	glucose	sodium alginate	5 day	6.00	(*65*)
Gluconacetobacter xylinus IFO 13773	sugar cane molasses	no	7 day	5.76	(*53*)
Gluconacetobacter sp. RKY5	glycerol	no	144 h	5.63	(*59*)
Co-culture of *Gluconacetobacter* sp. st-60–12 and *Lactobacillus mali* JCM1116	sucrose	no	72 h	4.20	(*60*)

Biosynthetic Pathway

Synthesis of bacterial cellulose is a precisely and specifically regulated multi-step process, involving a large number of both individual enzymes and complexes of catalytic and regulatory proteins, whose supramolecular structure has not yet been well defined. Pathways and mechanisms of uridine diphosphoglucose (UDPGlc) synthesis are relatively well known, whereas molecular mechanisms of glucose polymerization into long and unbranched chains still need exploring.

Biochemical reactions of cellulose synthesis by *A. xylinum* are extensively documented (*16,26*). It is a precisely and specifically regulated multi-step process, involving a large number of individual enzymes and complex of catalytic and regulatory proteins (Fig. 2). The process includes the formation of UDPGlc, which is the precursor in the formation of cellulose, followed by glucose polymerization into the β-1→4 glucan chain and a nascent chain which forms ribbon-like structure of cellulose chains formed by hundreds or even thousands of individual cellulose chains, their extrusion outside the

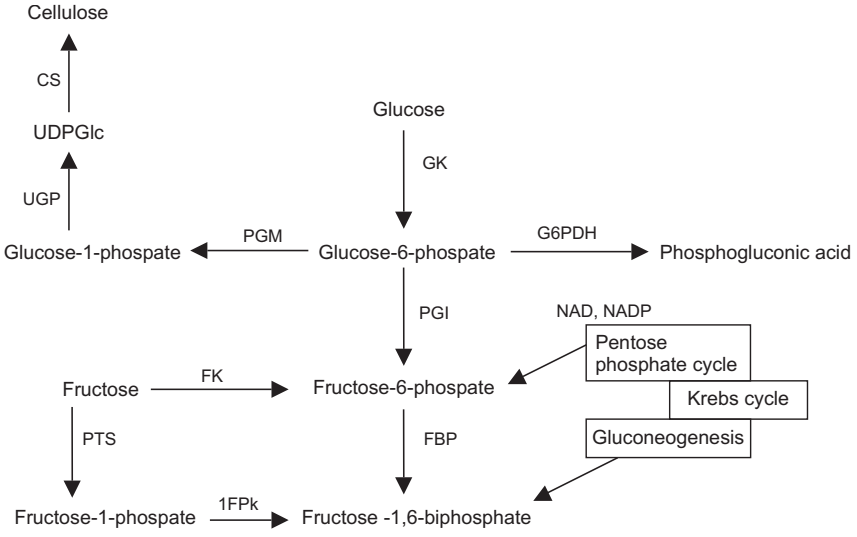

Fig. 2. Biochemical pathway for cellulose synthesis by *A. xylinum*. CS cellulose synthase, GK glucokinase, FBP fructose-1,6-biphosphate phosphatase, FK fructokinase, 1FPk fructose-1-phosphate kinase, PGI phosphoglucoisomerase, PMG phosphoglucomutase, PTS system of phosphotransferases, UGP pyrophosphorylase uridine diphosphoglucose, UDPGlc uridine diphosphoglucose, G6PDH glucose-6-phosphate dehydrogenase, NAD nicotinamide adenine dinucleotide, NADP nicotinamide adenine dinucleotide phosphate

cell, and self-assembly into fibrils (*23*). In *A. xylinum*, cellulose synthesis is tightly associated with catabolic processes of oxidation and consumes as much as 10 % of energy derived from catabolic reactions. Production of bacterial cellulose does not interfere with other anabolic processes, including protein synthesis. *A. xylinum* follows either pentose phosphate cycle or the Krebs cycle coupled with gluconeogenesis (*27,28*).

Synthesis of the Cellulose Precursor

A. xylinum converts various carbon compounds, such as hexoses, glycerol, dihydroxyacetone, pyruvate, and dicarboxylic acids, into cellulose, usually with about 50 % efficiency. Pyruvate and dicarboxylic acids enter the Krebs cycle and due to oxalacetate decarboxylation to pyruvate undergo a conversion to hexoses *via* gluconeogenesis, similarly to glycerol, dihydroxyacetone, and intermediates of the pentose phosphate cycle (Fig. 2). The direct cellulose precursor is UDPGlc, which is a product of a conventional pathway, common in many organisms, including plants, and involving glucose phosphorylation to glucose-6-phosphate (Glc-6-P), catalyzed by glucokinase, followed by isomerization of this intermediate to Glc-α-1-P, catalyzed by phosphoglucomutase, and conversion of the latter metabolite to UDPGlc by UDPGlc pyrophosphorylase. This enzyme seems to be the crucial one involved in cellulose synthesis, since some phenotypic cellulose-negative mutants (Cel^-) are specifically deficient in this enzyme (*29*), although they display cellulose synthase (CS) activity, which was confirmed *in vitro* by means of observation of cellulose synthesis, catalyzed by cell-free extracts of Cel^- strains (*30*). Furthermore, the pyrophosphorylase activity varies among different *A. xylinum* strains and the highest activity was detected in the most effective cellulose producers, such as *A. xylinum* ssp. *sucrofermentans* BPR2001 (*23*).

Cellulose synthase

Cellulose synthase of *A. xylinum* is a typical membrane-anchored protein having a molecular mass of 400–500 kDa. It is tightly bound to cytoplasmic membrane and appears to be very unstable (*31*). Isolation of cellulose synthase from membranes is carried out using digitonin (*31*) or detergents (Triton X-100) and a treatment with trypsin followed by its entrapment on cellulose. The conserved amino acids in cellulose synthases and other glycosyltranferases are present in a large globular region that is being actively analyzed. The globular region varies in size among the bacterial and plant cellulose synthases, with vascular plant cellulose synthases having at least two insertions. The globular region is predicted to be cytoplasmic, based on the analysis of the transmembrane segments and the presence of UDPGlc in cytosol (*32*). Cellulose biosynthesis in higher plants or in the prokaryotes is catalyzed by UDPGlc-forming cellulose synthase. This is basically a processing of 4-β-glucosyl transferase, since it transforms consecutive glucopyranose residues from UDPGlc to the newly found polysaccharide chain. Oligomeric cellulose synthase complexes are frequently called terminal complexes, which are responsible for β-1→4 glucan chain synthesis. The purified cellulose synthase preparations contain three different types of subunits, having molecular mass of 90, 67 and 54 kDa (*31*), but some researchers affirmed that there are only two polypeptides (83 and 93 kDa). Photolabelling affinity studies indicate that 83-kDa polypeptide is a catalytic subunit, displaying high affinity towards UDPGlc (*13*).

Fully acetylated carbohydrate derivatives were used as carbon sources instead of glucose for cultivation of *Acetobacter xylinum*, and resulting bacterial celluloses were analysed by IR-spectroscopy for possible incorporation of these substrates into the β-1→4-glucan chains. The feeding of acetylated glucose for the production of acetylated cellulose was not possible because of complicated active transport phenomenon, specificity of cellulose synthase, or the possibility for the formation of UDPGlc (*33*). The acetyl substituents found in the bacterial cellulose were established to be due to the acetylated derivatives that were associated with or entrapped in bacterial cellulose.

Mechanism of Biosynthesis

The synthesis of cellulose in *A. xylinum* or any other cellulose-producing organisms including plants follows two immediate steps: (*i*) formation of β-1→4 glucan chain with polymerization of glucose units, and (*ii*) assembly and crystallization of cellulose chain. The rate of polymerization is limited by the rate of assembly and crystallization.

Formation of β-1→4 glucan chain

Cellulose synthase catalyzes the cellulose biosynthesis by polymerizing the glucose units into the β-1→4 glucan chain. The formation of cellulose fibrils is shown in Fig. 1. The formation of the precellulosic polymer occurs in the cytoplasmic membrane. Two hypotheses for this mechanism in *A. xylinum* have been reported. The first hypothesis assumes that the polymerization of the β-1→4 glucan does not involve a lipid intermediate. The glucose residues were added to the nonreducing end of the polysaccharide, and those reducing ends were nascent polymer chains situated away from the cells (*32*). The torsion angle between the two adjacent glucose residues in cellulose molecule is 180° and the growing chain favours maintaining the twofold screw axis of the β-1→4 glucan. The second hypothesis is that the polymerization of β-1→4 glucan involves a lipid intermediate. The involvement of lipid intermediate in the synthesis of acetan, a soluble polysaccharide, has been proven (*34*). Studies have to be carried out to determine whether lipid intermediate plays any role in cellulose synthesis. The polymer synthesis is catalyzed by purified cellulose synthase in *A. xylinum* subunits, which does not contain the lipid component (*35*).

Assembly and crystallization of cellulose chains

The unique structure and properties of cellulose result from a course of extrusion of chains and their assembly outside the cells. The precellulosic polymer molecules synthesized in the interior of bacterial cell are spun out of the 'cellulose export components' to form a

protofibril of approx. 2–4 nm diameter, and the protofibrils are bundled in the form of a ribbon-shaped microfibril of approx. 80×4 nm (*36*).

Electron micrographs of the surface of the cell envelope indicate the presence of some 50 to 80 pore-like sites arranged in a regular row along the long axis of the cell and in evident combination with the extracellular cellulosic ribbon (*37*,*38*). These discrete structures of the lipopolysaccharide layer are presumed to be the sites of extrusion for precellulosic polymers. Aggregates of this size, rather than individual β-1→4 glucan chains, are postulated to be the initial form of the cellulosic product (*39*). The existence of such tactoidal aggregates, and of analogous structures in algal preparations (*39*), suggests that the synthesis of many β-1→4 glucan chains simultaneously at a spatially limited site is a common feature of the assembly of cellulose microfibrils in both higher and lower organisms (*40*). This process of assembly and crystallization of cellulose chains is usually described as cell directed because, although it occurs in the extracellular space, the mutual orientation and association of glucan chains, aggregates, microfibrils, bundles, and ribbon are apparently governed by the original pattern of extrusion sites (*27*).

Stronger aeration or the presence of certain substances that cannot penetrate inside the cells, but can form competitive hydrogen bonds with the β-1→4 glucan chains (carboxymethyl cellulose, fluorescent brightener, calcofluor white), bring significant changes in the supramolecular organization of cellulose chains. Instead of the ribbon-like polymer, *i.e.* cellulose I, the thermodynamically more stable amorphous cellulose II is formed. The differences in the assembly of cellulose I and cellulose II outside the cytoplasmic membrane are well defined in Fig. 1. Typical chain elongation rate, which is 2 μm/min, corresponds to a polymerization of more than 10^8 glucose molecules in the β-1→4 glucan (*23*).

Fermentative Production of Microbial Cellulose

In general, factors affecting cellulose production mainly include growth medium, environmental conditions and formation of byproducts. Generally, medium containing high carbon to limiting nutrient ratio (often nitrogen) is favourable for polysaccharide production. Conversion of 60–80 % of the utilized carbon source into crude polymer is commonly found in high yielding polysaccharide fermentations. The optimal design of the medium is very important for the growth of a microorganism and thus stimulating the formation of products. Nutrients required for the growth of a microorganism are carbon, nitrogen, phosphorus, sulphur, potassium and magnesium salts.

Effect of medium components

The fermentation medium contains carbon, nitrogen and other macro- and micronutrients required for the growth of organism. The changes in the medium components affect the growth and the product formation directly or indirectly. Secretion of exopolysaccharides is usually most noticeable when the bacteria are supplied with an abundant carbon source and minimal nitrogen source (*41*). Sometimes a complex medium supplying amino acids and vitamins is also used to enhance the cell growth and production (*42*). Effects of various medium components on microbial cellulose production are described in the following sections.

Carbon source

Usually, glucose and sucrose are used as carbon sources for cellulose production, although other carbohydrates such as fructose, maltose, xylose, starch and glycerol have also been tried (*43*). *G. hansenii* PJK (KCTC 10505 BP) produced 1.72 g/L of cellulose when glucose was provided as carbon source (*21*). *Acetobacter* sp. V6 strain isolated from the traditionally fermented vinegar produced 4.16 g/L cellulose in a complex medium containing glucose as a carbon source (*44*). The effect of initial glucose concentration on cellulose production is also important, since the formation of gluconic acid as a byproduct in the medium decreases the pH of the culture and ultimately decreases the production of cellulose. Cellulose yields at initial glucose concentrations of 6, 12, 24 and 48 g/L were studied, and the consumption of glucose was found to be 100, 100, 68 and 28 % of the initial concentration, respectively (*43*).

Ishihara *et al*. (*45*) used xylose as a carbon source for the production of cellulose by *A. xylinum* IFO 15606 and obtained a yield of 3.0 g/L. Sucrose, mannitol and glucose were found to be the optimal carbon sources for cellulose production by *A. xylinum* NCIM 2526 (*41*). *Gluconacetobacter xylinus* strain isolated from kombucha gave maximum cellulose production with mannitol as a carbon source (*46*).

Ethanol is used as additional carbon source and also to degenerate the cellulose non-producing cells of *G. hansenii* (Cel^-), which can appear under submerged culture conditions. Addition of ethanol increased cellulose production from 1.30 to 2.31 g/L in *G. hansenii* (*21*). Son *et al*. (*47*) studied the effect of the addition of ethanol on cellulose production by using newly isolated *Acetobacter* sp. A9 strain. It was observed that with the addition of 1.4 % (by volume) ethanol to the medium, cellulose production was 15.2 g/L, which was about four times higher than that without ethanol addition. Addition of ethanol was also found to eliminate spontaneous mutation of cellulose non-producing cells.

The problem associated with the use of glucose as a carbon source for cellulose production is the formation of gluconic acid as byproduct in the medium which decreases the pH of the culture and ultimately decreases the production of cellulose. Keshk and Sameshima (*48*) investigated the formation of gluconic acid and bacterial cellulose production in the presence of lignosulphonate. Gluconic acid production was decreased and bacterial cellulose production was increased when the medium was supplemented with lignosulphonate. This was attributed to the inhibition of gluconic acid formation in the presence of antioxidant and polyphenolic compounds in lignosulphonate.

The effect of the addition of organic acids to GPY--citric acid buffer medium was studied. Cellulose yield increased from 0.6 to 3.8 g/L in the presence of 20 g/L of acetic acid. The addition of organic acids other than acetic acid (succinic, lactic and gluconic acid) did not

increase the yield of cellulose. Acetic acid was consumed as monomeric raw material and mass flow of glucose catabolism was partly substituted by another carbon source (*e.g.* acetic acid) (*49*). Matsuoka *et al*. (*42*) observed that lactate had a stimulating effect on cellulose production when it was added with 4 % (by mass per volume) fructose containing corn steep liquor, yeast extract or peptone as a nitrogen source. However, time of initiation of bacterial cellulose production was different for different carbon sources. Naritomi *et al*. (*50*) also studied the effect of lactate on bacterial cellulose production from fructose in continuous culture by *Acetobacter xylinum* ssp. *sucrofermentans* BPR3001A. They reported that supplementing 12.5 g/L of lactate to the feed medium increased the cell concentration and fructose consumption at a steady state, resulting in a production rate of 0.90 g/(L·h) and a cellulose yield of 36 % at a dilution rate of 0.1 h^{-1}. The adenosine triphosphate (ATP) content of viable cells was maintained at a higher level by feeding with a lactate-supplemented medium rather than the unsupplemented corn steep liquor-fructose medium. They also indicated that lactate functioned as an energy source, not as a substrate for cellulose biosynthesis. Increased intracellular ATP resulting from lactate oxidation may have improved the fructose consumption and cellulose production in the continuous culture.

Bae and Shoda (*51*) studied the production of bacterial cellulose by *Acetobacter xylinum* BPR2001 using molasses medium in a jar fermentor. They reported that subjecting molasses to H_2SO_4-heat treatment gave a maximum cellulose concentration that was 76 % more than that achieved using untreated molasses, and also that the specific growth rate increased twofold. They also varied the initial sugar concentrations in the H_2SO_4-heat treated molasses from 23 to 72 g/L, and concluded that maintaining a lower concentration in the molasses is essential for efficient cellulose production in jar fermentors, the effect being attributed mainly to the complex nature of molasses. Bae and Shoda (*52*) studied the production of bacterial cellulose by intermittent and continuous fed-batch fermentation in molasses-based medium. They reported a production of 7.82 g/L when 200 mL of molasses medium was added five times by intermittent feeding, whereas maximum bacterial cellulose was obtained with a feeding rate of 6.3 g of sugar per h by continuous feeding. Keshk and Sameshima (*53*) investigated the production of bacterial cellulose using sugarcane molasses in a Hestrin-Schramm medium, and indicated it to be a better carbon source than glucose for cellulose production.

Premjet *et al*. (*54*) added components of sugarcane molasses such as sucrose, fructose, glucose, nitrogenous compounds, non-nitrogenous acids, nucleic acids, vitamins, other carbohydrates, minerals and black colour substances individually or in combined forms into Hestrin-Schramm medium, and investigated their effect on bacterial cellulose production by *Acetobacter xylinum* ATCC 10245. They concluded that the addition of vitamins, amino acids, other carbohydrates, minerals and black colour substances to the molasses in the Hestrin-Schramm medium with a mixture of sucrose and fructose as the carbon source increased the bacterial cellulose yield. The black colour substance was the most effective in increasing the production of bacterial cellulose.

Hornung *et al*. (*55*) studied the effect of the mass transfer rate of substrate on the microbial growth of the bacteria, cellulose formation, and the utilization of the substrate. A fundamental model for the diffusion of glucose through the growing cellulose layer was proposed. The model confirmed that the increase in diffusional resistance is indeed significant, but other factors should also be taken into account. Hornung *et al*. (*56*) noted that the growing cellulose is in contact with the wall of the box or beaker, and moves downwards into the nutrient broth with progress of time. They carried out experiments where this wall contact was eliminated and a constant rate of production over several weeks was found. This indicated the importance of understanding the role of the wall in the usual surface culture.

Hornung *et al*. (*57*) reported the use of a novel aerosol bioreactor working on a fed batch principle. This involved the generation of an aerosol spray of glucose and its even distribution to the living bacteria on the medium-air interface. The apparatus was built and operated up to eight weeks with a constant rate of cellulose production. The aerosol system provided the basis for an economic production of bacterial cellulose in surface culture.

Bae and Shoda (*51*) and Sun *et al*. (*58*) optimized the media for the production of bacterial cellulose by using statistical techniques. Sun *et al*. (*58*) used the one-factor-at-a-time and orthogonal array method for culture condition optimization. They reported the optimum medium composition of (in %): sucrose 5, protein peptone 1.5, citric acid 0.2, $Na_2HPO_4{\cdot}12H_2O$ 0.2, KH_2PO_4 0.2, $MgSO_4{\cdot}7H_2O$ 0.03, alcohol 1, pH=6.8, and cultivation at 28 °C for 6 days with maximum yield of 2 g/L of cellulose. Bae and Shoda (*51*) used Box-Behnken design to optimize the culture conditions and reported 14.3 g/L of bacterial cellulose production with 4.99 % fructose, 2.85 % corn steep liquor, 28.33 % dissolved oxygen, and 0.38 % agar.

Kim *et al*. (*59*) isolated *Gluconacetobacter* sp. RKY5 from persimmon vinegar and optimized the conditions for maximum cellulose production. The optimized medium composition for cellulose production was determined to be (in %): glycerol 1.5, yeast extract 0.8, K_2HPO_4 0.3, and acetic acid 0.3. In this optimized culture medium, *Gluconacetobacter* sp. RKY5 produced 5.63 g/L of cellulose after 144 h of shaken culture and 4.59 g/L were produced after 144 h of static culture. Seto *et al*. (*60*) studied the co-cultivation of *Gluconacetobacter xylinus* and *Lactobacillus mali* for maximum production of bacterial cellulose. A microbial colony that contained a marked amount of cellulose was isolated from vineyard soil. The colony was formed by the associated growth of two bacterial strains: a cellulose-producing acetic acid bacterium (st-60–12) and a lactic acid bacterium (st-20). Co-cultivation of the two organisms in corn steep liquor/sucrose liquid medium resulted in a threefold higher cellulose yield as compared to the st-60–12 monoculture. A similar enhancement was observed in a co-culture with various *L. mali* strains, but not with other *Lactobacillus* sp.

Nitrogen source

Nitrogen is a main component of proteins necessary in cell metabolism, and comprises 8–14 % of the dry cell mass of bacteria. The effect of various nitrogen sources on the production of bacterial cellulose has been reported; casein hydrolyzate gave yield of 5 g/L, and peptone gave yield of 4.8 g/L of cellulose in *A. xylinum* (*41*). The addition of extra nitrogen favours the biomass production, but diminishes cellulose production (*42*). Matsuoka *et al*. (*42*) observed that corn steep liquor had a stimulating effect on cellulose production when it was added at 0.15 % (by volume) to the medium with 4 % (by mass per volume) fructose. This was attributed to the presence of lactate in corn steep liquor, which is absent from other nitrogen sources.

The effect of yeast extract concentrations on cell growth and cellulose production in different carbon media has been studied. Yeast extract was added to the medium in the range from 5 to 60 g/L, while carbon sources comprised 20 g/L; the medium containing 40 g/L of yeast extract yielded maximum concentration (6.7 g/L) of cellulose (*61*).

Effect of precursors

The addition of precursor molecules is of considerable importance in the polysaccharide synthesis in terms of metabolic driving force. Amino acids have been used by some researchers as a nitrogen source or as a stimulator for improving biopolymer yield (*44*). Methionine has an important effect on the cellulose production by *Acetobacter xylinum* ssp. *sucrofermentans*, accounting alone for 90 % of cell growth and cellulose production (*42*). Nicotinamide has also been found to be important for cellulose production. Evaluation of nicotinamide in the fraction range of 0.00001 to 0.00008 % showed that maximal bacterial cellulose production was at 0.00005 % (*44*). Vitamins like pyridoxine, nicotinic acid, *p*-aminobenzoic acid and biotin were also found to be important for the cell growth and cellulose production, but vitamins like pantothenate and riboflavin were found to have contradictory effects (*42*).

The sugar nucleotides had an important effect on the cell growth and microbial cellulose production. UDPGlc has a stimulating effect on the production of microbial cellulose (*44,62*).

Effect of the addition of water-soluble polysaccharides

Bacterial cellulose production in a reactor is complex, mainly because the culture used consists of solid bacterial cellulose and cells, liquid medium and air gas. The heterogeneous coagulation of bacterial cellulose forms a large clump on the surface, with adhered cells and the subsequent deterioration of bacterial cellulose production. Therefore, a homogeneous and small bacterial cellulose suspension is essential for sufficient agitation and oxygen transfer. To minimize the clumps, several viscous water-soluble polysaccharides were supplemented to the medium. It is reported that the bacterial cellulose pellets became smaller in the medium supplemented with agar or acetan, which leads to improved bacterial cellulose production. Bacterial cellulose production in a 50-litre internal loop airlift reactor was increased from 6.3 to 8.7 g/L by the addition of 0.1 % agar (*63*). The effect of agar fractions ranging from 0 to 1 % (by mass per volume) was studied on the *Acetobacter xylinum* BPR 2001 by Bae *et al*. (*64*). The yield of bacterial cellulose increased from 8 to 12.8 g/L at an agar fraction of 0.4 %. The culture broth containing agar was more viscous and the free cell number was higher than that of the broth without the agar, suggesting that agar may hinder the coagulation of bacterial cellulose in the broth. The specific growth rate of the cellulose also increased in the presence of agar. Addition of agar was not associated with biochemical metabolism, but rather had a physicochemical effect on the medium (*64*).

Zhou *et al*. (*65*) studied the effect of the addition of sodium alginate on bacterial cellulose production by *Acetobacter xylinum* NUST4.1. It was observed that sodium alginate hindered the formation of large clumps of bacterial cellulose, and enhanced cellulose yield. The yield of bacterial cellulose reached 6.0 g/L after the addition of 0.04 % (by mass per volume) sodium alginate into the medium as compared to 3.7 g/L in the control.

Miscellaneous

The influence of lignosulphonate on bacterial cellulose productivity was studied by Keshk and Sameshima (*48*) using six strains of *G. xylinus*. The productivity of bacterial cellulose by all strains improved by almost 57 % in the presence of 1 % (by mass per volume) lignosulphonate. The stimulatory effect on the bacterial cellulose membrane production was attributed to the high molecular mass lignosulphonate fraction.

Calcofluor white ST, an optical brightener for cellulose, increased the rate of glucose polymerization into cellulose. *A. xylinum* bacterium normally produces a ribbon of cellulose that is a composite of crystalline microfibrils, but calcofluor white ST above 0.1 mM disrupts the assembly of crystalline cellulose I microfibrils and their integration into a composite ribbon by stoichiometric binding to glucose residues of newly polymerized cellulose polymer chains. Under these conditions, the rate of glucose polymerization increases up to 4 times the control rate, whereas oxygen uptake increases only 10–15 % (*39*). Addition of xylose isomerase to the medium containing D-xylose at the beginning of fermentation increased the production of cellulose from 1.2 to 3 g/L (*45*).

During continuous production of cellulose, *A. xylinum* cells, which produce large amounts of cellulose, are rather difficult to transfer from one inoculum to the next as the cells frequently become entangled in the thick cellulosic pellicle. A large inoculum is necessary for the large scale production of cellulose. Cellulase enzyme preparations added at appropriate concentrations do not impede cell growth but dissolve cellulose around the cells. Using this technique, dense cultures of cellulose-producing microorganisms may be produced and used for various purposes. Addition of cellulase at 0.000375 to 0.015 U/mL increased the rate of cellulose synthesis by *A. xylinum*. Brown (*66*) and Nakamura *et al*. (*67*) reported an increase in cellulose productivity by *A. xylinum* even in the presence of a small amount of heat-denatured cellulase. They also found that biosynthesis of cellulose in *A. xylinum* began at the earlier stage of fermentation as compared to the control.

Nguyen *et al*. (*46*) studied the effect of green tea on cellulose production by a *G. xylinus* strain isolated from kombucha. Green tea at a level of 3 g/L gave the highest cellulose yield of 3.34 g/L after 7 days of incubation. Tea infusions (caffeine and theophylline) function as stimulators of cellulose production by preventing bis-(3′--5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), the most important factor in cellulose synthesis, from being destroyed by the enzyme phosphodiesterase.

Effect of environmental factors

Microorganisms respond rapidly to environmental changes in many aspects such as induction and repression of protein synthesis, and changes in cell morphology. The main environmental parameters of interest are pH, temperature, dissolved oxygen, as well as stirrer speed. The physiology of *A. xylinum* has been studied by many researchers in shake flasks and in the 50-litre air lift bioreactor in order to understand how the microorganisms function and respond to the controlled environment, and to optimize process conditions for cellulose production. The bacterial cellulose produced by the airlift reactor formed a unique elliptical pellet different from the fibrous form that was produced in an agitated stirred tank fermentor (*68*).

Temperature

Temperature is a crucial parameter that affects both growth and cellulose production. In the majority of experiments, the maximal cellulose production was observed between 28 and 30 °C (*17*,*69*).

pH

The optimum pH of the culture medium for bacterial cellulose production is in the range of 4.0 to 6.0, the yield of cellulose decreasing below pH=4 (*43*). The pH decreases during fermentative production because of the accumulation of gluconic, acetic or lactic acids in the culture broth (*70*). Therefore, it is important to control the pH within the optimal range. It is actually difficult to autoregulate an optimal pH using a pH sensor as the viscous broth often attaches to the sensor, causing inaccurate pH readings. Even in a stirred tank reactor, a high broth viscosity causes inhomogeneous mixing, which results in the adsorption of broth onto the pH sensor (*4*). Noro *et al*. (*71*) used the buffering capacity of corn steep liquor (CSL) to maintain the pH, as CSL contains various buffering substances. When a CSL-fructose (CSL-Fru) medium with a buffering capacity was used, the pH could be maintained within the optimal range and high bacterial cellulose production was achieved. The value β, shown in Eq. 1, was introduced to evaluate the buffering capacity of CSL added to the medium:

$$\beta = \frac{\mathrm{d[OH]}}{\mathrm{dpH}} \qquad /1/$$

where β is the buffering capacity, d[OH] is the change in the molarity of the CSL-Fru medium after the addition of 0.2 M NaOH or 0.1 M H_2SO_4, and dpH is the change in pH.

Dissolved oxygen

The dissolved oxygen in the culture medium is an important factor affecting cellulose production. In static cultures, substrates have to be transported entirely by diffusion and as carbon sources are generally available, the oxygen availability might become the limiting factor for cell metabolism and could have a negative effect on cellulose production and quality of the cellulose (*72*).

Kouda *et al*. (*73*) studied the effects of oxygen and carbon dioxide pressures on the production of bacterial cellulose by *Acetobacter* in aerated and agitated culture. The production rate was dependent on the oxygen transfer rate, which declined as the broth viscosity increased. Although the cellulose production rate was not affected by higher oxygen pressure, it was reduced as the operating pressure was raised. The reduction in the production rate was reported to be due to the high carbon dioxide pressure, because carbon dioxide-enriched air also reduced the production rate, while the reduction was nullified by increasing the airflow rate.

The dissolved oxygen in the medium can be varied by changing the agitation speed. Tantratian *et al*. (*74*) found that too high dissolved oxygen in the medium increased the gluconic acid content when glucose was used as a carbon source and thus reduced the cellulose production, whereas too low dissolved oxygen in the medium did not provide enough oxygen for the culture to grow and cellulose production was again reduced. In the fed-batch cultures, the highest yield of cellulose concentration was obtained at 10 % saturation of dissolved oxygen (*75*).

Chao *et al*. (*76*) studied the production of bacterial cellulose under oxygen-enriched air at different fructose concentrations in a 50-litre, internal-loop airlift reactor. When the initial fructose concentrations were varied from 30 to 70 g/L, the highest production rate (0.22 g/L per h), and highest concentration of cellulose (10.4 g/L) were observed at 60–70 g/L of fructose. It was concluded that enhanced production was reflected as a decrease in both CO_2 evolution and the concentration of other unknown substances, suggesting the efficient utilization of energy for cellulose synthesis despite O_2 limitation.

Bacterial Cellulose Production Using Reactors

Bacterial cellulose has been conventionally produced by static and agitated culture method. Static culture method requires a long culture period and intensive manpower, thus resulting in a low productivity. On the other hand, agitated culture method converts bacterial cellulose-producing strains into cellulose-negative mutants, which become more enriched than the wild-type strain because of their rapid growth, thereby lowering the productivity of bacterial cellulose (*77*). In stirred tank or airlift reactors, the adhesion of bacterial cellulose culture broth to the inside wall and the upper part of the apparatus causes problems and reduces bacterial cellulose production (*78*). Therefore, it is necessary to develop a reactor for production of bacterial cellulose which requires less culture time and does not allow conversion of bacterial cellulose-producing strains into cel-

lulose-negative mutants. There are few reports in literature where different reactors have been used for bacterial cellulose production.

Rotating disk reactor

The rotating disk reactor is designed so that half of the surface of its disks is submerged in the medium broth while the other half is exposed to the atmosphere. As the disks rotate continuously, the surface of the disks alternates between the medium and the atmosphere. When this reactor is used for the production of bacterial cellulose, cells that are stuck to the disk surface take nutrients when they are immersed in the medium, and are exposed to oxygen in the atmosphere. Krystynowicz *et al*. (*78*) investigated the optimum conditions for the production of bacterial cellulose in a rotating disk reactor by changing the medium volume, rotation speed, and number of disks. Maximum production of bacterial cellulose was obtained when the rotation speed and the ratio of surface area to medium volume (S/V) were 4 rpm and 0.71 cm^{-1}, respectively.

Rotary biofilm contactor (RBC)

The RBC consists of a series of circular disks mounted on a horizontal shaft. The disks within the RBC are rotated, and alternatively exposed to the fermentation medium and air. Cellulose-producing bacteria cultivated in RBC do not face a strong shear stress and have an excellent oxygen transferability with which the microorganisms can readily come into contact with air, in comparison with stirred tank bioreactor. Kim *et al*. (*77*) used RBC for production of bacterial cellulose by *Gluconacetobacter* sp. Bacterial cellulose production was maximal at 5.52 g/L in RBC with eight disks. When the aeration rate was maintained at 1.25 vvm, bacterial cellulose production reached 5.67 g/L. The optimal rotation speed of the impeller in RBC was 15 rpm.

Bioreactor equipped with a spin filter

A fermentation system using a spin filter was developed by Jung *et al*. (*79*) and its performance characteristics were tested. Fermentations were carried out in a fermentor equipped with a 6 flat-blade turbine impeller and a spin filter consisting of a cylinder surrounded by stainless steel mesh and whose stainless steel bottom was attached to the agitator shaft. This reactor was used for the production of bacterial cellulose by *G. hansenii* PJK. In periodical perfusion culture, bacterial cellulose production reached 4.57 g/L after 140 h of cultivation, which was 2.9 times higher than that obtained in a conventional jar fermentor.

Reactor with silicone membrane

To increase the rate of production of bacterial cellulose by *A. pasteurianus* AP-1SK in static culture, Yoshino *et al*. (*22*) developed a system in which cellulose pellicles were formed on an oxygen-permeable synthetic membrane and on a liquid surface. The rate of bacterial cellulose production was doubled using the cylindrical vessel, the bottom end of which was covered with a silicone sheet of 100 μm thickness. A silicone air bag was also used to produce bacterial cellulose. The rate of cellulose production on silicone membrane depended strongly on the degree of roughness of the membrane surface. The rate of bacterial cellulose production was about five times higher on a glossy surface of a silicone membrane than on an embossed surface (*22*).

Static and submerged fermentation

The differences in the production of cellulose and its structure in static and submerged cultures have been reported by various researchers (*55–57,80–82*). The production of bacterial exopolysaccharide in submerged cultivation was accompanied by a substantial increase in the viscosity of fermentation broth, which impaired air distribution in the medium. The internal structures of bacterial cellulose strictly depend on the culture conditions. In the static culture, it is important to control the pH because the accumulation of gluconic, acetic or lactic acids in the culture broth decreases the pH far below the optimum for growth and polysaccharide production (*70*). The doubling time of *A. xylinum* in static culture is 8–10 h, while that in submerged culture is 4–6 h (*23*). The most important problem associated with cellulose synthesis under submerged culture conditions by *A. xylinum* is the generation of cellulose non-producing cells (*10,20*).

Kouda *et al*. (*83*) evaluated the effect of agitator configuration on bacterial cellulose productivity in aerated and agitated culture. They reported that: (*i*) the impellers such as Maxblend® and a gate with turbine were suitable for bacterial cellulose fermentation because they mixed culture broth well and had large K_La, (*ii*) the production rate and yield depended on K_La and the oxygen consumption rate, and (*iii*) the static gassing-out method to measure K_La was useful for characterization of the agitation conditions and the agitator configuration.

Morphological differences between the cellulose produced by static and agitated cultures contribute to varying degrees of crystallinity, different crystalline size and I_α cellulose content. The difference in I_α content between the cellulose produced under agitated and static conditions exceeds that of the crystallinity index. The crystallinity index is closely related to the I_α value (*82*). During the production of cellulosic films on non-agitated liquid media, the mass of the system does not change.

Under static immersed cultivation conditions, a biofilm of varying thickness (fleece) is produced which helps the colonized bacteria to maintain a high oxygen content near the surface, and which serves as a protective barrier against drying, natural inhibitors, and radiation (*84*). For most of the bacterial strains that produce cellulose, the pellicle is formed at a higher rate in a static culture than in shaken or submerged cultures where oxygen is supplied by forced aeration to enhance the bacterial respiration. Because the production rate of bacterial cellulose pellicle per unit of surface area of a static culture is almost constant, the production rate per culture volume can be increased by making the culture as shallow as possible. However, this requires a large area in which to place the culture vessel and is impractical for large-scale production.

The average thickness (or the grammage) of the final dried pellicle strongly affects its performance and hence must be carefully controlled. The fermentation process must be interrupted at a certain stage in order to obtain, after the final treatments, a dried pellicle presenting a grammage within a defined interval. Due to the relatively high complexity of the process, it is difficult to determine the time to stop the fermentation, leading to industrial losses that are frequently higher than 60 %. Borzani and De Souza (*85*) invented a simple method to control the bacterial cellulose production in static culture in order to obtain dried pellicles of desired average thickness. An equation was proposed to evaluate the volume of inoculated medium to be placed in a given tray, so that a completely dried film of a desired average thickness could be produced:

$$V = \frac{G \cdot A(1+\alpha)}{\sigma \cdot \rho} + \frac{R \cdot A}{\rho} t \qquad /2/$$

where G is the grammage of the dried film (g/m^2), A is the area of air/medium interface (m^2), α is a relative increase in film area due to pressing, ρ is the density of inoculated medium (g/mL), σ is dried content of wet film (g/g), t is time (day), and R is evaporation rate (L/(day·m^2).

Continuous production

The microbial cellulose can be produced under static conditions. Hestrin-Schramm medium was added to the smaller or larger culture pans. The depth of the culture medium in each pan was 3–4 mm. The media were inoculated with the subcultured *A. xylinum* at 28 °C under static conditions. After two days of incubation, the edge of the pellicle produced on the surface was picked up, passed through the sodium dodecyl sulphate (SDS) bath to denature the bacterial cell wall. After 2 or 3 days of static cultivation, thin bacterial cellulose gel was formed and the harvest was started on the roller system. The tensile strength of the filament was found to be significantly stronger than the ordinary cellulose fibres (*86*).

Genetic Modification for Production of Bacterial Cellulose

The Gram-negative bacterium *A. xylinum* has been studied as a model organism for cellulose biosynthesis. This organism has a cellulose synthase operon (*AxCes* operon) consisting of three or four genes (*87–90*). In addition, two genes, *cmcax* and *ccpax*, are located in the upstream region of the operon (*91*).

De Wulf *et al*. (*92*) attempted to improve cellulose productivity genetically by generating a mutant with restricted ketogluconate synthesis. They used UV mutagenesis to obtain a ketogluconate-nonproducing mutant from a parent strain, and its cellulose production increased from 1.8 g/L by the parent strain to 3.3 g/L after 10 days of shaking culture. This was supported by the fact that, when glucose or sucrose is used as the carbon source by *G. xylinus*, the main product is not cellulose, but ketogluconate, which decreases the pH, cell growth and cellulose production.

G. xylinus secretes not only water-insoluble cellulose, but also acetan, a viscous water-soluble polysaccharide which decreases fluidity of the culture broth adversely affecting the cellulose production. Acetan consumes UDPGlc for self-synthesis, which is also the starting material for cellulose synthesis. If acetan is not synthesized, the amount of UDPGlc used for cellulose synthesis is expected to increase, resulting in an increase in cellulose production. Ishida *et al*. (*93*) generated the acetan-nonproducing mutant strain, EP1, from the parental strain *G. xylinus* BPR2001. However, the cellulose production by EP1 decreased in the shake flask culture, although the productivity was maintained at the same level as that of the parent strain BPR2001 in a static culture. They found that the culture broth of EP1 became a heterogeneous suspension, containing large flocks formed by the aggregation of cells and cellulose, compared to that of the parent strain in the shake flask. This might be because acetan increases the viscosity of the culture, preventing the coagulation of cells and cellulose, resulting in an increased production. They also reported that when agar was added to the medium, similar effect to that of acetan was shown towards cellulose production. When agar was added at the start of cultivation, cellulose production was observed from the beginning of the experiments, and the cultivation time was reduced to two thirds as compared to that without the addition of agar.

The gene *dgc1* is known to be important for activating bacterial cellulose synthesis. Bae *et al*. (*62*) reported on cloning and sequencing the *dgc* gene associated with the regulation of c-di-GMP produced by *A. xylinum* BPR 2001 and determined the relationship between the structural characteristics and production of cellulose formed by the *dgc1* gene-disrupted mutant cultured using different cultivation methods. They unexpectedly found that the cellulose production by a mutant was almost the same as that of the parent strain in static and shake flask cultivations. Moreover, when the mutant was cultivated in the stirred tank reactor, the cellulose production increased by 36 % compared to that of the parent strain. They speculated that although *dgc1* was disrupted, cellulose production increased because other genes, *dgc2* and *dgc3*, which are functionally similar to those of *dgc1*, worked complementarily or were more stimulatory for cellulose synthesis. Therefore, they concluded that *dgc1* disruption is not critical for total bacterial cellulose production. However, Tal *et al*. (*94*) reported that cellulose production decreased with *dgc1* disruption. Although these two results seem to be inconsistent, the cultivation time used by Tal *et al*. (*94*) was too short to evaluate the final cellulose production, as the growth rate of the mutant was slower than that of the parental strain.

Kawano *et al*. (*95*) reported on cloning of cellulose synthesis related genes from *A. xylinum* ATCC23769 and ATCC53582. They cloned about 14.5 kb of DNA fragments from *A. xylinum* ATCC23769 and ATCC53582, and determined their nucleotide sequences. The sequenced DNA regions contained endo-β-1,4-glucanase, cellulose complementing protein, cellulose synthase subunits AB, C and D, and β-glucosidase genes. They found that cellulose production by ATCC53582 was 5 times greater than that of ATCC23769 during a 7-day incubation. In

A. xylinum ATCC53582, the synthesis of cellulose continued after glucose was consumed, suggesting that a metabolite of glucose, or a component of the medium other than glucose, may be a substrate for the production of cellulose. They concluded that the synthesis of cellulose and the growth of bacterial cells are contradictory.

To understand the structure–function relationships of endoglucanase gene (*CMCax*), which is involved in both cellulose hydrolysis and synthesis, Kawano *et al*. (*96*) studied crystallization and preliminary crystallographic analysis of the cellulose biosynthesis-related protein CMCax (EC 3.2.1.4) from *A. xylinum.* They overexpressed CMCax in *Escherichia coli*, then purified and crystallized it. CMCax, a protein coded by the *CMCax* gene, is an endoglucanase that has cellulose-hydrolyzing activity, while the *CCPax* product is suggested to be involved in cellulose crystallization (*97*). Kawano *et al*. (*90*) reported that over-expression of the *CMCax* gene in *A. xylinum* enhances cellulose production, so does the addition of the CMCax protein into the culture medium. Kawano *et al*. (*98*) revealed a regulation mechanism of *CMCax* expression in a cellulose non-producing mutant of *A. xylinum* using an enzyme assay and real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). They also studied condensation activity in *A. xylinum*, which produces β-glucodisaccharides from glucose. Furthermore, they investigated the *CMCax* gene expression in a wild-type strain using real-time qRT-PCR and concluded that gentiobiose induced *CMCax* expression and enhanced *CMCax* activity, suggesting that the concentrations of gentiobiose in the culture regulate cellulose production in *A. xylinum*.

Shigematsu *et al*. (*99*) cloned a gene fragment encoding a putative pyrroloquinoline quinone glucose dehydrogenase from *Gluconacetobacter xylinus* strain BPR2001, which was isolated as a high bacterial cellulose producer when using fructose as the carbon source. A glutamate dehydrogenase (GDH)-deficient mutant of strain BPR2001, namely GD-I, was generated *via* gene disruption using the cloned gene fragment. Strain GD-I produced no gluconic acid but it produced 4.1 g/L of cellulose aerobically in a medium containing glucose as the carbon source. The ability of strain GD-I to convert glucose to bacterial cellulose was approx. 1.7-fold higher than that of the wild type.

Nobles and Brown (*100*) reported on the functional expression of a partial cellulose synthase operon (acsABΔC) of *G. xylinus* in the unicellular cyanobacterium, *Synechococcus leopoliensis* strain UTCC 100 (synonym, *Synechococcus elongatus* strain PCC 7942), resulting in the production of non-crystalline cellulose. They sought to combine the prodigious cellulose biosynthetic capacity of *G. xylinus* with the photosynthetic ability of cyanobacteria.

Properties

Microbial cellulose possesses high crystallinity, high tensile strength, extreme insolubility in most of the solvents, moldability and high degree of polymerization (*101–103*). The thickness of cellulose fibrils is generally 0.1–10 μm, one hundred times thinner than that of cellulose fibrils obtained from plants with good shape retention. Its water holding capacity is over 100 times (by mass) higher. Microbial cellulose is far stronger than plant cellulose (*82*,*104*). Macroscopic morphology of cellulose strictly depends on the culture conditions, which can easily be tailored for the physicochemical properties. Wanichapichart *et al*. (*105*) demonstrated that cellulose fibre had the degree of polymerization of 793, with a corresponding molecular mass of approx. 142.73 kDa.

Cellulose is soluble in concentrated acids like sulphuric, hydrochloric or nitric acid. It is also soluble in 8.5 % NaOH solution. The solubility of cellulose in the alkali can be increased by adding 1 % of urea to the solution (*106*).

At higher temperatures (>300 °C) the biopolymer degrades, although the alkali-treated cellulose membrane is more stable (between 343 and 370 °C). Composites prepared by adding bacterial cellulose and microfibrillated cellulose (MFC) processed through fibrillation of kraft pulp were compared for mechanical properties and it was found that the bending strength increased up to 425 MPa, while the Young's modulus increased from 19 to 28 GPa, nearly retaining the modulus of the bacterial cellulose sheets (*107*,*108*). The mechanical properties of cellulose are due to the uniqueness of uniform nano-scalar network structure, which is oriented bi-dimensionally when compressed.

George *et al*. (*101*) studied the swelling property of cellulose under different conditions. NaOH at lower concentration caused greater swelling in fibres as compared to other alkalis at the same concentrations. The percentage mass gain by the cellulose membranes after immersion in different alkali solutions was found to be in the order of NaOH>KOH>Na_2CO_3>K_2CO_3. The pervaporation characteristics of deproteinated microbial cellulose membrane were investigated over a wide range of water-ethanol feed composition and it was found to be promising for dehydration of azeotropes of ethanol. It has a high selectivity towards water at a reasonable flux (*109*). The basic characteristics of cellulose membrane as a molecular separation medium in aqueous conditions, and with modification of the structure by chemical treatments for controlling its molecular permeation characteristics are well described (*110*). The most attractive feature of bacterial cellulose production is the ability to control and modify not only the physical characteristics, but also the chemical composition of the cellulose fibre (*72*). The structure of the cellulose assembly can be altered by using direct dyes (amide black, trypan red), fluorescent brightening agents (congo red) or derivatives like carboxymethyl cellulose (*111–113*). *A. xylinum* was cultured in Hestrin-Schramm medium (control medium) and Hestrin-Schramm medium containing acetyl glucomannan. The presence of acetyl glucomannan in the medium prevents the assembly of cellulose microfibrils and changes the crystal structure of cellulose (*86*). Cultivation of *A. xylinum* in Hestrin-Schramm medium containing glucuronoxylan (xylan medium) showed loose bundles of cellulose microfibrils in the medium. In contrast, cellulose ribbons were formed in the pectin medium. Glucuronoxylan in the medium prevented the assembly

of cellulose microfibrils and changed the crystal structure of cellulose, whereas pectin in the medium scarcely had an effect (*114*).

Chemical Structure

Cellulose is a homopolymer consisting of glucose glycosidically linked in a β-1→4 conformation (Fig. 3). The repeating unit of the polymer synthesis consists of two glucose molecules bonded together in such a way that one molecule is rotated 180 degrees with respect to the other.

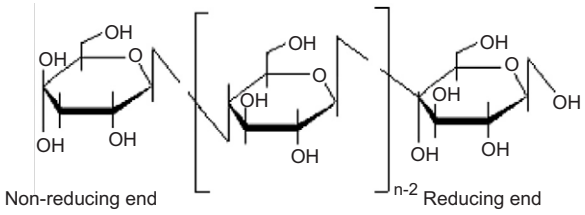

Fig. 3. Repeating units of cellulose

The chemical structure of bacterial cellulose is similar to that of plant cellulose but the degree of polymerization differs from about 13 000 to 14 000 for plant and 2000–6000 for bacterial cellulose (*115*). The glucose units in cellulose are bound together to produce a long straight unbranched polymer chain and the capacity to form intermolecular hydrogen bonds between adjacent glucan chains is extremely high. *A. xylinum* cellulose consists of ribbons of microfibrils generated at the surface of the bacterial cell. The dimensions of the ribbons are 3–4 nm thick and 70–80 nm wide. The shape of microbial cellulose sheet seems to be maintained by hydrophobic bonds. It is reported that in the course of time inter- and intramolecular hydrogen bonds initially occur in each cellulose sheet, and then the cellulose crystalline structure is formed with the development of hydrogen bonds between cellulose sheets (*23*).

The microfibrilar structure of microbial and plant cellulose under the scanning electron microscope (SEM) is shown in Fig. 4. The structural differences of cellulose produced by stationary and agitated culture were studied by using nuclear magnetic resonance (NMR) and Fourier transform infrared spectroscopy (FTIR) (*81,82*). The existence of tunnels as observed by scanning electron microscope (SEM) argues for some kind of coordination during the pellicle formation and a random formation of cellulose microfibrils (*116*). Two common crystalline forms of cellulose designated as I and II are distinguishable by X-ray diffraction (*117*).

The microbial cellulose observed under SEM showed a significant difference in appearance of the external and internal surfaces of the pellicles. The external surfaces had irregular clusters of fibrils, whereas internal surfaces were organized in fractured sections. At higher magnification, layers of tunnels in the bacterial cellulose of about 7 μm in diameter were found (*118*). The solid-phase nitration and acetylation processes for bacterial cellulose were studied using CP/MAS ^{13}C NMR spectroscopy together with wide-angle X-ray diffractometry and transmission electron microscopy (TEM). The relative reactivity of the OH groups in the glucose residues was found to decrease in the order of 6'OH>2'OH>3'OH. Moreover, the nitration rate greatly depends on the concentration of nitric acid in the reaction media. At lower concentrations, the 6'OH groups in the crystalline and disordered components were subjected to nitration at nearly the same rate, indicating that these two components were distributed almost randomly in the entire region of each microfibril. In contrast, all OH groups underwent nitration very rapidly at the higher concentration, although nitration levelled off to a certain extent for 3'OH groups. In solid-phase acetylation, no regioselective reactivity was observed among the three kinds of OH groups, which may be due to the characteristic reaction that precedes in a very thin layer between the acetylated and nonacetylated regions in each microfibril (*119*).

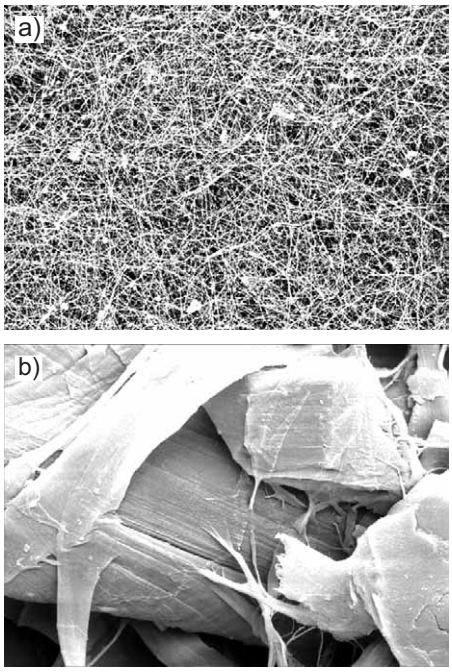

Fig. 4. Microfibrillar organization between (a) *Acetobacter* cellulose, and (b) wood pulp (5000×) (*7*)

Recovery and Purification

The microbial cellulose obtained after fermentation is not pure; it contains some impurities like cells and/or the medium components. Care must be taken in the interpretation of such yields, as crude products will often contain cells, which are bound to the polymer when it is recovered from fermentation broth (*23*). The fermented broth has to be purified to obtain pure cellulose. The process of isolation and purification of microbial cellulose is described in Fig. 5.

The most widely used process of purification of bacterial cellulose in the culture medium is the treatment with alkali (sodium hydroxide or potassium hydroxide), organic acids like acetic acid or repeated washing of the mixtures with the reverse osmosis water or hot tap wa-

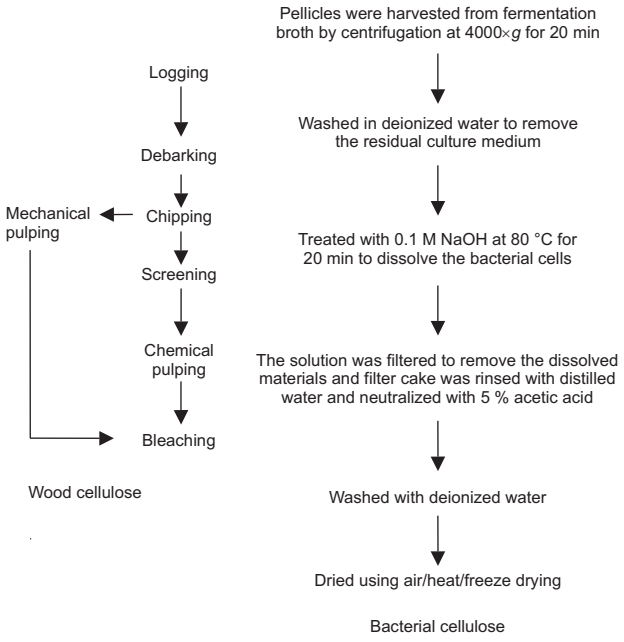

Fig. 5. Comparison of typical pulping processes for purification of cellulose from wood with a cleaning process for *Acetobacter* cellulose pellicles

ter for a period of time. The above purification steps can be used alone or in combination (*23*).

Bacterial cellulose containing entrapped cells was treated with solutions like NaOH/KOH/Na_2CO_3 at 100 °C for 15–20 min to lyse the microbial cells; thereafter the solution was filtered using an aspirator to remove the dissolved materials. The filter cake was repeatedly rinsed with distilled water until the pH of the filtrate became neutral. The dry mass of bacterial cellulose without any microbial cells was measured after drying for 4–6 h. As such, the dry cell mass was considered to be the difference between the mass of the dried bacterial cellulose containing the cells and the dried bacterial cellulose after the treatment with NaOH (*20*,*43*,*70*,*92*). The culture medium was treated with acetic acid after the addition of NaOH solution for neutralization and then with distilled water (*49*,*120*). The cells can be treated with aqueous solution of SDS and washed with aqueous NaOH, followed by neutralization with acetic acid or by repeated washing with distilled water and then drying in the air or at 60–80 °C to a constant mass (*3*,*20*,*86*).

Medical application of bacterial cellulose requires special procedure to remove bacterial cells and toxins which can cause pyrogenic reactions. One of the most effective protocols is gentle processing of cellulose pellicle between absorbent sheets to expel about 80 % of the liquid phase and then immersing the mat in 3 % NaOH for 12 h. This procedure is repeated 3 times and after that the pellicle is incubated in 3 % HCl solution, pressed and thoroughly washed in distilled water. The purified pellicle is sterilized in autoclave or by ^{60}Co irradiation. It performs excellently as a wound dressing as it contains only 1–50 ng of lipopolysaccharide endotoxins, whereas bacterial cellulose purified using conventional methods usually contains 30 μg or more of these substances (*23*).

Applications

Since bacterial cellulose has properties like high purity, high degree of crystallinity, high density, good shape retention, high water binding capacity, and higher surface area as compared to the native cellulose, it can be used in various areas including textile industry, paper, food, pharmaceutical, waste treatment, broadcasting, mining and refinery (*7*,*12*,*121*). The applications of the bacterial cellulose are summarized in the following categories.

Food applications

Chemically pure cellulose can be used in processed foods as thickening and stabilizing agent. The first use of microbial cellulose in the food industries was in nata de coco in the Philippines. The gel-like properties of microbial cellulose, combined with its complete indigestibility in the human intestinal tract, made this an attractive food base (*122*). In the 1970s, monacolin K (mevinolin), an important metabolite of *Monascus* sp., was identified and shown to inhibit the synthesis of cholesterol. Nata, a bacterial cellulose product, which is prepared from *Acetobacter xylinum*, is a popular snack in the Philippines and other countries. It is also widely used in food processing because of its distinctly soft texture and high fibre content. Monascus-nata complex, which combines the functional characteristics of monacolin K and bacterial cellulose, is a potential novel functional foodstuff (*123*,*124*).

In 1992, microbial cellulose was introduced into diet drinks in Japan. *Acetobacter* was grown along with yeast in the tea extract and sugar. This is consumed as a kombucha, or Manchurian tea, for improved health needs.

Pharmaceutical and medical applications

Microbial cellulose has high tensile strength, high porosity and microfibrillar structure. Chronic wounds such as venous leg ulcers, bedsores, and diabetic ulcers are difficult to heal, and they represent a significant clinical challenge both for the patients and for the healthcare professionals. The treatment of chronic wounds involves the application of various materials (hydrocolloids, hydrogels, biological or synthetic membranes) that provide a moist wound healing environment, which is necessary for optimal healing. According to the modern approaches in the field of wound healing, an ideal wound dressing system must display similarity to artificial skin, both structurally and functionally. The characteristics of modern wound dressing materials are: nontoxic, nonpyrogenic and biocompatible; ability to provide barrier against infection; ability to control fluid loss; ability to reduce pain during treatment; ability to create and maintain a moist environment in the wound; provide easy and close wound coverage; enable introduction or transfer of medicines into the wound; ability to absorb exudates during inflammatory phase; display high mechanical strength, elasticity and conformability; display an optional shape and surface area, and allow for easy and painless healing of the wound.

As microbial cellulose is a highly porous material, it allows the potential transfer of antibiotics or other medicines into the wound, while at the same time serving as

an efficient physical barrier against any external infection. It satisfies the requirements of modern wound dressing material (*7*). Bacterial cellulose has high water holding capacity and the size of fibrils is about 100 times smaller than that of plant cellulose, as shown under SEM in Fig. 4. A Brazilian company Biofill investigated its unique properties for wound healing (*125*), and produced two products, Bioprocess and Gengiflex (*126*), as dressings for extensive wounds. A bacterial cellulose preparation, Prima Cel™, produced by Xylos Corp. (USA), has been applied in clinical tests to heal ulcers and wounds. Tissue engineered blood vessels (TEBV) represent an attractive approach for overcoming reconstructive problems associated with vascular diseases by providing small calibre vascular grafts, and bacterial cellulose exhibits properties that are promising for use as a scaffold for tissue engineered blood vessels (*127*).

Other applications

The unique physical and mechanical properties of microbial cellulose such as high reflectivity, flexibility, light mass and ease of portability, wide viewing angles, and its purity and uniformity determine the applications in the electronic paper display (*121*). Fragmented bacterial cellulose has promising prospects in papermaking, so test pieces of flexure-durable papers and high filler--content papers, which are ideal for banknote paper and bible paper, are being prepared (*36*).

Microbial cellulose also has applications in mineral and oil recovery. There is a patented invention related to the use of bacterial cellulose in hydraulic fracturing of geological formations at selected levels of wells drilled for recovery of hydrocarbons. Addition of relatively small quantities of bacterial cellulose to hydraulic fracturing fluids improves their rheological properties and the friction through well casings is significantly reduced, resulting in lower pumping energy requirements. Computer models also indicate that formation fractures will be propagated for greater distances as will the propped portion of the fracture. Normally only about 0.60–1.80 g of bacterial cellulose per litre of fracturing fluid is needed (*128*).

Addition of cellulose microfibrils obtained by acid hydrolysis of cellulose fibres at low concentrations to polymer gels and films as reinforcing agents showed significant changes in tensile strength and mechanical properties (*107*). Based on the tensile strength, low oxygen transmission (barrier property) rate and its hydrophilic nature, the processed cellulose membrane appears to be of great relevance for its application as packaging material in food packaging, where continuous moisture removal and minimal oxygen transmission properties play a vital role (*102*).

The unique dimensional stability of microbial cellulose gives rise to a sound transducing membrane which maintains high sonic velocity over a wide frequency ranges, thus being the best material to meet the rigid requirements for optimal sound transduction. Sony Corporation (Japan), in conjunction with Ajinimoto (Japan), developed the first audio speaker diaphragms using microbial cellulose. However, the production of the speaker membrane by using bacterial cellulose is not justifiable for the market because of the high costs (*129*).

Producers of Microbial Cellulose

Xylos Corp. (USA) produces Prima Cel™, a type of cellulose used for wound dressing in clinical trials for healing ulcers (*23*). Other commercial preparations of *A. xylinum* cellulose are Biofill™ and Bioprocess™. Fzmb GmbH (former fzmb e.V.), Germany is one of the biggest producers of cellulose sheets for an application in cosmetics and the medicinal area. Cellulose is used in food industry as nata de coco in the Philippines (*122*,*124*). Sony Corporation (Japan) in conjunction with Ajinimoto (Japan) developed the first audio speaker diaphragms using microbial cellulose (*129*). Ajinomoto sells 1 kg of wet *Acetobacter* cellulose for 1000 yen.

Future Perspectives and Conclusions

Bacterial cellulose is of commercial interest for many of the same reasons that cotton fields and forests attract the industrialists' attention. It is likely that the maximum capacity of bacteria such as *A. xylinum* to produce cellulose has not reached full expression, although reports on industrial production do indicate an ultimate potential that is both greater and more obtainable than expected.

Economical feasibility of bacterial cellulose is primarily dependent on its productivity. The choice of fermentor design is particularly critical because it must withstand vigorous mechanical agitation of the rapidly growing *A. xylinum* (obligate aerobe) culture, and also prevent the cellulose fibril and fibrillar matrix from mechanical disruption. Agitated culture results in highly branched, three-dimensional, reticulated structure, whereas static culture produces a normal cellulose pellicle with a lamellar structure and less significant branching. Desirable modifications in the fibrillar and/or macroscopic nature of the cellulosic product may be achieved by varying fermentor design factors such as the shape of vessels and agitating impellers. The advantage of the stirred tank reactor is its ability to prevent the heterogenity of the culture broth by strong mechanical agitation, while its drawback is high energy cost for generating the mechanical power. On the contrary, the energy cost of an airlift reactor is one sixth of that of a stirred tank reactor. However, the agitation power of an airlift reactor is limited, resulting in low fluidity of the culture broth, especially at high cellulose concentrations. To meet different demands, the combined use of an airlift and a stirred tank reactors, or continuous cultivation, may be a possible solution. Use of some modified reactors like rotating disk reactor, rotary biofilm contactor, bioreactors equipped with a spin filter or reactor with silicone membrane can also be a useful solution.

One of the major obstacles encountered in the industrial adaptation of *A. xylinum* is the wasteful and noxious accumulation of metabolic byproducts from otherwise desirable carbon sources and the marked tendency of wild-type strains to revert to cellulose non-producing mutants under the oxygen-enriched conditions

of stirred tank fermentations. The isolation of a genetically stable strain with a substantially reduced ability to form gluconic acid has been achieved by relatively standard techniques of mutagenesis and selection; this strain is reported to allow rapid and reliable culture on a glucose substrate, from which the major product is fibrillar cellulose.

Another approach to increase the bacterial cellulose production is to genetically modify the bacteria. *G. xylinus* has a long doubling time compared to most other bacteria, such as *E. coli* and *B. subtilis*. Since their growth rates are relatively faster than that of *G. xylinus*, genetic modification of these bacteria will also be one possible means of increasing bacterial cellulose production. A very promising line of advance toward obtaining industrially valuable strains is in the direct genetic manipulation of the genes coding for the catalysts of cellulose synthesis, their adjunctive regulatory enzymes, and the relevant associated membrane structures such as the postulated extrusion pores. However, bacterial cellulose obtained from genetically modified organisms may face regulatory restrictions in medical and food industry.

References

1. J.F. Wilkinson, The extracellular polysaccharides of bacteria, *Bacteriol. Rev. 22* (1958) 46–73.
2. I.W. Sutherland, Structure-function relationship in microbial exopolysaccharides, *Biotechnol. Adv. 12* (1994) 393–448.
3. I.W. Sutherland, Novel and established applications of microbial polysaccharides, *Trends Biotechnol. 16* (1998) 41–46.
4. M. Shoda, Y. Sugano, Recent advances in bacterial cellulose production, *Biotechnol. Bioprocess Eng. 10* (2005) 1–8.
5. A.J. Brown, On an acetic ferment which forms cellulose, *J. Chem. Soc. Trans. 49* (1886) 432–439.
6. F. Yoshinaga, N. Tonouchi, K. Watanabe, Research progress in production of bacterial cellulose by aeration and agitation culture and its application as a new industrial material, *Biosci. Biotechnol. Biochem. 61* (1997) 219–224.
7. W. Czaja, A. Krystynowicz, S. Bielecki, R.M. Brown Jr., Microbial cellulose – The natural power to heal wounds, *Biomaterials, 27* (2006) 145–151.
8. K. Mühlethaler, The structure of bacterial cellulose, *Biochim. Biophys. Acta, 3* (1949) 527–535.
9. X. Yu, R.H. Atalla, Production of cellulose II by *Acetobacter xylinum* in the presence of 2,6-dichlorobenzonitrile, *Int. J. Biol. Macromol. 19* (1996) 145–146.
10. E.J. Vandamme, S. De Baets, A. Vanbaelen, K. Joris, P. De Wulf, Improved production of bacterial cellulose and its application potential, *Polym. Degrad. Stabil. 59* (1998) 93–99.
11. Y. Nishi, M. Uryu, S. Yamanaka, K. Watanabe, N. Kitamura, M. Iguchi, S. Mitsuhashi, The structure and mechanical properties of sheets prepared from bacterial cellulose, *J. Mater. Sci. 25* (1990) 2997–3001.
12. R.L. Legge, Microbial cellulose as a specialty chemical, *Biotechnol. Adv. 8* (1990) 303–319.
13. P.A. Richmond: Occurrence and Functions of Native Cellulose. In: *Biosynthesis and Biodegradation of Cellulose*, C.H. Haigler, P.J. Weimer (Eds.), Marcel Dekker, Inc. New York, USA (1991) pp. 5–23.
14. S. Isizawa, M. Araragi: Chromogenicity of Actinomycetes. In: *Actinomycetes: The Boundary Microorganisms*, T. Arai (Ed.), Toppan Co., Tokyo, Japan (1976) pp. 43–65.
15. W.D. Bellamy, Single cell proteins from cellulosic wastes, *Biotechnol. Bioeng. 16* (1974) 869–880.
16. R.M. Brown Jr., The biosynthesis of cellulose, *Food Hydrocolloids, 1* (1987) 345–351.
17. Z. Gromet-Elhanan, S. Hestrin, Synthesis of cellulose by *Acetobacter xylinum*. VI. Growth on citric acid-cycle intermediates, *J. Bacteriol. 85* (1963) 284–292.
18. U. Geyer, D. Klemm, H.P. Schmauder, Kinetics of the utilization of different C sources and the cellulose formation by *Acetobacter xylinum*, *Acta. Biotechnol. 14* (1994) 261–266.
19. U. Geyer, T. Heinze, A. Stein, D. Klemm, S. Marsch, D. Schumann, H.P. Schmauder, Formation, derivatization and applications of bacterial cellulose, *Int. J. Biol. Macromol. 16* (1994) 343–347.
20. J.Y. Jung, J.K. Park, H.N. Chang, Bacterial cellulose production by *Gluconoacetobacter hansenii* in an agitated culture without living non-cellulose producing cells, *Enzyme Microb. Technol. 37* (2005) 347–354.
21. J.K. Park, J.Y. Jung, Y.H. Park, Cellulose production by *Gluconacetobacter hansenii* in a medium containing ethanol, *Biotechnol. Lett. 25* (2003) 2055–2059.
22. T. Yoshino, T. Asakura, K. Toda, Cellulose production by *Acetobacter pasteurianus* on silicone membrane, *J. Ferment. Bioeng. 81* (1996) 32–36.
23. S. Bielecki, A. Krystynowicz, M. Turkiewicz, H. Kalinowska: Bacterial Cellulose. In: *Polysaccharides and Polyamides in the Food Industry*, A. Steinbüchel, S.K. Rhee (Eds.), Wiley-VCH Verlag, Weinheim, Germany (2005) pp. 31–85.
24. V.P. Puri, Effect of crystallinity and degree of polymerization of cellulose on enzymatic saccharification, *Biotechnol. Bioeng. 26* (1984) 1219–1222.
25. C. Wiegand, D. Klemm, Influence of protective agents for preservation of *Gluconacetobacter xylinus* on its cellulose production, *Cellulose, 13* (2006) 485–492.
26. D.P. Delmer, Y. Amor, Cellulose biosynthesis, *Plant Cell, 7* (1995) 987–1000.
27. P. Ross, R. Mayer, M. Benziman, Cellulose biosynthesis and function in bacteria, *Microbiol. Rev. 55* (1991) 35–58.
28. N. Tonouchi, T. Tsuchida, F. Yoshinaga, T. Beppu, S. Horinouchi, Characterization of the biosynthetic pathway of cellulose from glucose and fructose in *Acetobacter xylinum*, *Biosci. Biotechnol. Biochem. 60* (1996) 1377–1379.
29. S. Valla, D. H. Coucheron, E. Fjaervik, J. Kjosbakken, H. Weinhouse, P. Ross, D. Amikam, M. Benziman, Cloning of a gene involved in cellulose biosynthesis in *Acetobacter xylinum*: Complementation of cellulose-negative mutant by the UDPG pyrophosphorylase structural gene, *Mol. Gen. Genet. 217* (1989) 26–30.
30. I.M. Saxena, R. M. Brown Jr.: Cellulose Biosynthesis in *Acetobacter xylinum*: A Genetic Approach. In: *Cellulose and Wood – Chemistry and Technology*, C. Schuerch (Ed.), John Wiley & Sons, Inc., New York, USA (1989) pp. 537–557.
31. F.C. Lin, R.M. Brown Jr., J.B. Cooper, D.P. Delmer, Synthesis of fibrils *in vitro* by a solubilized cellulose synthase from *Acetobacter xylinum*, *Science, 230* (1985) 822–825.
32. R.M. Brown Jr., I.M. Saxena, Cellulose biosynthesis: A model for understanding the assembly of biopolymers, *Plant Physiol. Biochem. 38* (2000) 57–67.
33. L. Einfeldt, D. Klemm, H.P. Schmauder, Acetylated carbohydrate derivatives as C-sources for *Acetobacter xylinum*, *Nat. Prod. Res. 2* (1993) 263–269.
34. N.I. De Iannino, R.O. Couso, M.A. Dankert, Lipid-linked intermediates and the synthesis of acetan in *Acetobacter xylinum*, *J. Gen. Microbiol. 134* (1988) 1731–1736.
35. F.C. Lin, R.M. Brown Jr.: Purification of Cellulose Synthase from *Acetobacter xylinum*. In: *Cellulose and Wood: Chemistry and Technology*, C. Schuerch (Ed.), John Wiley & Sons, Inc., New York, USA (1989) pp. 473–492.

36. M. Iguchi, S. Yamanaka, A. Budhiono, Bacterial cellulose – A masterpiece of nature's arts, *J. Mater. Sci. 35* (2000) 261–270.

37. R.M. Brown Jr., J.H.M. Willison, C.L. Richardson, Cellulose biosynthesis in *Acetobacter xylinum*: Visualization of the site of synthesis and direct measurement of the *in vivo* process, *Proc. Natl. Acad. Sci. USA, 73* (1976) 4565–4569.

38. K. Zaar, Visualization of pores (export sites) correlated with cellulose production in the envelope of the Gram-negative bacterium *Acetobacter xylinum*, *J. Cell Biol. 80* (1979) 773–777.

39. M. Benziman, C.H. Haigler, R.M. Brown Jr., A.R. White, K.M. Cooper, Cellulose biogenesis: Polymerization and crystallization are coupled processes in *Acetobacter xylinum*, *Proc. Natl. Acad. Sci. USA, 77* (1980) 6678–6682.

40. D.P. Delmer, Cellulose biosynthesis, *Ann. Rev. Plant Physiol. 38* (1987) 259–290.

41. K.V. Ramana, A. Tomar, L. Singh, Effect of various carbon and nitrogen sources on cellulose synthesis by *Acetobacter xylinum*, *World J. Microbiol. Biotechnol. 16* (2000) 245–248.

42. M. Matsuoka, T. Tsuchida, K. Matsushita, O. Adachi, F. Yoshinaga, A synthetic medium for bacterial cellulose production by *Acetobacter xylinum* subsp. *sucrofermentans*, *Biosci. Biotechnol. Biochem. 60* (1996) 575–579.

43. S. Masaoka, T. Ohe, N. Sakota, Production of cellulose from glucose by *Acetobacter xylinum*, *J. Ferment. Bioeng. 75* (1993) 18–22.

44. H.J. Son, H.G. Kim, K.K. Kim, H.S. Kim, Y.G. Kim, S.J. Lee, Increased production of bacterial cellulose by *Acetobacter* sp. V6 in synthetic media under shaking culture conditions, *Bioresour. Technol. 86* (2003) 215–219.

45. M. Ishihara, M. Matsunaga, N. Hayashi, V. Tišler, Utilization of D-xylose as carbon source for production of bacterial cellulose, *Enzyme Microb. Technol. 31* (2002) 986–991.

46. V.Y. Nguyen, B. Flanagan, M.J. Gidley, G.A. Dykes, Characterization of cellulose production by a *Gluconacetobacter xylinus* strain from kombucha, *Curr. Microbiol. 57* (2008) 449–453.

47. H.J. Son, M.S. Heo, Y.G. Kim, S.J. Lee, Optimization of fermentation conditions for the production of bacterial cellulose by a newly isolated *Acetobacter* sp. A9 in shaking cultures, *Biotechnol. Appl. Biochem. 33* (2001) 1–5.

48. S. Keshk, K. Sameshima, Influence of lignosulfonate on crystal structure and productivity of bacterial cellulose in a static culture, *Enzyme Microb. Technol. 40* (2006) 4–8.

49. K. Toda, T. Asakura, M. Fukaya, E. Entani, Y. Kawamura, Cellulose production by acetic acid-resistant *Acetobacter xylinum*, *J. Ferment. Bioeng. 84* (1997) 228–231.

50. T. Naritomi, T. Kouda, H. Yano, F. Yoshinaga, Effect of lactate on bacterial cellulose production from fructose in continuous culture, *J. Ferment. Bioeng. 85* (1998) 89–95.

51. S. Bae, M. Shoda, Statistical optimization of culture conditions for bacterial cellulose production using Box-Behnken design, *Biotechnol. Bioeng. 90* (2005) 20–28.

52. S. Bae, M. Shoda, Bacterial cellulose production by fed-batch fermentation in molasses medium, *Biotechnol. Progr. 20* (2004) 1366–1371.

53. S. Keshk, K. Sameshima, The utilization of sugar cane molasses with/without the presence of lignosulfonate for the production of bacterial cellulose, *Appl. Microbiol. Biotechnol. 72* (2006) 291–296.

54. S. Premjet, D. Premjet, Y. Ohtani, The effect of ingredients of sugar cane molasses on bacterial cellulose production by *Acetobacter xylinum* ATCC 10245, *Sen-i Gakkaishi, 63* (2007) 193–199.

55. M. Hornung, M. Ludwig, A.M. Gerrard, H.P. Schmauder, Optimizing the production of bacterial cellulose in surface culture: Evaluation of substrate mass transfer influences on the bioreaction (Part 1), *Eng. Life Sci. 6* (2006) 537–545.

56. M. Hornung, M. Ludwig, A.M. Gerrard, H.P. Schmauder, Optimizing the production of bacterial cellulose in surface culture: Evaluation of product movement influences on the bioreaction (Part 2), *Eng. Life Sci. 6* (2006) 546–551.

57. M. Hornung, M. Ludwig, H.P. Schmauder, Optimizing the production of bacterial cellulose in surface culture: A novel aerosol bioreactor working on a fed batch principle (Part 3), *Eng. Life Sci. 7* (2007) 35–41.

58. D.P. Sun, J.D. Zhang, L.L. Zhou, M.Y. Zhu, Q.H. Wu, C.Y. Xu, Production of bacterial cellulose with *Acetobacter xylinum* 1.1812 fermentation, *J. Nanjing Univ. Sci. Technol. 29* (2005) 601–604.

59. S.Y. Kim, J.N. Kim, Y.J. Wee, D.H. Park, H.W. Ryu, Production of bacterial cellulose by *Gluconacetobacter* sp. RKY5 isolated from persimmon vinegar, *Appl. Biochem. Biotechnol. 131* (2006) 705–715.

60. A. Seto, Y. Saito, M. Matsushige, H. Kobayashi, Y. Sasaki, N. Tonouchi, T. Tsuchida, F. Yoshinaga, K. Ueda, T. Beppu, Effective cellulose production by a coculture of *Gluconacetobacter xylinus* and *Lactobacillus mali*, *Appl. Microbiol. Biotechnol. 73* (2006) 915–921.

61. Y.K. Yang, S.H. Park, J.W. Hwang, Y.R. Pyun, Y.S. Kim, Cellulose production by *Acetobacter xylinum* BRC5 under agitated condition, *J. Ferment. Bioeng. 85* (1998) 312–317.

62. S.O. Bae, Y. Sugano, K. Ohi, M. Shoda, Features of bacterial cellulose synthesis in a mutant generated by disruption of the diguanylate cyclase 1 gene of *Acetobacter xylinum* BPR 2001, *Appl. Microbiol. Biotechnol. 65* (2004) 315–322.

63. Y. Chao, M. Mitarai, Y. Sugano, M. Shoda, Effect of addition of water-soluble polysaccharides on bacterial cellulose production in a 50-L airlift reactor, *Biotechnol. Progr. 17* (2001) 781–785.

64. S. Bae, Y. Sugano, M. Shoda, Improvement of bacterial cellulose production by addition of agar in a jar fermentor, *J. Biosci. Bioeng. 97* (2004) 33–38.

65. L.L. Zhou, D.P. Sun, L.Y. Hu, Y.W. Li, J.Z. Yang, Effect of addition of sodium alginate on bacterial cellulose production by *Acetobacter xylinum*, *J. Ind. Microbiol. Biotechnol. 34* (2007) 483–489.

66. M.R. Brown, Use of cellulase preparations in the cultivation and use of cellulose-producing microorganisms. *European patent 19870307513* (1993).

67. T. Nakamura, K. Tajima, M. Fujiwara, M. Takai, J. Hayashi, Cellulose production by *Acetobacter xylinum* in the presence of cellulose, *Use of Minerals in Papermaking* (1998) 3–8.

68. Y. Chao, T. Ishida, Y. Sugano, M. Shoda, Bacterial cellulose production by *Acetobacter xylinum* in a 50L internal-loop airlift reactor, *Biotechnol. Bioeng. 68* (2000) 345–352.

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

70. S. Kongruang, Bacterial cellulose production by *Acetobacter xylinum* strains from agricultural waste products, *Appl. Biochem. Biotechnol. 148* (2008) 245–256.

71. N. Noro, Y. Sugano, M. Shoda, Utilization of the buffering capacity of corn steep liquor in bacterial cellulose production by *Acetobacter xylinum*, *Appl. Microbiol. Biotechnol. 64* (2004) 199–205.

72. A. Shirai, M. Takahashi, H. Kaneko, S. Nishimura, M. Ogawa, N. Nishi, S. Tokura, Biosynthesis of a novel polysaccharide by *Acetobacter xylinum*, *Int. J. Biol. Macromol. 16* (1994) 297–300.

73. T. Kouda, T. Naritomi, H. Yano, F. Yoshinaga, Effects of oxygen and carbon dioxide pressures on bacterial cellulose

production by *Acetobacter* in aerated and agitated culture, *J. Ferment. Bioeng. 84* (1997) 124–127.

74. S. Tantratian, P. Tammarate, W. Krusong, P. Bhattarakosol, A. Phunsri, Effect of dissolved oxygen on cellulose production by *Acetobacter* sp., *J. Sci. Res. Chula Univ. 30* (2005) 179–186.

75. J.W. Hwang, Y.K. Yang, J.K. Hwang, Y.R. Pyun, Y.S. Kim, Effects of pH and dissolved oxygen on cellulose production by *Acetobacter xylinum* BRC5 in agitated culture, *J. Biosci. Bioeng. 88* (1999) 183–188.

76. Y. Chao, Y. Sugano, M. Shoda, Bacterial cellulose production under oxygen-enriched air at different fructose concentrations in a 50-liter, internal-loop airlift reactor, *Appl. Microbiol. Biotechnol. 55* (2001) 673–679.

77. J.Y. Kim, J.N. Kim, Y.J. Wee, D.H. Park, H.W. Ryu, Bacterial cellulose production by *Gluconacetobacter* sp. RKY5 in a rotary biofilm contactor, *Appl. Biochem. Biotechnol. 137* (2007) 529–537.

78. A. Krystynowicz, W. Czaja, A. Wiktorowska-Jezierska, M. Gonçalves-Miśkiewicz, M. Turkiewicz, S. Bielecki, Factors affecting the yield and properties of bacterial cellulose, *J. Ind. Microbiol. Biotechnol. 29* (2002) 189–195.

79. J.Y. Jung, T. Khan, J.K. Park, H.N. Chang, Production of bacterial cellulose by *Gluconacetobacter hansenii* using a novel bioreactor equipped with a spin filter, *Korean J. Chem. Eng. 24* (2007) 265–271.

80. W. Czaja, D. Romanovicz, R.M. Brown, Structural investigations of microbial cellulose produced in stationary and agitated culture, *Cellulose, 11* (2004) 403–411.

81. C. Tokoh, K. Takabe, M. Fujita, H. Saiki, Cellulose synthesized by *Acetobacter xylinum* in the presence of acetyl glucomannan, *Cellulose, 5* (1998) 249–261.

82. K. Watanabe, M. Tabuchi, Y. Morinaga, F. Yoshinaga, Structural features and properties of bacterial cellulose produced in agitated culture, *Cellulose, 5* (1998) 187–200.

83. T. Kouda, H. Yano, F. Yoshinaga, Effect of agitator configuration on bacterial cellulose productivity in aerated and agitated culture, *J. Ferment. Bioeng. 83* (1997) 371–376.

84. D. Klemm, B. Heublein, H.P. Fink, A. Bohn, Cellulose: Fascinating biopolymer and sustainable raw material, *Angew. Chem. Int. Edit. 44* (2005) 3358–3393.

85. W. Borzani, S.J. De Souza, A simple method to control the bacterial production of cellulosic films in order to obtain dried pellicles presenting a desired average thickness, *World J. Microbiol. Biotechnol. 14* (1998) 59–61.

86. N. Sakairi, H. Asano, M. Ogawa, N. Nishi, S. Tokura, A method for direct harvest of bacterial cellulose filaments during continuous cultivation of *Acetobacter xylinum, Carbohydr. Polym. 35* (1998) 233–237.

87. H.C. Wong, A.L. Fear, R.D. Calhoon, G.H. Eichinger, R. Mayer, D. Amikam, M. Benziman, D.H. Gelfand, J.H. Meade, A.W. Emerick, R. Bruner, A. Ben-Bassat, R. Tal, Genetic organization of the cellulose synthase operon in *Acetobacter xylinum, Proc. Natl. Acad. Sci. USA, 87* (1990) 8130–8134.

88. I.M. Saxena, K. Kudlicka, K. Okuda, R.M. Brown Jr., Characterization of genes in the cellulose-synthesizing operon (acs operon) of *Acetobacter xylinum*: Implications for cellulose crystallization, *J. Bacteriol. 176* (1994) 5735–5752.

89. T. Nakai, A. Moriya, N.Tonouchi, T. Tsuchida, F. Yoshinaga, S. Horinouchi, Y. Sone, H. Mori, F. Sakai, T. Hayashi, Control of expression by the cellulose synthase (*bcsA*) promoter region from *Acetobacter xylinum* BPR 2001, *Gene, 213* (1998) 93–100.

90. S. Kawano, K. Tajima, H. Kono, T. Erata, M. Munekata, M. Takai, Effects of endogenous endo-β-1,4-glucanase on cellulose biosynthesis in *Acetobacter xylinum* ATCC23769, *J. Biosci. Bioeng. 94* (2002) 275–281.

91. R. Standal, T.G. Iversen, D.H. Coucheron, E. Fjaervik, J.M. Blatny, S. Valla, A new gene required for cellulose production and a gene encoding cellulolytic activity in *Acetobacter xylinum* are colocalized with the bcs operon, *J. Bacteriol. 176* (1994) 665–672.

92. P. De Wulf, K. Joris, E.J. Vandamme, Improved cellulose formation by an *Acetobacter xylinum* mutant limited in (keto)gluconate synthesis, *J. Chem. Technol. Biotechnol. 67* (1996) 376–380.

93. T. Ishida, Y. Sugano, T. Nakai, M. Shoda, Effects of acetan on production of bacterial cellulose by *Acetobacter xylinum, Biosci. Biotechnol. Biochem. 66* (2002) 1677–1681.

94. R. Tal, H.C. Wong, R. Calhoon, D. Gelfand, A.L. Fear, G. Volman, R. Mayer, P. Ross, D. Amikam, H. Weinhouse, A. Cohen, S. Sapir, P. Ohana, M. Benziman, Three *cdg* operons control cellular turnover of cyclic di-GMP in *Acetobacter xylinum*: Genetic organization and occurrence of conserved domains in isoenzymes, *J. Bacteriol. 180* (1998) 4416–4425.

95. S. Kawano, K. Tajima, Y. Uemori, H. Yamashita, T. Erata, M. Munekata, M. Takai, Cloning of cellulose synthesis related genes from *Acetobacter xylinum* ATCC23769 and ATCC53582: Comparison of cellulose synthetic ability between strains, *DNA Res. 9* (2002) 149–156.

96. S. Kawano, Y. Yasutake, K. Tajima, Y. Satoh, M. Yao, I. Tanaka, M. Munekata, Crystallization and preliminary crystallographic analysis of the cellulose biosynthesis- related protein CMCax from *Acetobacter* xylinum, *Acta Crystallogr. F, Struct. Biol. Cryst. Commun. 61* (2005) 252–254.

97. T. Nakai, Y. Nishiyama, S. Kuga, Y. Sugano, M. Shoda, ORF2 gene involves in the construction of high-order structure of bacterial cellulose, *Biochem. Biophys. Res. Commun. 295* (2002) 458–462.

98. S. Kawano, K. Tajima, H. Kono, Y. Numata, H. Yamashita, Y. Satoh, M. Munekata, Regulation of endoglucanase gene (cmcax) expression in *Acetobacter xylinum, J. Biosci. Bioeng. 106* (2008) 88–94.

99. T. Shigematsu, K. Takamine, M. Kitazato, T. Morita, T. Naritomi, S. Morimura, K. Kida, Cellulose production from glucose using a glucose dehydrogenase gene (*gdh*)-deficient mutant of *Gluconacetobacter xylinus* and its use for bioconversion of sweet potato pulp, *J. Biosci. Bioeng. 99* (2005) 415–422.

100. D.R. Nobles Jr., R.M. Brown Jr., Transgenic expression of *Gluconacetobactor xylinus* strain ATCC 53582 cellulose synthase genes in the cyanobacterium *Synechococcus leopoliensis* strain UTCC 100, *Cellulose, 15* (2008) 691–701.

101. J. George, K.V. Ramana, S.N. Sabapathy, A.S. Bawa, Physico-mechanical properties of chemically treated bacterial (*Acetobacter xylinum*) cellulose membrane, *World J. Microbiol. Biotechnol. 21* (2005) 1323–1327.

102. J. George, K.V. Ramana, S.N. Sabapathy, H.J. Jambur, A.S. Bawa, Characterization of chemically treated bacterial (*Acetobacter xylinum*) biopolymer: Some thermo-mechanical properties, *Int. J. Biol. Macromol. 37* (2005) 189–194.

103. A.R. White, R.M. Brown Jr., Enzymatic hydrolysis of cellulose: Visual characterization of the process, *Proc. Natl. Acad. Sci. USA, 78* (1981) 1047–1051.

104. S. Schrecker, P. Gostomski, Determining the water holding capacity of microbial cellulose, *Biotechnol. Lett. 27* (2005) 1435–1438.

105. P. Wanichapichart, S. Kaewnopparat, K. Buaking, W. Puthai, Characterization of cellulose membranes produced by *Acetobacter xylinum, J. Sci. Technol. 24* (2002) 855–862.

106. B. Łaszkiewicz, Solubility of bacterial cellulose and its structural properties, *J. Appl. Polym. Sci. 67* (1998) 1871–1876.

107. W.J. Orts, J. Shey, S.H. Imam, G.M. Glenn, M.E. Guttman, J.F. Revol, Application of cellulose microfibrils in polymer nanocomposites, *J. Polym. Environ. 13* (2005) 301–306.

108. A.N. Nakagaito, S. Iwamoto, H. Yano, Bacterial cellulose: The ultimate nano-scalar cellulose morphology for the production of high-strength composites, *Appl. Phys. A: Mater. Sci. Process. 80* (2005) 93–97.

109. V. Dubey, C. Saxena, L. Singh, K.V. Ramana, R.S. Chauhan, Pervaporation of binary water-ethanol mixtures through bacterial cellulose membrane, *Sep. Purif. Technol. 27* (2002) 163–171.

110. H. Shibazaki, S. Kuga, F. Onabe, M. Usuda, Bacterial cellulose membrane as separation medium, *J. Appl. Polym. Sci. 50* (1993) 965–969.

111. R.M. Brown, C.H. Haigler, J. Suttie, A.R. White, E. Roberts, C. Smith, T. Itoh, K. Cooper, The biosynthesis and degradation of cellulose, *J. Appl. Polym. Sci. 37* (1983) 33–78.

112. A. Kai, The structure of the nascent fibril produced by *Acetobacter xylinum*: The lattice spacing of cellulose produced in the presence of a fluorescent brightener, *Macromol. Rapid Commun. 5* (1984) 653–655.

113. S.K. Cousins, R.M. Brown Jr., X-ray diffraction and ultrastructural analyses of dye-altered celluloses support van der Waals forces as the initial step in cellulose crystallization, *Polymer, 38* (1997) 897–902.

114. C. Tokoh, K.J. Takabe, M. Fujita, Cellulose synthesized by *Acetobacter xylinum* in the presence of plant cell wall polysaccharides, *Cellulose, 9* (2002) 65–74.

115. R. Jonas, L.F. Farah, Production and application of microbial cellulose, *Polym. Degrad. Stabil. 59* (1998) 101–106.

116. H. Shibazaki, S. Kuga, F. Onabe, R.M. Brown Jr., Acid hydrolysis behavior of microbial cellulose II, *Polymer, 36* (1995) 4971–4976.

117. S. Kuga, S. Takagi, R.M. Brown Jr., Native folded chain cellulose II, *Polymer, 34* (1993) 3293–3297.

118. N.S. Thompson, J.A. Carlson, H.M. Kaustinen, K.I. Uhlin, Tunnel structures in *Acetobacter xylinum, Int. J. Biol. Macromol. 10* (1988) 126–127.

119. H. Yamamoto, F. Horii, A. Hirai, Structural studies of bacterial cellulose through the solid-phase nitration and acetylation by CP/MAS ^{13}C NMR spectroscopy, *Cellulose, 13* (2006) 327–342.

120. S.M.A.S. Keshk, K. Sameshima, Evaluation of different carbon sources for bacterial cellulose production, *Afr. J. Biotechnol. 4* (2005) 478–482.

121. J. Shah, R.M. Brown Jr., Towards electronic paper displays made from microbial cellulose, *Appl. Microbiol. Biotechnol. 66* (2005) 352–355.

122. A. Budhiono, B. Rosidi, H. Taher, M. Iguchi, Kinetic aspects of bacterial cellulose formation in *nata-de-coco* culture system, *Carbohydr. Polym. 40* (1999) 137–143.

123. S.R. Stephens, J.A. Westland, A.N. Neogi, Method of using bacterial cellulose as a dietary fiber component. *US patent 4960763* (1990).

124. C.C. Ng, Y.T. Shyu, Development and production of cholesterol-lowering Monascus-nata complex, *World J. Microbiol. Biotechnol. 20* (2004) 875–879.

125. L.F.X. Farah, Process of the preparation of cellulose film, cellulose film produced thereby, artificial skin graft and its use. *US patent 4912049* (1990).

126. D.C. Johnson, A.N. Neogi, Sheeted products formed from reticulated microbial cellulose. *US patent 4863565* (1989).

127. H. Bäckdahl, G. Helenius, A. Bodin, U. Nannmark, B.R. Johansson, B. Risberg, P. Gatenholm, Mechanical properties of bacterial cellulose and interactions with smooth muscle cells, *Biomaterials, 27* (2006) 2141–2149.

128. J.A. Westland, G.S. Penny, R.S. Stephens, A.R. Winslow, Method of supporting fractures in geologic formations and hydraulic fluid composition for same. *US patent 5350528* (1994).

129. M. Iguchi, S. Mitsuhashi, K. Ichimura, Y. Nishi, M. Uryu, S. Yamanaka, K. Watanabe, Bacterial cellulose-containing molding material having high dynamic strength. *US patent 4742164* (1988).