学位論文内容の要旨

博士の専攻分野の名称　博士（工学）　　氏名　　田島　健次

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Studies on Bacterial Cellulose Composites

（バクテリアセルロースコンポジットに関する研究）

現在環境汚染を引き起こす廃棄合成高分子プラスチックの処理が、大きな社会問題になっている。また近年環境問題に対する関心が高まっており、生分解性高分子素材の開発が強く要望されている。

セルロースは自然界でもっとも豊富に存在する生分解性高分子で、主に高等植物によって合成されるが、ある種の藻類や微生物によっても合成される。この内、微生物によって合成されるセルロースはバクテリアセルロース（BC）と呼ばれ、特に酢酸菌によって合成されるBCは機械的強度が高く、しかも生分解性もあることから、新規の生分解性材料として注目されている。

本論文は酢酸菌 *Acetobacter xylinum*(*A. xylinum*)のBCを用いた高機能性生分解性材料の開発を目的とし、BCCの合成条件、構造、機械的強度、生分解性に関する詳細な検討を行ったものであり、6章から構成されている。

第1章は緒論であり、本研究の目的を明らかにした。

第2章では種々の水溶性高分子（WSP）を用いた添加培養によってBCCを合成したところ、BCC合成のためのWSPとしてセルロース誘導体が適していた。次に置換基のBCC合成への影響について検討したところ、置換度が小さいカルボキシメチルセルロース（CMC)、と置換基自体が小さいメチルセルロース（MC）がBCCをより形成しやすく、収量は最大で1.8倍にまで増加した。構造解析の結果から、MC、CMCは分子レベルで取り込まれていると考えられた。またBCC収量と含有量の比較から、BC生産自体が増強されていることを見出した。そして特にCMCによる増強効果が大きいことから、この機構について第2章-2で詳細に検討した。

CMCはある種の微生物におけるセルラーゼ誘導物質であることが報告されている。また近年種々の酢酸菌におけるセルラーゼの存在が報告されており、これらのBC生産における役割の解明は、収率の向上と物性のコントロールにおいて非常に重要であると考えられる。そこでCMCによるBC生産の増強に着目し、第2章-2ではCMCによるBC生産増強のメカニズムを解明することを目的とした。まずカビ由来のセルラーゼを用い、セルラーゼにBC生産の増強効果があること、およびBC生産における最適セルラーゼ濃度が存在することを確認した。次に酢酸菌ATCC23769のCMCase遺伝子断片をPCR法によって増幅し、CMCase遺伝子導入菌でCMCase活性の増加とBC生産の増強を確認した。また野生株のセルラーゼ活性が、CMCによって誘導・増強されることを見出した。酢酸菌におけるセルロースフィブリル形成は、①重合→②グルカン鎖の集合・サブエレメンタリーフィブリルの形成・結晶化→③フィブリルサブユニットの形成→④BCリボンの形成の4つの過程によって進行す

る。CMCはこの内の③→④の過程を阻害し、これによってBC生産速度が増加することから、③→④の過程はフィブリル形成における律速段階の一つであると考えられている。またCMCaseの添加によって細いBCリボンが形成されることから、CMCによるBC生産の増強は、CMCそれ自身ばかりでなく、CMCによって誘導されたセルラーゼによる③→④の過程の相加あるいは相乗的な阻害によると結論した。

第3章ではセルロースと構造が非常に類似しているキチン・キトサンに着目し、キチン・キトサン誘導体（WSChD)、および水溶性キトサンオリゴマー（WSChO）の添加培養によるBCCの合成を行った。収量・含有量の比較からWSChDはBCCを形成し、BC生産を増強することが解った。またこれらは分子レベルで含有されていることが確認された。一方、WSChOについてはBCCを形成しないにも関わらずBC生産を増強した。収量・グルコース消費・菌体増殖・pHの時間経過から、WSChOによるBC生産の増強は、定常期におけるBC生産性の増加に基づいていることが考えられた。

第4章ではより簡便にBCCを合成する方法として混合培養を試行した。

第4章-1では酢酸菌 *A. xylinum* NCI1005が合成する菌体外水溶性多糖のキャラクタリゼーションを行なった。GPCによる分子量測定、酸加水分解による構成糖の分析、NMRによる詳細な構造解析の結果、この水溶性多糖が分子量数万～数十万のレバンであることを見出した。第4章-2ではBC合成酢酸菌としてATCC10245とNCI1051、WSP合成酢酸菌としてNCI1005を用いて混合培養を行った。スクロースを基質とした混合培養によってBCCを合成することに成功した。またスクロースを基質としたBC合成酢酸菌の単独培養に比べBC生産が増強され、これはNCI1005のレバンスクラーゼによるスクロースの加水分解によって、BC合成酢酸菌の低いスクロース資化性が補われたためであると結論した。

第5章ではBCCの機械的強度と生分解性に関する検討を行った。機械的強度は膜の動的ヤング率を測定することによって評価した。ヤング率の測定結果から、MC、CMCが分子レベルでBC中に取り込まれることによって機械的強度が2～3倍に飛躍的に増加した。分解性についてはセルラーゼに対する分解性および、土壌中における分解性によって評価した。セルラーゼ受容能を有するCMCを含有させたBCCは分解速度は低下するが、通常のBCと同様にセルラーゼによってほとんど分解された。一方、セルラーゼ受容能を有さないMCを含有させたBCCは、セルラーゼによって20%程度しか分解されなかった。土壌中における分解試験ではBCC（MC)・BCC（CMC）ともに分解性がNBCに比べて若干減少するが、4週間でかなりの部分が分解された。つまりWSPを含有させることよって、BCの生分解性制御が可能であり、BCCにおけるヤング率の増加、および分解性の低下は、WSPがフィブリルサブユニット表面およびリボン間に含有されることによる膜内の水素結合の増加と緻密化に起因していると考えた。

第6章は総括であり、本研究によって高機能性・高強度を有し、生分解性制御可能な新規材料の合成が可能となったことを結論した。

Studies on Bacterial Cellulose Composites

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CONTENTS

CHAPTER I

General Introduction

Synthetic polymers have low weight and high mechanical strength, and they are one of the representative instances of technical renovations in 20th century. At present, a large quantity of synthetic polymers are synthesized from petroleum and consumed. In general, biodegradability is inversely proportional to strength, and the environmental disruption by waste synthetic polymers with low biodegradability have became one of the big social problems. Recently, peoples have became more and more concerned with the environmental problems, and from an environmental viewpoint, better biodegradable polymers to replace synthetic polymers widely are sought in the material industries. In order to establish methods by which both biodegradabilities and physical properties of polymer materials can be controlled, many researchers have promoted various investigations in the whole world. Polymer materials investigated as biodegradable polymers are divided into three categories, (1) polymer synthesized by microorganisms, (2) polymer from green plants, and (3) biodegradable chemical synthetic polymer.

(1) In polymer from microorganisms, poly-β-hydroxyalkanoates (PHA) have been investigated, widely. A wide range of microorganisms accumulated PHA as an intracellular energy reserve material when grown under conditions of nutrient limitation (Lemoigne, 1926, Merrick, 1978). Controlled microbial fermentation experiments have shown that >70 different hydroxyacids can be incorporated into these storage polymer granules, and PHAs provide a range of natural, renewable, biodegradable thermoplastics with a broad range of useful materials properties (Steinbüchel, 1991). (2) As to natural polymer from green plants, composites of starch and biodegradable synthetic polymers have been investigated, widely. The future problems in the biodegradable polymers using starch are developments of materials with wide range properties from hydrophilic to hydrophobic. (3) About chemical synthetic polymer, aliphatic polyesters occupy the attention of the whole world. Investigation of aliphatic polyesters in their applications had not been performed because of their low melting points (<60°C), but, recently, one with high melting point and mechanical strength was developed. There are huge data in their fundamental research, and further developments are expected.

Cellulose is the most abundant biodegradable polymer in nature, and the amount of production per year is estimated at sixty four billion ton. Cellulose is a polymer of β-linked D-glucose, which is synthesized by algae, bacteria, and fungi as well as green plants. The structure and physical property of cellulose differ depending on the origin, effecting on its reactivity. Cellulose is the principle constituent of cell walls in higher plants, forming the main structural element. There are four allomorphs in cellulose crystallites, I, II, III(I/II), IV(I/II) (Delmer, 1983), the crystal type of natural cellulose is I, mostly. Cellulose I converts to II, irreversibly, with

treatments of high concentration alkaline solution or high temperature. Recently, solid CP-MASS 13CNMR spectra of natural cellulose provides the existence of two allomorphs in cellulose I, Ia and Ib (Atalla, 1984), and cellulose Ia converts to Ib (Yamamoto, 1993), irreversibly, with annealing likely to cellulose I and II. The average degree of polymerization determined by viscosity measurement was 5,700 (Takai, 1975).

Cellulose produced by bacteria is called Bacterial Cellulose (BC) in general. In particular, BC produced by *Acetobacter xylinum* (*A. xylinum*) has excellent properties such as great mechanical strength and biodegradability in comparison with green plant cellulose or synthetic polymers, and it is noted as one of novel new biodegradable polymers.

Acetobacter strains are familiar bacterium, which have been used in brewage of vinegar. Some species of *Acetobacter* are able to synthesize cellulose as an extracellular polysaccharide (Brown, 1886), whereas other species are able to produce either water-soluble polysaccharides (Minakami, 1984, Tayama, 1985, Tayama, 1986) or both cellulose and water-soluble polysaccharides (Couso, 1982, Valla, 1981). *A. xylinum* is a gram-negative, aerobic bacterium that secrets cellulose fibrils as part of its normal metabolic activity. The substrate for cellulose synthesis is glucose, which is the major constituent of the medium in which *A. xylinum* is cultivated. *Acetobacter* species are obligate aerobes usually found on fruits and vegetables and in vinegar, fruit juice, and alcoholic beverages (Williams, 1989).

Pathway of carbon metabolism in *A. xylinum* was shown in Fig. I-1 (Ross, 1991). The presence of five enzyme steps in direct polymerization has been suggested (Ross, 1991); (1) active transport of glucose by glucose permease, (2) phosphorylation of glucose by glucokinase, (3) the transphosphorylation of glucose-6-phosphate (G-6-P) to glucose-1-P (G-1-P) by phosphoglucomutase, (4) the synthesis of uridine-5'-diphosphoglucose (UDPG) by pyrophosphorylase, and (5) the polymerization of UDPG by cellulose synthase. Besides the direct polymerization, there are three pathways in phosphorylation of glucose; pentose cycle, gluconeogenesis pathway, and Enter-Douboroff pathway. Cellulose polymerization is a membrane associated event, cellulose synthase is located in plasma membrane (Bureau, 1987). Cyclic-di-GMP (c-di-GMP; Fig. I-2) is referred to as an allosteric effector of the cellulose synthase, in the sense that it binds directly to the enzyme in a reversible manner at a regulatory site distinct from that of the catalytic, or substrate-binding sites (Fig. I-2, (Ross, 1987)). The c-di-GMP is essential for the high activity, by which cellulose synthesis is regulated. Recently, Koyama et al. (Koyama, 1997) suggested that *Acetobacter* synthesized and polymerizes cellulose chains with their reducing ends pointing. That is, cellulose biosynthesis occurs by addition of sugar residues onto the nonreducing ends of growing cellulose chains.

The polymerization of glucose is the first of many steps in cellulose biogenesis, most of which appear to take place in the space immediately exterior to the cell surface. The use of cellulose-binding agents which alter microfibril assembly at different phases has been the principal experimental methods for characterization of the fibrillar subunit and ribbon assembly process in *A. xylinum* . From the microscopic observations of fibrillar subunits and ribbons altered by

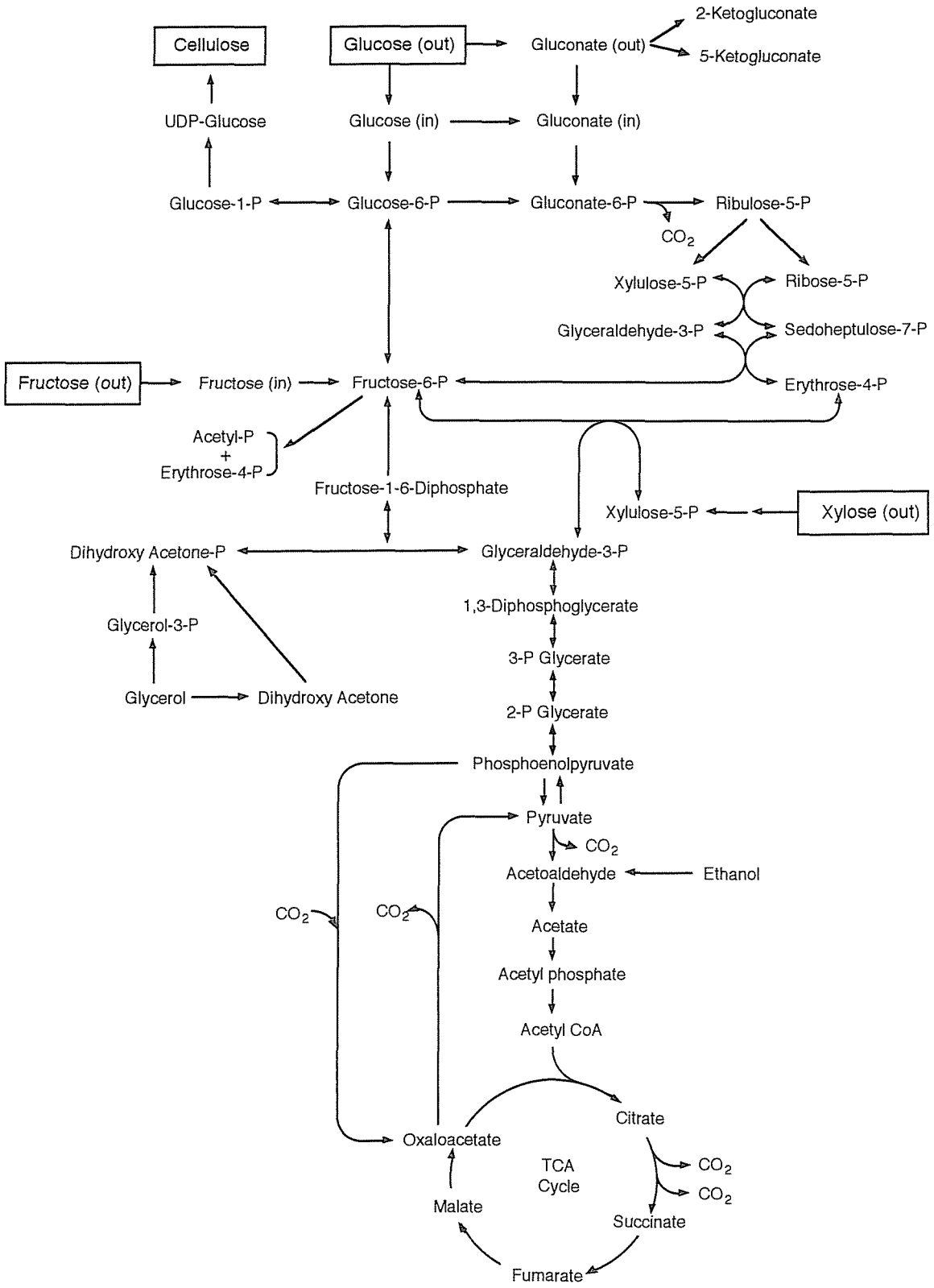


Fig. I-1 Pathways of carbon metabolism in *A. xylinum*.

such agents, Haigler et al. (Haigler, 1985) concluded that the hierarchical assembly process for BC synthesis contains four steps: (1) the synthesis and extrusion of tactoidal aggregates of parallel glucan chains; (2) the crystallization of adjacent glucan aggregates into 3-4 nm wide microfibrils; (3) the formation along slightly separated groups of extrusion pores of the fibrillar subunits that contain the crystallites; and (4) the fasciation of the subunits to form the composite ribbon (Fig.I-3).

When *A. xylinum* is cultivated statically in broth culture, a pellicle of cellulose is formed and cells of the bacterium are trapped within it, and it has been proposed that cellulose serves to hold the bacterium in an aerobic environment (Cook, 1980, Schramm, 1954). Williams et al. reported that cellulose pellicles may have multiple functions (UV protection, enhancement of colonization, and protection from competitors) in the growth and survival of the organism in nature. The hierarchically assembled ribbons form the fine network structure in the pellicle, and the air-dried one has high mechanical strength by hydrogen bondings among the narrow microfibrils and ribbons. As Hayashi et al. reported (Hayashi, 1995), BC was degraded by cellulases, easily, in comparison withe other crystallized celluloses. Although the reasons has not been cleared, it was thought that the high degradability of BC could be due to the helix structures of the ribbons constructed by orientated many microfibrils.

Thus, BC seems to be one of the favored material sources for biodegradable polymer with multifunctions based on its previously mentioned suitable characteristics. It also is known that BC can be modified easily during synthesis. For example, we have succeeded in preparing a new functional BC, which was incorporated with a water-soluble polymer (WSP) and called BC composite (BCC). This was done by the incubation of *A. xylinum* in a medium containing a WSP such as carboxymethyl cellulose (CMC), methyl cellulose (MC), and so forth (Takai, 1991). We found that these BCCs could be used as ultrafiltration membranes (Takai, 1991). As mentioned above, the cellulose assembly of *A. xylinum* was altered by the adsorption or incorporation of direct dyes or cellulose derivatives (CMC and others), and until now, many researchers (Benhayyim, 1965, Benziman, 1980, Haigler, 1980, Brown, 1982, Haigler, 1982a, Haigler, 1982b, Brown, 1983, Haigler, 1985b, Haigler, 1988) have used them for clarifying the process of cellulose assembly. Significant progress in explaining the mechanism and control of cellulose fibril biogenesis has been made by studying the normal and altered cellulose ribbon assembly in *A. xylinum*. However, the biosynthesis, structures, and biodegradabilities of these BCCs as functional materials have not been examined. In this thesis, we reported our studies on the synthesis, structure, mechanical strength, and biodegradability of BCC in detail.

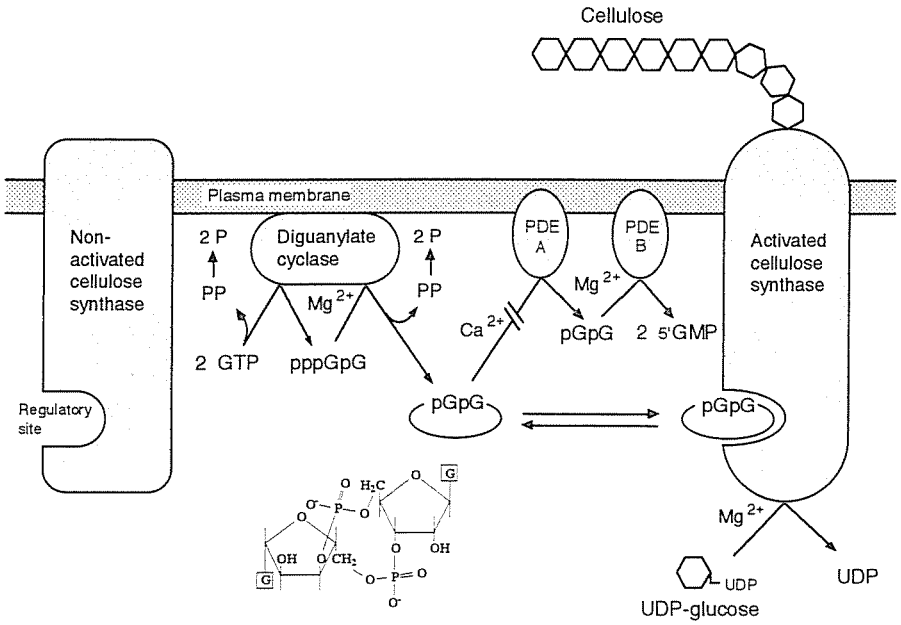


Fig I-2 Proposed model for regulation of cellulose synthesis in *A. xylinum* and structure of c-di-GMP.

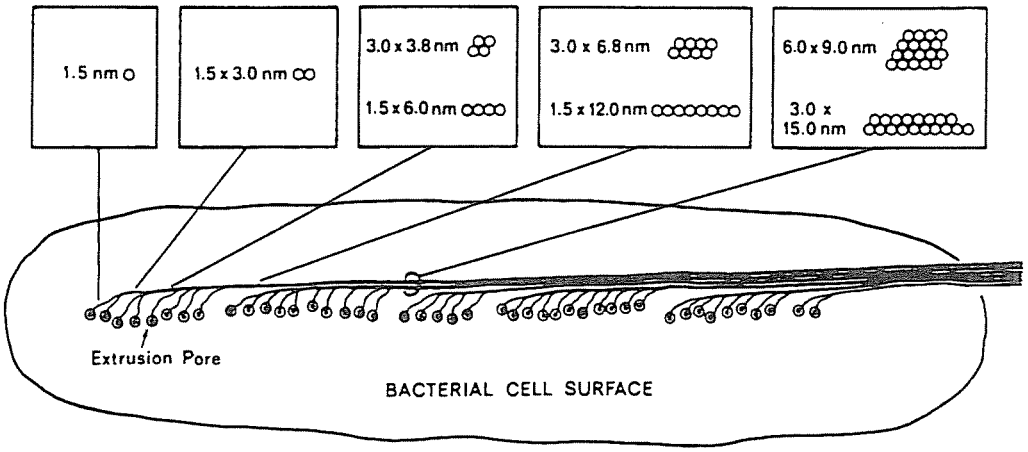


Fig I-3 Proposed model of cellulose ribbon assembly in *A. xylinum*.

CHAPTER II

Synthesis of Bacterial Cellulose Composites by Addition of Cellulose Derivatives

Introduction

In a liquid medium containing various carbon sourses, *Acetobacter xylinum* (*A. xylinum*) produces pure cellulose in pellicle form, which is called bacterial cellulose (BC). BC can be modified easily during synthesis by addition of reagents into a medium. For example, we have succeeded in preparing a new functional BC, which was incorporated with a cellulose derivative, such as carboxymethyl cellulose (CMC), methyl cellulose (MC), and so forth), and called BC composite (BCC) (Takai, 1991. We found that these BCCs could be used as ultrafiltration membranes (Takai, 1991, Tajima, 1995). However, the biosynthesis and the structures of these BCCs have not been examined. In ChapterII-1, we report our studies of the biosynthesis and the structures of BCCs.

From the comparion of the BCC yields and the BC contents, we found that CMC molecules enhanced BC production more effectively than MC. CMC is known to be one of cellulase inducers in fungi (Mandels, 1957, Pardo, 1996, Rao, 1988) and bacteria (Breuil, 1976, Godden, 1989). Recently, few endoglucanase and β-glucosidase type enzymes have been purified from some *A. xylinum*. (Standal, 1994, Oikawa, 1997, Tahara, 1997, Okamoto, 1994). Although their biological function in *A. xylinum* have not been known so far, in Rhizobium meliloti, it was reported that (Becker, 1993) the protein encoded by a gene (exoK) located on a 22 kb gene cluster involved in the biosynthesis of exopolysaccharide I displays homology to secreted endo-b-1,3-1,4-glucanases and that an exoK mutant strain produced reduced quantities of exopolysaccharide I. Tonouchi et al. reported (Tonouchi, 1995) that addition of a small amount of endoglucanase enhanced BC production by *A. xylinum*. These results suggest the possibility that endoglucanase plays important role in the cellulose production of *A. xylinum*.

In chapter II-2, the function of endoglucanase (CMCase) of *A. xylinum* on the BC production was reported, in detail.

CHAPTER II-1

Synthesis of Bacterial Cellulose Composites and Their Structures

Introduction

Until now, many researchers (Ben-hayyim, 1965, Benziman, 1980, Haigler, 1980, Brown, 1982, Haigler, 1982a, Haigler, 1982b, Brown, 1983, Haigler, 1985b, Haigler, 1988) have used cellulose-binding agents for clarifying the process of cellulose assembly. The cellulose assembly of *A. xylinum* was altered by the adsorption or incorporation of direct dyes or cellulose derivatives, and significant progress in explaining the mechanism and control of cellulose fibril biogenesis has been made by studying the normal and altered cellulose ribbon assembly in *A. xylinum*. However, the biosynthesis and the structures of these BCCs have not been examined. In Chapter II-1, we reported our studies of the biosynthesis and the structures of BCCs.

Materials and Methods

Materials

The carboxymethyl celluloses (sodium salts) (CMC) were the products of Kanto Chemical Co., Inc., Nacalai Tesque, Inc. and Wako Pure Chemical Industries Ltd. The methyl celluloses (MC) and polyethylene glycols (PEG) were the products of Nacalai Tesque, Inc. and Wako Pure Chemical Industries Ltd., respectively. The hydroxyethyl cellulose (HEC) and hydroxypropyl celluloses (HPC) were gifted from Daicel Chemical Industries, Ltd. Other WSPs, pullulan, starch, sodium alginate, and dextran, were purchased from Wako Pure Chemical Industries Ltd.

The molecular weights and degrees of substitution (DS) of the water-soluble polymers (WSP) used are summarized in Table II-1-1. Cellulase ONOZUKA R-10 was the product of Yakult Pharmaceutical Ind. Co., Ltd. The Glucose Test C II WAKO, which is a kit to determine the concentration of glucose, was purchased from Wako Pure Chemical Industries, Ltd.

Preparations of normal bacterial cellulose and bacterial cellulose composite

Two bacterial strains of *A. xylinum* NCI 1051 (Nakano Vinegar Central Institute, Nakano Vinegar Co. Ltd.) or ATCC 10245 were used for BC production. For the preparation of normal BC (NBC), 0.5 ml of a preculture of *A. xylinum* NCI 1051 or ATCC 10245 incubated at 28 °C for 72 h was inoculated into 15 ml of Hestrin and Schramm's medium (Hestrin, 1954) (HS; 2 % D-glucose, 0.5 % bactopeptone, 0.5 % yeast-extract, 0.115 % citric acid and, 0.27 % disodium hydrogen phosphate anhydride in distilled water, pH 6.0). For BCC, the same preculture was followed in inoculating into HS medium containing 0.5 (w/v) % WSP, MC, CMC, HEC, HPC, PEG, or other polysaccharides. The inoculated cultures were incubated in static at 28 °C for seven days. Membranes in greatly swollen conditions were taken from the surfaces of the cultures. They were soaked for seven days in 1 % NaOH solutions to remove all alkali-soluble components, and then washed with distilled water and 1 (w/v) % acetic acid to neutralize the last traces of alkali for three days. Finally, they were washed with large volumes of distilled water and air-dried at room temperature on glass plates or were lyophilized.

Determination of WSP content in BCC

The carbon percent (C %) of the BC and the WSPs were different; therefore, incorporation of WSP changes the C % of BC. That is, the C % of the BCC(MC)s are more than that of NBC. The calibration curves were obtained previously by the C % measurement of the mixture of cellulose and WSP mechanically mixed at a given mix ratio, and the weight percent of WSP was estimated from the C % of BCC determined by elemental analysis with calibration curves. The calibration curve for MC#8,000 is shown in Fig. II-1-1.

The contents of WSPs with the just or almost same C% as that of cellulose were estimated by HPLC analysis of their acidic or enzymatic hydolysates.

Table II-1-1 Molecular weight, DS[MS] and substituents of water-soluble polymers.

Additives	MW	DS(MS)	Substituent
MC#15	15,000	1.79-1.83	OCH3
MC#25	17,000	1.79-1.83	OCH3
MC#100	26,000	1.79-1.83	OCH3
MC#400	41,000	1.79-1.83	OCH3
MC#1500	63,000	1.79-1.83	OCH3
MC#4000	86,000	1.79-1.83	OCH3
MC#8000	110,000	1.79-1.83	OCH3
CMC(Nakalai)	13,500	0.62-0.68	OCH2COONa
CMC(Kanto)	50,000	0.48-0.60	OCH2COONa
CMC(Wako)	100,000-110,000	0.80	OCH2COONa
HEC	120,000	1.0[2.0]	OCH2CH2OH
HPC(SL)	30,000-40,000	-	OCH2CH2CH2OH
HPC(L)	55,000-70,000	[2.7]	OCH2CH2CH2OH
HPC(M)	110,000-150,000	[2.8]	OCH2CH2CH2OH
PEG200	200	-	-
PEG1000	1,000	-	-
PEG6000	6,000	-	-
PEG20000	20,000	-	-
PEG50000	50,000	-	-
PEG500000	500,000	-	-
Pullulan	50,000-100,000	-	-
Starch	-	-	-
Alginate 3	-	-	-
Alginate 2	-	-	-
Alginate 1	-	-	-
Dextran 3	200,000-300,000	-	-
Dextran 2	100,000-200,000	-	-
Dextran 1	60,000-90,000	-	-

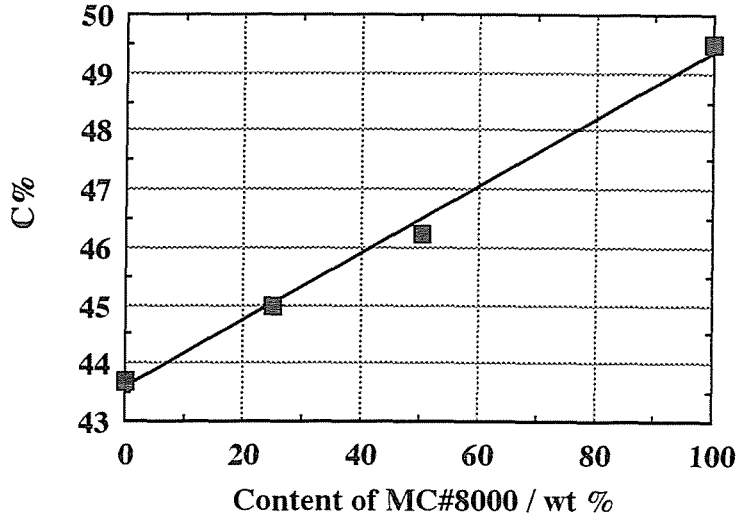


Fig. II-1-1 Calibration curve for determine of WSP contents; MC#8,000.

Measurement of culture absorbance, pH and glucose concentration

Culture pH was measured with a Horiba pH meter M8L. Glucose concentration in culture was determined with the Glucose Test C II WAKO (Wako Pure Chemical Industries Ltd.). After measurements of pH and glucose concentrations, cellulase (ONOZUKA R-10) solution was added to give a 1 mg/ml at a final concentration, and the culture was incubated at 50 °C and 120 strokes/min until cellulose pellicle was dissolved. After cellulose pellicle was degraded completely, the culture absorbance at 590 nm was measured with a spectrophotometer (Nippon InterMed K. K.; IMUMNO-MINI NJ-2300) to be used as a index of cell growth.

Infrared spectrum

Infrared (IR) data were used for confirming of the existence of WSP in BCC. IR spectra were obtained with a JASCO A-202 and Hershel FT-IR350 spectrophotometer by direct use of the film as a sample.

X-ray diffractions

X-ray diffraction patterns were obtained by the reflection method using nickel-filtered CuKa radiation from a Rigaku Denki automatic diffractometer operated in the ω–2θ scanning mode between 5 ° and 30 ° (2θ). The flat surface of the membrane was set parallel to the reflecting surface. Slit systems were 1 ° for divergence, 0.15 mm for receiving, and 1 ° for scatter.

X-ray diffractgrams of membranous samples were obtained in a flat-film camera with the beam perpendicular or parallel to the membrane surface. Nickel-filtered CuKα radiation from a High Power Rota Unit 3V system (Rigaku Denki Co., Ltd.) was used with a specimen-to-film distance of 40 mm.

Transparency of BCC

Transparency was calculated using the following equation:

Transparency = d / ABS(at 500 nm),

where d: thickness of membrane,

ABS: absorbance at 500 nm.

Absorbance of membrane at 500 nm was determined using a Hitachi U-2000A spectrophotometer (Hitachi, Co., Ltd.) by direct use of membrane as a sample.

Scanning electron microscope observation

The scanning electron microscope (SEM) observations were made after lyophilization or air-drying of the membranous samples. The surface views of BCCs, which were coated with vacuum-evaporated gold of a 200 Å thickness, were observed using a Hitachi SEM S-430. All photographs were taken at a 15 kV accelerating voltage.

Results and Discussion

Yield and WSP content

The yields of BCC(MC)s, BCC(CMC)s, and BCC(HEC)s were 1.3-1.7 times those of NBC(Control), whereas BCC(HPC)s, BCC(PEG)s, and BCC(other polysaccharide)s were almost the same as those of NBC (Table II-1-2). For MC and CMC, no effects of the molecular weights on the BCC yields were observed.

The WSP contents in the BCCs were estimated from the elemental analysis. BCC(MC)s, BCC(CMC)s, and BCC(HEC)s were estimated to contain 11-23 wt % WSP (Table II-1-2). In contrast, BCC(HPC)s, BCC(PEG)s, and BCC(other poly saccharides)s contained only slight amount of WSP (Table II-1-2).

The weights of cellulose in the BCCs were almost equal to or greater than those in NBC. Ben-Hayyim and Ohad (Ben-hayyim, 1965) reported that CMC increases the rate of polymerization of glucose in vivo. Haigler also reported the same result. The hierarchical assembly process for BC synthesis contains four steps: (Haigler, 1985b) (1) the synthesis and extrusion of tactoidal aggregates of parallel glucan chains; (2) the crystallization of adjacent glucan aggregates into 3-4 nm wide microfibrils; (3) the formation along slightly separated groups of extrusion pores of the fibrillar subunits that contain the crystallites; and (4) the fasciation of the subunits to form the composite ribbon. For explanation of this CMC inducing effect, Haigler and others reported that CMC prevents the fasciation of the larger fibrillar subunits (Haigler, 1985b) and that the ribbon assembly is a slightly rate limiting step (Haigler, 1982a, Brown, 1983). Thus, it was considered that the prevention of the ribbon assembly by the adsorption of MC or CMC molecules on the fibrillar subunits could be one of the causes in the increase of the BC productivity.

The yields in the medium containing CMC and MC with the same molecular weights at given ratios were suggested in Fig. II-1-2. As the ratio of CMC increased, the yield increased, and this suggests that CMC enhances BC production more effectively than MC. CMC is known to be one of cellulase inducers in fungi (Mandels, 1957, Pardo, 1996, Rao, 1988) and bacteria (Breuil, 1976, Godden, 1989). Recently, few endoglucanase and β-glucosidase type enzymes have been purified from some *A. xylinum.* (Standal, 1994, Oikawa, 1997, Tahara, 1997, Okamoto, 1994). Although their biological function in *A. xylinum* have not been known so far, in *Rhizobium meliloti*, it was reported that (Becker, 1993) the protein encoded by a gene (exoK) located on a 22 kb gene cluster involved in the biosynthesis of exopolysaccharide I displays homology to secreted endo-β-1,3-1,4-glucanases and that an exoK mutant strain produced reduced quantities of exopolysaccharide I. Tonouchi et al. reported (Tonouchi, 1995) that addition of a small amount of endoglucanase from *Bacillus subtilis* enhanced BC production by *A. xylinum.* These results suggest the possibility that endoglucanase plays important role in the cellulose production of *A. xylinum.*

The function of endoglucanase (CMCase) of *A. xylinum* on the BC production was reported in chapter II-2, in detail.

Table II-1-2 Yields of BCCs and contents of WSPs.

Sample	Yields(Relative Yields)	Contents
NBC(Control)	33.2(1.00)	-
BCC(MC#15)	50.2(1.51)	15.9
BCC(MC#25)	51.0(1.54)	22.5
BCC(MC#100)	50.7(1.53)	11.0
BCC(MC#400)	52.3(1.58)	15.8
BCC(MC#1500)	50.3(1.52)	21.5
BCC(MC#4000)	50.7(1.53)	19.5
BCC(MC#8000)	44.4(1.34)	21.3
NBC(Control)	32.3(1.00)	-
BCC(CMCKanto)	52.9(1.64)	14.6
BCC(CMCNakalai)	55.5(1.72)	18.5
BCC(CMCWako)	53.7(1.66)	15.8
NBC(Control)	35.0(1.00)	-
BCC(HEC)	54.4(1.55)	11.5
NBC(Control)	35.0(1.00)	-
HPC(SL)	39.4(1.13)	2.42
HPC(L)	37.3(1.07)	1.14
HPC(M)	34.4(0.98)	3.93
NBC(Control)	30.5(1.00)	-
BCC(PEG200)	34.5(1.13)	3.62
BCC(PEG1000)	31.3(1.03)	-
BCC(PEG6000)	31.0(1.03)	-
BCC(PEG20000)	29.4(0.96)	-
BCC(PEG50000)	33.8(1.11)	-
BCC(PEG500000)	29.9(0.98)	1.34
NBC(Control)	31.5(1.00)	-
BCC(Starch)	30.7(0.97)	-
BCC(Pululan)	33.7(1.07)	-
BCC(Alginate3)	31.2(0.99)	-
BCC(Alginate2)	31.1(0.99)	-
BCC(Alginate1)	31.2(0.99)	-
BCC(Dextran3)	32.8(1.04)	-
BCC(Dextran2)	33.3(1.06)	-
BCC(Dextran1)	33.8(1.07)	-

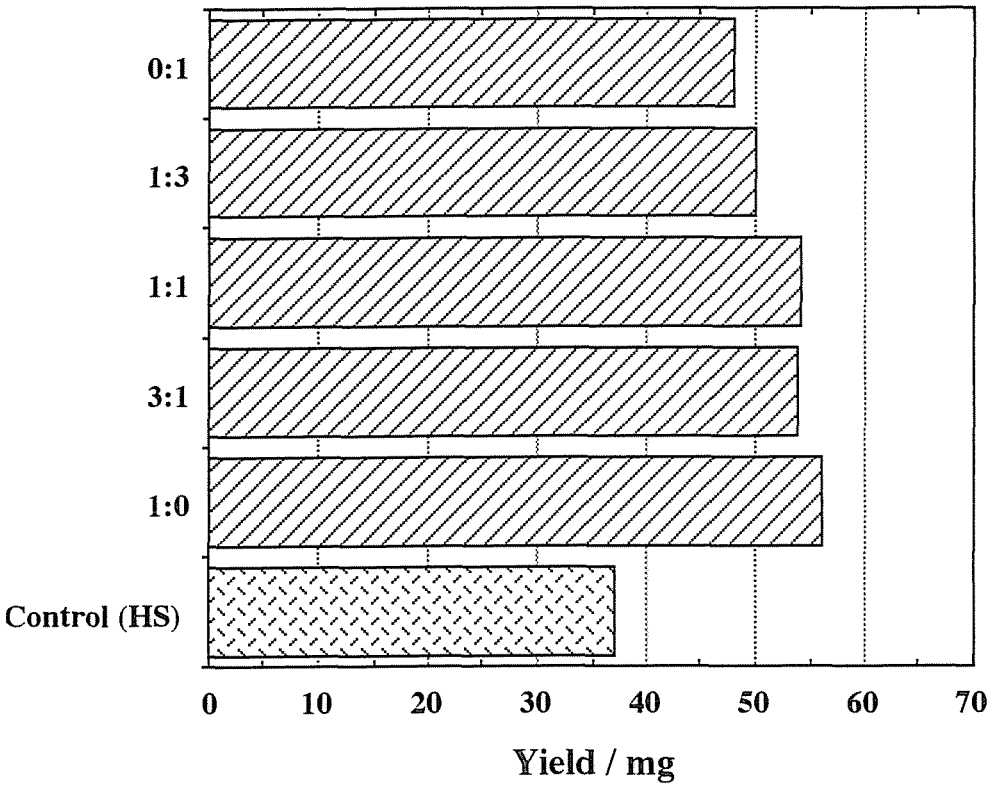


Fig. II-1-2 Yields of BCCs in HS medium containing CMC(Kanto) and MC#1500 at various ratios.
Bacterial strains was ATCC 10245.

Effect of glycosidic linkage, molecular weight and substituent of a water-soluble polymer on BCC synthesis

The a glycosidic-linked polysaccharides, pullulan, starch, alginate, and dextran were scarcely incorporated irrespective of their molecular weights, while the b glycosidic linked polysaccharides, MC, CMC, and, HEC were incorporated (Table II-1-2). These results could suggest that the incorporation of a polymer related to the configuration of the glycosidic linkage. Since cellulose is a β(1→4) glycosidic linked polysaccharide, β(1→4) glycosidic linked polysaccharides may be more favorable than a glycosidic-linked ones in formation of hydrogen bonding with cellulose.

The order in the contents of the cellulose derivatives in the BCCs was CMC, MC>HEC>HPC, and their contents were decreased with the increase of the bulkiness in the substituent. The CMCs have the smaller DS than other cellulose derivatives. The hydrogen bondings among β(1→4) glucan chains occur between OH at C6 and at C3', and the CMCs with low degree of substitution could make stronger hydrogen bonding with cellulose. Whereas, a part of OH groups at C3 carbons in the HECs and HPCs were substituted by OCH2CH2OH and OCH2CH2CH2OH, respectively, and this resulted in the prevention of hydrogen bondings between these WSPs and cellulose.

PEG was scarcely incorporated, and the content of PEG was 1.34-3.62 wt% (Table II-1-2). The small content of PEG might be derived from the weak interaction between cellulose and PEG, because the structure of PEG is not suitable for forming strong hydrogen bonds with the cellulose molecule.

Thus the incorporation of WSP is related to the DS, the position of the substitution, the bulkiness in the substituent, and the configuration of the glycosidic linkage.

Mechanism of BCC synthesis

The difference in the production rate between NBC and BCC(CMC) at the middle stage (2-5 days) was larger than those at the other stages (1-2 and 5-7 days) (Fig. II-1-3). While the timecourse change in the production rate of BCC(PEG) was similar with that in NBC throughout the incubation time, and this suggested that PEG had no effect on the cellulose synthesis. The time course changes in the glucose concentrations of the all cultures were similar (Fig. II-1-4). Glucose in the cultures was completely consumed in five days. The pHs in the all cultures rapidly decreased with the incubation time (Fig. II-1-5). The pHs in the HS and the HS medium with PEG (HS(PEG)) was steady (pH 3.3) after three days, while the pH in the HS medium with CMC (HS(CMC)) slightly increased after three days. Because *A. xylinum* has the ability to synthesize cellulose from gluconic acid and cellulose was synthesized even after the complete consumption of glucose in the culture, and we suspected that the pH increase in the culture of HS(CMC) was due to the utilization of acidic substances formed in metabolic pathways. The cell growth rate and cell number in the HS(CMC) medium were slightly lower than those in HS and HS(PEG) medium (Fig. II-1-6). The decrease in cell growth and cell number could be due to

the low diffusion velocity of oxygen derived from the high viscosity of CMC solution. The results of yield and cell number suggest that BC productivity of a cell in the HS(CMC) medium was greater than that in HS medium.

The content of CMC in BCC(CMC) at the first stage was highest, and it decreased rapidly with the incubation time (Fig. II-1-7). After middle stage, the value was constant. Because cellulose is synthesized at the upper region in membrane, CMC molecules have to diffuse from culture to the upper region in membrane to make hydrogen bonding with cellulose. In addition, BCC had more minute structure than that of NBC. Thus, the decrease in the CMC content with the incubation time could be due to the low diffusion velocity of CMC molecules in BCC(CMC).

From these results, the mechanism of BCC(CMC) synthesis was suspected as follows. (1) Bacteria grow. Glucose is metabolized and pH in culture decreases rapidly. (2) Cellulose is synthesized with growth of bacteria, then CMC molecules adsorb onto fibrillar subunits and ribbons. Rate of cellulose synthesis of a cell in HS medium with CMC is faster than that in HS culture, which is derived from the prevention of the ribbon assembly by the adsorption of CMC molecules on the fibrillar subunits. (3) Cellulose synthesis gradually decreases with suppression of bacterial growth derived from consumption of nutrient and accumulation of metabolic substances.

Structural analysis of BCC by IR spectroscopy and X-ray diffractometry

The IR spectra of NBC and BCC are shown in Fig. II-1-8. The IR spectra of BCC(MC) and BCC(PEG) were very similar to that of NBC, except a shoulder peak which could be derived from the β(1, 4) glycosidic bond was observed at 950 cm-1 in BCC(MC). On the other hand, in the IR spectra of BCC(CMC), the peak derived from the carbonyl group was observed at 1,730 cm-1. The existence of the two crystalline allomorphs (Iα and Iβ) in cellulose I was first deduced by VanderHart and Atalla (Atalla, 1984, VanderHart, 1984) from high-resolution CP/MAS 13C NMR experiments, and this was confirmed by diffraction data (Sugiyama, 1990). Sugiyama et al. (Sugiyama, 1991) reported that absorption bands near 3240 and 750 cm-1 were assigned to the Iα phase whereas bands near 3270 and 710 cm-1 corresponded to the Iβ phase. FT-IR spectra of NBC and BCCs in the range 800-650 cm-1 are shown in Fig. II-1-9. The ratio of the absorption band at 750 cm-1 corresponding to Iα phase and that at 710 cm-1 corresponded to the Iβ phase in BCC(CMC) was lower than that in NBC. Recently, Yamamoto et al. (Yamamoto, 1994) reported that the fraction of Iα was decreased by the addition of CMC. Considering morphological and crystallographic alteration of BC, they concluded that the cause of the decrease in the level of Iα is associated with difference in crystallization induced in fibrillar units with different sizes. Our result agreed with that of them, the ratio of Iα phase decreased with the increase of CMC concentrations (0-1.0 wt%). Thus, these results suggest that CMC affected on the crystal structure of BC.

The X-ray diffractograms of air-dried NBC and BCCs are shown in Fig. II-1-10. Three peaks, corresponding to $(1\bar{1}0)$, (110) and (020), were observed in X-ray diffractograms of air-

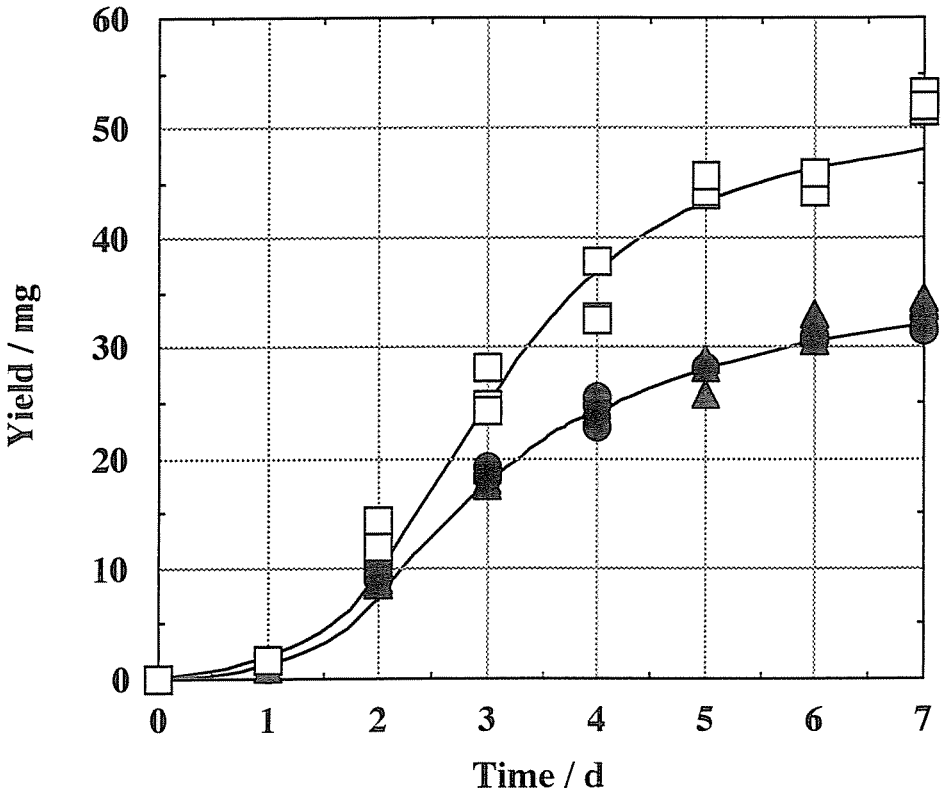


Fig. II-1-3 Time course changes of BCC yields;
▲ ; HS; ● ; HS(PEG); □ ; HS(CMC).

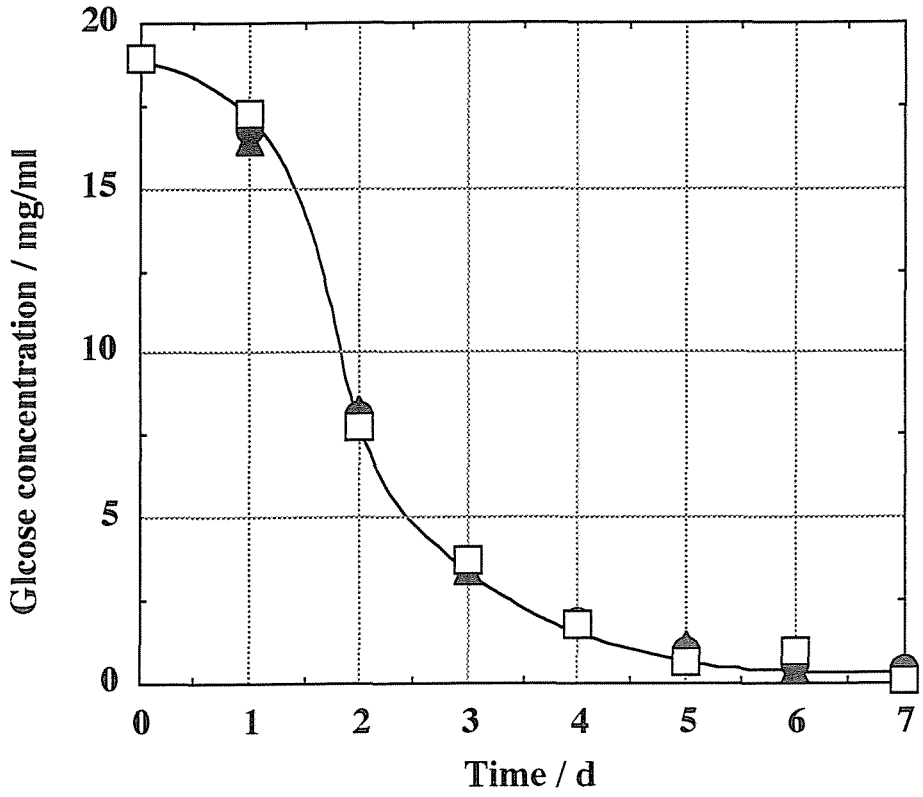


Fig. II-1-4 Time course changes of glucose concentrations;
▲; HS; ●; HS(PEG); □; HS(CMC).

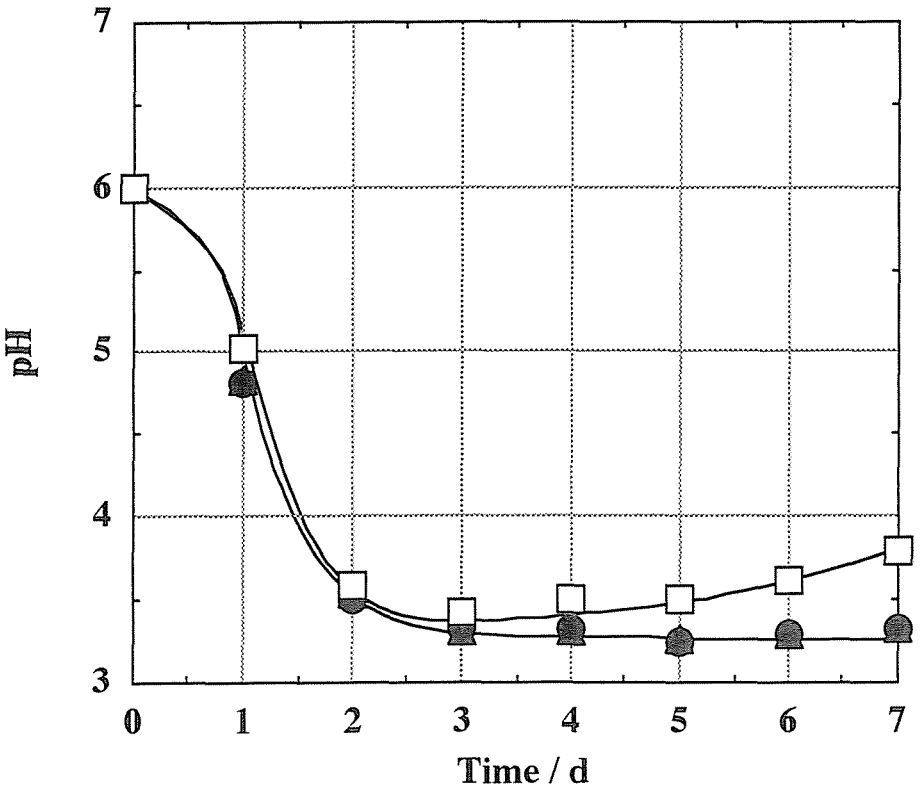


Fig. II-1-5 Time course changes of pH;
▲; HS; ●; HS(PEG); □; HS(CMC).

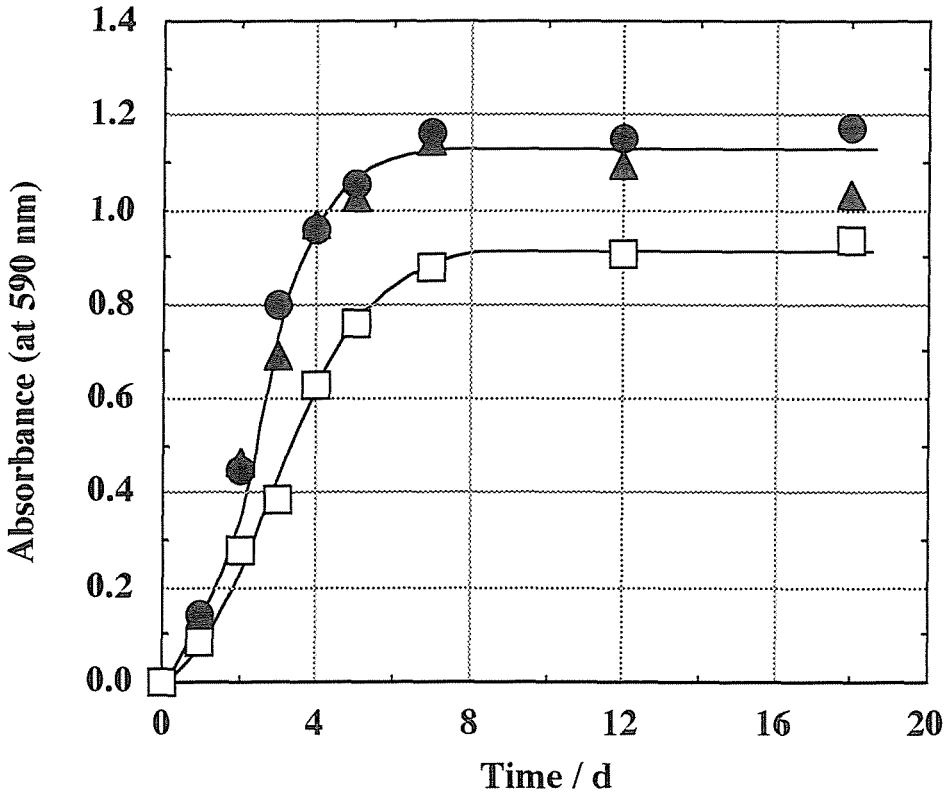


Fig. II-1-6 Time course changes of culture absorbances;
▲; HS; ● ; HS(PEG); □ ; HS(CMC).

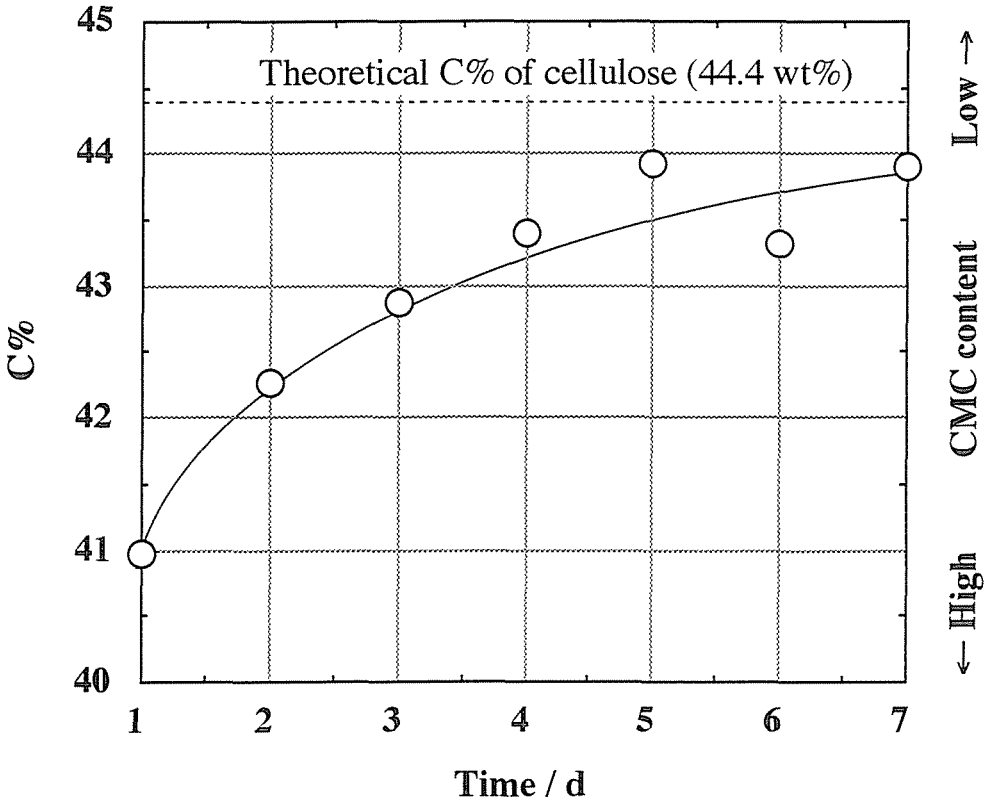


Fig. II-1-7 Time course change in carbon percent (C%) of BCC (CMC) .

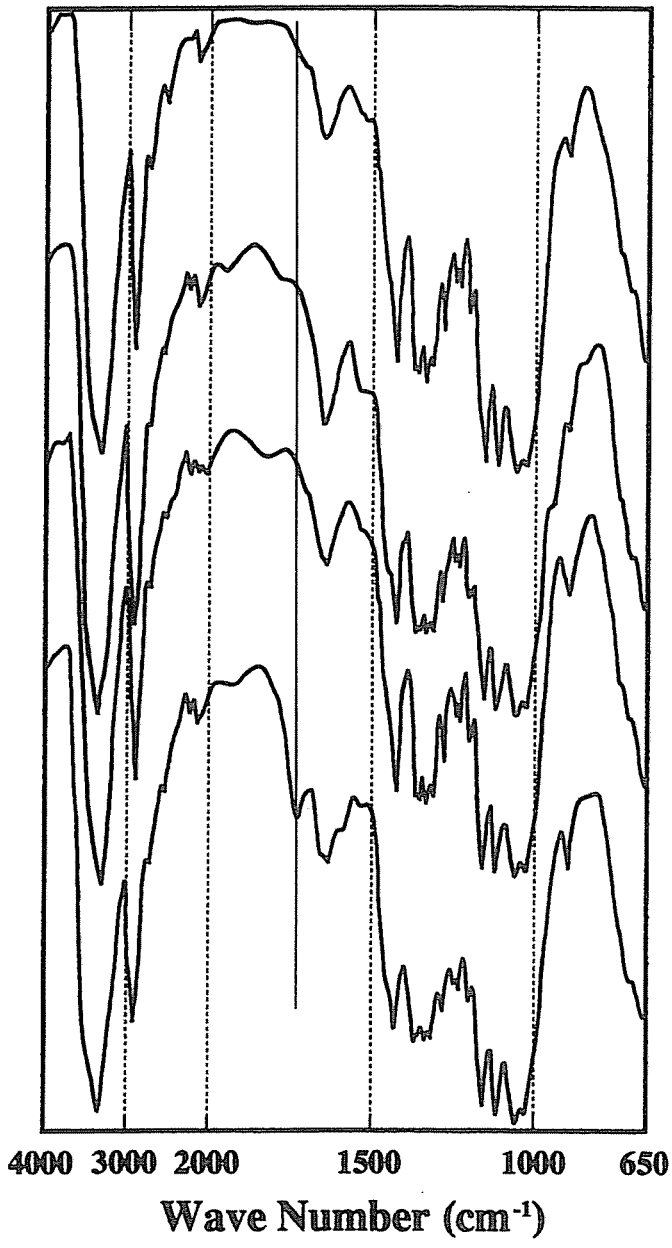


Fig. II-1-8 IR spectra of air-dried NBC and BCC(10245)s; 1: NBC, 2: BCC(MC#400), 3: BCC(PEG50,000), 4: BCC(CMC(Kanto)).

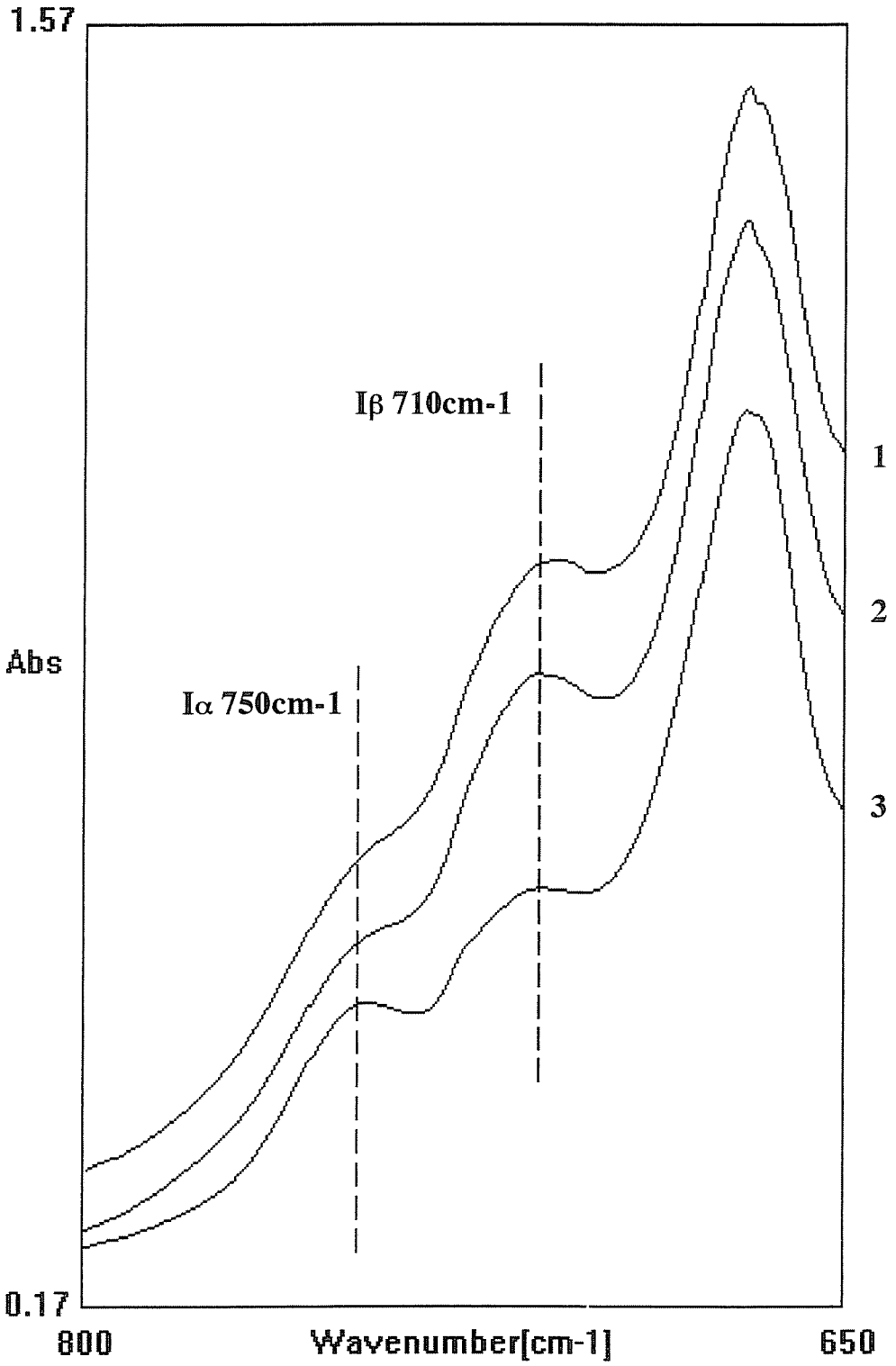


Fig. II-1-9 Details of the FT-IR spectra in the region 800-650 cm-1 of 1; BCC(MC#1500), 2; BCC(CMC(Nakalai)), and 3; NBC(10245).

dried NBC and BCCs. Generally, air-dried BC provides high uniplanar orientation (Takai, 1975), which is indicated by the ratio of the $(1\bar{1}0)$ peak intensity to the (020) peak intensity. The index ($(1\bar{1}0)/(020)$) for preferential orientation of air-dried and lyophilized NBCs and BCCs are shown in Table II-1-3. The index of BCC(MC), BCC(CMC), and BCC(HEC) were smaller than that of NBC. The index of air-dried NBCs were larger than that of lyophilized ones, while those of air-dried and lyophilized BCC(MC) and BCC(CMC) with high WSP content were almost same each other. These results could suggest that the preferential orientation of $(1\bar{1}0)$ planes increased during drying process, and that CMC molecules prevented the preferential orientation of $(1\bar{1}0)$ planes in ribbons and the increase of preferential orientationality during drying process. Their values and the differences in the index ($(1\bar{1}0)/(020)$) of air-dried and lyophilized samples decreased in direct proportion to their WSP contents, supporting the opinion mentioned above. The half width value of the peak corresponding to $(1\bar{1}0)$ in BCC(MC), BCC(CMC), and BCC(HEC) was lager than that in NBC, and this suggests that crystal size in the $(1\bar{1}0)$ plane direction of them was smaller than that of NBC.

In contrast with BCC(MC), BCC(CMC), and BCC(HEC), the index ($(1\bar{1}0)/(020)$) for preferential orientation of BCC(HPC)s, BCC(PEG)s, and BCC(other polysaccharides)s (data are not shown) were similar with those of NBCs, suggesting that HPC, PEG, and other polysaccharides used in the experiment had no effect in crystal structure of BC.

In the case of NBC membrane dried with a flat of the membrane parallel to a glass plate, it was recognized that the membrane provided a typical selective uniplanar orientation (Takai, 1975). The X-ray photographs of NBC and BCC(CMC) dried with a flat surface of the membrane parallel to a glass plate are shown in Figs. II-1-11, 12, respectively. In the perpendicular patterns of NBC and BCC(CMC), the random orientated patterns were obtained with line $(1\bar{1}0)$ missing and (110) present. In the parallel pattern of NBC, line $(1\bar{1}0)$ was present as a intensive arc on the equator, and line (110) was present as a weak arc on the meridian. While, in the parallel pattern of BCC(CMC), line $(1\bar{1}0)$ was present as a weak intensive arc on the equator, and the random orientated patterns of (110) was obtained. These results suggest that the degree of the selective uniplanar orientation of $(1\bar{1}0)$ plane in the BCC(CMC) membrane was lower than that in the NBC membrane.

Transparency of BCC

The transparency of BCC(CMC) was quite greater than that of NBC, while that of BCC(PEG) was almost the same as that of NBC (Table II-1-4). Since transparency reflects homogeneity of membranes, these results suggest that BCC(CMC) had the more homogeneous structure than that of NBC. This was supported by the results of SEM observation (Fig. II-1-13). Many pores were observed on the air-dried NBC, while a few pores were observed on the air-dried BCC(CMC). Therefore, CMC molecules were incorporated among ribbons as well as fibrillar subunits, thereby, pores were decreased and membrane was made homogeneous.

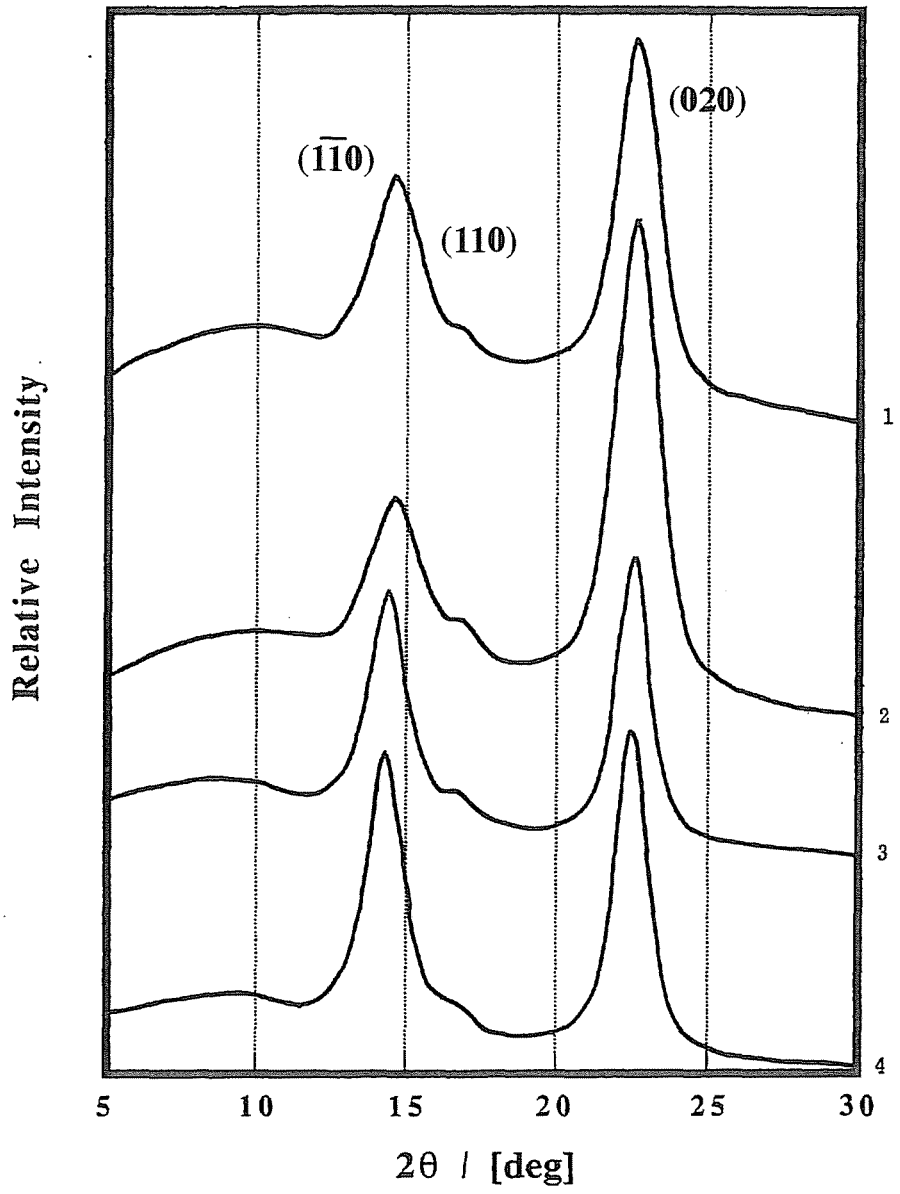


Fig. II-1-10 X-ray diffractgrams of air-dried NBC and BCC(10245)s;
1: BCC(MC#400), 2: BCC(CMC(Kanto)),
3: BCC(PEG 50,000), 4: NBC.

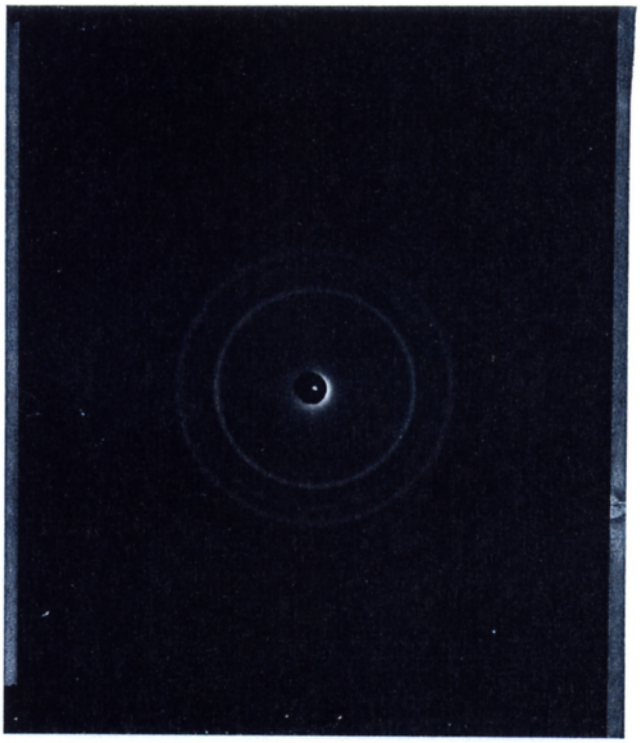
Table II-1-3 Peak intensity ratio of $(1\bar{1}0)/(020)$ in X-ray patterns.

Sample	$(1\bar{1}0)$ / (020)*)	
	air-drying	freeze-drying
NBC	0.811	0.596
BCC(PEG200)	0.803	0.542
BCC(PEG1000)	0.831	0.533
BCC(PEG6000)	0.837	0.555
BCC(PEG20,000)	0.846	0.540
BCC(PEG50,000)	0.840	0.567
BCC(PEG500,000)	0.886	0.576
NBC	0.899	0.658
BCC(MC#15)	0.397	0.390
BCC(MC#25)	0.394	0.397
BCC(MC#100)	0.450	0.431
BCC(MC#400)	0.437	0.417
BCC(MC#1500)	0.376	0.344
BCC(MC#4000)	0.456	0.426
BCC(MC#8000)	0.473	0.479
BCC(CMCNakalai)	0.365	0.307
BCC(CMCKanto)	0.349	0.305
BCC(CMCWako)	0.353	0.359
NBC	0.915	0.676
BCC(HEC)	0.521	0.435
BCC(HPC(SL))	0.872	0.628
BCC(HPC(L))	0.779	0.611
BCC(HPC(M))	0.732	0.620

Strain; *Acetobacter xylinum* ATCC 10245

*) Peak intensity ratio of $(1\bar{1}0)$ to (020) in X-ray patterns.

perpendicular to the membrane surface



parallel to the membrane surface

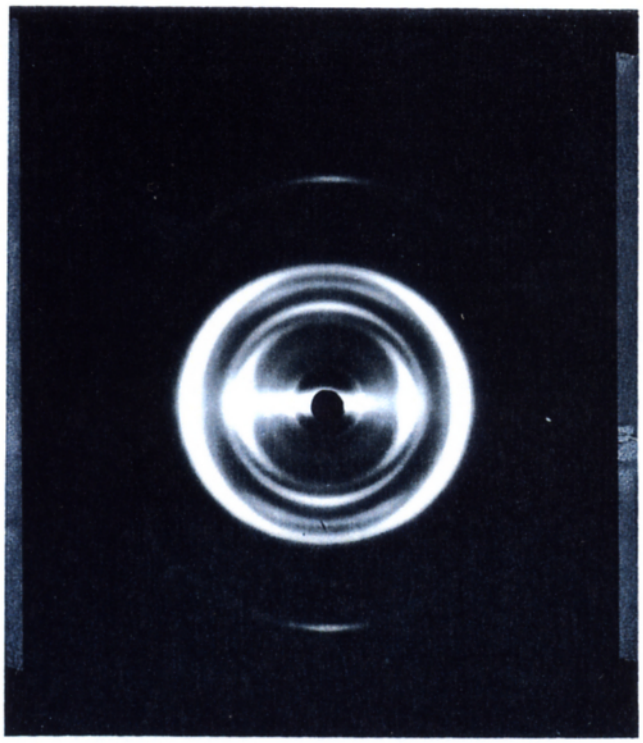
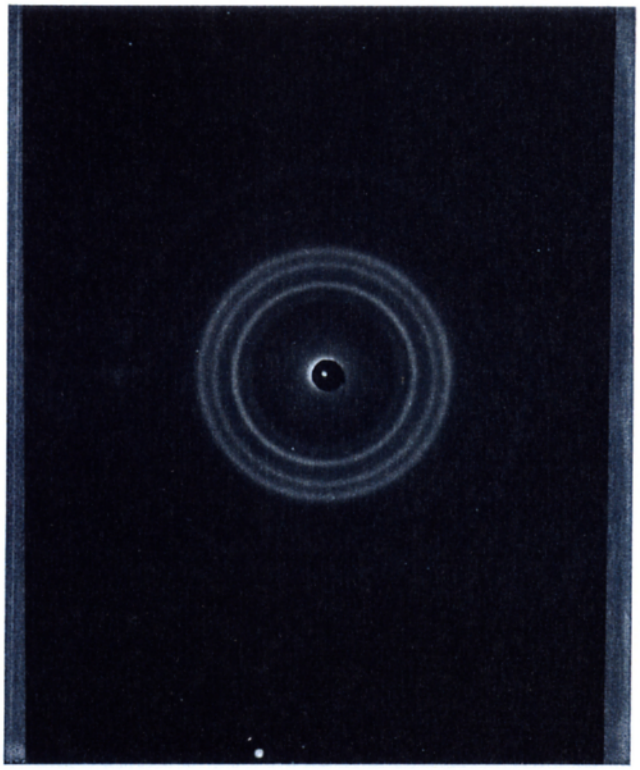


Fig. II-1-11 X-ray phtographs of NBC dried with a flat surface parallel on a glass plate

perpendicular to the membrane surface



parallel to the membrane surface

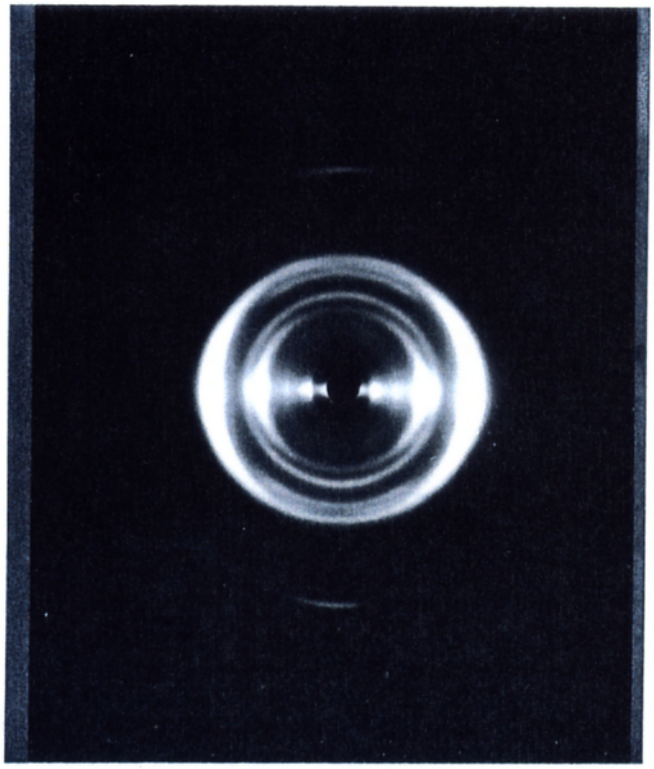


Fig. II-1-12 X-ray phtographs of BCC(CMC) dried with a flat surface parallel on a glass plate

Table II-1-4 Relative transparencies of BCCs to NBC membrane.

Sample	Thickness d (cm)	Relative transparency*
NBC	0.0019	100
BCC(PEG200)	0.0021	120
BCC(PEG1000)	0.0019	120
BCC(PEG6000)	0.0020	110
BCC(PEG20,000)	0.0019	120
BCC(PEG50,000)	0.0018	110
BCC(PEG500,000)	0.0018	120
BCC(CMC(Nakalai))	0.0023	250
BCC(CMC(Kanto))	0.0022	400
BCC(CMC(Wako))	0.0023	280

Strain; Acetobacter xylinum ATCC 10245

*) Transparency (T) = d / ABS(500nm)

Relative transparency (RT) = T(BCC) / T(NBC) × 100

SEM observations

The clear difference between the surface views of air dried (AD-) and lyophilized (LP-) NBC was observed, while it was not observed in BCC(CMC) (Fig. II-1-13). The difference between AD- and LP-NBC could suggest that ribbons fused each other during drying process, thereby the clear ribbon networks disappeared. The differences in surface views of AD- and LP-samples corresponded to the results of preferential orientations observed in X-ray diffractgrams.

The clear network structures of the composite ribbons were observed on LP-NBC, while the same structure was not observed at all and only small pores were observed on LP-BCC(MC)s and LP-BCC(CMC)s. Under the lyophilization process of BC, the fusion of cellulose ribbons by hydrogen bonding through water molecules could not happen because water molecules in the solid state are sublimed directly to the gaseous state. Therefore, cellulose ribbons seem to remain completely in the lyophilized samples. In the cases of the BCC(MC)s and BCC(CMC)s, the fusion of the ribbons and the disappearances of the clear ribbon networks may be caused by hydrogen bonding through WSP molecules incorporated among the ribbons instead of water. This could seem to be the positive evidence of the incorporation of WSP into the spaces among the ribbons. In all kinds of air-dried BCCs, no significant differences on SEM views were observed.

The thick ribbons compared with normal ribbons were observed in some places on the BCC(PEG)s. It is suspected that these thick ribbons were normal ribbons covered with PEG. The difference in molecular weights of the WSPs did not morphologically affect the surface of the lyophilized BCC for any WSP.

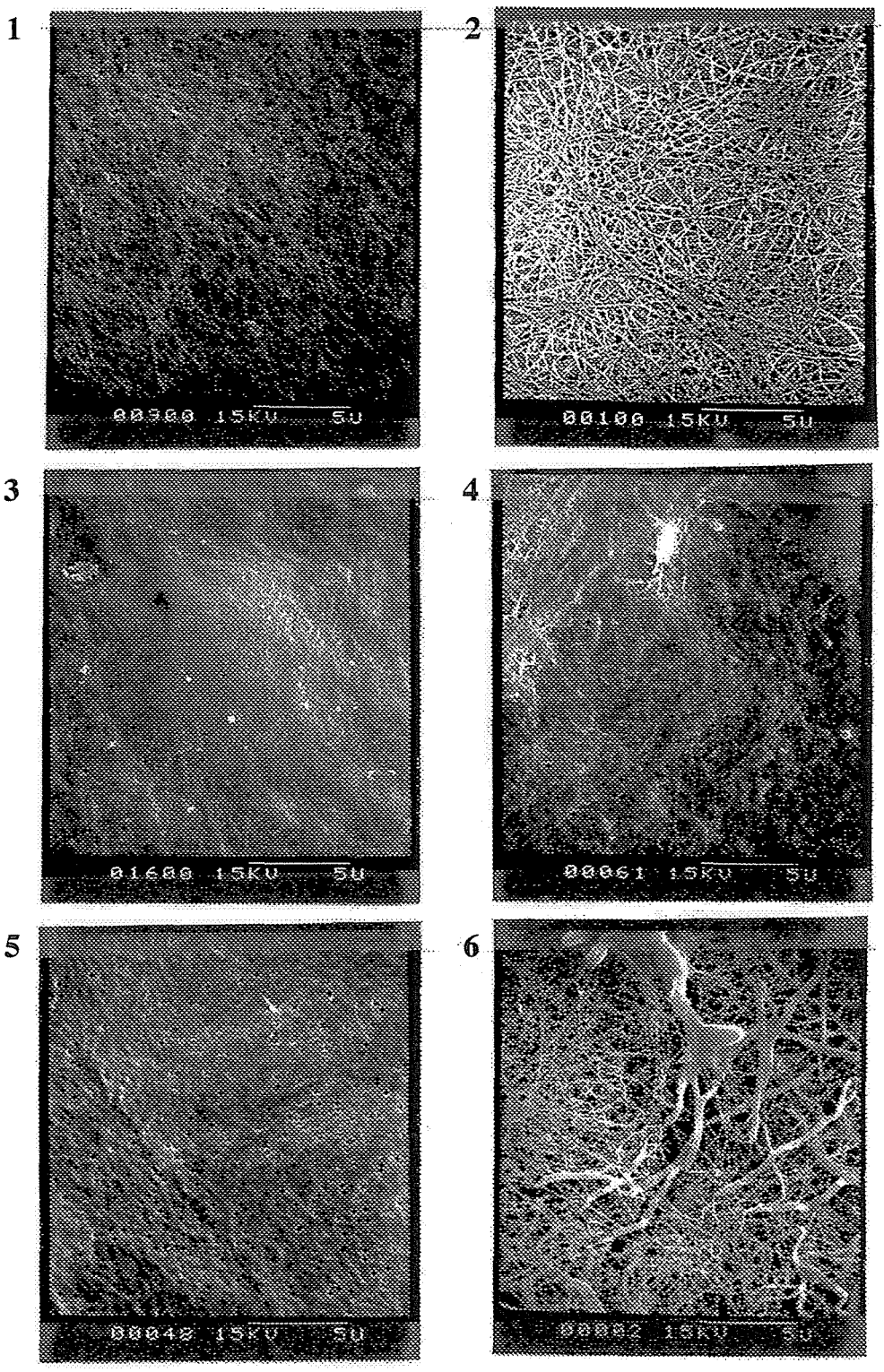


Fig. II-1-13 SEM phtographs of air-dried(AD) and lyophilized(LP) NBCs and BCCs; 1: AD-NBC, 2: LP-NBC, 3: AD-BCC(CMC(Wako)), 4: LP-BCC(CMC(Wako)), 5:LP-BCC(MC#8,000), 6: LP-BCC(PEG50,000).

Conclusions

The relative yields, the WSP contents, and the peak ratio of $(1\bar{1}0)/(020)$ are summarized in Table II-1-5, and we obtained the following results: (1) cellulose derivatives with low DS or small substituents were suitable for the formation of WSP-BC composites. (2) WSP contents in BCCs decreased with the increase of the bulkiness in the substituents. (3) BC synthesis was enhanced by addition of cellulose derivatives. (4) WSPs were incorporated into BC at molecular level, and they effected on the crystal structure of BC. (5) WSPs existed both on fibrillar subunits and among ribbons in BCCs, and BCCs had fine structure compared with that of NBC.

From these results, it was suggested that physical properties of BC and BC productivity of *A. xylinum* could be controlled by the incorporation of water-soluble cellulose derivatives with various substituents and DS.

Table II-1-5. Relative yields, contents, and peak ratios of $(1\bar{1}0)/(020)$.

Samples	Relative yields	Contents/wt %	Peak ratio of $(1\bar{1}0)/(020)$
NBC	1	-	≅1
BCC(MC)	1.2-1.6	11-23	<0.5
BCC(CMC)	1.6-1.8	15-19	<0.5
BCC(HEC)	1.5	11.5	<1
BCC(HPC)	1	≅5	≅1
BCC(PEG)	1	≅4	≅1
BCC(other polysaccharides)	1	-	≅1

CHAPTER II-2

Effect of CMCase on Bacterial Cellulose Production

Introduction

In Chapter II-1, it was reported that the bacterial cellulose production by *A. xylinum* was enhanced by water-soluble cellulose derivatives, especially carboxymethyl cellulose (CMC). CMC is a water-soluble cellulose derivative and one of cellulase inducers in fungi. Recently, few endoglucanase and β-glucosidase type enzymes have been found in some strains of *A. xylinum*. Although their biological function in *A. xylinum* have not been known so far, in *Rhizobium meliloti*, it was reported that the protein encoded by a gene (exoK) located on a 22 kb gene cluster involved in the biosynthesis of exopolysaccharide I displays homology to secreted endo-β-1,3-1,4-glucanases and that an exoK mutant strain produced reduced quantities of exopolysaccharide I. Tonouchi et al. reported that addition of a small amount of endoglucanase enhanced BC production by *A. xylinum*, and these results could suggest the possibility that endo-glucanases play important role in the cellulose production.

In this chapter, the induction of CMCase by CMC and the effect of CMCase on the BC production were investigated, in detail.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used are described in Table II-2-1.

Commercial cellulase

The commercially available cellulase complex used, Meicelase P-1 and ONOZUKA R-10 was purchased from Meiji Seika Kaisha Ltd and Yakult Pharmaceutical Ind. Co. Ltd, respectively.

Growth conditions

A. xylinum strains were grown in HS medium at 28°C either statically or on a rotary shaker. For preparation of *A. xylinum* cells that were not attached to cellulose product, cellulase (0.1% Cellulase ONOZUKA R-10) was added to the culture medium to give a uniform cell suspension., while E. coli cells were grown in Luria-Bertani broth at 37°C on a rotary shaker. For selection of resistance maker, antibiotic (ampicillin) were used at the concentration of 100 υg/ml.

DNA techniques

Total genomic DNA from *A. xylinum* was isolated by a standard procedure. Isolation of plasmid DNA from *E. coli* and transformation were performed by standard protocols. Plasmids were isolated from *A. xylinum* with Quantum Prep Plasmid Miniprep Kit (Bio-Rad Laboratories). Large-scale purified plasmid DNA was prepared according to the procedure whose description was supplied with the Qiagen plasmid kit.

Preparation of *Acetobacter* CMCase gene

Standal et al. reported (Standal, 1994) the nucleotide sequence of CMCase in *A. xylinum* ATCC 23769, and the oligonucleotide primers were designed on the basis of their sequence. The sense and antisense primer contained *Eco*RI and *Bam*HI sites, respectively. A genomic DNA fragment (1.3kb) encoding the *A. xylinum CMCase* gene was amplified by PCR.

Preparations of shuttle vector and plasmids

The shuttle vector was constructed by the method described by Fujiwara et al (Fujiwara, 1992). Plasmid pFF6 isolated from *A. xylinum* IFO 3288 was linearized with *Acc*I. The linear plasmid was treated with DNA blunting kit (Takara Biomedicals) and ligated to *Hinc*II-digested pUC18. The ligation mixture was introduced into *E. coli* JM109 and composite plasmid was recovered from ampicillin resistant colonies. The plasmid, named pUA18, has unique cloning sites derived from pUC18. Cloned genes are expected to be expressed in *A. xylinum* ATCC 23769 under the lac promoter (Wong, 1990). The plasmid was introduced into ATCC 23769 by

Table II-2-1 Bacterial strains and plasmids.

Strains

E. coli

JM109 F'*traD36proABlacIqΔ(lacZ)*M15/*e14-(mcrA)recA1endA1gyrA96thi-1hsdR17*(rk-mk+)*supE44relA1Δ(lac-proAB)*

JM109(pUA18); Apr

JM109(pAC1849); Apr CMCase+

A. xylinum

ATCC 23769 Wild type; Cel+

23769(pUA18); Cel+ Apr

23769(pAC1849); Cel+ Apr CMCase+

IFO 3288 Wild type; Cel- source of pFF6

Plasmids

pUC18 Apr; α-*lac*Z/MCS Plac

pFF6; indigenous plasmid in *A. xylinum* IFO3288

pUA18; pFF6 (*AccI* cutted) with blunt end cloned in the *Hinc*II site of pUC18

pAC1849; *Eco*RI-*Bam*HI fragment containing CMCase gene cloned in pUA18

the electroporation method described by Hall et. al (Hall, 1992).

The 1.3kb *Eco*RI-*Bam*HI fragment encoding the *A. xylinum CMCase* gene was ligated to pUA18 (pAC1849). The ligation mixture was introduced into *E. coli* JM109 and composite plasmid was recovered from ampicillin resistant colonies which were able to hydrolyze CMC. The colonies were selected by plate assay. The procedure of plate assay was described in CMCase assay.

Purification of CMCase and Assay

For measurements of CMC-hydrolyzing activity in *E. coli*, cells were harvested and washed twice in 25 mM sodium phosphate buffer (pH 6.0). The washed cells were sonicated in the same buffer and then centrifuged at 15,000×g for 60 min. The supernatants were used directly as a source of crude enzyme for measurements of CMC-hydrolyzing activity.

Preparation of extracellular CMCase: Cells were removed from the medium by centrifugation at 12,000×g for 5 min. Proteins were precipitated by solid ammonium sulfate to 60% saturation at 0°C. The precipitates were dissolved in 5 ml of 25 mM sodium phosphate buffer (pH 6.0) and dialyzed overnight against the Milli-Q water at 4°C. The cellulolytic activity was reprecipitated at between 40 and 55% saturation with ammonium sulfate at 0°C. The precipitates were dissolved in 2 ml of 25 mM sodium phosphate buffer (pH 6.0) and filtered with a ultrafilter membrane whose fractionation molecular weight was 10,000. The pertains were dissolved in 500 ul of 25 mM sodium phosphate buffer (pH 6.0) and the solution s were used as a source of crude enzyme for measurements of CMC-hydrolyzing activity.

Preparation of intracellular CMCase: The cells centrifuged were washed twice in 25 mM sodium phosphate buffer (pH 6.0). The washed cells were sonicated in the same buffer and then centrifuged at 15,000×g for 60 min. The supernatants were used directly as a source of crude enzyme for measurements of CMC-hydrolyzing activity.

CMC-hydrolyzing activities were determined by measuring the generation of reducing sugar ends. Twenty micro liter of enzyme preparations were mixed with 180 ml of 1% CMC (Kanto Chemical Co., Inc.)-50 mM sodium acetate solution (pH 4.5) , and those were incubated at 30°C or 45°C for given times. The reaction solutions (200 ml) were mixed with an equal volume of the Somogi reagent. After being 100°C for 10 min, the tubes were cooled on ice, 200 ml of Nelson reagent was added, and the optical density at 490 nm of the solutions was finally determined. Glucose was used as a standard.

For plate assays, clones were replicated onto LB plates containing 0.5% CMC and 100 ug/ml ampicillin. Following an overnight period of colony formation at 37°C, the plates were incubated at 45°C for 1 day and then stained with Congo red (Okamoto, 1994).

TLC chromatography was performed according to the method described by Tahara et al (Tahara, 1997). The reaction mixture consisted of 0.5%(W/V) of each cellooligosaccharide (G1-G6) and enzymes. Reaction conditions were pH 4.5 and 30°C. Aliquots (2ul) of the reaction mixtures were applied onto a TLC plate (Silica gel 60, Merck). A chloroform-methanol-water

(90:65:15, V/V) solvent system was used and spots were detected by heating the plate at 110°C for 15 min after splaying it with a solution of phenol and H2SO4 (phenol 3g; conc. H2SO4 5ml; ethanol 95ml). Lanes: S, standard; G1, glucose; G2, cellobiose; G3, cellotriose; G4, cellotetraose; G5, cellopentaose; G6, cellohexaose.

Preparation and characterization of bacterial cellulose

BCs were prepared as described in Chapter II-1. The BCs prepared were characterized with IR spectrophotometer, X-ray diffractmeter, and scanning electron microscope.

Results and Discussion

Expression and Characterization of CMCase from *A. xylinum* ATCC23769

Fig. II-2-1 shows CMCase activity in cell extracts prepared from *E. coli* JM109 carrying plasmids pAC1849 and pUA18, respectively. This result suggests that CMCase was expressed in cells containing pAC1849, while no such activity was detected in the corresponding extracts prepared from cells containing pUA18 without the insert.

Table II-2-2 shows the character of CMCase expressed in *E. coli* JM109 from *A. xylinum* ATCC23769, KU-1, and IFO3288. No difference between CMCases from *A. xylinum* strains was observed, except for the values of Km and Vmax. These results suggested the substrate affinity and reaction rate of CMCase from ATCC23769 was higher than that from KU-1. The detailed investigations on the relationships between CMCase activity and BC production are required in future.

Effect of cellulase (ONOZUKA R-10) concentration on BC production

Figure II-2-2 shows the BC yields in HS medium containing various concentrations of cellulase ONOZUKA R-10. The yield increased with concentration increase of cellulase in low concentration region (up to 5mg/l). In the concentrations more than 30 mg/l, the yields were lower than that in HS medium without cellulase, in contrast. This result suggests the possibility of the existence of a optimum cellulase concentration for the optimum BC production.

Induction of CMCase by CMC and Expression of CMCase from pAC1849 in *A. xylinum*

Thin-layer chromatogram of hydrolysates formed by extra- and intra-cellular enzymes, which were prepared from *A. xylinum* ATCC23769, incubated in HS medium without or with 0.5% CMC, incubated with cellooligosaccharides (G1-G6) were shown in Fig. II-2-3. In both extra- and intra-cellular enzymes, increases of hydrolytic activities were observed, and this could suggest the possibility of cellulase induction by CMC. Although CMC has been known to be one of cellulase inducers in fungi (Mandels, 1957, Pardo, 1996, Rao, 1988) and bacteria (Breuil, 1976, Godden, 1989), cellulase induction of CMC in *A. xylinum* has not been reported. The biological function of cellulase in *A. xylinum* is not known, and the cellulase induction by CMC is very interesting from the point of the comprehension in function of cellulases on BC production and degradation.

CMCase activities and SDS-PAGE of the extracellular proteins of ATCC23769(WT), 23769(pUA18), and 23769(pAC1849) were shown in Table II-2-3 and Fig. II-2-4, respectively. From these results, the over expression of CMCase in *A. xylinum* 23769(pAC1849) was confirmed, then, BC production by this strain was performed.

BC production by *A. xylinum* 23769(pAC1849)

The BC yields of *A. xylinum* 23769(pUA18) and 23769(pAC1849) in HS medium without

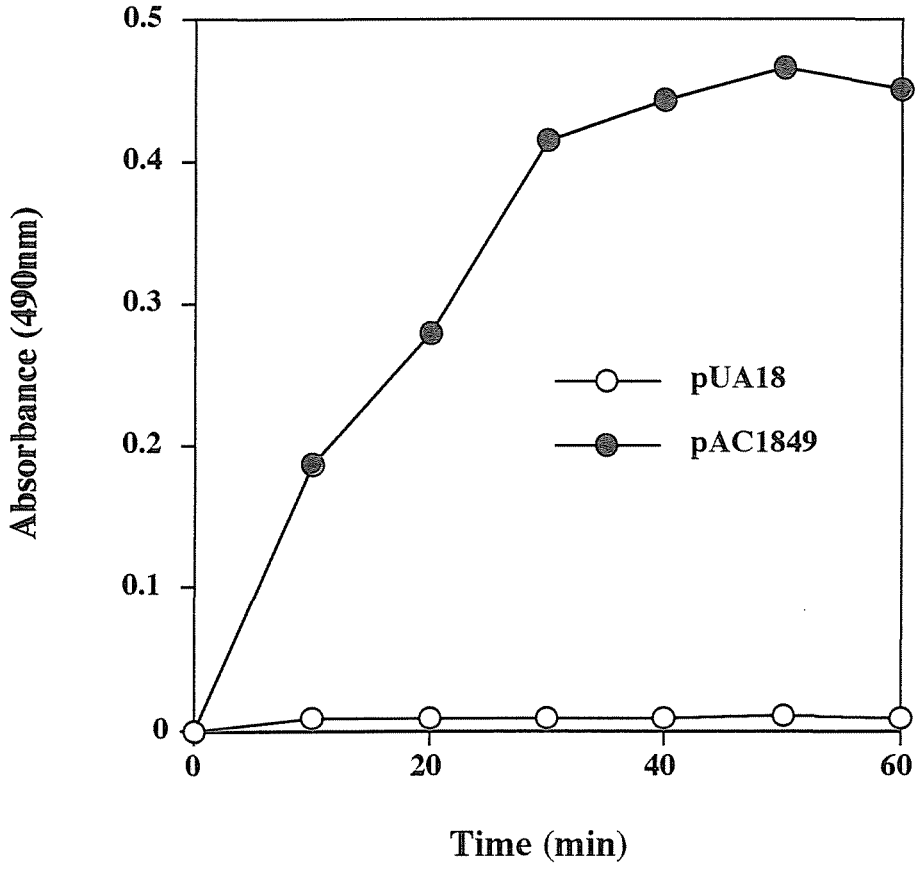


Fig. II-2-1 CMCase activity in cell extracts prepared from *E. coli* JM109 carrying plasmids pUA18 and pAC1849, respectively.

Table II-2-2 Characters of CMCases from *A. xylinum* strains.

	Strains (*A. xylinum*)		
	ATCC23769	KU-1	IFO3288
Molecular weight (kDa)	35.6	39	24
Optimal temperature(°C)	50	50	50
Optimal pH	4.5-5.0	5.0	4.5
Recognition unit of cellooligosaccharide	≥5	≥5	ND
Km (mM)	0.148	0.222	ND
(mg/ml)	7.40	30	ND
Vmax(uM/min)	37.2	1.2	ND
Hydrolysis of air-dried BC	-	ND	ND

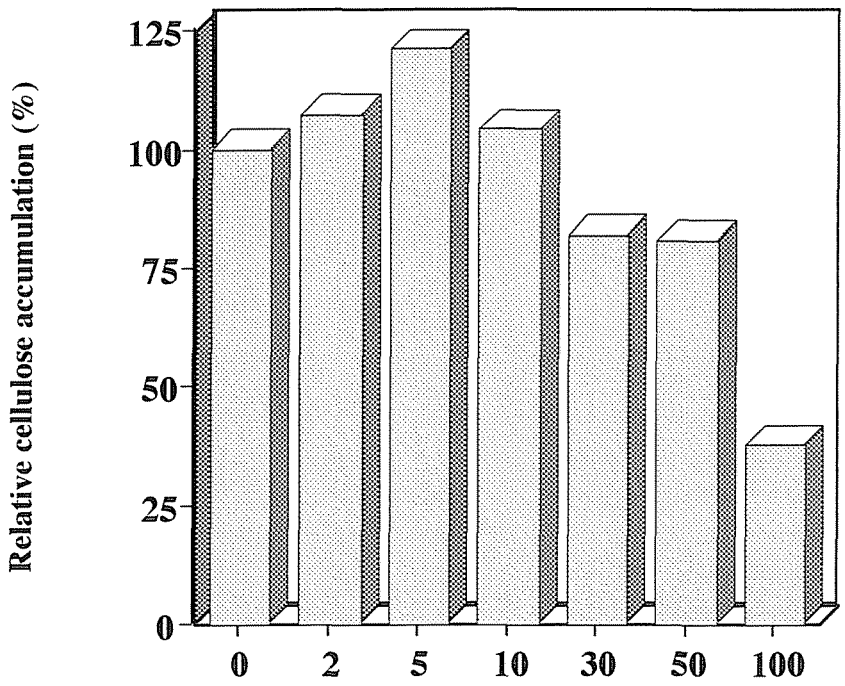


Fig. II-2-2 BC yields in HS medium containing various concentrations of cellulase ONOZUKA R-10.

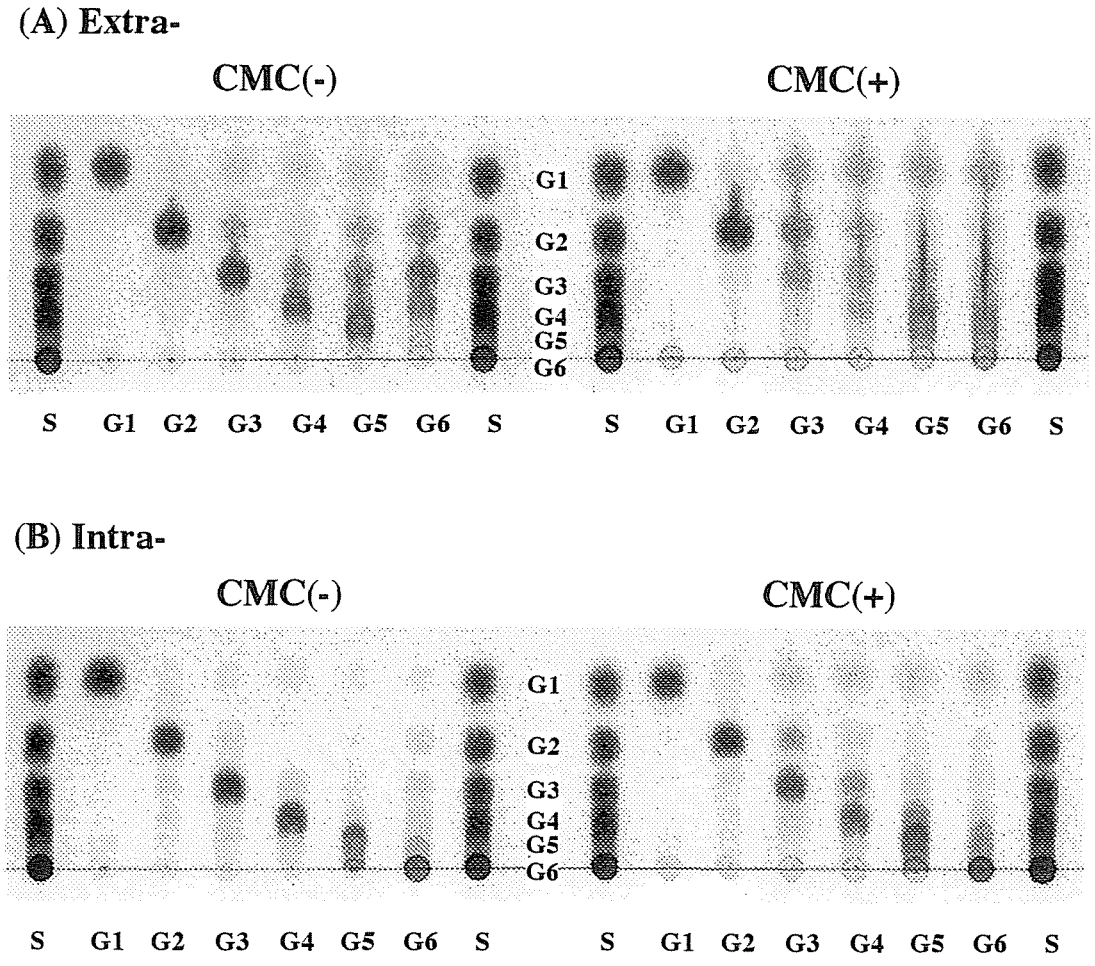


Fig. II-2-3 Thin-layer chromatograms of hydrolysates formed by (A) extra- and (B) intra-cellular enzymes incubated with cello-oligosaccharides (G1-G6). These enzymes were prepared from *A. xylinum* ATCC23769 incubated in HS medium without or with 0.5% CMC.

Table II-2-3 CMCase activities of the extracellular proteins prepared from the cultures of *A. xylinum* ATCC23769(WT), 23769(pUA18), and 23769(pAC1849).

Strain *A. xylinum*	CMCase activities (U/mg-protein)
23769(WT)	0.119
23769(pUA18)	0.097
23769(pAC1849)	0.148

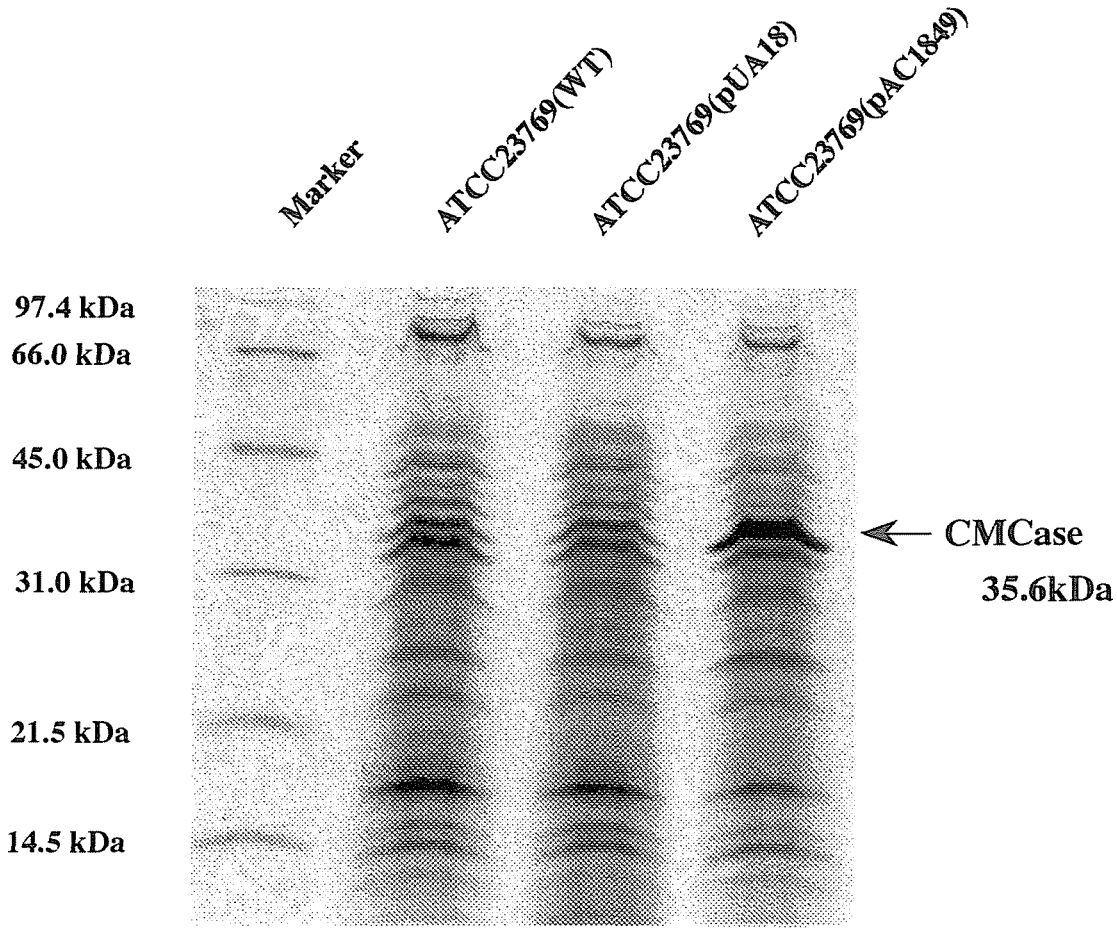


Fig. II-2-5 SDS-PAGE of the extracellular proteins prepared from the cultures of *A. xylinum* ATCC23769(WT), 23769(pUA18), 23769(pAC1849).

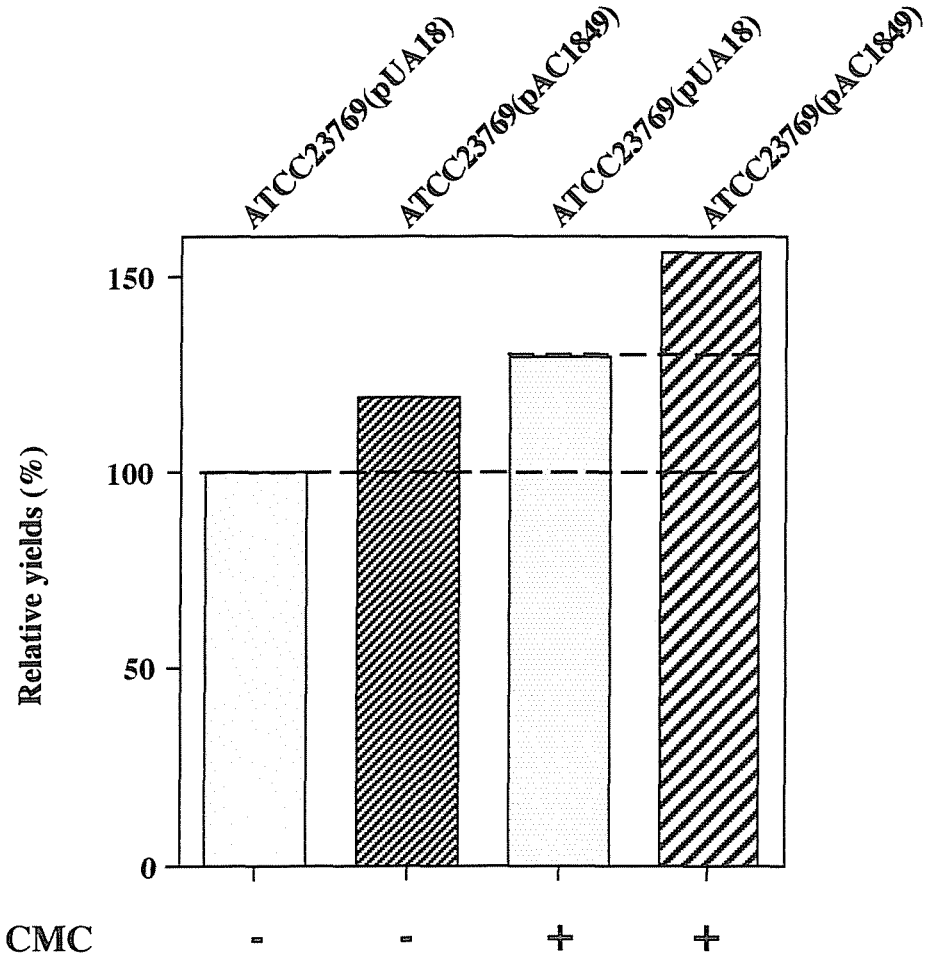
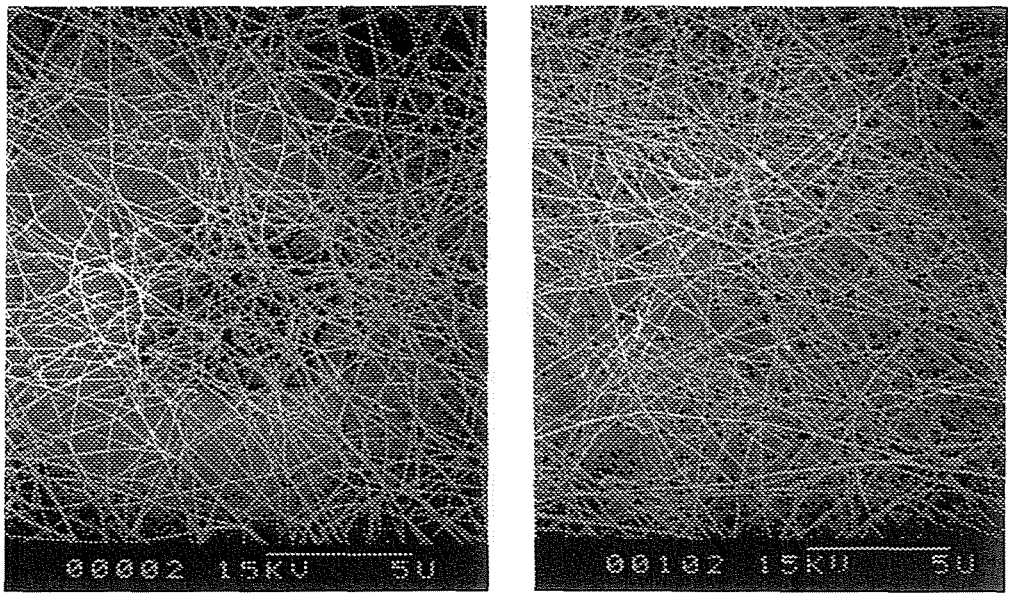


Fig. II-2-5 The BC yields of *A. xylinum* ATCC23769(pUA18) and 23769(pAC1849) in HS medium without or with 0.5% CMC.

and with 0.5% CMC were shown in Figure. II-2-5. In both media, the yields of 23769(pAC1849) were higher than those of 23769(pUA18).

No differences in IR spectra and X-ray diffraction patterns of BCs synthesized by 23769(pUA18) and 23769(pAC1849). While their SEM observations suggested that the ribbons from 23769(pAC1849) became more narrow than those from 23769(pUA18) (Fig. II-2-6). White et al. (White, 1981) reported that endo-1,4-β-D-glucanase produced some splaying of ribbons into fibrillar subunits. As mentioned in Chapter II-1, the ribbon assembly is a slightly rate limiting step, and prevention of the ribbon assembly by CMCase could be one of the causes in the increase of the BC productivity of 23769(pAC1849) similarly to cellulose derivatives.



ATCC23769(pUA18) **23769(pAC1849)**

Fig. II-2-5 SEM photographs of lyophilized BCsynthesized by *A. xylinum* ATCC23769(pUA18) and 23769(pAC1849) in HS medium.

Conclusions

We obtained the following results: (1) possibility of cellulase induction by CMC molecules in *A. xylinum* ATCC23769 was suggested, (2) there is a optimum cellulase concentration for the optimum BC production, (3) overexpression of extracellular endoglucanase (CMCase) increased BC yield. From these results, the mechanism of the increase of BC production in HS medium containing CMC was suggested as follow: (1) CMC induced cellulases, (2) cellulases prevented the ribbon assembly which is a rate limiting step, (3) BC production was increased by the dissolution of the rate limiting step.

CHAPTER III

Synthesis of Bacterial Cellulose Composite by Addition of Chitin-Chitosan Derivatives and Oligosaccharides

Introduction

Chitin, a glucosaminoglycan of β(1→4) glycoside linkage, is known to have a very tight crystalline structure owing to rather strong hydrogen bonds formed by acetamide groups of N-acetylglucosamine (GlcNAc) residues and known also as a native mucopolysaccharide with biodegradability in animals (Carlstrem, 1957). Chitosan, a polymer of β(1→4) linked aminoglucose, is one of a few natural cationic polysaccharides that can be derived from crustanceans or various fungi. Chitin and Chitosan have amide or amino group which have high reactivities than that of hydroxyl group, and they were used as precursors of functional polymers, carriers of bacteria or enzymes, and so on. Hosokaw et al. (Hosokawa, 1990, Hosokawa, 1991) reported that the combination of chitosan and fine cellulosic fiber results in the formation of various kinds of strong, gas-barrier, and water-resistant composite films by only cast drying the material without any complicated treatment. Therefore, BC-water-soluble chitin/chitosan derivative (WSChD) composites could be expected to be materials having multifunctions such as biodegradability, high mechanical strength, and high reactivity.

In contrast with that chitin and chitosan with high molecular weights are insoluble in water, chitin and chitosan oligomers (WSChOs) are soluble, and many investigators (Hadwiger, 1983, Hadwiger, 1984, Alan, 1979, Walker-Simmons, 1983, Walker-Simons, 1984, Hirano, 1987, Mauch, 1984, Roby, 1987, Nichols, 1980, Hadwiger, 1986) have reported that they activated plant cells and functioned as elicitors which enhance the synthesis of chitinase, chitosanase, and phytoalexins or callose. We have found that WSChOs enhanced the BC productivity of *A. xylinum* in the experiments in the synthesis of BCC using various natural polymers.

In this chapter, we have reported the synthesis of BC-WSChD composites and the effects of water-soluble chitosan oligomers (WSChOs) on BC production.

Materials and Methods

Materials

The WSChO-W and the poly-L-lysine hydrobromide (molecular weight (M.W.) 100,000-150,000) were purchased from Wako Pure Chemical Industries, Ltd. Chitin and chitosan were the products of Katakura Chikkarin Co., Ltd. and the chitosan oligomers ML and MH, were from Pias Co., Ltd. The chitosan oligomers are termed WSChO-ML and WSChO-MH. The molecular weight, degree of substitution (DS) and degree of deacetylation of water-soluble chitosans are shown in Table III-1. Cellulase ONOZUKA R-10 was the product of Yakult Pharmaceutical Ind. Co., Ltd. The cellulase was used after small molecular weight materials were removed by salting-out and ultrafiltration. Glucose Test C II WAKO to determine the concentration of glucose was purchased from Wako Pure Chemical Industries, Ltd.

BC synthesis of *A. xylinum* in Hestrin-Schramm (HS) medium with water-soluble chitosan

Two bacterial strains of *A. xylinum* NCI 1051 (Nakano Vinegar Central Institute, Nakano Vinegar Co., Ltd.) and ATCC 10245 were used for BC production. A half milliliter of the preculture of *A. xylinum* NCI 1051 or ATCC 10245 incubated at 28 °C for 72 h was inoculated into 20 ml of standard HS medium (2 (w/v) % D-glucose, 0.5 (w/v) % bactopeptone, 0.5 (w/v) % yeast-extract, 0.115 (w/v) % citric acid, and 0.27 (w/v) % disodium hydrogen phosphate anhydride in distilled water, pH 6.0) to which 0.5 (w/v) % of WSChO or WSChD was added. These are summarized in Table 1. The inoculated cultures were statically incubated at 28 °C for seven days. Membranes in a greatly swollen condition were taken from the surface of the culture. They were soaked for seven days in a 1 % NaOH solution to remove all of the alkali-soluble components, then washed with distilled water and 1 (v/v)% acetic acid to neutralize the last traces of alkali for three days. Finally, they were washed with large volumes of distilled water and air-dried at room temperature on glass plates or lyophilized.

Preparation of WSChD

Chitin and chitosan were carboxymethylated as described by Tokura and others (Tokura, 1983) and by Muzzarelli and others (Muzzarelli, 1982), respectively. The N-trimethylation of chitosan was performed as described by Domard and others (Domard, 1986). These chitin and chitosan derivatives were water-soluble.

Determination of WSCh content in BCC

The chitosan molecule has nitrogen atoms compared with that of BC which has none. Therefore, the nitrogen percent (N%) in BCC(WSCh) is proportional to the WSCh content. The calibration curves were obtained by the N% measurement of the mixture of cellulose and WSCh mechanically mixed at a given mixing ratio, and the weight percent of WSCh was determined by element analysis with calibration curves.

Table III-1 Molecular weights, DSs and degrees of deacetylation of chitosan oligomers and chitosan and chitin derivatives.

Natural Polymers	Molecular weights (MW)	DSs*1)	Degrees of deacetylation*2)
WSChO-W	3,000-30,000	-	-
WSChO-ML	< 4,000 70.1%, ≥ 4,000 29.9%	-	-
WSChO-MH	< 4,000 33.6%, ≥ 4,000 66.4%	-	-
Chitosan 1000	470,000	-	83.16 (82.6)%
Chitosan 500	390,000	-	82.51 (85.0)%
Chitin 1000	-	-	7.95%
Chitin 500	-	-	5.77%
CM-chitosan 1000	470,000	0.624	83.16 (82.6)%
CM-chitosan 500	390,000	0.574	82.51 (85.0)%
CM-chitin 1000	-	0.678	7.95%
CM-chitin 500	-	0.856	5.77%
NTM-chitosan 1000	470,000	-	83.16 (82.6)%
NTM-chitosan 500	390,000	-	82.51 (85.0)%

*1) DS; Degrees of substitution determined by IR method.

*2) Degrees of deacetylation determined by elemental analysis (determined by IR method).

X-ray Diffraction, Infrared spectrum, Scanning electron microscopy, Measurement of culture absorbance, pH, and glucose concentration

These procedure were described in **Material and Methods** of Chapter 2-1.

CP-MAS ^{13}C-NMR (Cross polarization-magic angle spinning Carbon 13 nuclear magnetic resonance) spectroscopy

The air-dried samples were cut into small pieces (1 mm X 1 mm), and packed into a MAS rotor. CP/MAS 13C NMR measurements were performed using a Bruker MSL300 spectrometer operating at 75 MHz for 13C and 300 MHz for 1H (proton). Cross-polarization time was 1 ms.

Results and Discussion

The effects of WSChO and WSChD on BC productivity

BCC yields in HS medium containing 0.5 (w/v) % of WSChO were 1.4-1.5 times that of the control (Table III-2). No influence of the molecular weight of WSChO on BC productivity was observed. Fig. III-1 shows the weights of BC and WSChOs in BCCs. WSChOs were scarcely incorporated into BCC(WSChO)s. Therefore, the yield increase was not due to the incorporation of WSChO but to the enhancement of BC productivity by WSChO.

BCC yields in HS medium containing 0.5 (w/v) % WSChD were 1.2-1.4 times for carboxymethyl (CM) chitosans, 1.6 times for CM-chitins, 0.9-1.0 times for N-trimethylated chitosans (NTMCs) and 0.7 times for native chitosans as those of the control (Table III-2). The contents of CM-chitosans or CM-chitins in BCCs were 7-8 and 12-16 wt%, respectively. Fig. III-2 shows the weights of BC and WSChDs in BCCs. The weights of BC in BCCs were larger than that in NBC. This suggests that the yield increase for BCC(CM-chitosans) and BCC(CM-chitins) was not only due to the incorporation of WSChD but to the enhancement of BC productivity by WSChD. On the other hand, it was suggested that N-trimethylated chitosans did not enhance the BC productivity, because the weights of cellulose in the BCC(NTMC)s were almost the same as that of NBC (Fig. III-2). The small yield in HS containing water-insoluble chitosan could be due to the decrease in free glucose by the Maillard reaction because the glucose concentration decreased and the color of chitosan changed from off-white to dark-brown during sterilization.

As described in Chapter 2-1, Haigler and others (Haigler, 1985, Haigler, 1982a) and Brown and others (Brown, 1983) reported that CMC prevented the fasciation of the larger fibrillar subunits and that the polymerization rate of glucose was increased by the prevention of the fasciation which is one of rate limiting steps. We have suspected that yield increases for BCC(CM-chitin) and BCC(CM-chitosan) might be caused by the prevention of the ribbon assembly by CM-chitin or CM-chitosan molecules as well as BCC(CMC), because BCC(CM-chitosan) and BCC(CM-chitin) had similar structures as that of BCC(CMC) from the results of X-ray diffractograms (Figs. III-4-1, 2) and SEM observations (Fig. III-3).

WSChO and WSChD enhanced the BC productivity. However, BCC(WSChO) and BCC(WSChD) were different in their contents (Table III-2) and structures (Fig. III-3). These results suggest that each of WSChO and WSChD enhanced the BC productivity by different mechanisms. Many investigators (Hadwiger, 1983, Hadwiger, 1984, Alan, 1979, Walker-Simmons, 1983, Walker-Simons, 1984, Hirano, 1987, Mauch, 1984, Roby, 1987, Nichols, 1980, Hadwiger, 1986) have reported that chitin and chitosan and their oligomers functioned as elicitors of green plant cells. Elicitors are substances which activate cells and which enhance the synthesis of chitinase, chitosanase, phytoalexins or callose. Hadwiger and others hypothesized that: (1) chitosan fragments (oligomers of seven monomer units or longer) entering a green plant cell may influence the chromatin structure via their ability to form a complex with deoxyribonucleic

Table III-2. Yields of BCC(WSCh)s and contents of WSChs.

Samples	Yields / mg	Contents / wt%
NBC(Control)	31.5	-
BCC(WSChO-W)	44.9	0.44
BCC(WSChO-ML)	43.8	2.19
BCC(WSChO-MH)	48.5	0.34
NBC(Control)	31.5	-
BCC(native chitosan 1000)	23.1	-
BCC(native chitosan 500)	22.8	-
BCC(CM-chitosan 1000)	45.6	7.10
BCC(CM-chitosan 500)	39.3	7.84
BCC(CM-chitin 1000)	49.1	12.3
BCC(CM-chitin 500)	49.8	16.1
NBC(Control)	37.1	-
BCC(NTMA-chitosan 1000)	36.0	7.94
BCC(NTMA-chitosan 500)	35.0	8.89

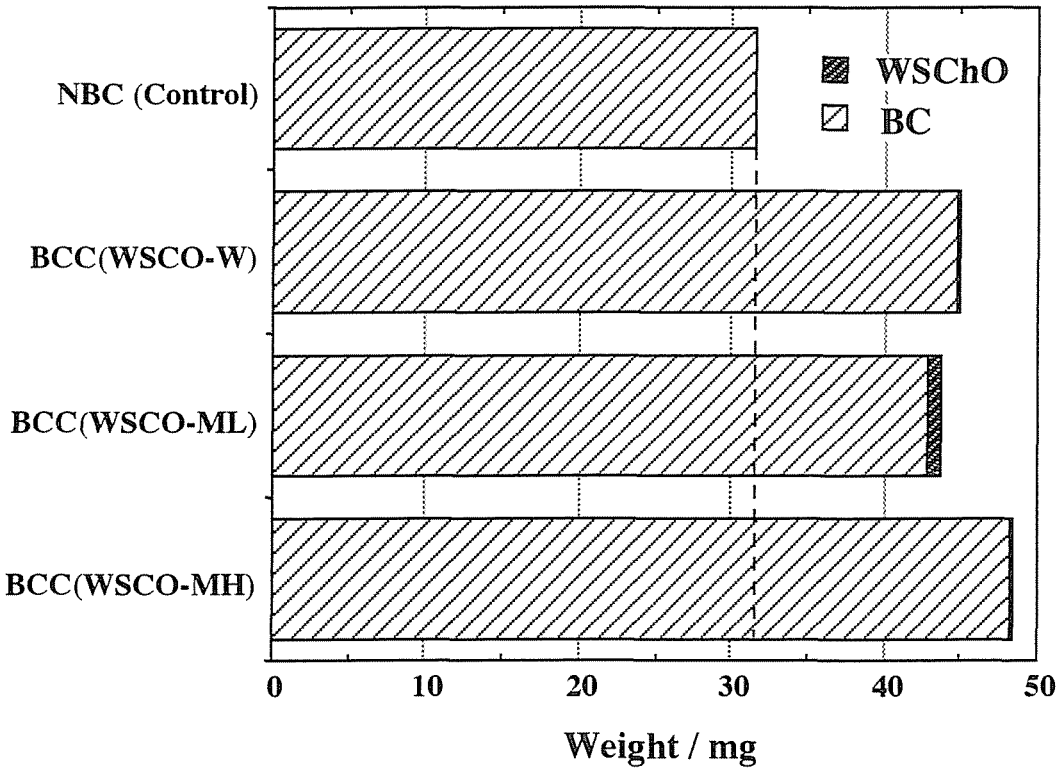


Fig. III-1. Weights of BC and WSChOs in BCC(WSChO)s.

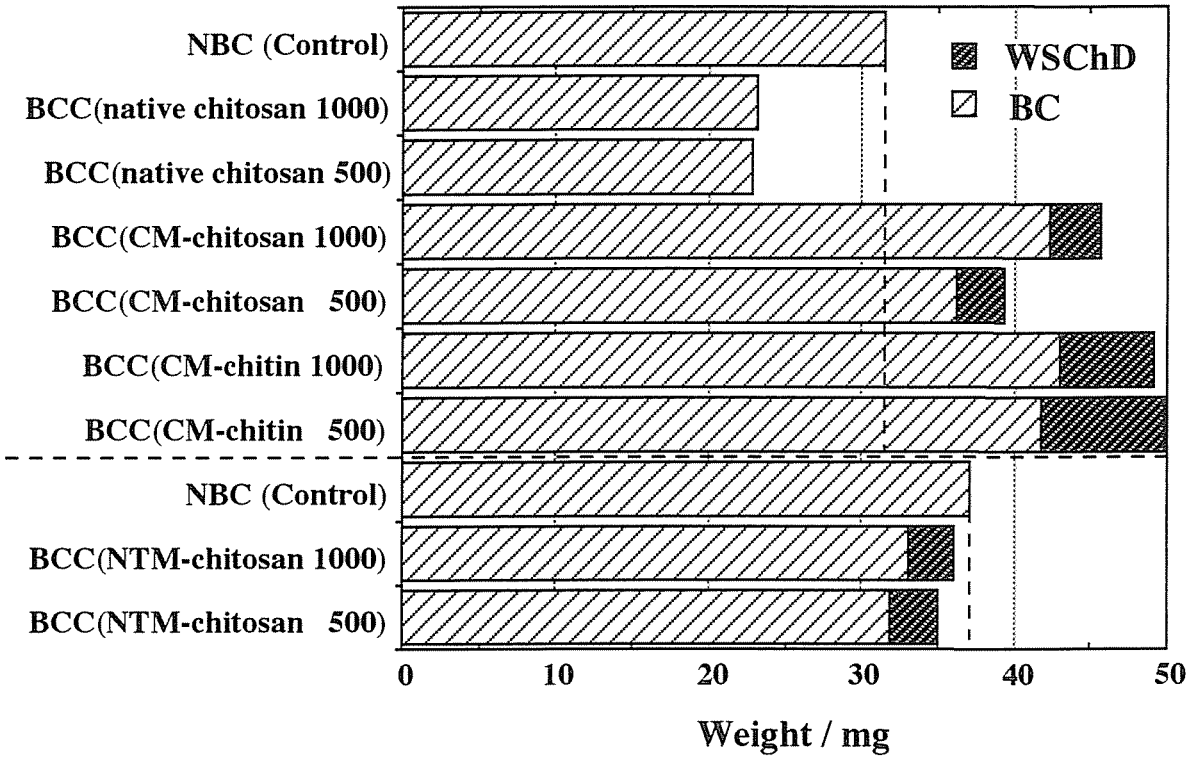


Fig. III-2. Weights of BC and WSChDs in BCC(WSChD)s.

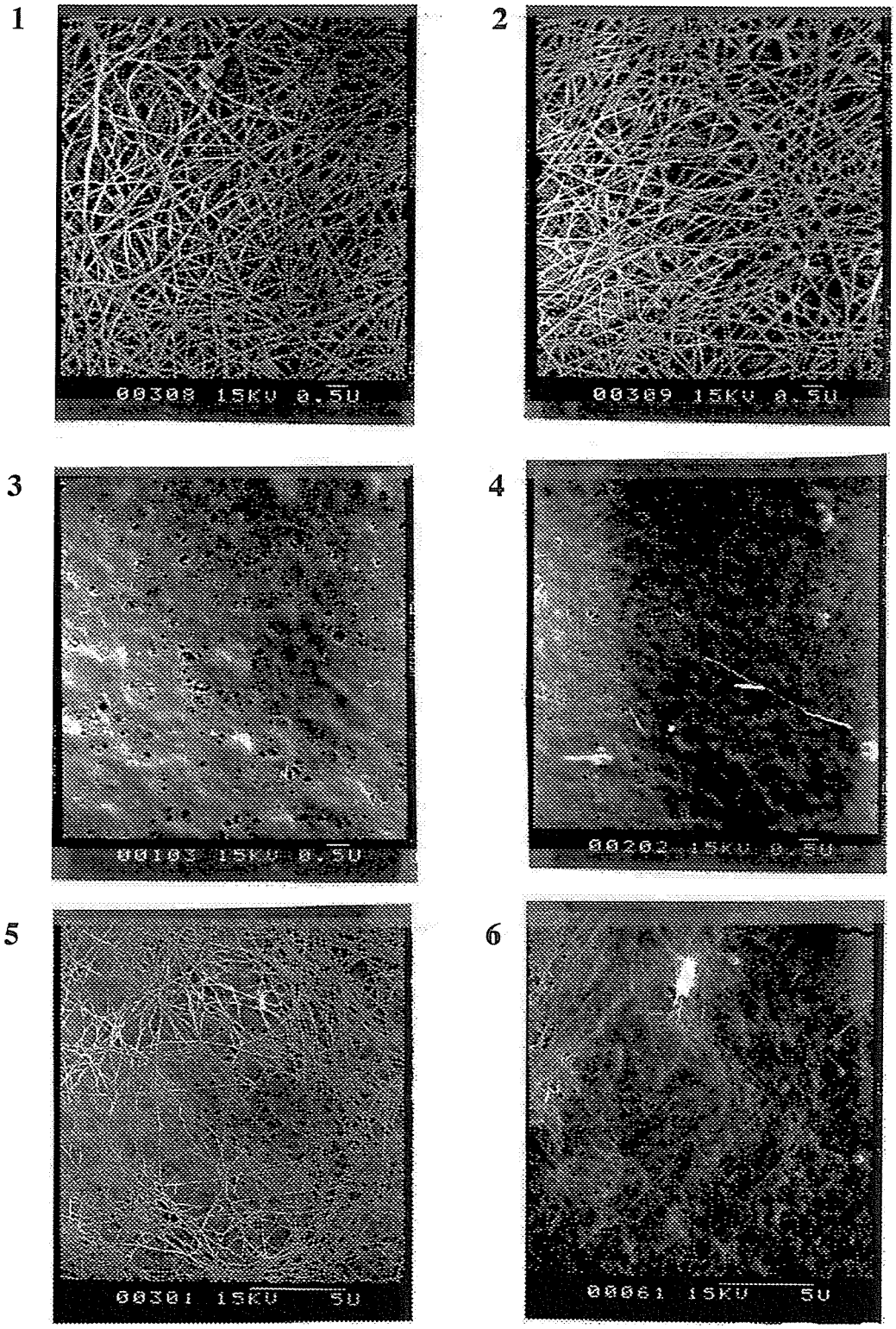


Fig. III-3. SEM photographs of lyophilized NBC and BCCs: 1: NBC, 2: BCC(WSChO-W), 3: BCC(CM-chitosan500), 4: BCC(CM-chitin500), 5: BCC(NTM-chitosan500), 6: BCC(CMC). Bacterial strain was ATCC10245.

acid (DNA). (2) The messenger ribonucleic acids (mRNAs) from some of the activated genes produce proteins which enhance the activity of the phenylpropanoid pathway which in turn produce phenolic compounds potentially adverse to the pathogen such as phytoalexins or lignin-like compounds (Hadwiger, 1986). At the present time, we suppose that WSChO activated the bacteria and that, as a result, the BC productivity was enhanced.

Characterization of BCC(WSChO)

IR spectra, X-ray diffractograms, CP-MAS ^{13}C-NMR spectra of NBC and BCC(WSChO-W) were very similar to each other. A clear difference between the surface views of lyophilized NBC and BCC(WSChO-W) was not observed (Fig. III-3). The clear network structure of the ribbons was observed on both NBC and BCC(WSChO-W) compared with the ribbon network structure that could not be observed in BCC(CM-chitosan) and BCC(CM-chitin) (Fig. III-3). These results suggests that BCC(WSChO-W) has the same structure as that of NBC. This is attributable to the small content of WSChO-W in BCC.

Characterization of BCC(WSChD)

In the IR spectra of BCC(CM-chitin)s, the peak derived from a carbonyl group was observed at 1,730 cm-1, suggesting the presence of CM-chitin in BCC. The X-ray diffractograms of NBC and BCC(CM-chitin)s or BCC(CM-chitosan)s are shown in Figs. III-4-1 and 4-2. The three peaks corresponding to $(1\bar{1}0)$, (110), and (020) were observed in all samples. Generally, BC provides great uniplanar orientation, which was indicated by the ratio of the $(1\bar{1}0)$ peak intensity to the (020) peak intensity. The small $(1\bar{1}0)/(020)$ ratio suggests that BCC(CMchitin)s and BCC(CM-chitosan)s had little uniplanar orientation and these results were similar to those of BCC(CMC) and BCC(MC). Consequently, similarly to BCC(CMC), the small uniplanar orientation in BCC(CM-chitin) and BCC(CM-chitosan) could be due to the prevention of direct hydrogen bonding between the $(1\bar{1}0)$ planes during the air-drying process by the adsorption of CM-chitin or CM-chitosan molecules onto $(1\bar{1}0)$ planes, whereas the X-ray diffractograms of the BCC(NTMC)s were very similar to that of NBC (data are not shown). This suggests that N-trimethylated chitosan did not decrease the uniplanar orientation in BCC and could be related to the enhancement of the BC productivity.

SEM observations

The clear differences between the surface views of lyophilized NBC and each BCC(CM-chitosan) or BCC(CM-chitin) were observed (Fig. III-3). The clear network structure of the composite ribbons was observed in NBC, but the structure was not observed at all and only small pores were observed in BCC(CM-chitosan) and BCC(CM-chitin). During the lyophilization process of BC, the adhesion of cellulose ribbons by hydrogen bonding through water molecules could not occur because water molecules are sublimed directly. Therefore, the clear network structure of the composite ribbons seems to remain completely in the lyophilized samples. In the

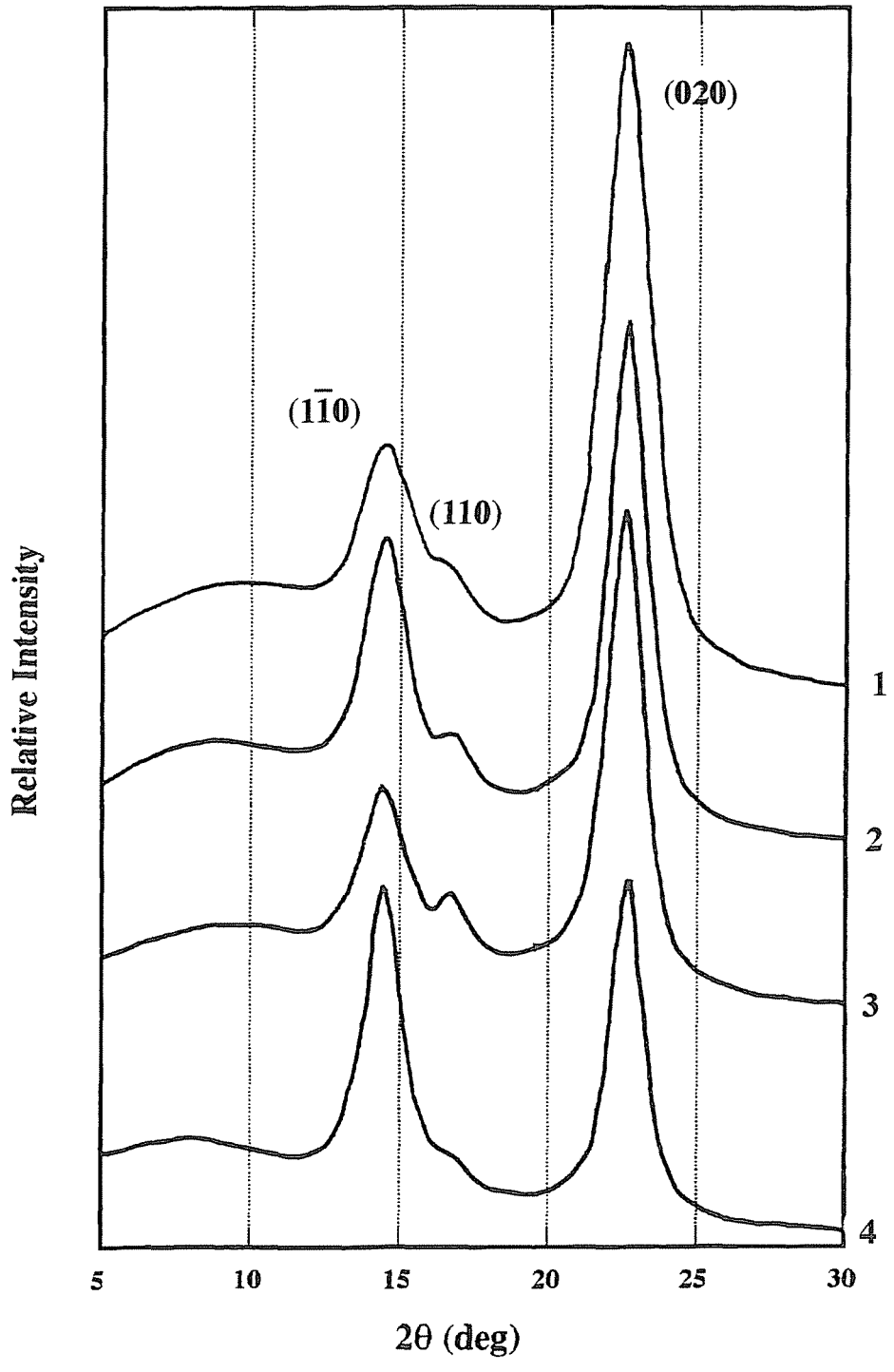


Fig. III-4-1. X-ray diffractgrams of 1: BCC(CMC), 2: BCC(CM-chitin1000), 3: BCC(CM-chitin500), 4: NBC.
Bacterial strain was 10245.

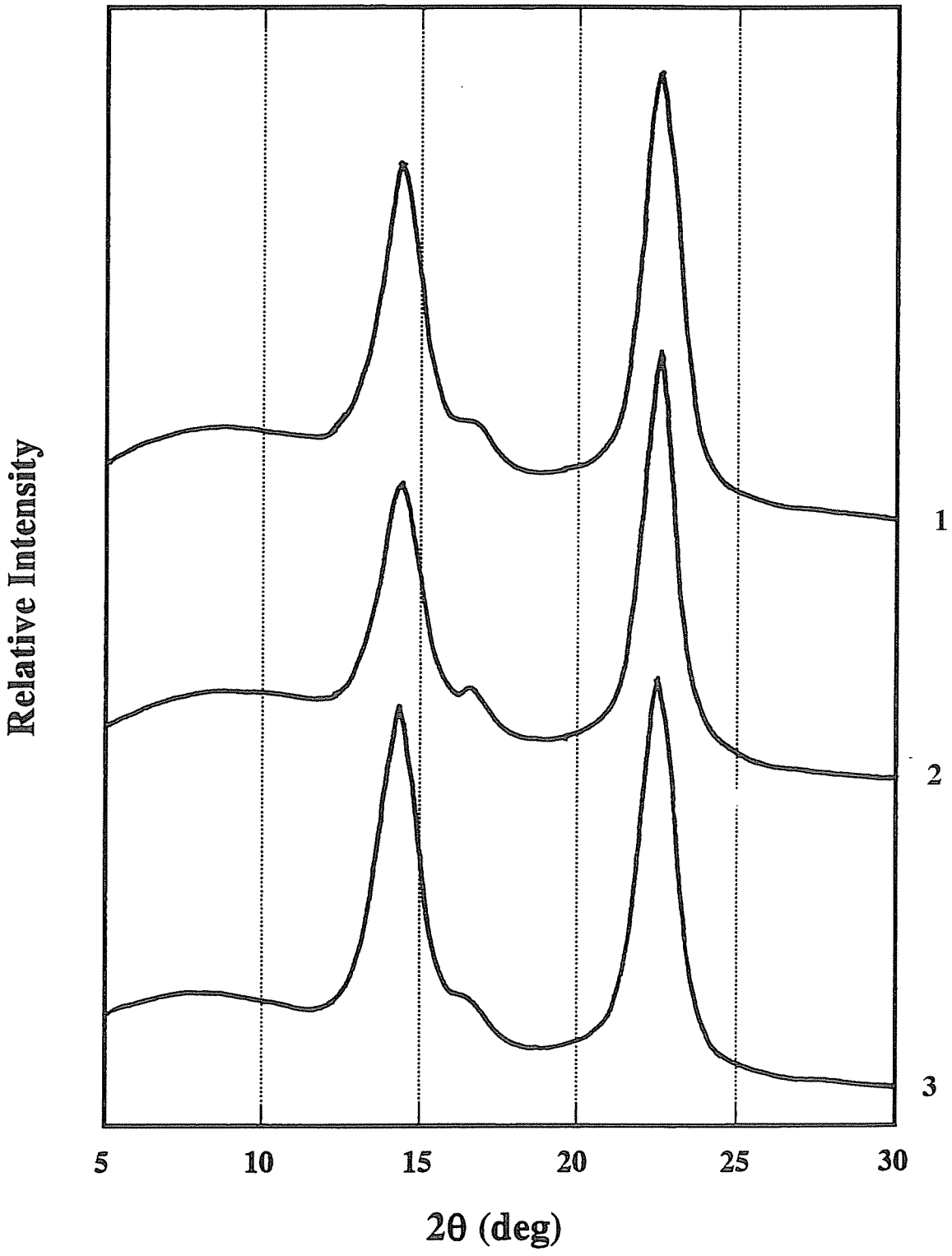


Fig. III-4-2. X-ray diffractgrams of 1: BCC(CM-chitosan1000), 2: BCC(CM-chitosan500), 3: NBC.
Bacterial strain was 10245.

cases of the BCC(CM-chitosan) and BCC(CM-chitin), the adhesion of ribbons and the disappearance of the clear ribbon network may be caused by hydrogen bonding through WSChD molecules incorporated among the ribbons instead of water. Also, this could be positive evidence of the incorporation of water-soluble chitosan derivative into the spaces between the ribbons.

The effect of WSChO concentration on BC productivity

To study the concentration effect of WSChO on the BC productivity, *A. xylinum* was cultivated in HS medium containing 0.005-0.5 (w/v) % of WSChO-W. After incubation, BCC yields, WSChO-W contents and final pH in the cultures were measured. Table III-3 shows them in HS medium with WSChO-W at various concentrations.

The minimum WSChO-W concentration to increase the BCC yield was 0.25 (w/v) % for NCI 1051 and 0.10 (w/v) % for ATCC 10245. The yields of BCCs were 1.4-1.5 times those of the NBCs. No relationships between the contents and concentrations of WSChO-W were observed, and the contents were very small (Table III-3). This could be due to the small molecular weight of WSChO-W. From these results, it is suggested that water-soluble chitosan enhanced the BC productivity even at the small concentrations.

The final pH of the culture rose rapidly at WSChO-W concentrations of more than 0.1 (w/v) % (Table III-3). The tendency for increases in the final pH of WSChO-W concentrations (more than 0.1 (w/v) %) was similar to that of the BCC yield. From these results and the fact that A. *xylinum* has the ability to metabolize gluconic acid, it can be concluded that the rise in final pH could be due not only to the basicity of WSChO but to the utilization of gluconic acid.

The effect of glucose concentration on BC productivity

To study the concentration effect of glucose in cultures with WSChO on the BC productivity, *A. xylinum* was cultivated in HS medium containing 0.5 (w/v) % of WSChO-W at various glucose concentrations, 1, 2, 3, 5, and 10 (w/v) %. Fig. III-5 shows the BCC yields and final pHs at various glucose concentrations. The BCC yield increased with the increase in glucose concentration, and reached a maximum value at 2 (w/v) % glucose concentration. The further addition of glucose induced the yield decrease, and at concentrations of more than 5 (w/v) %, the yields were steady. Premjet and others.22) reported that the largest yield was obtained at a 2 (w/v) % glucose dosage in HS medium with 0.5 (w/v) % CP powder ("SAN PEARL CP", Nippon Paper Indstries), commercially available wood based sulfite pulping waste liquor fraction isolated by ultra-filtration and spray drying. Our result agreed with that of Premjet and others (Premjet, 1994). The final pH of a culture decreased rapidly with the increase in glucose concentration. At a concentration of more than 5 (w/v) %, the pH was steady (Fig. III-5). The yield decrease with the increase in the glucose dosage could be due to the increases and accumulations of acidic metabolic substances such as gluconic acid. From these results, it was concluded that the optimum glucose concentration in this system was about 2 (w/v) % and that the presence of excessive glucose induced a lessening of pH resulting in a yield decrease.

Table III-3. Yields of BCCs, contents of WSChO-W and final pHs in HS medium with WSChO-W at various concentrations.

WSChO-W	Yields / mg		Contents / wt%		Final pHs	
concentrations / (w/v)%	1051	10245	1051	10245	1051	10245
0.000	35.9	31.1	-	-	3.78	3.72
0.005	31.2	29.0	1.51	1.30	3.82	3.73
0.050	34.4	30.9	3.89	0.65	3.90	3.85
0.100	37.0	37.3	2.59	0.65	3.93	3.90
0.250	44.0	38.3	3.89	0.00	4.19	4.09
0.500	49.2	44.6	4.97	2.39	4.55	4.41

The effect of poly L-lysin on BC productivity

The chitosan oligomer is cationic in water solutions. Köhle and others (Köhle, 1985) reported that poly-L-lysine hydrobromide, a kind of cationic polymer, as well as chitosan, has the ability to promote callose synthesis in soybean cells. This would be due to the cationic nature. To study the effect of cationic water-soluble polymer on BC productivity, *A. xylinum* was cultivated in HS medium containing 0.1 (w/v) % poly-L-lysine hydrobromide. No enhancing effect of the poly-L-lysine hydrobromide on BC productivity was observed (Fig. III-6). This suggests that the factors enhancing the BC productivity were not only the cationic nature of WSChOs.

Time course changes of yield, cell number, pH, and glucose concentration

The BCC yield in the medium containing WSChO-W increased throughout the incubation time, whereas the NBC yield in the standard medium reached a constant value after four days (Fig. III-7). The BCC yield at seven days was 1.7 times that of the control. The time course changes of glucose concentrations in HS and HS(WSChO-W) cultures were similar to each other (Fig. III-8). The glucose concentrations decreased rapidly from the first to the middle stage, and approached zero after five days. The cell growth curves in HS and HS(WSChO-W) media were similar to each other (Fig. III-9). The cell number increased with incubation time, then reached a constant value after five days. This result suggests that WSChO-W did not enhance the cell growth. The pH of the culture decreased rapidly after the beginning of incubation; then the pH in the HS(WSChO-W) culture gradually increased after passing the minimum value, whereas the pH in the HS culture was steady (Fig. III-10). The pH increase of the HS(WSChO-W) culture could be due to the utilization of acidic substances, such as gluconic acid, formed in the metabolic pathway because *A. xylinum* has the ability to synthesize cellulose using gluconic acid as a substrate, and BC was synthesized after five days at which time the glucose concentration approached zero (Figs. 7 and 8). In general, bacteria growth is a function of incubation time, and there are four periods in bacterial growth: (1) the induction phase, (2) the logarithmic growth phase, (3) the stationary growth phase, and (4) the death phase. In the stationary growth phase, bacterial activity decreases from the accumulation of metabolic substances, the consumption of nutrients and so on; then, the bacteria die and autolyze. In HS medium containing WSChO-W, BC was synthesized even after the stationary growth phase, whereas in the standard medium, BC production terminated after four days. From these results, we have concluded that WSChO activated the bacteria by some actions and that as a result, BC productivity is enhanced.

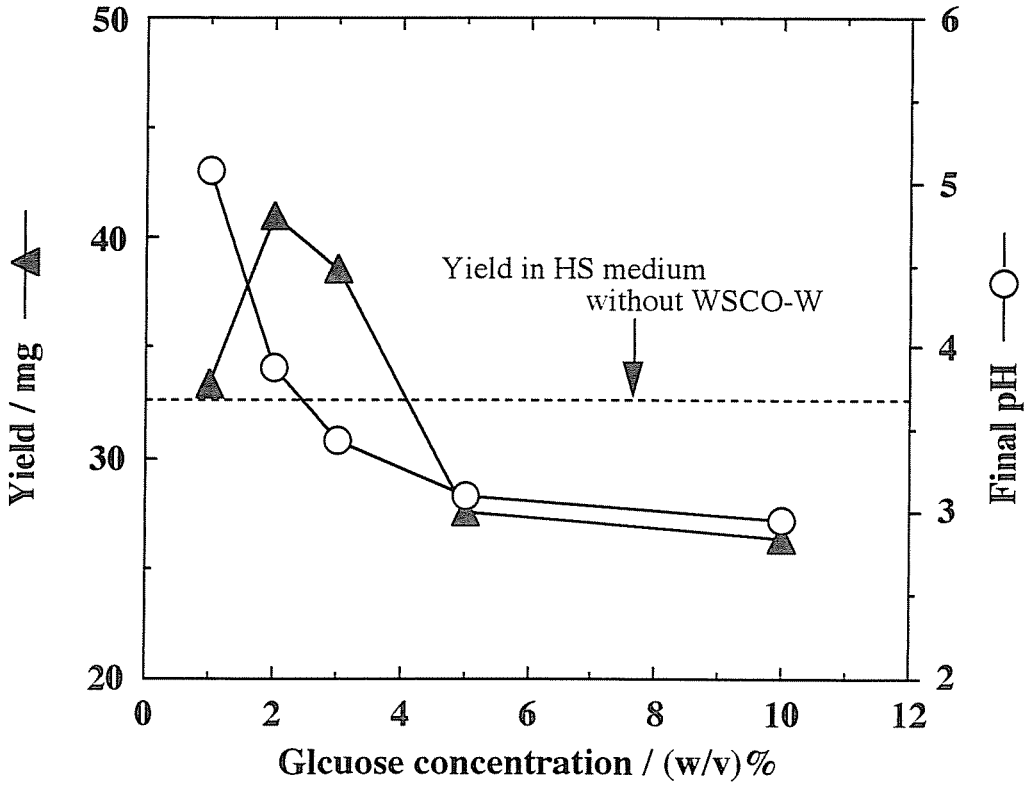


Fig. III-5. Yields and final pHs in HS medium containing 0.5 % WSChO-W at various glucose concentrations.

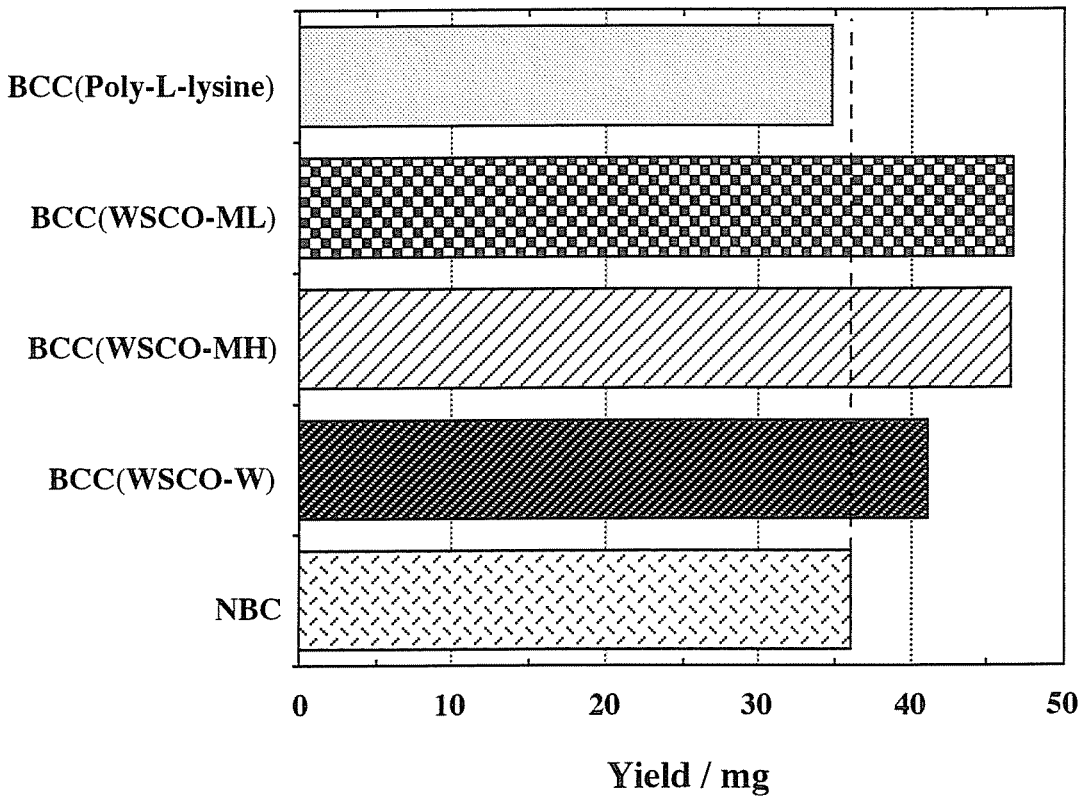


Fig. III-6. BCC yields in HS medium containing 0.1 (w/v) % poly-L-lysin, WSChO-ML, - MH, or -W.

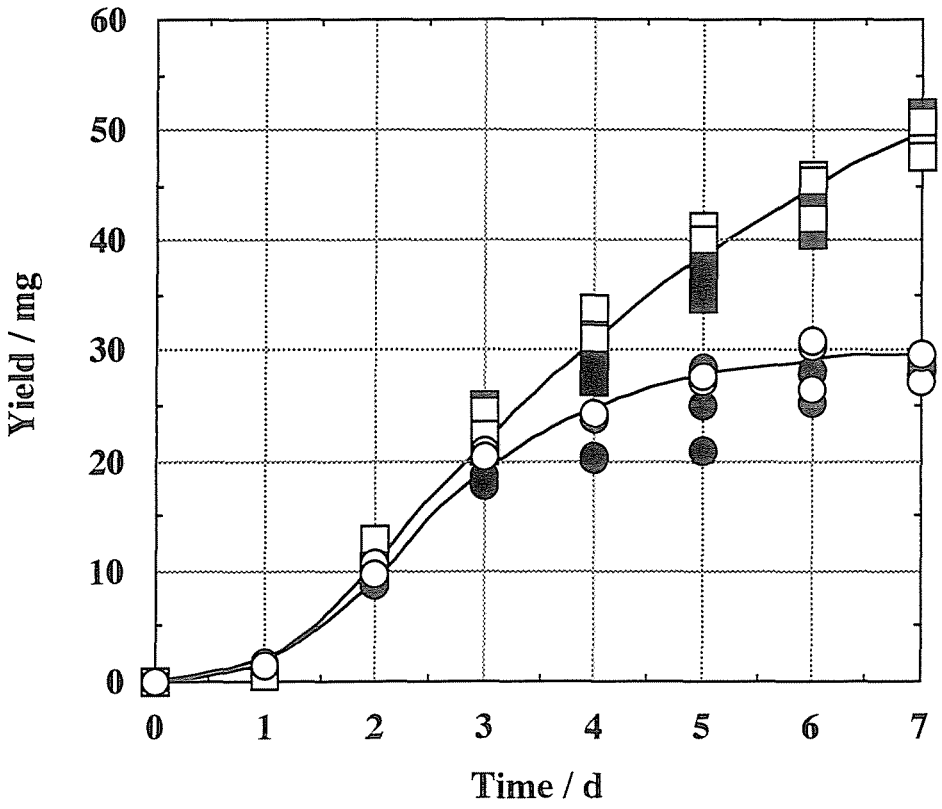


Fig. III-7. Time course changes of BCC yields,
○ : HS(1051), □ : HS(chitosan, 1051),
● : HS(10245), ■ : HS(chitosan, 10245) .

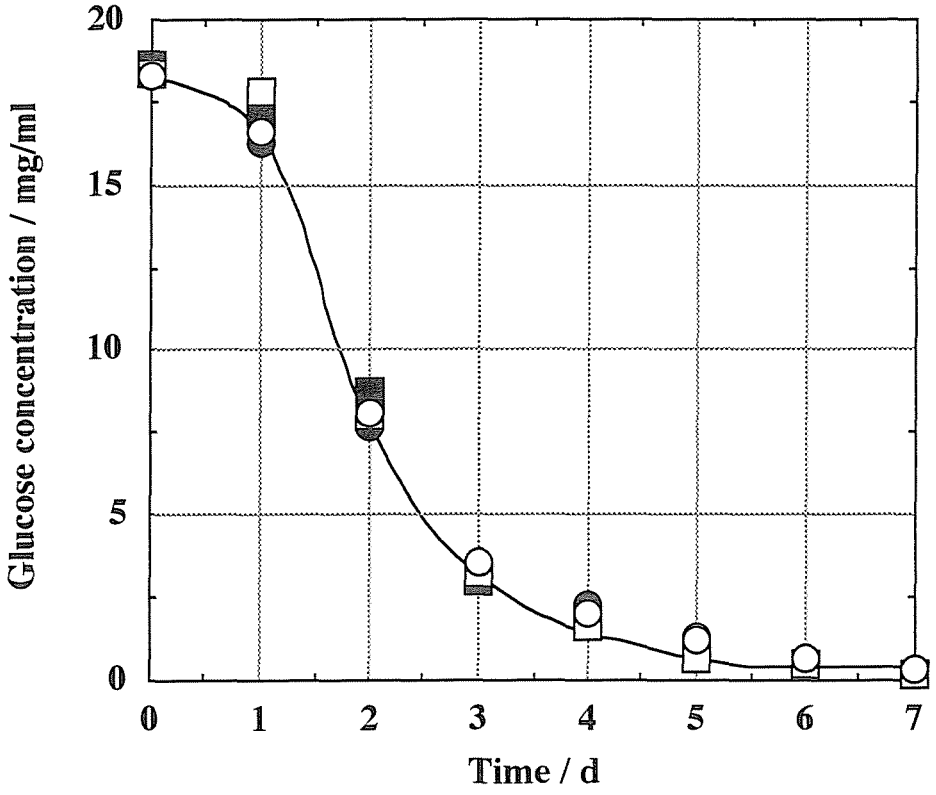


Fig. III-8. Time course changes of glucose concentrations:
○ : HS(1051), □ : HS(chitosan, 1051),
● ; HS(10245), ■ : HS(chitosan, 10245) .

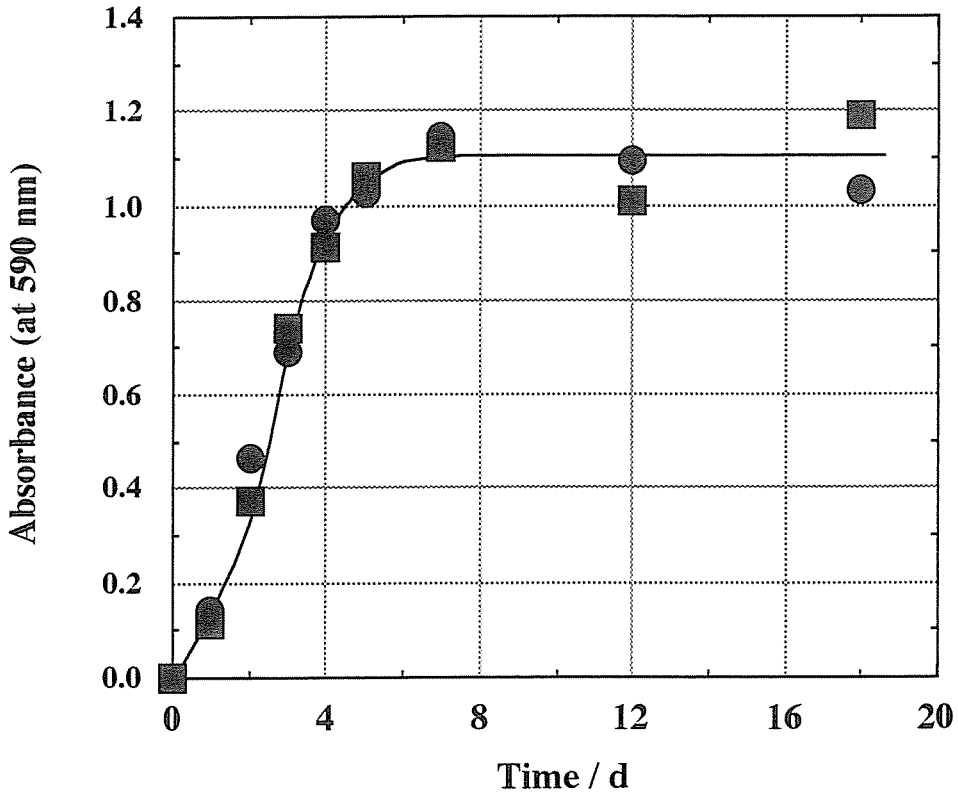


Fig. III-9. Time course changes of clture absorbance:
● : HS(10245), ■ : HS(chitosan, 10245) .

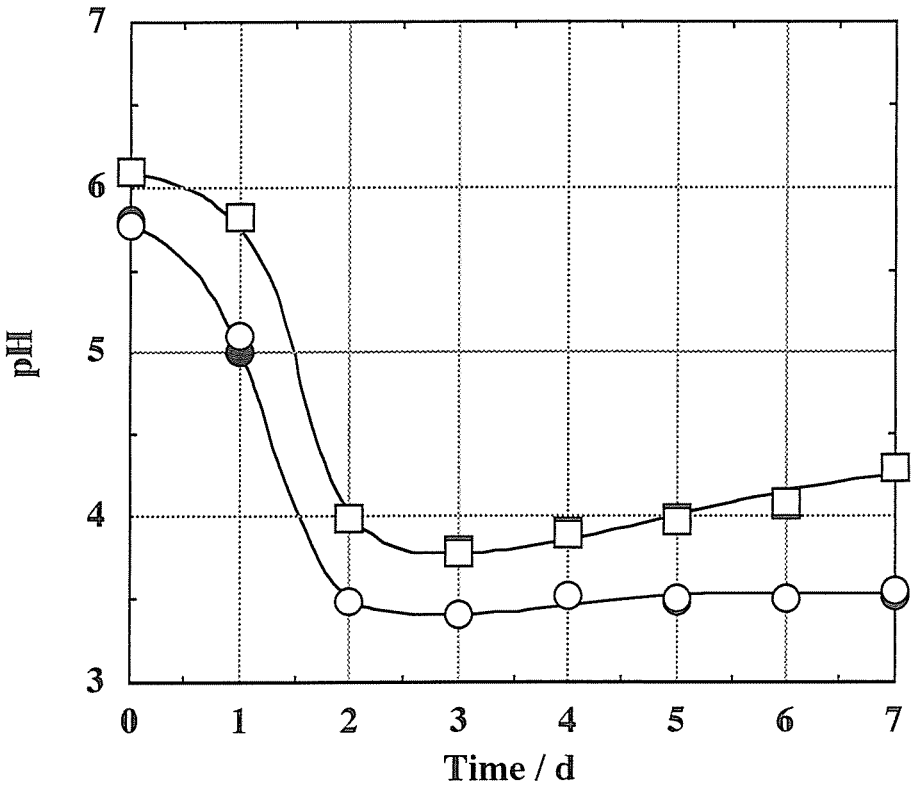


Fig. III-10. Time course changes of pH: ○ : HS(1051), □ : HS(chitosan, 1051), ■ : HS(10245), ● : HS(chitosan, 10245).

Conclusions

We obtained the following results: (1) BCC(WSChO) and BCC(WSChD) yields were about 1.3-1.7 and 1.0-1.6 times that of the control, respectively (2) the contents of WSChOs and WSChDs were 0-5 (w/w) % and 8-17 (w/w) %, respectively, and their structures were quite different, (3) in HS medium containing WSChO-W, BC was synthesized even after the stationary growth phase. From these results, WSChOs and WSChDs enhanced BC production with different mechanism each other, (2) WSChOs activated the bacteria by some actions and that, as a result, BC productivity was enhanced.

CHAPTER IV

Synthesis of Bacterial Cellulose Composite by Co-cultivation of *Acetobacter* Species

Introduction

In Chapters 2 and 3, BCCs were synthesized by addition of various water-soluble polymers. In this method, however, previous preparations of water-soluble polymers added are necessary. In order to synthesize BCC more conveniently, co-cultivation of bacterium which produces BC and one produces water-soluble polymer was attempted. Though various bacteria synthesize water-soluble polymer extracellularly, in this report, *Acetobacter* was used as a producer of WSP with similarity of incubation conditions in mind.

The production of water-soluble polysaccharides by *A. xylinum* NCI 1005 and their structural analysis by NMR spectroscopy and the BCC synthesis by co-cultivation of two *Acetobacter* species were discussed in Chapter IV-1 and 2, respectively.

CHAPTER IV-1

The Production of a New Water-Soluble Polysaccharide by *Acetobacter xylinum* NCI 1005 and Its Structural Analysis by NMR Spectroscopy

Introduction

Some species of the Gram-negative aerobe *A. xylinum* are able to synthesize cellulose as an extracellular polysaccharide (Brown, 1886), whereas other species of *Acetobacter* are able to produce either water-soluble polysaccharides (Minakami, 1984, Tayama, 1985, Tayama, 1986) or both cellulose and WSPs (Couso, 1982, Valla, 1981). Tayama et al. (Tayama, 1986) have reported that, when grown on glucose, *A. xylinum* NCI 1005 produces a new type of water-soluble acidic heteropolysaccharide, tentatively designated as AM-1. The polysaccharide AM-1 is composed of D-glucose, D-galactose, D-mannose, and D-glucuronic acid. We have observed that *A. xylinum* NCI 1005 produces a WSP which is structurally different from AM-1 when grown on a sucrose medium. These results suggest that the synthesis of a WSP can be controlled by changing the carbon source of the medium.

In chapter IV-1, the structure of the WSP from NCI 1005 has been analyzed in detail by nuclear magnetic resonance (NMR) spectroscopy.

Materials and Methods

Preparation of the water-soluble polysaccharide

A. xylinum NCI 1005 was available from Nakano Vinegar Co. Ltd. Fifty milliliters of a 72-h seed culture of *A. xylinum* NCI 1005 was inoculated into 1,500 mL of SYP medium (6% sucrose, 1% yeast extract, and 0.5% polypeptone in distilled water) and incubated in a shaking culture at 120 strokes/min or in a static culture at 28 °C for 7 days. Cells were removed by centrifugation at 8,000 rpm. for 15 min. Ethanol (four times the volume as that of the supernatant) was added to the supernatant and maintained at 4 °C overnight. A precipitate formed and was collected by centrifugation at 8,000 rpm for 15 min, and the precipitated WSP was dissolved in 15 mL of deionized water. Activated charcoal was added into the solution to remove residual organic compounds, and the solution was stirred for 10 min. The activated charcoal was removed by centrifugation and ultrafiltration, and the filtrate was dialyzed against deionized water at 4 °C for 2 days. The dialyzed solution was lyophilized.

Acidic hydrolysis of the water-soluble polysaccharide

The purified polysaccharide was hydrolyzed with 0.5 N H2SO4 solution at 70 °C for 3 h. The hydrolysate was filtered through a 0.45-mm membrane filter, and the filtrate was analyzed and fractionated by high-performance liquid chromatography (HPLC) with a SP 8010 column (Showa Denko Co., Ltd.) and G4000PW column (Tohso Co., Ltd.), respectively.

Structural analysis of the water-soluble polysaccharide by NMR spectroscopy

NMR spectra of the hydrolysate and WSP were obtained at 100 MHz for 13C and 400 MHz for proton with a Bruker MSL400 spectrometer. 13C NMR spectra were accumulated with a 90° pulse, 5.3 ms, 25,000 Hz spectral width, and a 2 s repetition rate. 1H decouplded NMR spectra were accumulated with a 90° pulse, 4.0 ms, 3,000 Hz spectral width, and a 4 s repetition rate.

Homonuclear shift-correlated (COSY) spectra were obtained using spectra widths of 850 Hz and 425 Hz, with 1K and 256 data points in the F2 and F1 dimensions respectively, and zero-filled to 2 K and 512 respectively. The recycle time was 2 s and the total accumulation time was approximately 1 h on a D2O sample solution in 5 millimeter outside-diameter spinning sample tubes at 20 °C. Heteronuclear shift-correlated two-dimensional (2D) experiments were carried out using an F2 spectral width of 2500 Hz and an F1 width of 250 Hz; 64 time points were accumulated with 1K and 128 data points in F2 and F1, respectively. The recycle time was 1.5 s. The accumulation time was over 2 h, and the 2D matrix was zero-filled (256 Å~ 2 K) and converted with a Gaussian and Lorentz function in the F1 and F2 dimensions, respectively.

Determination of molecular weight

The molecular weight of polysaccharide was determined by gel-permeation chromatography

(GPC) with G4000PWXL + G2500PWXL (Tohso Co., Ltd.).

Determination of the culture component

The concentration of each component in the culture was determined by HPLC with a SP 8010 column (Showa Denko Co., Ltd.).

Results and Discussion

Acidic hydrolysis of a water-soluble polysaccharide

In the HPLC pattern of the acidic hydrolysate of the WSP, only one peak was observed (data are not shown). The retention time for the peak was the same as that of fructose. By comparison of the 1H NMR spectra for fructose and the hydrolysate, it was confirmed that the hydrolysate was fructose (data are not shown). These results suggest that the WSP synthesized by *A. xylinum* NCI 1005 from sucrose is a homopolymer of fructose, polyfructan. Polyfructans are generally subdivided into two main classes: levan, in which the main linkage is β(2→6), and inulin, in which the main linkage is β(2→1). To determine the structure of the polyfructan, 1H, 13C, 1H-1H, and 13C-1H NMR spectra were measured.

Structural analysis of water-soluble polysaccharides by NMR spectroscopy

Figure IV-1-1 shows 1H NMR spectra of the WSP and levan from Erwinia herbicola (Sigma Co., Ltd.). No difference was observed between these spectra, except that the spectrum of levan was broadened slightly due to its high molecular weight. Peaks corresponding to the glycosidic proton were not observed in the 1H NMR spectrum of WSP, which suggests that there are very few or possibly no glycosidic protons occurring in the WSP. The 13C NMR spectra of the WSP and of levan are the same (Fig. IV-1-2). There are six peaks in the spectra, and these peaks correspond to the carbons constructing the fructose residues. The simplicity of the spectra reflects that the WSP is a homopolymer with fructose. From the comparison of the proton-decoupled and undecoupled 13C NMR spectra of the WSP, it could be suggested that the glycosidic carbon (106.88 ppm) has no linkage with the hydrogen. This is consistent with the absence of an apparent glycosidic proton in the 1H NMR spectra of WSP. The peaks at 82.97, 79.00, and 77.89 ppm and those at 66.08 and 62.62 ppm correspond to the CH and CH2 carbons, respectively.

To determine the assignments for the 1H, 1H chemical shift correlation, the NMR spectrum (COSY) was measured (Fig. IV-1-3). The peak at the highest field appears to be the 6"-proton, and the assignment of the 6'- and 5'-protons can be determined by correlation with the 6"-protons. In the same manner, the assignment of 4'- and 3'-protons can be determined by their correlation with the 5'- and 4'-protons, respectively. The peaks at 3.6-3.8 ppm do not correlate with the other protons, and therefore, they can be assigned to the 1'- and 1"-protons, respectively. The difference in chemical shift between the 6'- and 6"-protons is very large, which could be due to the shielding effect of the electron cloud of oxygen. Oxygen in the fructose residue and glycosidic oxygen takes an sp2 hybrid orbital, and the peak for the 6"-proton appears at the highest field due to the shielding of the 2pz-electrons in the glycosidic oxygen.

Figure IV-1-4 shows the 13C-1H 2D NMR spectrum of the WSP. From the correlation between C-H, each peak has been assigned as follows: C-1 (62.6 ppm), C-6 (66.0 ppm), C-4 (77.9 ppm), C-3 (79.0 ppm) and C-5 (83.0 ppm). C-1 and C-6 each correlate with two protons,

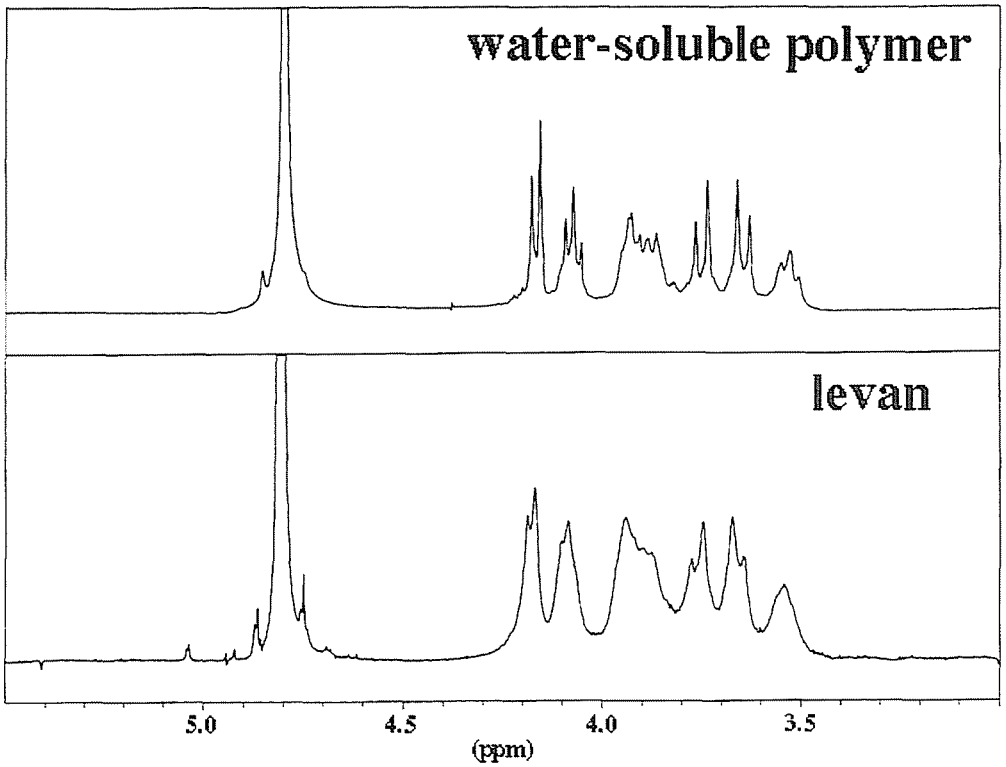


Fig. IV-1-1 1H NMR spectra of the water-soluble polymer and levan from Erwinia herbicola.

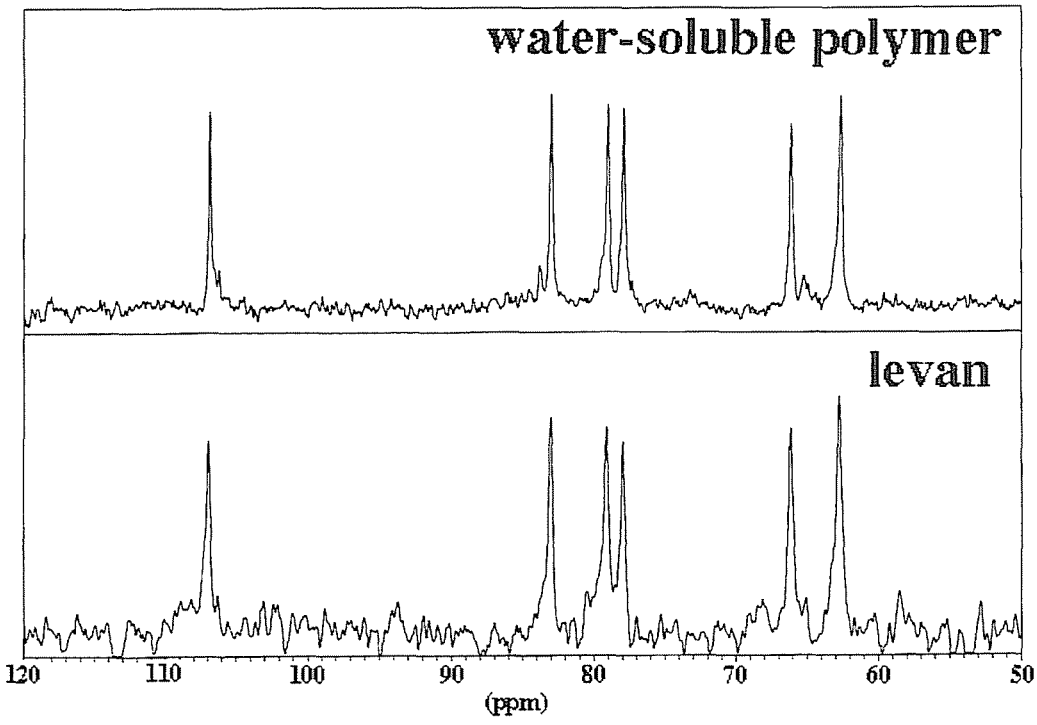


Fig. IV-1-2 13C NMR spectra of the water-soluble polymer and levan from Erwinia herbicola.

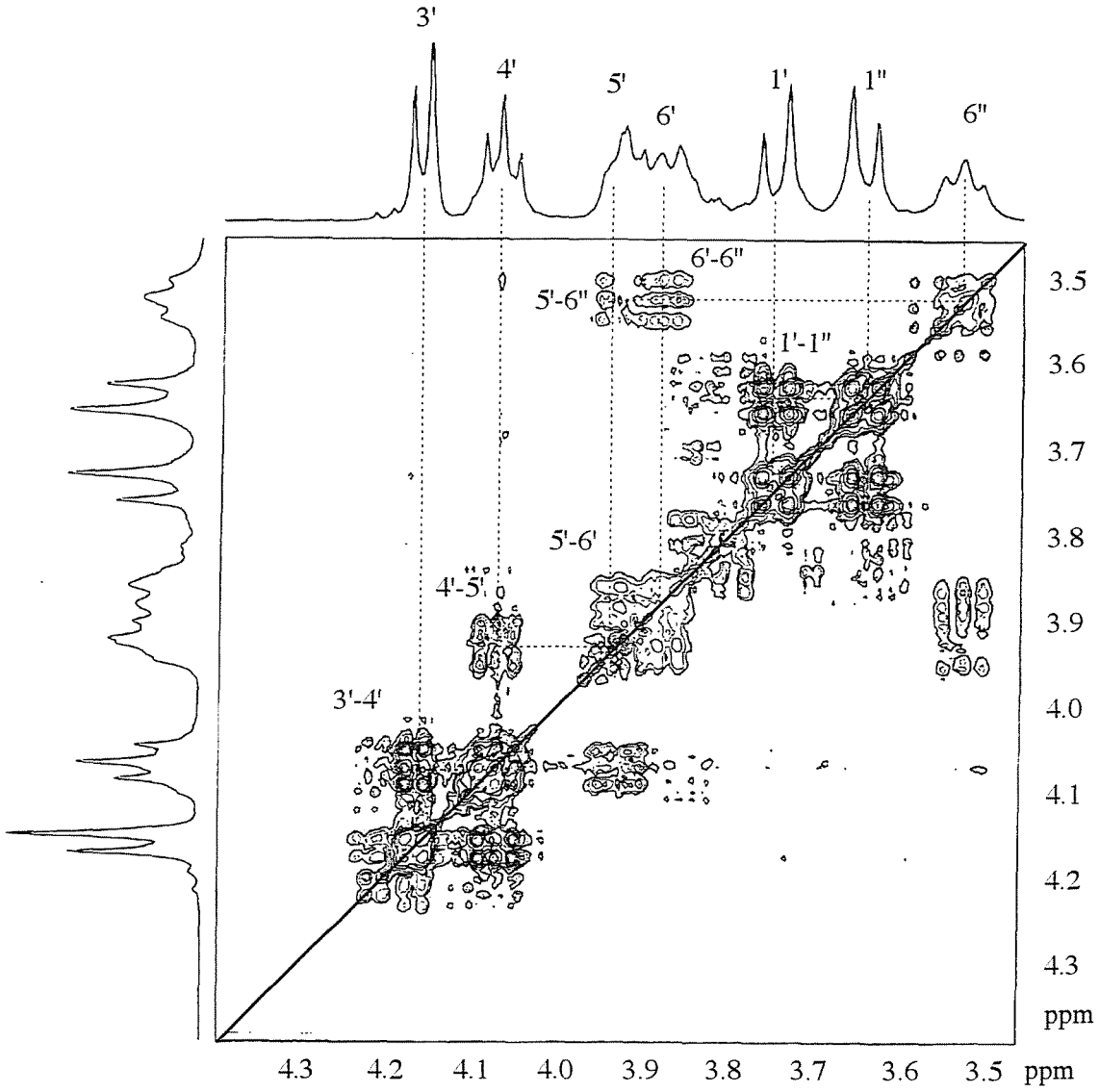


Fig. IV-1-3 1H-1H chemical shift correlation NMR spectrum of the water-soluble polymer.

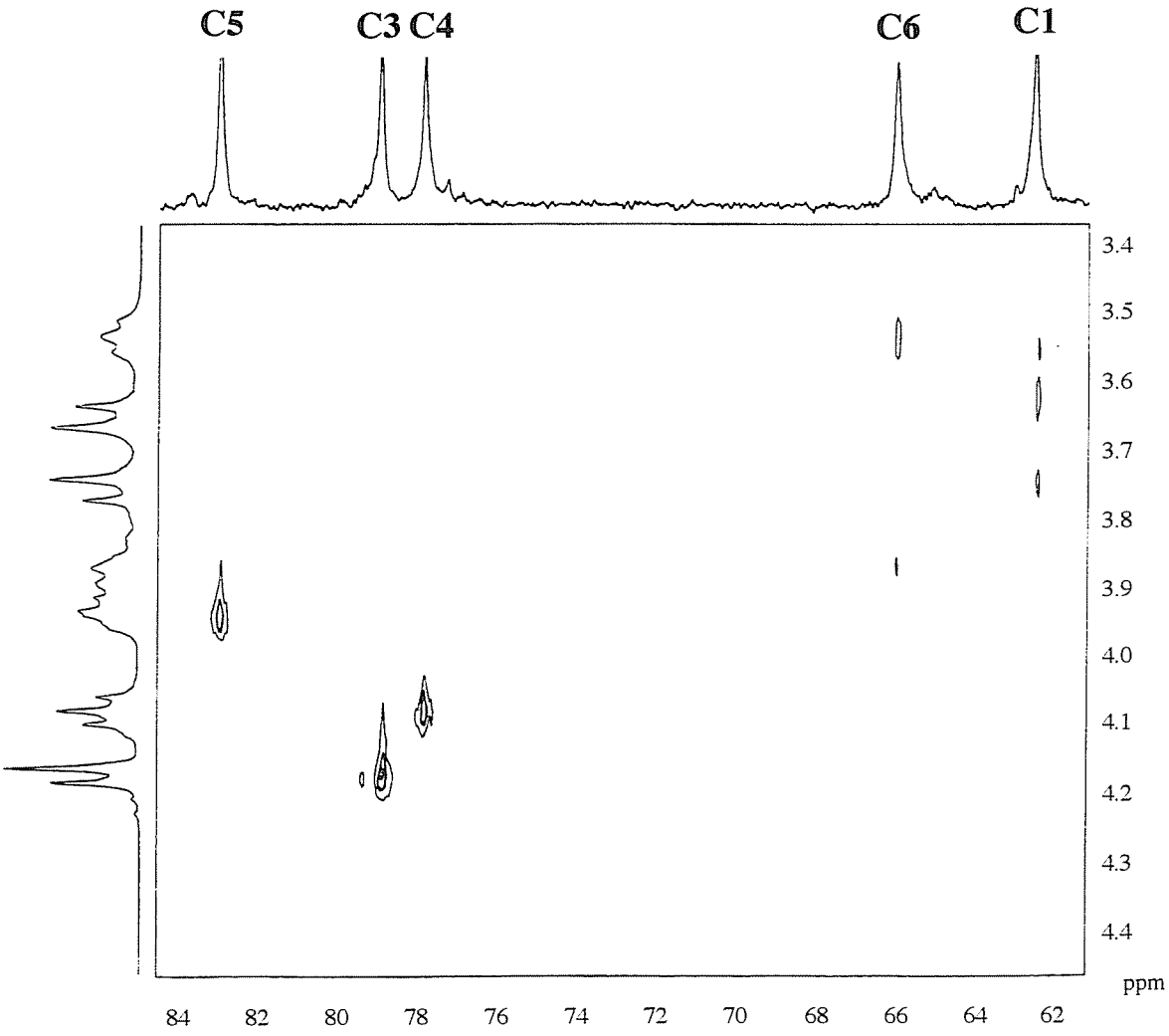


Fig. IV-1-4 13C-1H NMR spectrum of the water-soluble polymer.

which corresponds with results from the non proton-decoupled 13C NMR spectrum. C-4, C-3 and C-5 each correlate with one proton.

From these results, the structure and the peak assignments for the 13C NMR spectra of the WSP have been determined, as shown in figure IV-1-5 and Table IV-1-1, respectively. The differences in the chemical shifts of C-5 in inulin, β(2→1) polyfructan, and levan, β(2→6) polyfructan, were the most distinct, with a different of 6.1 ppm, and the chemical shift of the WSP from NCI 1005 was closer to that of levan. Thus, a combination of NMR techniques has demonstrated that the WSP synthesized by *A. xylinum* NCI 1005 from sucrose is a β(2→6)-linked polyfructan, namely levan.

Production of a water-soluble polysaccharide

Figure IV-1-6 shows the time-course changes in the pH, the absorbance at 540 nm which is a index of cell number, and the concentration of each component (levan, fructose, glucose, and sucrose) in the culture of *A. xylinum* NCI 1005. Levan, glucose, and fructose appear, with a rapid decrease in sucrose. That glucose and fructose were detected in the culture suggests that sucrose was hydrolyzed by extracellular or membrane-binding enzymes. The sucrose levels decreased rapidly, and levan began to appear in 3 days. The levels of glucose and levan increased gradually and reached their maximal concentrations in 5 and 7 days, respectively. After reaching its maximum, the amount of levan decreased gradually, while, the glucose disappeared in 9 days. The fructose levels remained constant throughout the incubation time, and it was observed that the concentrations of glucose and fructose in the culture were not the same. Because sucrose is composed of both glucose and fructose residues, the difference in the time-course changes of their concentrations suggests that their metabolic pathways in the bacterium are different. The culture pH fell from 6.8 to 3.0, indicating the formation of acidic substances. The levan had two components, with low and high molecular weight fractions. The high molecular weight fraction disappeared at an earlier period, while the low molecular weight fraction remained even at later periods. The molecular weight and the yield of levan decreased with the incubation time after 5 and 7 days, respectively. This could be due to the hydrolysis of levan by acidic metabolic substances secreted from the bacterium, because levan is hydrolyzed rapidly in an acidic solution (pH 3.0), and the bacterium has a weak ability to metabolize levan.

Many researchers have reported that levan is produced from sucrose by plants (Carpita, 1991) as well as a variety of microorganisms such as *Streptococcus salivarius* (Simss, 1990), *Bacillus polymyxa* (Han, 1990), *Zymomonas mobilis* (Barrow, 1984), *Acetobacter diazotrophicus* (Hernandez, 1995), and so forth. These bacteria have levansucrase (EC 2.4.1.10), which specifically reacts with sucrose, synthesizing levan and releasing glucose. In our experiment, it was observed that levan, glucose, and fructose appeared in the culture as sucrose levels rapidly decreased, suggesting that *A. xylinum* NCI 1005 expresses an extracellular or membrane-binding levansucrase.

Tayama et al. (Tayama, 1986) have reported that *A. xylinum* NCI 1005 synthesizes a acidic

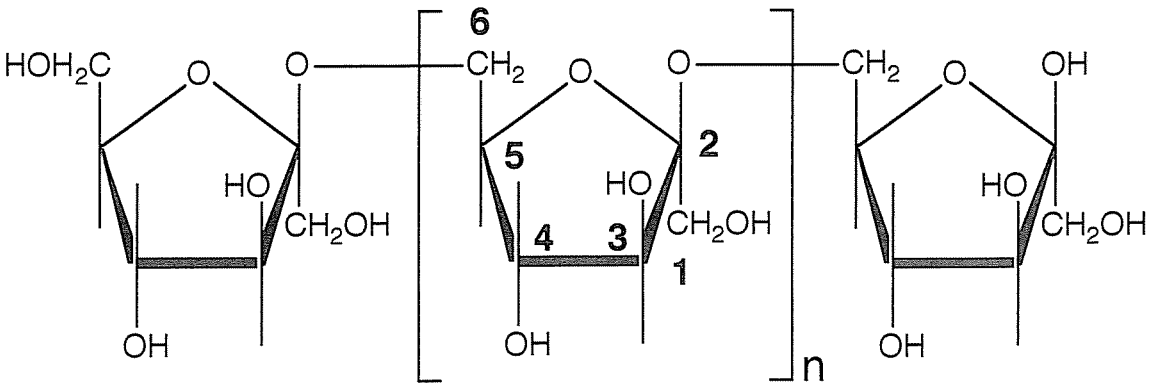


Fig. IV-1-5 Structure of the water-soluble polymer.

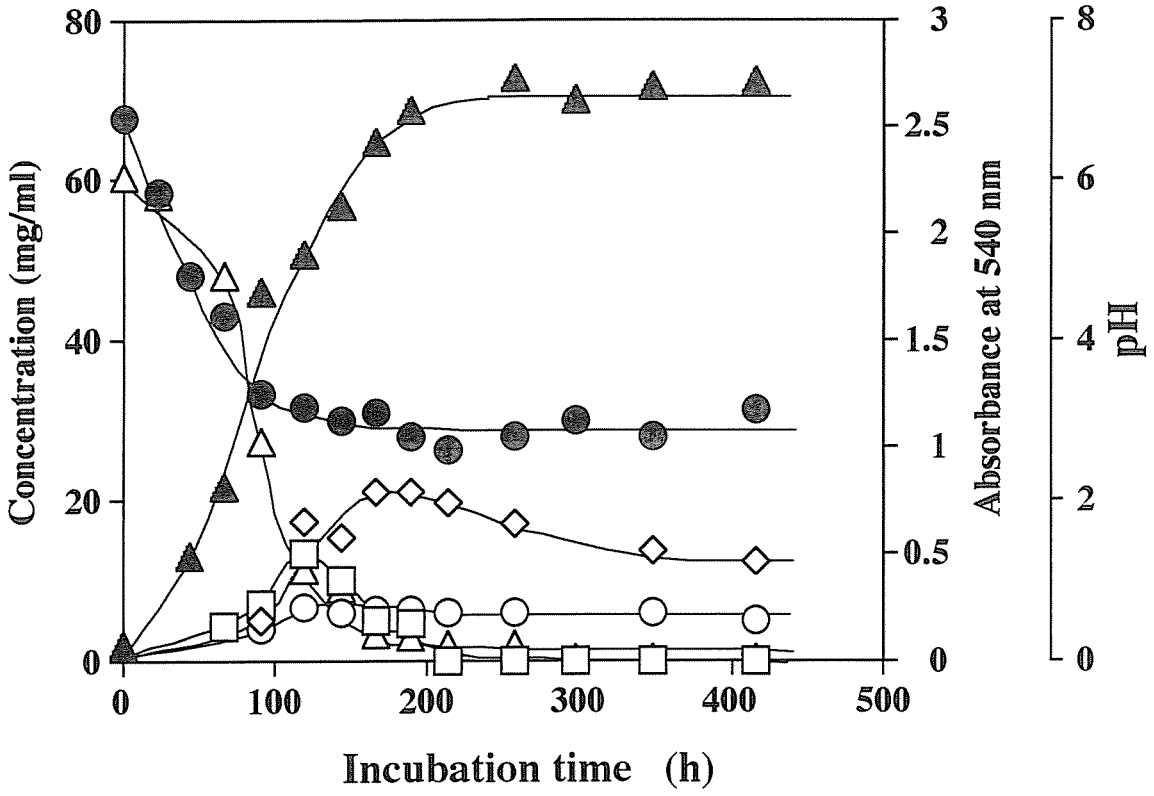


Fig. IV-1-6 Time-course changes in the pH(●), the absorbance at 540 nm, which is a index of cell number(▲), and the concentration of each component (levan(◇), fructose(○), glucose(□), and sucrose(△)) in the culture of *A. xylinum* NCI 1005.

water-soluble heteropolysaccharide (AM-1, Fig. IV-1-7, 8) from glucose. Similarly some quantity of the AM-1 was observed in our experiment, although the amount was smaller than that from glucose. This could, however be due to a low concentration of glucose in the culture when sucrose was used as a carbon source. We have observed that *A. xylinum* NCI 1005 produces levan which is structurally different from AM-1 when grown on a sucrose medium. Therefore, it is clear that *A. xylinum* NCI 1005 is a very interesting bacterium which can be produce two kinds of water-soluble polymers, suggesting that the synthesis of WSP can be controlled by changing the carbon source of the medium.

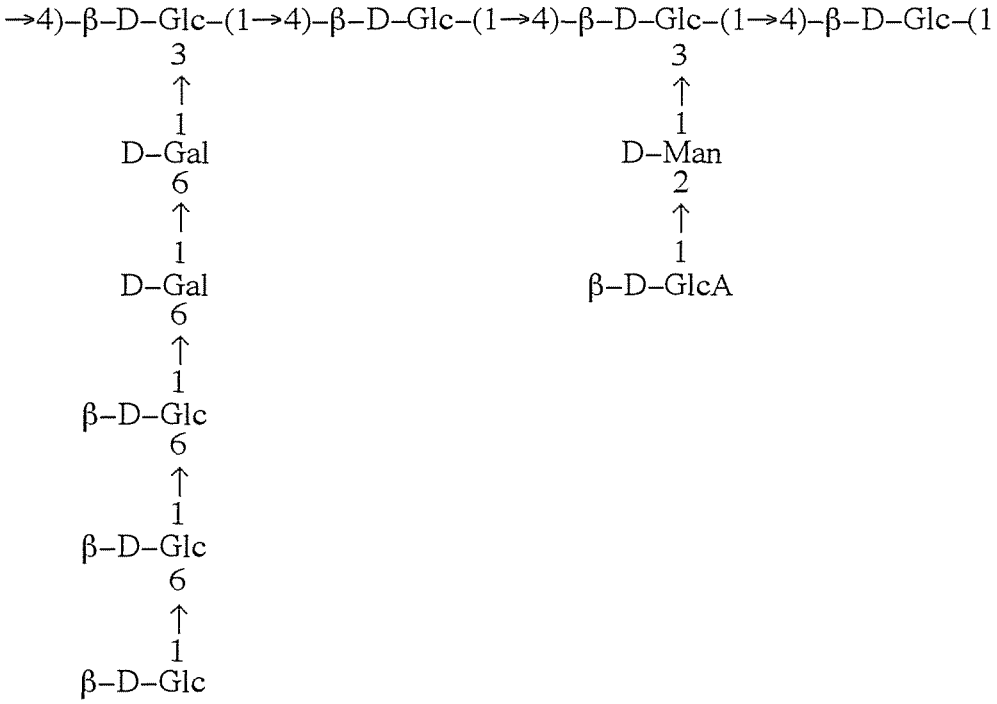


Fig. IV-1-7 Proposed structure of the water-soluble polymer from sucrose and glucose (AM-1).

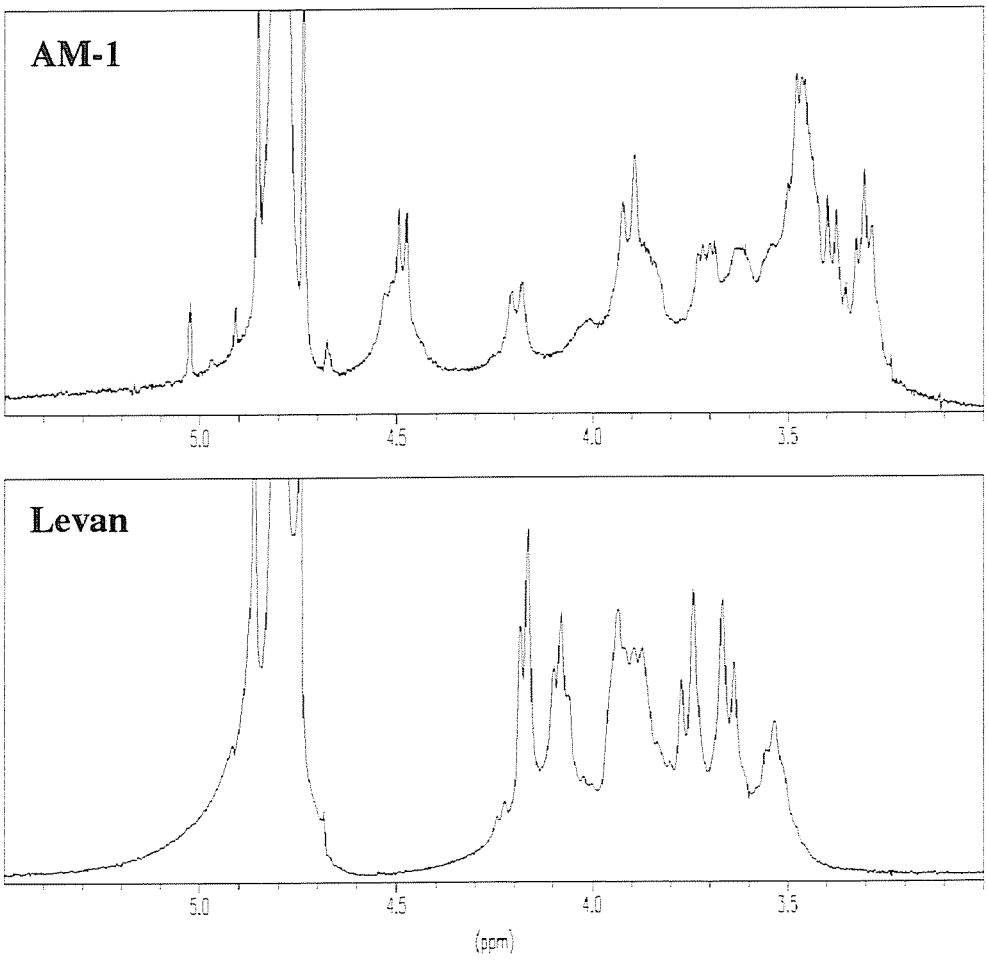


Fig. IV-1-8 1H NMR spectra of the water-soluble polymer from sucrose and glucose (AM-1).

Conclusions

A new water-soluble polysaccharide was isolated from a culture of *A. xylinum* NCI 1005 grown on sucrose. The structure of the WSP was analyzed by nuclear magnetic resonance spectroscopy and determined to be a β(2→6)-linked polyfructan, which is structurally different from the polymer synthesized from glucose instead of sucrose by the same strain. The discovery of this new polysaccharide has revealed that the bacterium is able to synthesize two different kinds of water-soluble polysaccharides.

In Chapter IV-2, the BCC synthesis by co-cultivation of two *Acetobacter* species, NCI 1005 and ATCC 10245 or NCI 1051, were reported.

CHAPTER IV-2

Co-Cultivation of *Acetobacter* Species

Introduction

As discussed in Chapters 2 and 3, BCCs could be synthesized by addition of various water-soluble polymers. In this method, however, previous preparations of water-soluble polymers added are necessary. In addition, the BC cost is very high and the application of BC is limited to high-value products, because the general carbon source, glucose is pretty expensive. The use of the cheaper carbon sources might be able to directly reduce the production cost. Sucrose easily obtained from sugar beet or sugar cane seems to be a better source for BC production. Sucrose is composed of glucose and fructose, and fructose is also a good carbon source. However, the BC yield from sucrose is only half that from glucose. It is due to the low metabolic activity for sucrase in BC-producing *A. xylinum*. If sucrose is efficiently hydrolyzed, it would increase the quantity of BC produced by the microorganism. And sucrose would be able to be a reasonable carbon source for BC production.

In order to synthesize BCC more conveniently and cheaply, co-cultivation of bacterium producing BC and one producing water-soluble polymer was attempted. Though various bacteria synthesize water-soluble polymers extracellularly, *Acetobacter* sp. was used as a producer of WSP with similarity of incubation conditions in mind. Many researchers have reported that some species of *Acetobacter* are able to produce water-soluble polysaccharides (Minakami, 1984, Tayama, 1985, Tayama, 1986) and other species produce both cellulose and water-soluble polysaccharides (Colvin, 1977, Sandermann, 1979, Valla, 1981, Couso, 1982, De Iannino, 1988). Tayama et al. (Tayama, 1986) have reported that, when grown on glucose, *A. xylinum* NCI 1005 produces a water-soluble β(1→4) linked glucan (AM-1). As shown in Chapter II-1, we have found that *A. xylinum* NCI 1005 produced a water-soluble polymer which was structurally different from AM-1 when grown on a sucrose medium, and the structure of the polymer was determined to be a β(2→6)-linked polyfructan.

In this chapter, we have tried to synthesize BCC by co-cultivation of BC-producing and WSP-producing *A. xylinum*. And we have examined the yield of the BCC synthesized in this method and characterized them, carefully.

Material and Methods

Preparation of Normal Bacterial Cellulose and Bacterial Cellulose Composite

A. xylinum NCI 1051 (Nakano Vinegar Central Institute, Nakano Vinegar Co. Ltd.) and ATCC 10245, which utilize glucose as a carbon source, were used for the BC production. *Acetobacter xylinum* NCI 1005, which utilizes sucrose as a carbon source, was used for the WSP production. Five hundred microliters of a 72 hour seed culture of *A. xylinum* (NCI 1051 or ATCC 10245) and 0.5, 0.25, 0.125, 0.05, 0.005 ml of a 72 hour seed culture of *A. xylinum* (NCI 1005) were inoculated into 18.5 ml of modified Hestrin & Schramm's medium (2% carbon source, 0.5 % bactopeptone, 0.5 % yeast extract, 0.115 % citric acid and 0.27 % disodium hydrogen phosphate anhydrous in distilled water, pH 6.00). It was then incubated in a static culture at 28 °C for 7 days. Membranes in a highly swollen condition were taken from the surface of the culture. They were soaked in 1 % NaOH solution for 7 days to remove all alkali-soluble components, then washed with distilled water and 1 (v/v)% acetic acid to neutralize the last traces of alkali for 3 days. Finally they were washed with large volumes of distilled water for 4 days and dried at room temperature on glass plates.

NBC was obtained by the similar method besides using only BC-producing bacterium

Enzymatic hydrolysis

NBC and BCC were degraded in 5 ml of citric acid-phosphate buffer containing 5 mg cellulase (Onozuka R-10; Yakult Honsha Co., Ltd.) at 120 strokes / min for 48 hours at 50 °C. The degraded samples were filtered through a 0.45 mm Tosoh filter, and 20 ml of the samples was chromatographed by HPLC on a SP 0810 column (8 mm × 300 mm; Showa Denko Co., Ltd.). The concentrations of glucose released from NBC or BP-BC composites were determined using the Glucose CII Test Wako.

X-ray Diffraction, IR spectrum

These procedure were described in Chapter II-1.

Results and Discussion

Selection of carbon source

In order to determine the most suitable carbon source for BCC synthesis, co-cultivations in media containing various carbon sources were attempted. Table IV-2-1 shows growth of NCI 1005, contents of WSP, and BCC yields in media containing various carbon sources. The WSP contents and BCC yields from sucrose were larger than those from the other carbon sources, and, therefore, sucrose was used as a carbon source to synthesize BCC in the subsequent experiments

Yield of BCC and content of water-soluble polymer in BC composite

The yields of the NBCs and BCCs are shown in Fig. IV-2-1. The BCC yields increased with a decrease in the inoculation volume of NCI 1005, and all yields were larger than those of NBC (HS(Sucrose)) and NBC(HS).

In order to determine the contents of levan in the BCCs, enzymatic hydrolysis and liquid chromatography analysis were performed. Liquid chromatograms of the enzymatic hydrolysates of the BCCs showed the peak of levan in addition to that of glucose (Fig. IV-2-2)). The contents of levan in BCCs estimated from the liquid chromatograms of enzymatic hydrolysates were 20-30 wt% in BCC(levan)s from 1051 and 5-15 wt% in BCC(levan)s from 10245. The contents of levan in BCCs from 1051 were larger than those from 10245, while the cellulose yields were similar in all conditions, suggesting that levan did not enhance the BC production. The weight of cellulose in the BCC was greater than that synthesized from sucrose by mono-cultivation (Fig. IV-2-3), and it was suggested that, in sucrose medium, the BC-productivity of *A. xylinum* for co-cultivation was superior to that for mono-cultivation. This would be due to the hydrolysis of sucrose by levansucrase of NCI 1005. Thus, it was concluded that the co-cultivation of the two types of *Acetobacter*s, one hydrolyzing sucrose and another one synthesizing cellulose, will be a useful method for the BCC synthesis and the efficient BC-production using sucrose as the carbon source.

Hydrolysis of sucrose by *Acetobacter*s

As shown in Figure IV-1-5, sucrose was rapidly hydrolyzed, and, at the same time, levan, glucose, and fructose were formed. Glucose and fructose reached maximal concentrations after three days, and then gradually decreased. From these results, it could be suggested that glucose and fructose formed by the reaction of levansucrase. Figure IV-2-4 shows the time course change of the sucrose concentration in the culture medium in which the species of acetic acid bacterium was cultivated. For NCI 1051 and ATCC 10245, sucrose was not almost metabolized during the first stage. After 5 days, small amounts of glucose and fructose were then released. These glucose and fructose could be formed upon hydrolysis of levansucrase. For NCI 1005, sucrose was rapidly metabolized. Thus, the low BC-productivities of ATCC 10245 and NCI 1051 using sucrose

Table IV-2-1 Growth of NCI 1005, contents of WSPs, and BCC yields in medium containing various carbon sources.

Carbon source	Inoculated strains	Growth of NCI1005	Contents of WSPs	BCC Yields
Glucose	1051	-	-	++
Glucose	1051+1005	-	-	++
Glycerol	1051+1005	+	-	-
Sucrose	1051+1005	+	+	++
Glucose	10245	-	-	++
Glucose	10245+1005	-	-	++
Glycerol	10245+1005	+	-	-
Sucrose	10245+1005	+	+	++

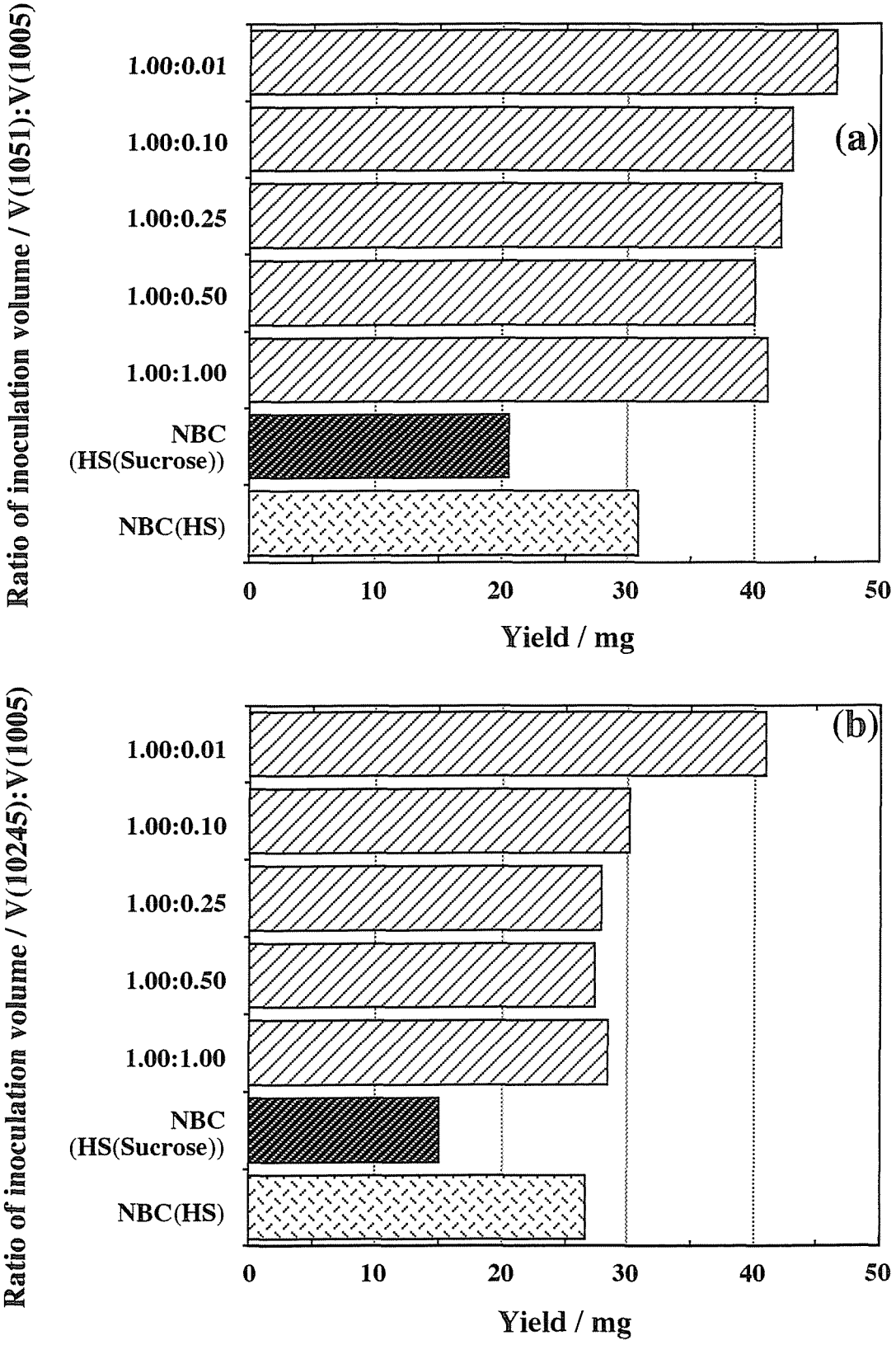


Fig. IV-2-1 Yields of BCC: (a); NCI1051+NCI1005; (b); ATCC10245+NCI1005

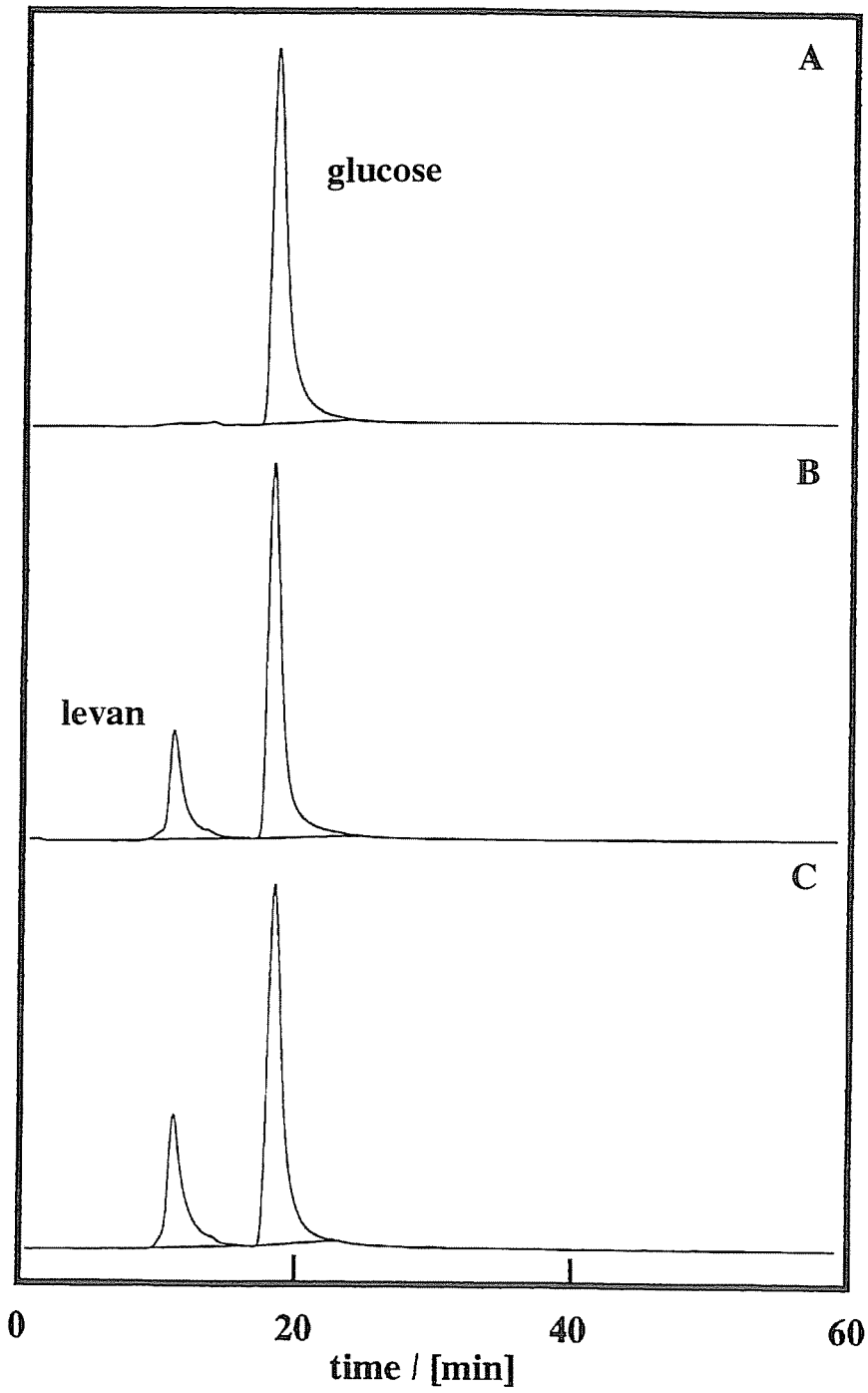


Fig. IV-2-2 HPLC patterns of the enzymatic hydrolysates: A; NBC; B; BCC(1.00:1.00); C; BCC(1.00:0.01). Strains: NCI 1051 and NCI 1005.

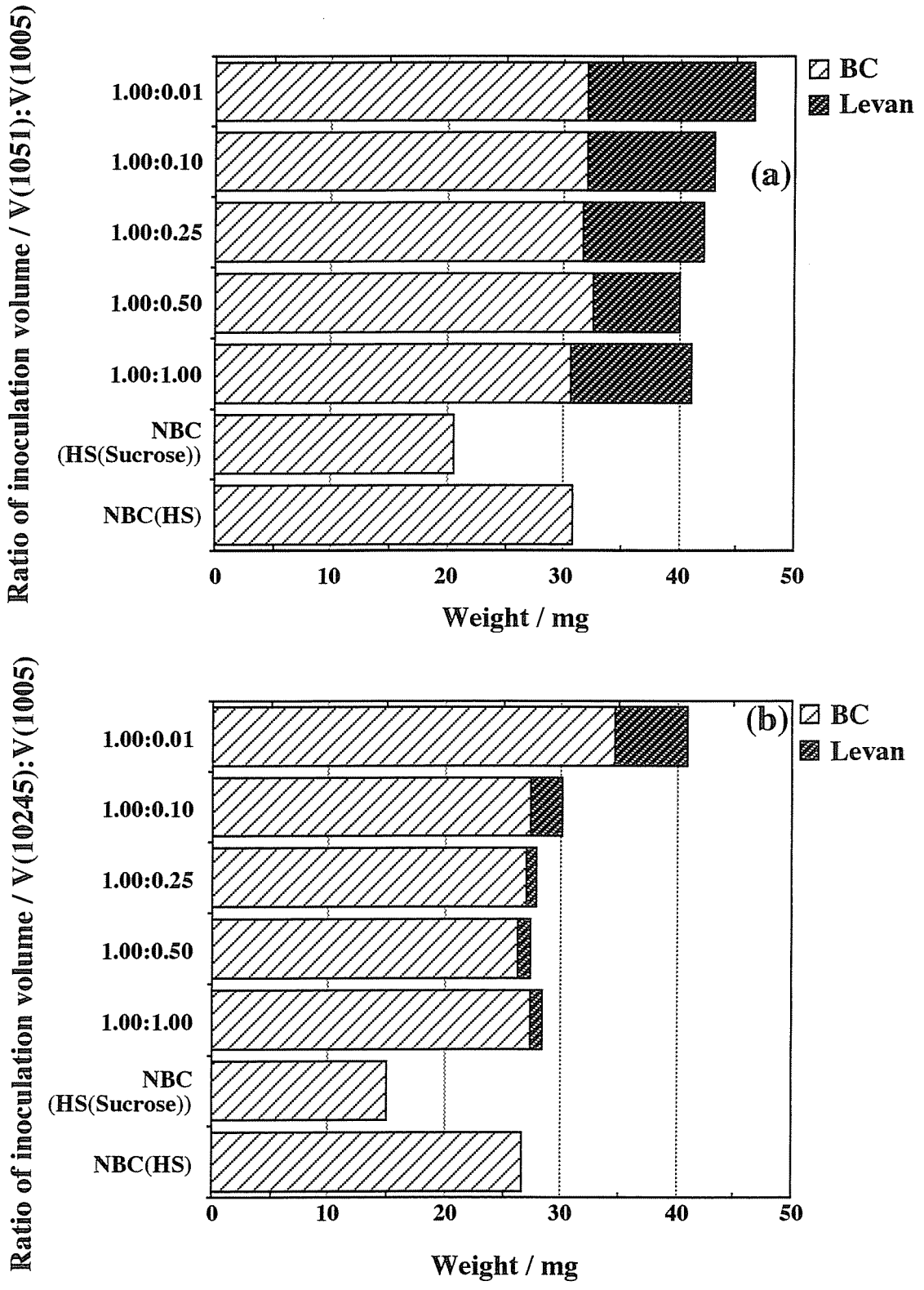


Fig. IV-2-3 Weights of BC and levan in NBC or BCC: (a); NCI1051+NCI1005; (b) ATCC10245+NCI1005

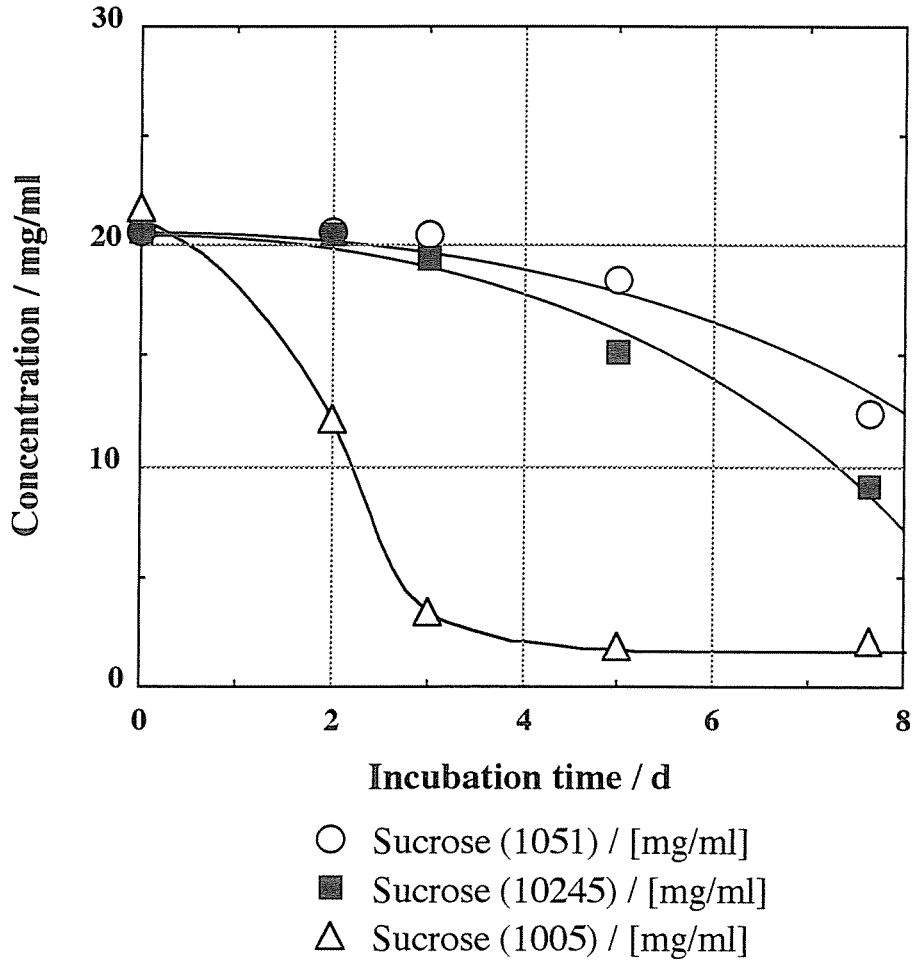


Fig. IV-2-4 Time course change of sucrose concentraion in culture medium.

as the carbon source could be based on their low metabolic abilities for sucrose.

Characterization of BCC synthesized by co-cultivation

The IR spectra of NBC, levan and BCC(levan) are shown in Fig. IV-2-5. The peak derived from levan was observed at 925 cm-1 on each BCC.

The X-ray diffractograms of a BCC synthesized with various mix ratios showed almost a similar pattern as that of NBC (Fig. IV-2-6). The three peaks respectively corresponding to $(1\bar{1}0)$, (110) and (020) were observed. The index $((1\bar{1}0)/(020))$ for preferential orientation of NBC's and BCCs are shown in Table IV-2-2. Usually BC has high preferential orientation, and a significant decrease in the index was observed by the addition of a water-soluble polymer such as carboxymethyl cellulose (CMC) or methyl cellulose (MC) into the HS medium. Whereas, the index $((1\bar{1}0)/(020))$ for preferential orientation of all BCC(levan)s were similar to those of NBC. This could be due to the differences in the way the water-soluble polymer was incorporated into BC.

The surface views of the lyophilized NBC and BCC(levan)s are shown in Fig. IV-2-7. There is a clear difference between the NBC and BCCs from SEM observations. Clear microfibrils were observed on the surface of the NBCs. However, they were not observed on the surface of any BCC, but only small pores are visible. This was seem as positive evidence for the incorporation of levan into BC and that levan incorporated into the space of the ribbons.

Model for synthesis of BCC by co-cultivation

The model for the synthesis of the BCC by co-cultivation was proposed in Fig. IV-2-8. After inoculation, NCI 1005 expresses levansucrase, and then synthesizes levan and released glucose into the culture. Two types of bacteria (1051 or 10245 and 1005) multiply and then cellulose is synthesized by BC-producing bacterium from glucose. Since the *Acetobacter*s used are aerobe bacteria, they need oxygen for metabolism. BC-producing *A. xylinum* is usually retained by cellulose pellicle in the upper area, and multiplies, which then produces more cellulose. In this co-culture, the levan-producing bacterium is also retained by the cellulose pellicle, in which it multiplies, expresses levansucrase and produces levan. Finally, the BC incorporated with levan, BCC(levan) are formed.

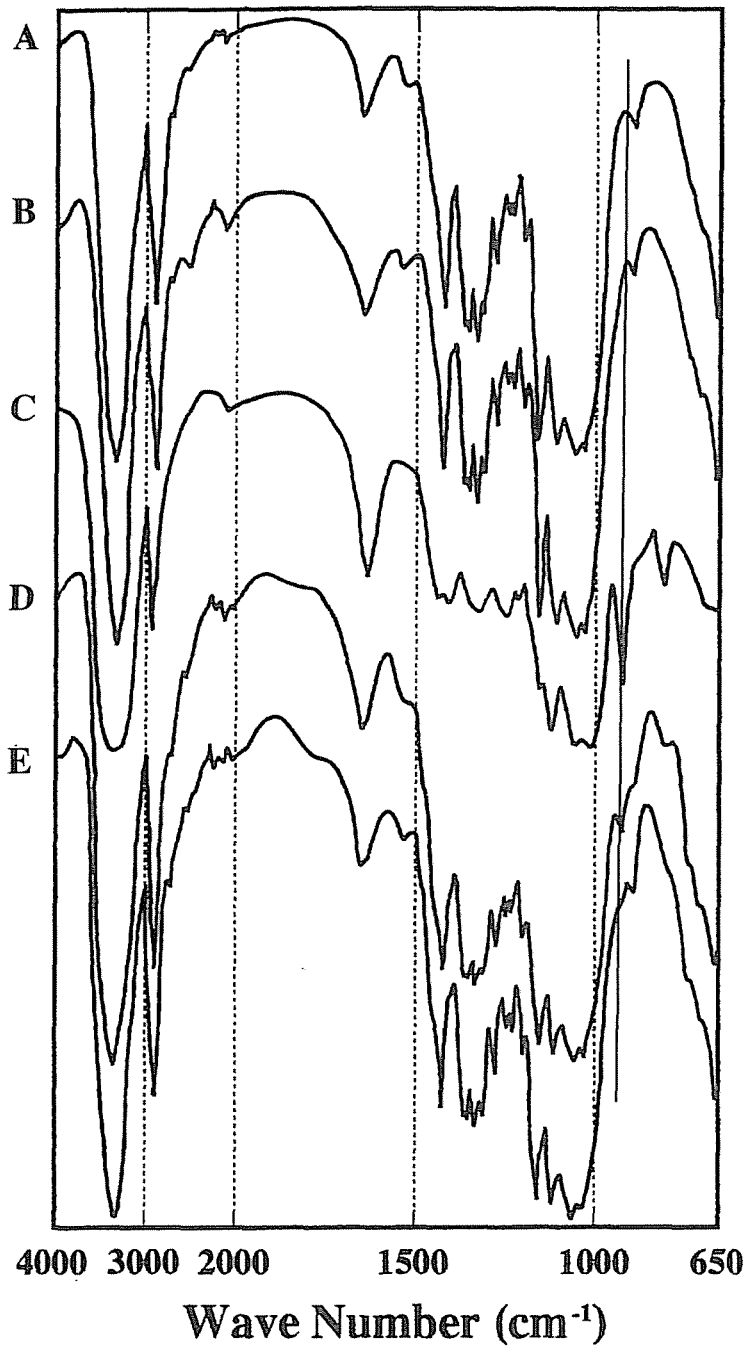


Fig. IV-2-5 IR spectra of NBC and BCC:; A; NBC(NCI1051); B; NBC(ATCC10245); C; levan; D; BCC(NCI1051+1005); E; BCC(ATCC10245+NCI1005).

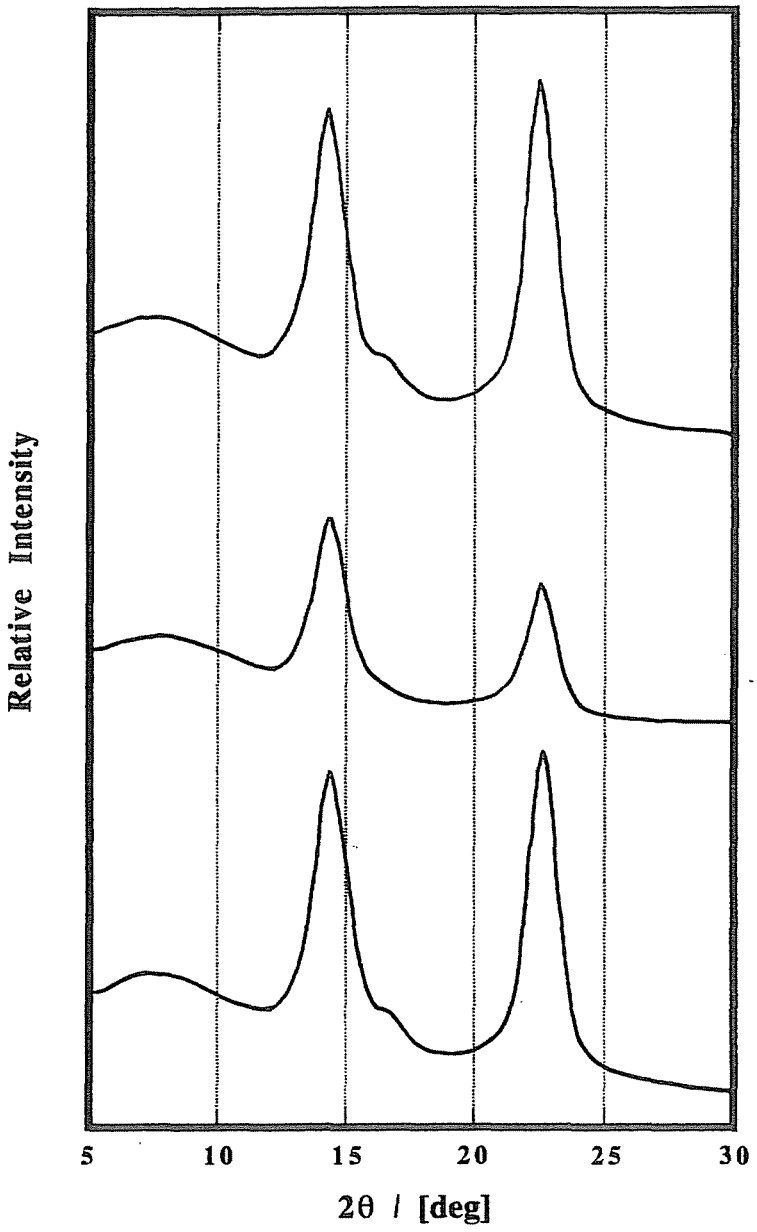


Fig. IV-2-5 X-ray diffractgrams of NBC and BCC: A; NBC(HS); B; NBC(HS(Sucrose)); C; BCC(1.00:1.00). Strain: NCI1051 and ATCC10245.

Table IV-2-2 Peak intensity ratio of $(1\bar{1}0)/(020)$ in X-ray patterns.

Sample	Inoculated strains (mixed raio)	$(1\bar{1}0)/(020)$
NBC(HS)	NCI1051	1.076
BCC	NCI1051 + NCI1005 (1.00:1.00)	1.230
(HS(Sucrose))	NCI1051 + NCI1005 (1.00:0.50)	1.276
	NCI1051 + NCI1005 (1.00:0.25)	1.283
	NCI1051 + NCI1005 (1.00:0.10)	1.314
	NCI1051 + NCI1005 (1.00:0.01)	1.340
NBC(HS)	ATCC10245	0.805
BCC	ATCC10245 + NCI1005 (1.00:1.00)	0.812
(HS(Sucrose))	ATCC10245 + NCI1005 (1.00:0.50)	0.804
	ATCC10245 + NCI1005 (1.00:0.25)	0.850
	ATCC10245 + NCI1005 (1.00:0.10)	0.932
	ATCC10245 + NCI1005 (1.00:0.01)	1.099

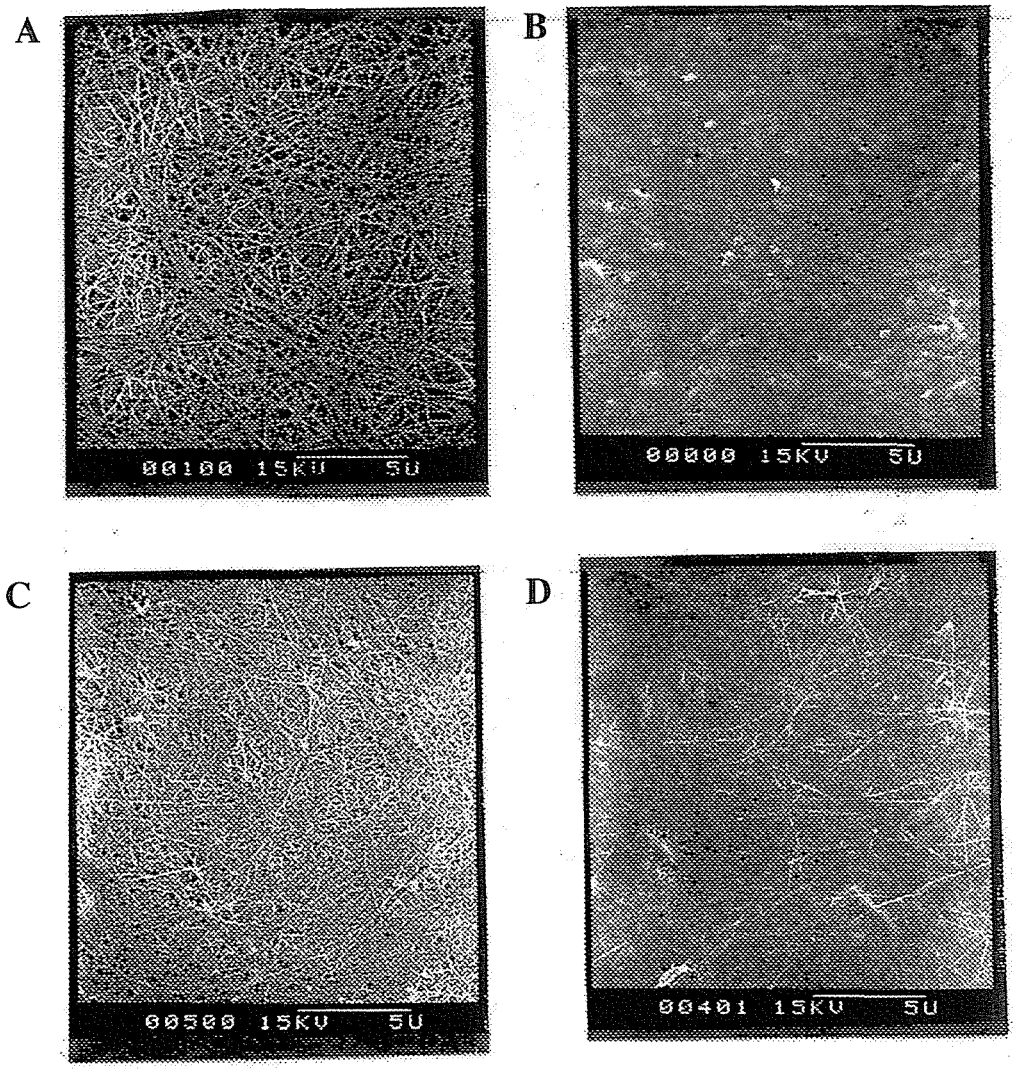


Fig. IV-2-5 SEM photographs of lyophilized NBC and BCC:
A; NBC(NCI1051); B; BCC(NCI1051+1005);
C; NBC(ATCC10245); D; BCC(ATCC10245+NCI1005).

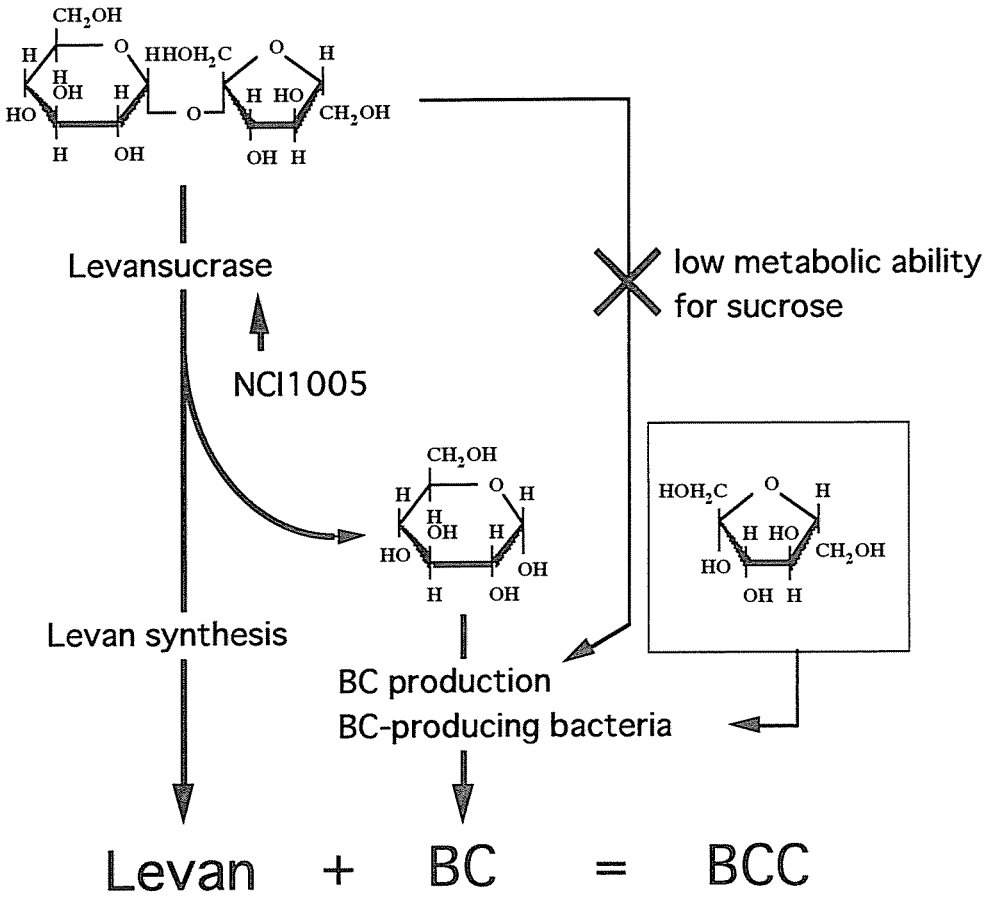


Fig. IV-2-5 Proposed model of BCC synthesis by co-cultivation.

Conclusions

We obtained the following results, (1) the BC-productivity of *A. xylinum* for co-cultivation was superior to that for mono-cultivation, (2) BCC could be synthesized without the addition of a WSP into the medium by the co-cultivation of the two strains (NCI 1051 or ATCC 10245 and 1005) on the HS(Sucrose) medium, (3) *A. xylinum* NCI 1005 synthesized levan and released glucose into medium from sucrose. From these results, it is suggested that; (1) the co-cultivation of *Acetobacter* sp. NCI 1005 enhances the cellulose productivity of *A. xylinum* ATCC 10245 or NCI 1051 using sucrose as a carbon source, and (2) this preparation method will be applied to more convenient synthesis of a new type of BC having both high biodegradability and other functions.

CHAPTER V

Mechanical Strength and Biodegradability of Bacterial Cellulose Composite

Introduction

BC has great mechanical strength and biodegradability in comparison with green plant cellulose or synthetic polymers. At present, from an environmental viewpoint, better biodegradable polymers to replace synthetic polymers widely are sought in the material industries, and BC seems to be one of the favored material sources for such a polymer based on its previously mentioned suitable characteristics. BC can be modified easily during synthesis, we have prepared BCCs, which were incorporated with various water-soluble polymers, by the additive cultivation (Chapter II and III) and co-cultivation (Chapter IV). The cellulose assembly of *A. xylinum* was altered by the adsorption or incorporation of cellulose derivatives, and until now, many researchers (Ben-hayyim, 1965, Benziman, 1980, Haigler, 1980, Brown, 1982, Haigler, 1982a, Haigler, 1982b, Brown, 1983, Haigler, 1985b, Haigler, 1988) have used them for clarifying the process of cellulose assembly. However, mechanical strength, biodegradabilities, and applications of these BCCs as functional materials have not been examined.

In this chapter, we reported mechanical strengths and biodegradabilities of BCCs prepared in this study and discussed the possibility of their utilization as biodegradable materials.

Materials and Methods

Materials

The NBCs and BCCs prepared in Chapter II, III, and IV were used as samples. Cellulase ONOZUKA R-10 was the product of Yakult Pharmaceutical Ind. Co., Ltd. The Glucose Test C II WAKO, which is a kit to determine the concentration of glucose, was purchased from Wako Pure Chemical Industries, Ltd.

Scanning electron microscope observation

The procedure was described in Materials and Methods of Chapter II-1.

Young's modulus

The dynamic Young's modulus was measured using the vibrating reed method, and was estimated from the real part of the following equation:

$$\text{Young's modulus / Pa} = \frac{48\,\pi^2 \times l^4 \times \rho}{a^4 \times d^2}\,[f_r + 1/8(\Delta f)^2],$$

where d : thickness of sample (cm),
l : length of sample (cm),
ρ : density of sample (g/cm3),
a : constant = 1.874,
fr : frequency for maximum vibrating width (Hz),
and Df : frequency difference for $1/\sqrt{2}$ vibrating width (Hz).

Biodegradability tests

Two biodegradability tests were performed; one was enzymatic degradation by cellulase, and the other was degraded in native soil. Biodegradability was estimated using the following equation:

degradability /% = (w(inital) - w(final)) /w(inital) × 100,
where w(inital) : initial sample weight (before degradation) (g)
and w(final) : final sample weight (after degradation) (g)

For enzymatic degradation, NBC and BCC (25 mg) were incubated in 5 ml of citric acid-phosphate buffer containing 5 mg of cellulase ONOZUKA R-10 at 120 strokes/min at 50 °C for 48 h. After incubation, the components of the enzymatic hydrolysates were separated and identified using a high performance liquid chromatography system (Tosoh Co., Ltd.; SC 8010) with a Shodex SUGAR SP 0810 column (Showa Denko K. K.). The concentrations of glucose released from the NBC and BCC were determined using the Glucose Test C II WAKO.

The biodegradation tests of NBC and BCC were performed by incubation in native soil in a beaker at 30 °C for 28 days. The soil was collected from the forest at Hokkaido University. After incubation, their forms were observed visually.

Results and Discussion

Mechanical strength of BCCs

Mechanical strength was estimated by the measurements of the dynamic Young's moduli (Tables V-1, 2, and 3). Dynamic Young's moduli were measured using the vibrating reed method. The Young's moduli of BCC(MC and CMC)s were larger than those of NBCs (Table V-1), and the maximal values were about three times those of NBCs. The difference in molecular weights of the WSPs did not affect the Young's moduli of the BCCs. On the other hand, the Young's moduli of BCC(PEG)s were no different compared to that of NBC, and these results would reflect the small contents of the PEGs in BCC(PEG)s. The mechanical strength of BC would be due to the number of hydrogen bonds among the cellulose molecules, so that, Young's modulus of BC significantly depends upon the method of drying. Heat dried BC has the largest Young's modulus value, the second is air dried BC, and the least is lyophilized BC. Cellulose ribbons in greatly swollen BC form hydrogen bonds through water molecules, and the removal of water during drying might fuse the cellulose ribbons which leads to reinforced BC. Also, rapid vaporization of water increases the hydrogen bonding among the cellulose molecules. The large Young's modulus of BCC(MC and CMC) can be explained by increments of hydrogen bonding among the ribbons based on the filling of WSP. This speculation is supported by the SEM observation of BCC(MC and CMC) (Fig. II-1-13).

The Young's modulus of BCC(WSChO-W) was 1.7 times that of NBC in spite of its low content in the BCC. Hosokawa and others (Hosokawa, 1990, Hosokawa, 1991) reported that a novel composite film was derived from chitosan and fine cellulosic fiber and that the film formation was accompanied by a cross-linking between the carbonyl group in the cellulose and the amino group in the chitosan. Similarly, the slight increase in Young's modulus of BCC(WSChO-W) could be due to the cross-linking between trace amounts of carbonyl groups in the cellulose and amino groups in the incorporated WSChO.

The Young's moduli of BCCs synthesized by co-cultivaton were the same as those of NBCs (Tale V-3). The content of WSP (levan) in the BCCs were 20-30 wt% in BCC1051(1005) and 5-15 wt% in BCC10245(1005),and these values were the almost same as those of MC and CMC. The remarkable differences between them were the WSPs (levan or cellulose derivatives) incorporated. From the results of X-ray diffractograms, levan which is a β(2→6) polyfructan did not alter the crystal structure, while MC and CMC which are β(1→4) glucan did. Therefore, to increase the mechanical strength of BC membrane, it was essential to incorporate WSP into ribbons and alter the crystal structure.

Biodegradability of BCC

The ratio of the weight of a sample (insoluble part) before and after treatment with cellulase is defined as biodegradability. NBC, BCC(CMC)s, BCC(PEG)s, BCC(WSCh-W), BCC(CM-chitosan)s, BCC(CM-chitin)s, and BCC(levan)s were degraded completely by the hydrolysis

Table V-1 Young's moduli of NBCs, BCC(MC)s, BCC(CMC)s, and BCC(PEG)s.

Samples	Young's moduli / GPa	
	Strains	
	NCI 1051	ATCC 10245
NBC(Control)	33(1.0)	32(1.0)
BCC(MC#15)	91(2.8)	59(1.8)
BCC(MC#25)	87(2.6)	53(1.7)
BCC(MC#100)	88(2.7)	52(1.6)
BCC(MC#400)	75(2.3)	59(1.8)
BCC(MC#1,500)	73(2.2)	61(1.9)
BCC(MC#4,000)	80(2.4)	85(2.6)
BCC(MC#8,000)	72(2.2)	73(2.3)
NBC(Control)	30(1.0)	32(1.0)
BCC(CMCKanto)	66(2.2)	68(2.1)
BCC(CMCNakalai)	74(2.5)	88(2.8)
BCC(CMCWako)	80(2.7)	84(2.6)
NBC(Control)	30(1.0)	32(1.0)
BCC(PEG200)	29(≈1)	40(1.3)
BCC(PEG1,000)	31(≈1)	42(1.3)
BCC(PEG6,000)	35(1.2)	39(1.2)
BCC(PEG20,000)	39(1.3)	39(1.2)
BCC(PEG50,000)	34(1.1)	40(1.3)
BCC(PEG500,000)	35(1.2)	43(1.3)

Table V-2 Young's moduli of NBC and BCC(WSChO-W).

Samples	Young's moduli / GPa
	A. xylinum ATCC 10245
NBC(Control)	33(1.0)
BCC(WSChO-W)	55(1.7)

Table V-3 Young's moduli of NBCs, BCCs synthesized by cocultivation.

Sample / media / mono- or co-cultivation	Young's moduli / GPa	
	Strains (*A. xylinum*)	
	NCI 1051	ATCC 10245
NBC / HS /mono	33(1.0)	33(1.0)
NBC / HS(Sucrose) / mono	28(≈1)	27(≈1)
BCC / HS(Sucrose) / co (1.00:1.00)	30(≈1)	29(≈1)
BCC / HS(Sucrose) / co (1.00:0.01)	33(≈1)	43(1.3)

Table V-4 Biodegradabilities of NBCs, BCC(MC)s, BCC(CMC)s, and BCC(PEG)s.

Samples	Biodegradabilities / %	
	Strains	
	NCI 1051	ATCC 10245
NBC(Control)	≅100	≅100
BCC(MC#15)	31.8	33.9
BCC(MC#25)	23.3	25.4
BCC(MC#100)	25.8	31.4
BCC(MC#400)	28.4	29.7
BCC(MC#1,500)	26.7	32.6
BCC(MC#4,000)	26.3	27.1
BCC(MC#8,000)	28.4	38.6
NBC(Control)	≅100	≅100
BCC(CMCKanto)	≅100	≅100
BCC(CMCNakalai)	≅100	≅100
BCC(CMCWako)	≅100	≅100
NBC(Control)	≅100	≅100
BCC(PEG200)	≅100	≅100
BCC(PEG1,000)	≅100	≅100
BCC(PEG6,000)	≅100	≅100
BCC(PEG20,000)	≅100	≅100
BCC(PEG50,000)	≅100	≅100
BCC(PEG500,000)	≅100	≅100

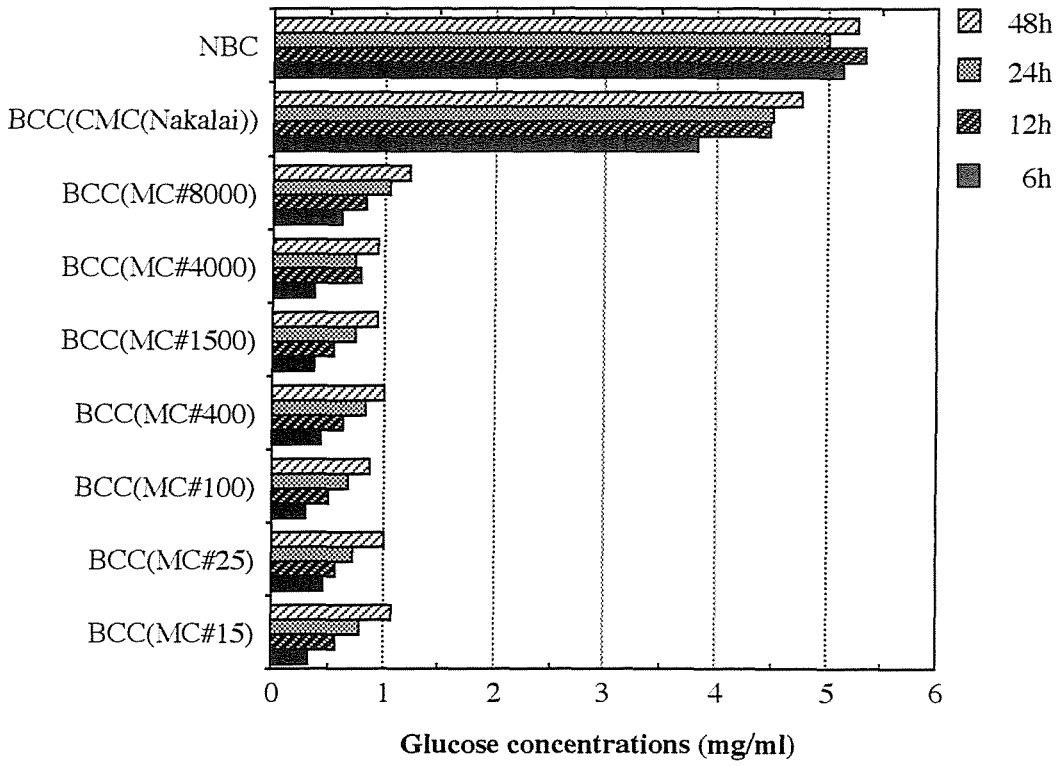


Fig. V-1 Timecourse changes of glucose concentrations in the enzymic hydrolysates of NBC, BCC(CMC(Nakalai)), and BCC(MC)s.

Table V-5 Biodegradabilities of NBCs, BCC(WSChO-W)s, BCC(CM-chitosan)s, and BCC(CM-chitin)s.

Samples	Biodegradabilities / %
	A. xylinum ATCC 10245
NBC(Control)	≅100
BCC(WSChO-W)	≅100
NBC(Control)	≅100
BCC(CM-chitosan1000)	94.6
BCC(CM-chitosan 500)	95.5
BCC(CM-chitin1000)	94.6
BCC(CM-chitin 500)	70.2

Table V-6 Biodegradabilities of NBCs, BCCs synthesized by co-cultivation.

Sample / media / mono- or co-cultivation	Biodegradabilities / %	
	Strains (*A. xylinum*)	
	NCI 1051	ATCC 10245
NBC / HS /mono	≅100	≅100
NBC / HS(Sucrose) / mono	≅100	≅100
BCC / HS(Sucrose) / co (1.00:1.00)	≅100	≅100
BCC / HS(Sucrose) / co (1.00:0.01)	≅100	≅100

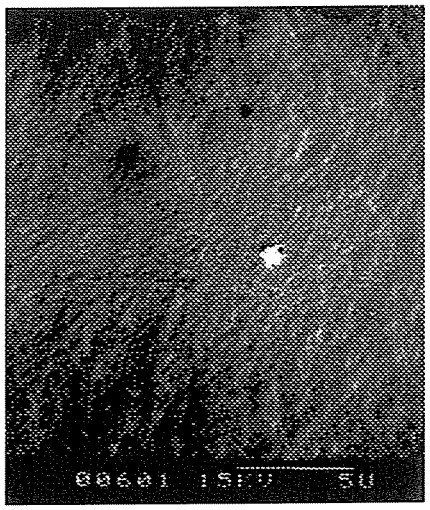
action of cellulase except for BCC(CM-chitin500) with high content (Tables V-4, 5, and 6). On the other hand, the BCC(MC)s were not able to be degraded fully and only the 20-40 wt % were degraded by cellulase (Table V-4 and Fig. V-1). This difference could be considered as follows: when cellulase degrades cellulose, first of all, it attaches to the surface of the cellulose, and then degrade the cellulose molecule. However, in BCC, WSP molecules cover the surface of the cellulose, and furthermore, cellulase can not diffuse easily inside, because WSP fills the spaces among the cellulose ribbons as observed in the SEM photographs. Thus, cellulase has to attack and degrade the WSP molecules covering the cellulose as the first step, and after degradation of the WSP molecules, it can attack directly the cellulose. In the case of BCC(PEG)s, BCC(WSCh-W), BCC(CM-chitosan)s, and BCC(CM-chitin)s, the cellulose efficiently was degraded because of their low content. In the case of BCC(CMC), the cellulose efficiently was degraded because CMC was degradable by cellulase as well as cellulose though the degradation rate was slower than that of NBC (Fig. V-1). On the other hand, MC showed resistance to degradation by cellulase because of their large degree of substitution (DS) (data are not shown). SEM photographs (Fig. V-2) of the surface of BCC(MC#8,000) before and after degradation and in which cellulose ribbons can be observed clearly on the surface even after degradation can support this speculation. This would suggest that cellulose ribbons are covered with MC which is not degraded by cellulase.

In the biodegradation test in soil, BCC(MC) and BCC(CMC) significantly lost their original shapes after four weeks, whereas NBC was completely degraded (Fig. V-3). BCC(MC) and BCC(CMC) had fine structure compared with that of NBC (Fig. II-1-13), and the decreases in the degradation rates for the BCC(MC)s could be due to the decreases in the diffusion rates of cellulosic enzymes secreted by microorganisms in the native soil.

Proposed model of BCC and degradation mechanism of BCC by cellulase

Many workers have reported that cellulase is composed of three types of enzymes, Endo-, Exo-cellulases, and b-glucosidase, and that cellulose is degraded synergically by these enzymes Wood, 1979, Gritzail, 1979, Takai, 1983) Figure V-3 is a proposed model of BCC(MC) and the degradation mechanism of BCC(MC) by cellulase. The small wavy lines are MC molecules bound to cellulose by hydrogen bonding, and which are incorporated among the fibrillar subunits and ribbons. For BCC degradation by cellulase as well as NBC, first, Endo-cellulase attacks the crystalline and noncrystalline regions and breaks glycosyl bonds within a glucan chain. Then, Exo-cellulase attacks the non- reducing end and releases cellobiose. For NBC, Exo-, and Endo-cellulase successively attack the cellulose and release cellooligomers which are hydrolyzed into glucose by b-glucosidase. Whereas, for BCC, the degradation mechanism is different depending upon the degradability of the incorporated WSP in the cellulase solution. That is, if the incorporated WSP such as CMC is degraded by cellulase, BCC also would be degraded, and, in contrast, if the incorporated WSP, such as MC, is not degraded by cellulase, the degradation would be stopped at some step (Table V-5 and Fig. V-2).

1



2

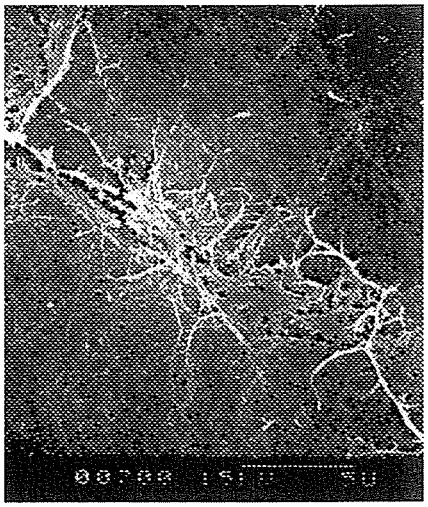


Fig. V-2 SEM photographs of the surface of the air-dried BCC(MC#8,000): (1); before and (2); after degradation with cellulase for 48 hours.
Bacterial strain was *A. xylinum* NCI1051.

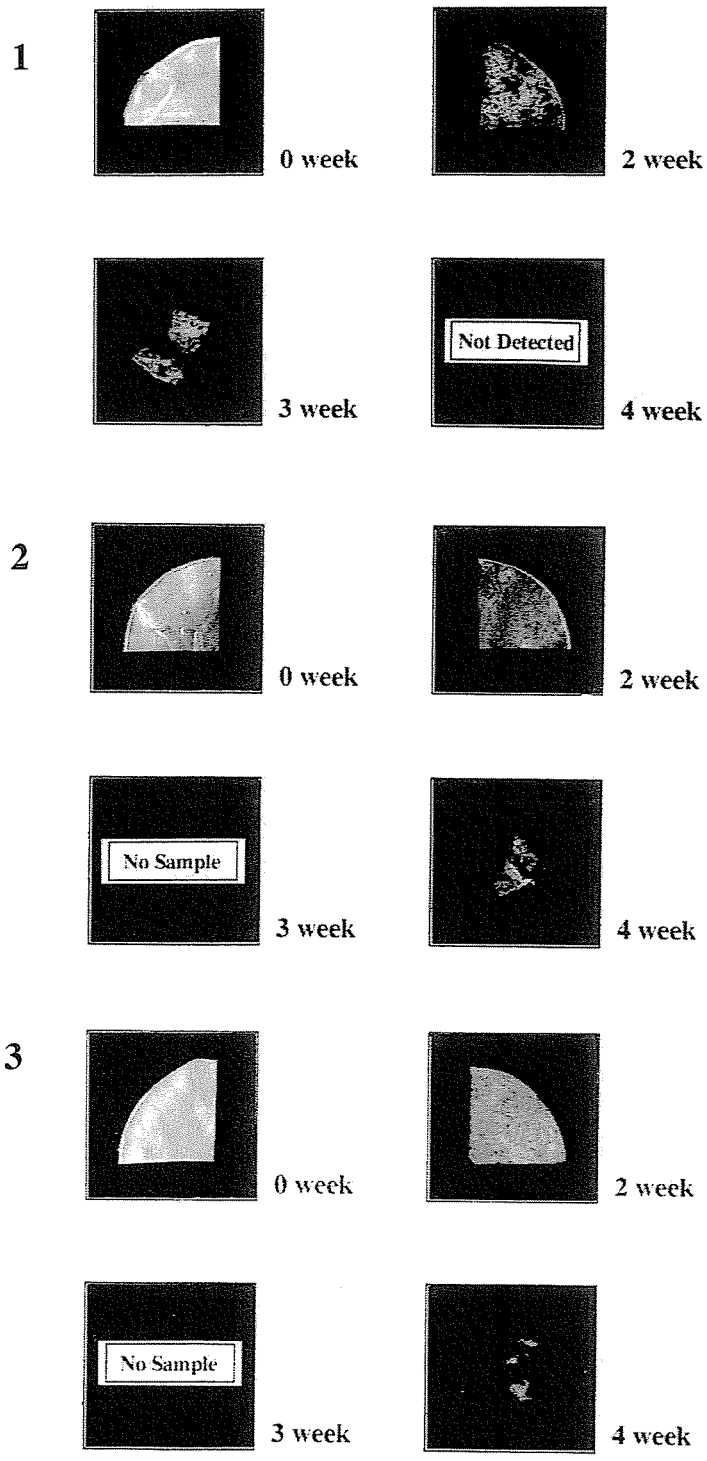


Fig. V-3 Photographs of the air-dried NBC and BCCs degraded in native soil: 1: NBC, 2: BCC(MC#8,000), 3: BCC(CMC(Nakalai)).
Bacterial strain was *A. xylinum* NCI1051.

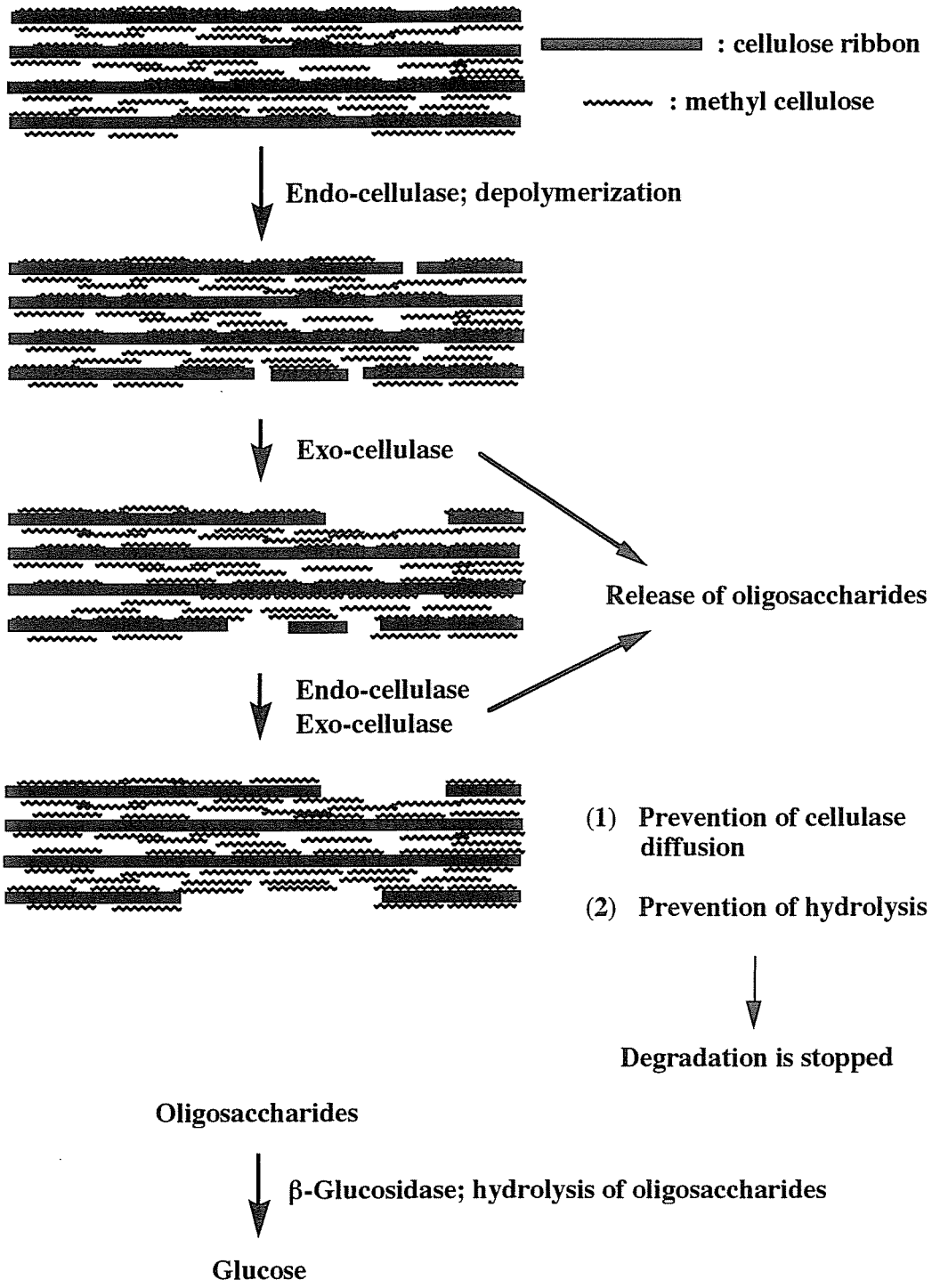


Fig. V-4 Proposed degradation mechanism and structure of BCC(MC) by cellulase.

Conclusions

The yields, the WSP contents, and the characterization results are summarized in Table V-7, and we obtained the following results: (1) BCC(CMC)s had great mechanical strength and biodegradability by cellulase, (2) BCC(MC)s had great mechanical strength and little biodegradability by cellulase, and (3) BCC(PEG)s and BCC(other polysaccharides)s had little mechanical strength and great biodegradability by cellulase. From these results, it is suggested that (1) BCC(CMC) can be used as a biodegradable material, (2) biodegradability of BC can be controlled by the incorporation of WSP with different degradability, and (3) this production method will be applied to synthesize a new type of BC having great mechanical strength, various biodegradabilities, and other functions.

Table V-7 Relative young's moduli and biodegradabilities.

Samples	Relative young's moduli	Biodegradabilities (%)
NBC	1	≅100
BCC(MC)	2-3	20-40
BCC(CMC)	2-3	≅100
BCC(PEG)	1-1.3	≅100
BCC(WSChO-W)	1.7	≅100
BCC(CM-chitosan)	ND	≅100
BCC(CM-chitin)	ND	70-100
BCC(cocultivation)	1-1.3	≅100

Chapter VI
Concluding Remarks

Chapter II

In Chapter II-1, we obtained the following results: (1) cellulose derivatives with low DS or small substituents were suitable for the formation of WSP-BC composites. (2) WSP contents in BCCs decreased with the increase of the bulkiness in the substituents. (3) BC synthesis was enhanced by addition of cellulose derivatives. (4) WSPs were incorporated into BC at molecular level, and they effected on the crystal structure of BC. (5) WSPs existed both on fibrillar subunits and among ribbons in BCCs, and BCCs had fine structure compared with that of NBC. From these results, it was suggested that physical properties of BC and BC productivity of *A. xylinum* could be controlled by the incorporation of water-soluble cellulose derivatives with various substituents and DS.

In Chapter II-2, we obtained the following results: (1) possibility of cellulase induction by CMC molecules in *A. xylinum* ATCC23769 was suggested, (2) there is a optimum cellulase concentration for the optimum BC production, (3) overexpression of extracellular endoglucanase (CMCase) increased BC yield. From these results, the mechanism of the increase of BC production in HS medium containing CMC was suggested as follow: (1) CMC induced cellulases, (2) cellulases prevented the ribbon assembly, which is a rate limiting step, (3) BC production was increased by the dissolution of the rate limiting step.

Chapter III

In Chapter III, we obtained the following results: (1) BCC(WSChO) and BCC(WSChD) yields were about 1.3-1.7 and 1.0-1.6 times that of the control, respectively (2) the contents of WSChOs and WSChDs were 0-5 (w/w) % and 8-17 (w/w) %, respectively, and their structures were quite different, (3) in HS medium containing WSChO-W, BC was synthesized even after the stationary growth phase. From these results, WSChOs and WSChDs enhanced BC production with different mechanism each other, (2) WSChOs activated the bacteria by some actions and that, as a result, BC productivity was enhanced.

Chapter IV

In Chapter IV-1, we obtained the following results: (1) a new water-soluble polysaccharide was isolated from a culture of *A. xylinum* NCI 1005 grown on sucrose, (2) the structure of the WSP was analyzed by nuclear magnetic resonance spectroscopy and determined to be a β(2→6)-linked polyfructan, which is structurally different from the polymer synthesized from glucose instead of sucrose by the same strain. The discovery of this new polysaccharide has revealed that the bacterium is able to synthesize two different kinds of water-soluble polysaccharides.

In Chapter IV-2, we obtained the following results, (1) the BC-productivity of *A. xylinum*

for co-cultivation was superior to that for monocultivation, (2) BCC could be synthesized without the addition of a WSP into the medium by the co-cultivation of the two strains (NCI 1051 or ATCC 10245 and 1005) on the HS(Sucrose) medium, (3) *A. xylinum* NCI 1005 synthesized levan and glucose into medium from sucrose. From these results, it is suggested that; (1) the co-cultivation of *Acetobacter* sp. NCI 1005 enhanced the cellulose productivity of *A. xylinum* ATCC 10245 or NCI 1051 using sucrose as a carbon source, and (2) this preparation method will be applied to more convenient synthesis of a new type of BC having both high biodegradability and other functions.

Chapter V

In Chapter V, we obtained the following results: (1) BCC(CMC)s had great mechanical strength and biodegradability by cellulase, (2) BCC(MC)s had great mechanical strength and little biodegradability by cellulase, and (3) BCC(PEG)s and BCC(other polysaccharides)s had little mechanical strength and great biodegradability by cellulase. From these results, it is suggested that (1) BCC(MC, CMC) can be used as a biodegradable material, (2) biodegradability of BC can be controlled by the incorporation of WSP, and (3) this production method will be applied to synthesize a new type of BC having great mechanical strength, various biodegradabilities, and other functions.

From this study, it was concluded that:

(1) BCC can be synthesized by addition of a WSP in to medium of *A. xylinum* and co-cultivation of two *Acetobacter* species, BC-producing and WSP-producing strains, and besides BC productivity increased.

(2) Cellulose, chitin, and chitosan derivatives are suitable for the formation of WSP-BC composites, and formation of composite increases mechanical strength of BC without decrease in biodegradability.

(3) I have succeeded in preparing a new biodegradable materials with high mechanical strenth and noble functions.

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