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특집 : 미생물 자원을 이용한 신소재 개발 및 응용

Production and Application of Functional Bacterial Cellulose

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INTRODUCTION

Biopolymers are a diverse and versatile class of materials that have potential applications in various industry. In general, biopolymers fall into two principal categoreis : polymers that are produced by biological systems such as microorganism, plants, and animals ; and polymers that are synthesized chemically but are derived from biological starting materials such as amino acids, sugars, natural fats, or oil (1). Currently, many biopolymers are still in the developmental stage, but important applications are beginning to emerge in the areas of packaging, cosmetics, food additives, industrial plastics and biosensors and medical materials (1).

Microbial exopolysaccharides are long-chain, high-molecular-mass polymers that are secreted into the environment by a large variety of different bacteria. These polymers are believed to play a protective role in the native state (2). Microbial exopolysaccharide have many commercial applications such as paper coating, drug delivery, metal recoveries, cosmetic products, food products and separation. The great diversity exhibited by exopolysaccharides can be attributed to differences in their chemical composition and structure. Generally, functional properties of exopolysaccharides are largely influenced by their molecular weight, chemical composition, linkage-type, and functional side chains (3). Recently, tailored biopolymers have been considered as means of enhancing the functional properties. The following aspects are especially suitable for variation in order to create 'tailored biopolymers' : 1) microorganism selection ; 2) selection of the carbon source for growth and polymer accumulation ; 3) accumulation conditions during fermentation (e.g. focused limitations) ; 4) selection of the isolation methods for biopolymers ; 5) treatment of the isolated product (4).

Several bacteria such as the genera *Agrobacterium*, *Pseudomonas*, *Rhizobium* and *Sarcina* are in conditions to produce cellulose as a bacterial polysaccharide (5). In particular, the gram-negative *Acetobacter xylinum* extracellularly secrete the synthesized cellulose as fibers. Bacterial cellulose is a homopolysaccharide consisting of linear β(1-4) glucose chains (Fig. 1).

Bacterial cellulose is synthesized in a process whereby the polymer material is extruded from the bacterial cells. Most cellulose-producing bacteria (e.g. *Acetobacter*) extrude cellulose as a ribbonlike product from a single fixed site on the cell surface. This results in the formation of a network of interlocking fibers. Fermentor design and the degree of aeration are important factors in optimizing yield. Bacterial cellulose is essentially a high-value biopolymer with specific applications and usage. The fibrils form a unique ribbon 3~8 nn thick and approximately 100 nm wide, which differs in morphology from other native celluloses (6). Due to its purity and unusual physico-chemical characteristics, a wide range of speciality applications of bacterial cellulose can be envisaged in the food, bio-industry and medical field (Table 1).

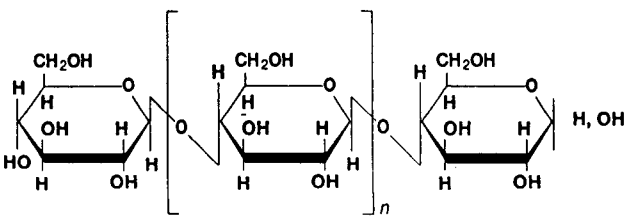

Fig. 1. The structure of bacterial cellulose.

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Table 1. Applications of bacterial cellulose

Material	Application
• Temporary artificial skin	Therapy of burns, ulcers, dental implants
• Sensitive diaphragms	Stereo headphones
• Bacterial cellulose	Immobilization of proteins Food additive Stabilizer of emulsions in cosmetics, food Coating compositions

Bacterial cellulose is produced under conditions of agitated fermentation. In particular, cellulose membrane is obtained from the static culture. Yield of cellulose is in general dependent upon the medium composition, environmental factors. High polymer production rates occur when the growth medium contains glucose, salts, corn steep liquor, iron chelators, and various productivity enhancers. Current yields are more than 0.2 g of cellulose per gram of glucose. Although the bacterial cellulose has high potential value, the current price of bacterial cellulose remains too high to make it commercially attractive. Indeed, bacterial cellulose production is a fairly inefficient process so far. Improved fermentation processes, based on an increased insight into the biochemical and genetic background of cellulose biosynthesis might enhance an economical process for bacterial cellulose production.

SYNTHESIS OF BACTERIAL CELLULOSE

Carbon metabolism in *A. xylinum*

Carbohydrate metabolism in *A. xylinum* involved in two main pathways: the pentose phosphate cycle for the oxidation of carbohydrates and the Krebs cycle for the oxidation of organic acids and related compounds (7). As shown in Fig. 2, hexose phosphate is regarded as a common intermediate in cellulose synthesis of *A. xylinum*, and it arises directly by phosphorylation of exogenous hexose. The hexose phosphate would be activated as UDP-hexose in the metabolic pathway of the bacteria. Cellulase synthease will play an important role in the production of cellulose fiber. The precursor for the polymerization reaction is uridine diphosphoglucose, and the organism can synthesize this molecule from a variety of carbon sources, ranging from different hexoses, pyruvate or the dicarboxylic acid intermediates of the

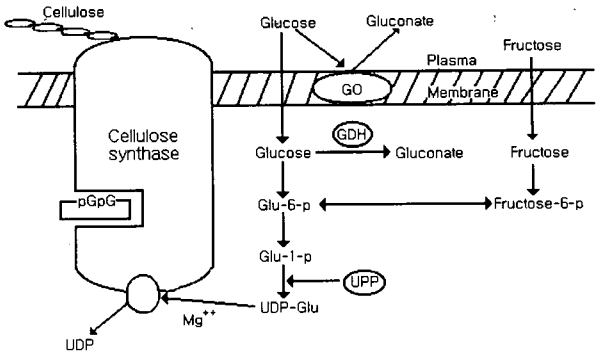

Fig. 2. Proposed biochemical pathway for cellulose synthesis in *Acetobacter xylinum*.
GO : glucose oxidase, GDH : glucose dehydrogenase
UPP : UDPG-pyrophosphorylase, pGpG : d-ci-GMP

citrate cycle (8). Although cellulose synthesis might be considered as a simple polymerization reaction from UDPG due to β-(1,4) linear linkage, cellulose biosynthesis is a complex process. Bacterial polysaccharide synthesis may be subject to complex regulatory circuits, e.g. alginate synthesis in *Pseudomonas aeruginosa* (9).

In general, the compound cyclic diguanylic acid (c-di-GMP) is a key element in the up-to-now best characterized regulatory system involved in the control of cellulose synthesis in *A. xylinum*. It functions as an allosteric activator of membrane-bound cellulose synthase (UDP-glucose ; 1,4-β-glucosyltransferase), binding at the different site from the catalytic or the substrate-binding site. In the absence of c-di-GMP, cellulose synthase is inactive (7). *In vitro*, the polymerization reaction is stimulated by a factor of 50~200 by the addition of c-di-GMP at submicromolar concentrations (10). Diguanylate cyclase catalyzes the synthesis of c-di-GMP from two molecules of GTP via the linear intermediate pppGpG. However, the degradation of positive regulator is carried out by two enzymes, phosphodiesterases A and B. PDE-A cleaves the c-di-GMP to from pGpG, which is then rapidly degrade to produce two molecules of 5′-GMP. Both enzymes are dependent on Mg^{2+} for their activity, and PDE-A is selectively inhibited by Ca^{2+} ions (7).

Assembly of the cellulose fibril

Bacterial cellulose membrane is a masterpiece of nature's arts. No other organism have the events of microfibril assembly observed so directly as in *A. xylinum*

(7). The surface of the cell envelope indicated the presence of some 50 to 80 porelike sites arranged in a regular low along the long axis of the cell (11,12) (Fig. 3).

The cellulose-binding agents such as the fluorescent brighteners Calcofluor White (Tinopal) and Congo red or the water-soluble, high-molecular-weight cellulose derivative CMC binds to β-glucan in a definable, reversible manner (13). A high level of assembly in which large bundles of crystalline microfibrils fascinate to form the final, twisted-ribbon product is prevented by the presence of CMC. The microfibrillar nature of bacterial cellulose produced by *A. xylinum* was modified by various chemical reagents in a culture medium. For example, nalidixic acid and chloramphenicol induced elongation of bacteria, resulting in the formation of wider cellulose ribbons or aggregates of ribbons (14). This may allow to produce bacterial cellulose with superior mechanical properties or other physical properties.

Surface or submerged fermentation for bacterial cellulose

A. xylinum in static culture produces a readily visible film of cellulose which covers the surface of the growth medium. Under suitable conditions, 50% of the supplied carbon substrate may be assimilated into a thick, leather-like white pellicle at the air-liquid interface of the culture. Generally, bacteria become entrapped in the pellicle. The process of cellulose formation by *Acetobacter* under static conditions is controlled by the supply of air from the medium surface and the yield depends only moderatly on the concentration of saccharides (15). When a broth culture is shaken or stirred. *A. xylinum* grows more rapidly as a result of the increased oxygen tension in the medium (8). Under these conditions, a well-organized pellicle is not formed but, rather, round balls of cellulose are typically observed. With the aim of enhancing the productivity, production of cellulose has been investigated in agitated conditions in which the product is obtained in the form of slurry or small fragments (15). Upon agitated culture, a highly branched, three-dimensional, reticulated structure may result, which is suitable for the production of high-quality paper (8). It is designated as acetan compared with cellulose. Interestingly, the acetan produced by *A. xylinum* is structurally related to the commercially important polymer, xanthan. Fig. 4 indicated the structure of the repeat units of acetan produced by *A. xylinum* and mutant strain.

Other researchers optimized a two-stage fermentation process, with cell growth in a first stage in agitated culture and cellulose formation in a second stage in static surface cultures (7). In agitated submerged cultures resulting in sufficient oxygen transfer, *A. xylinum* produced cellulose mass. Sometimes bacterial cellulose productivity dramatically drops (7). Aeration of cultures of most *A. xylinum* strains by shaking gives rise to spontaneous non-cellulose-producing mutants (16). Moreover, the spontaneous selection of cellulose negative variants also contributes to a reduced cellulose yield.

Although for the production process new bioreactor have been developed, static cultures are still preferred. A large surface area is important for a good productivity (15). In static culture, a normal cellulose pellicle with

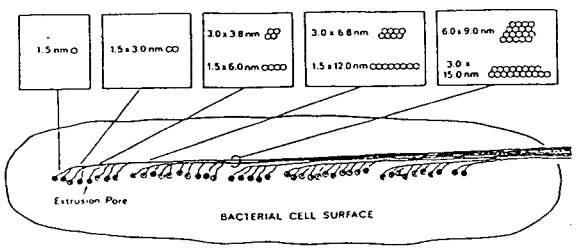

Fig. 3. Generalized model of ribbon assembly in *A. xylinum*, showing a possible mechanism of microfibrils on bacterial cell surface.

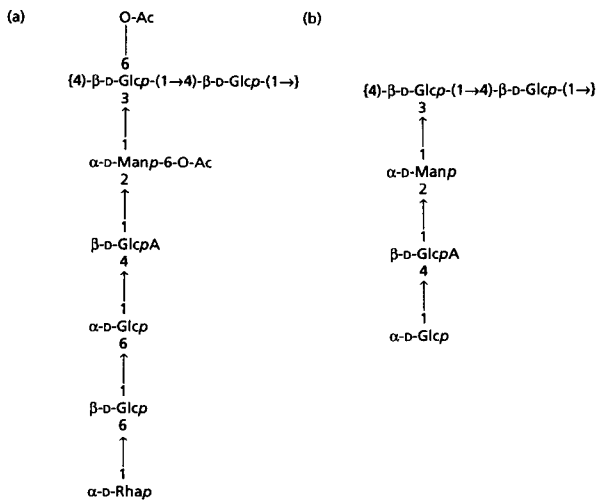

Fig. 4. Structure of the repear units of acetan (a) produced by *A. xylinum* and (b) P2 polysaccharide produced by the mutant strain of *A. xylinum*.
Glc, glucose; Man, mannose; GlcA, glucuronic acid; Rha, rhamnose; O-Ac, O-acetyl group; Pyr, pyruvate ketal.

a lamellar structure and less significant branching tends to be produced, which is ultimately usable as a bio-film. It has been suggested that the route to 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 (17). During the last decade, attention has been focused on the development of fermentation technology to overcome limitations in cellulose production under large-scale and submerged process conditions. Multiple adhesion sites in the culture vessels by supplying the medium with water-insoluble microparticles, ranging from diatomaceous earth, silica gel, sea sand, small glass beads enhanced cellulose production (7). Furthermore, specific fermentation media have now been developed which lead to increased cellulose levels, especially in combination with the use of cellulose overproducing mutant strains (7).

IMPROVED PRODUCTION OF BACTERIAL CELLULOSE

Medium composition

Bacterial cellulose production is generally performed with sucrose or glucose as a carbon source for growth and polysaccharide formation. However, many other carbon substrates such as mannitol, fructose, sucrose, arabitol, glycerol can also be applied for cellulose production. The medium composition, and more particularly the type of carbon source is important factor for bacterial cellulose production (Table 2).

With exception of two sugar alcohols, arabitol and mannitol, all sources are less active than glucose (5). Cellulose production was reported to be stimulated by addition of lactic acid, methionine, tea infusion and corn steep liquor. Relatively low glucose concentrations also gave better productivity and yields than higher ones. D-glucose as a carbon source is actively converted by membrane-bound *Acetobacter* dehydrogenases into ketogluconic acid. This feature not only lowers the overall cellulose yield, but also lowers the medium pH to suboptimal levels for cell viability and cellulose synthesis. A high initial glucose concentration does not increase the cellulose production because the excess glucose is converted into ketogluconic acids with a concomitant lowering of the pH (Fig. 2). In this respect, the use of a pH-controlled fermentation process is inevitable (7).

Table 2. Effect of carbon sources on the production of cellulose by *Acetobacter xylinum*

Carbon source	Cellulose yield (%)
Monosaccharides	
D-glucose	100
D-Fructose	92
D-Galactose	15
Disaccharides	
Lactose	16
Maltose	7
Sucrose	33
Alcohols	
Ethanol	4
Ethylene glycol	1
D-mannitol	380
D-Arabitol	620
Ogranic acids	
Citrate	20
succinate	12
L-Malate	15

Glucose was set as 100% yield.

In agitated culture, mixture of glucose and fructose increased cellulose production compared with addition of glucose or fructose. Carbon ratio (1 : 1) of glucose and sucrose produced cellulose (2.78 g/L).

In cellulose production using tea fungus, honey as a carbon source enhanced the cellulose production compared with other carbon sources such as glucose and sucrose (unpublished results).

pH

Acetobacter is able to convert glucose to gluconic acid and ketogluconic acids. The production of gluconic acid removes glucose from the medium at the expense of cellulose production. Also, the pH of culture affected the cellulose production.

It is generally accepted that the optimal pH range for cellulose production by *A. xylinum* is 4~7. Most authors used pH 5 or 6 in their research work. For the industrial production of Bio-fill® and Gengiflexl®, a pH between 4 and 4.5 gave better results, especially to avoid contaminations (5). In jar fermentor, the conversion of glucose to gluconic acid leads to a significant drop in pH of the medium in batch culture resulting in affecting cell growth and cellulose production. The glucose oxidase (GOD) in the cells showed higher activity at pH 4.0 and

is favorable for conversion of glucose to gluconic acid. It has been reported that NAD^+ independent glucose dehydrogenase (GDH) catalyzes direct oxidation of D-glucose to D-gluconate at the outer surface of the cytoplasmic membrane of acetic acid bacteria. The enzymes favor production of gluconate at acidic pH (18). Therefore, the pH shift from 4.0 to 5.5 by addition of NaOH resulted in the best yield, 5.95 g/L of cellulose (19). *Acetobacter* sp. LMG1518 using acetic acid as a co-substrate indicated that the initial medium pH 5.5 could be kept constant throughout the fermentation period, illustrating the beneficial buffering capacity, and a static surface culture gave rise to 28.4 g/L of cellulose (7).

In kombucha fermentation, tea fungus formed bacterial cellulose in below pH 4.0 (unpublished results). The optimal growth temperature for cellulose production is 25~30°C (5).

Nitrogen sources

The basic medium for cellulose production is developed by Hestrin and Schramm, which contains yeast extract and peptone, 0.5% each. CSL was the most effective. Methionine had an important effect on the cell growth and cellulose production. Fontana et al. used plant extract infusions, especially from black tea, as a stimulator for cellulose production. But bacterial cellulose produced in media containing tea infusion resulted in the interferences in the quality of the cellulose formed.

Antibiotics and biomaterial

Cellulose biosynthesis can be induced in different ways, for instance, by inhibition of protein synthesis. A spontaneous Cel^- mutant of *A. xylinumn* resulted in restoration of the original Cel^+ phenotype in the presence of tetracycline (12). *Z. ramigera* 115SL with rifampicin resistant indicated the better production of exopolysaccharide in the presence of rifampicin (unpublished results). Recently, it has been reported that the production of secondary metabolites was enhanced by the modification of ribosome by adding antibiotics. This ribosomal engieering is one of the efficient way to produce secondary metabolites. Therefore, *A. xylinum* resistant with certain antibiotics allowed to increase the cellulose production. Recent improvements of the cellulose productivity related to the addition of water-soluble chitosan to the Hestrin-Schramm medium and the addition of small amounts of endoglucanase to the production culture (20).

Kombucha/tea fungus

Kombucha, an acetic acid flavored fermented tea beverage, is made from sweetened tea by the a fungus fermentation (21). The symbiotic culture of *A. xylinum* and yeasts in tea fungus is grown traditionally on black tea with sucrose and gives a pleasantly sour and sparkling kombucha beverage under aerobic conditions (22). In particular, *A. xylinum* synthesizes a floating cellulose pellicle in which the cells are embedded. The film produced is essentially a microbial cellulose similar to the "mother of vinegar" that forms on the surface of wines or ciders. Kombucha beverages were prepared with various tea extracts and tea fungus starters. Kombucha fermentation was carried out under a static culture and cellulose production was dependent upon the type of tea fungus. Oriental tea fungus and cellulose-producing strain isolated have been used for the production cellulose in tea infusion and SH medium (23). Kombucha broth and cellulose pellicle were applied as starter for cellulose production. The addition of ethanol facilitate the cellulose production in static or agitate cultures (unpublished results). Various form of cellulose membrane was obtained from kombucha fermentation. The tubular form of cellulose pellicle was prepared from kombucha fermentation.

PRODUCTION OF NOVEL POLYSACCHARIDES

The bacterial cellulose produced by *A. xylinum*, the formation of microfibrils and gel layer is a masterpiece of nature's arts (24). The structure of bacterial cellulose could be modified using various chemicals in culture medium. For example, an *A. xylinum* adapted to a medium containing N-acetylglucosamine (GlcNAc) has been used to prepare a novel polysaccharide containing residual GlcNAc in cellulose. Several ammonium salts were also found to be effective for the incorporation of GlcNAc residues when the incubation system was converted to rotatory and aerobic incubation from static incubation (25). The novel polysaccharide was expected to be a

multifunctional polymer with both chitinous and cellulosic properties. The amounts of incorporated GlcNAc residues in the pellicle was achieved in SH medium containing D-glucose and the phosphorylated chitin in the ration of 3 : 1. It also had a higher orienting tendency and Young's modulus than those of bacterial cellulose (26). Fig. 5 showed the how the GlcNAc residue is transferred into the (1-4)β-D-glucan produced.

Recently Takai and his co-workers reported that the production of bacterial cellulose was increased by addition of CMC and the product had stronger mechanical strength (27). Novel cellulose-like polysaccharides bearing carboxymethyl groups have been synthesized by incubation of *A. xylinum* in mixed culture medium containing D-glucose and CMC. It indicates CMC is depolymerized into oligomeric CMC and is taken into the metabolic cycle of the bacteria. The different physicochemical properties and biological properties are expected from the hetero-polysaccharides. A further area for potential development lies in the applications for cellulose-synthetic copolymers (6).

Genetics in bacterial cellulose synthesis

The analysis of the genetics of cellulose biosynthesis in *A. xylinum* has become possible because of the isolation of Cel^- mutants, the development of gene transfer systems, and the development of an efficient system for cell-free synthesis of β-1,4-glucan (12). An operon encoding four proteins required for bacterial cellulose synthesis (*bcs*) in *A. xylinum* has been isolated by genetic complementation with Cel^- strain lacking cellulose synthase activity. The structure of the *bcs* operon (9217 bp) with *bcs*A, B, C and D, including flaking regions

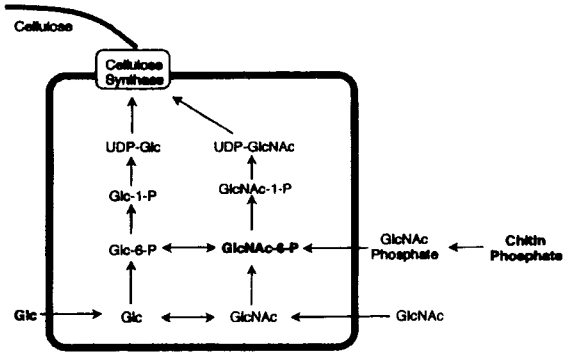

Fig. 5. Proposed flow of GlcNAc phosphate in the metabolic pathway of *A. xylinum*.

was reported. The *bcs*B gene product is the catalytic subunit of cellulose synthase (28). Results from genetic complementation tests and gene disruption analyses demonstrate that all four genes are essential for maximal cellulose synthesis. The *bcs* upstream region encodes a protein product required for cellulose biosynthesis in *A. xylinum*.

Genetic modification of bacterial cellulose

The functional properties of an exopolysaccharide are determined by its primary structure, which dictates the subsequent folding and association under various environmental conditions. In particular, cellulose membrane produced by *A. xylinum* also can be manipulated by the modification of primary structure. It is possible to produce novel polymers which are variants of the common structure by manipulating sugar unit/linkage/substituent (29). It is envisaged that novel polymers could be produced either by inactivation of a selected sugar-transferase gene or by heterologous expression of transferase genes from other bacterial systems (15).

A wild-type *Acetobacter pasteurianus* was subjected to chemical mutagenesis for the induction and isolation of a cellulose overproducing strain. A mutagenized strain produced double amounts of cellulose with the same physico-chemical properties compared to the wild type (30). By recombinant technology, the expression of sucrose synthase in *A. xylinum* not only changed sucrose metabolism but also enhanced cellulose production, in which UDP-glucose was efficiently formed from sucrose (31). (Keto) gluconate non-producing mutants derived from *A. xylinum* by UV-mutagenesis produced an amount of cellulose, double that of the wild-type. Mutant strain was able to convert efficiently glucose into cellulose, by limiting the conversion of D-glucose into the oxidative metabolites D-gluconic acids, 2-keto-gluconic acid, 5-ketogluconic acid and 2,5-keto-gluconic acid (7).

To date, eight different proteins have been established to participate directly in the biosynthetic pathway and its regulation. The cellulose synthease appears to be the rate-limiting enzyme. UDPG-pyrophoshorylase also appears to be a key-and possibly rate-determining enzyme in cellulose biogenesis (8). The expression of genes encoding these proteins will be enhanced by genetic engineering and ultimately allow to increase the cellulose

production. The metabolic engineering of *A. xylinum* is one of the powerful means to manipulate the structure and production of cellulose. Kamoun *et al* have reported the method for the direct disruption in *Xanthomonas campestris* (32). A gene encoding PHB polymerase in a gram-negative, *Z. ramigera* 115SLR was successfully disrupted by homologous recombination (33). However, genetic manipulation of *Acetobacter* strains is widely known to be impaired by the low frequencies of transformation. *A. xylinum* could not be transformed by either chemical-based protocols (34) or by conjugation using broad-host-range mobilizable vector (35). Recently, It reported that mutagenized strains of *Acetobacter* sp. that is more amenable to electrotransformation was essential prerequisite to the use of a suicide delivery vehicle for gene disruption experiments (2).

Many of the genes involved in acetan biosynthesis have been cloned and sequenced from the bacterial chromosome (29). Based on the identification and sequencing of *ace*P, a new gene encoding a glycosyl transferase involved in acetan biosynthesis, the disruption of the *ace*P gene in *Acetobacter* strain was performed and resulted in production of a novel polysaccharide with a pentasaccharide repeat unit. Fig. 6 showed the strategy for the construction of an *ace*P$^-$ strain of *A. xylinum*.

Therefore, with mutant strain of *A. xylinum* and characterization of genes involved in cellulose biosynthesis will allow us to manipulate the structure of cellulose for enhancing functional properties.

Current and potential applications

Bacterial cellulose offers a wide range of applications due to its high purity and special physico-chemical characteristics. Considerable progress has been made in the field of bacterial cellulose synthesis in the past few years. Bacterial cellulose is now available in limited quantities from Weyerhaeuser in the United States and Ajinomoto in Japan. The most prevalent application of bacterial cellulose exploit its very large surface area and its ability to absorb liquids. Consequently, very low concentrations of bacterial cellulose can be used to create excellent binding, thickening, and coating agents. Fig. 7 showed the Cellulon® engineered bacterial cellulose fiber produced by Weyerhaeuser Company.

Ionas et al. (5) reported applications in cases of skin transplants for both sides, donor and receptor. Cases of second and third degree burns, ulcers and decubitus could be treated successfully with Biofill® and Bioprocess ®. The product 'BioFill®' has a number of potential uses and is manufactured in the form of wound dressings for patients with burns, chronic skin ulcers or other extensive loss of tissue. The cellulose acts as a temporary skin substitute with high mechanical strength in the wet state. the high water capacity of the oxygen-permeable film appears to stimulate regrowth of skin tissue, while at the same time limiting infection. Recently, this biosynthetic cellulose was successfully applied in experiments with dogs to substitute the dura mater in the brain (5). Bacterial cellulose also forms the basis for high-quality acoustic-diapharagm membranes. The large-

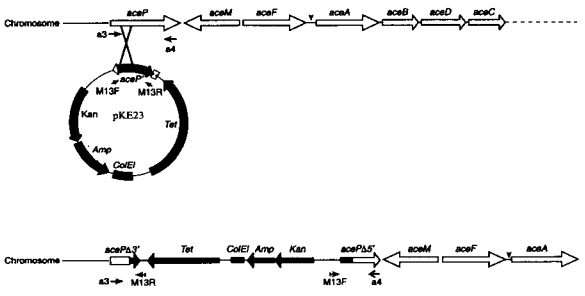

Fig. 6. Strategy for the construction of an *ace*P$^-$ strain of *A. xylinum*.

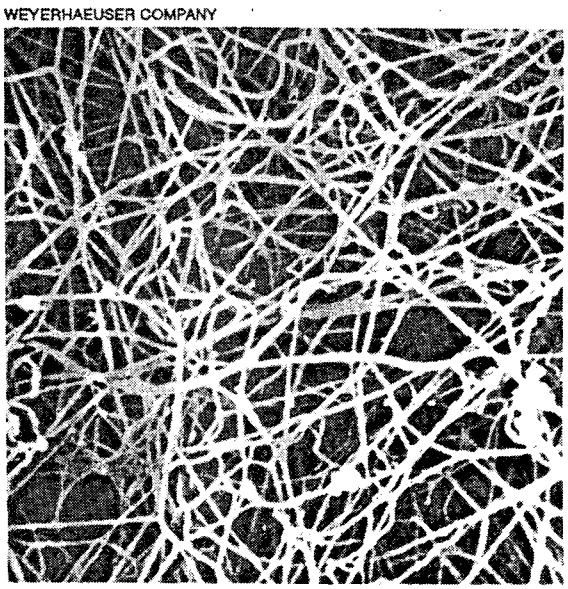

Fig. 7. Microstructure of Cellulon® engineered bacterial cellulose fiber.
The fibers have a typical diameter of 0.1 microns while some wood pulp fibers have a diameter of 25~35 microns.

scale production of bacterial cellulose by Weyerhaeuser Co. (Tacoma, Washington, USA) and Cetus Co. (Emeryville, California, USA) has led to the development of Cellulon®, a bulking agent with a broad spectrum of applications (7). Many different application of bacterial cellulose are possible and an open field for new applications.

CONCLUSIONS

A. xylinum have shown that the degree of polymerization in the cellulose varies within the cell cycle. Furthermore, its has been shown that a highly branched reticulated cellulose matrix can be produced under agitated culture conditions and this material seems to be suitable for production of high-quality paper. In static culture, the matrix structure is more lamellar and with less branching, and this type of material has been suggested for use in wound dressings. The culture condition may be used as one way of manipulating the qualitative properties of bacterial cellulose. The composition of medium enhanced the yield of bacterial cellulose as well as affected the structure with different physicochemical properties. The modification of bacterial cellulose using chitosan oligosaccharide, CMC resulted in the heteropolysaccharide with highly potential application as biomaterial in the future.

Recombinant DNA technology will also almost certainly be used in the future for process improvement in the industrial production of bacterial cellulose. The genetic and metabolic engineering of bacterial cellulose synthesis, as well as the construction of highly efficient *Acetobacter* (mutant or recombinant) strains, shall be necessary to arrive at an economic production process for bacterial cellulose.

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