CHAPTER 1

INTRODUCTION

1. General introduction

Cellulose is the most abundant earth biopolymer and recognized as the major component of plant biomass, but also a representative of microbial extra-cellular polymers. The structure of cellulose is presented in Figure 1.

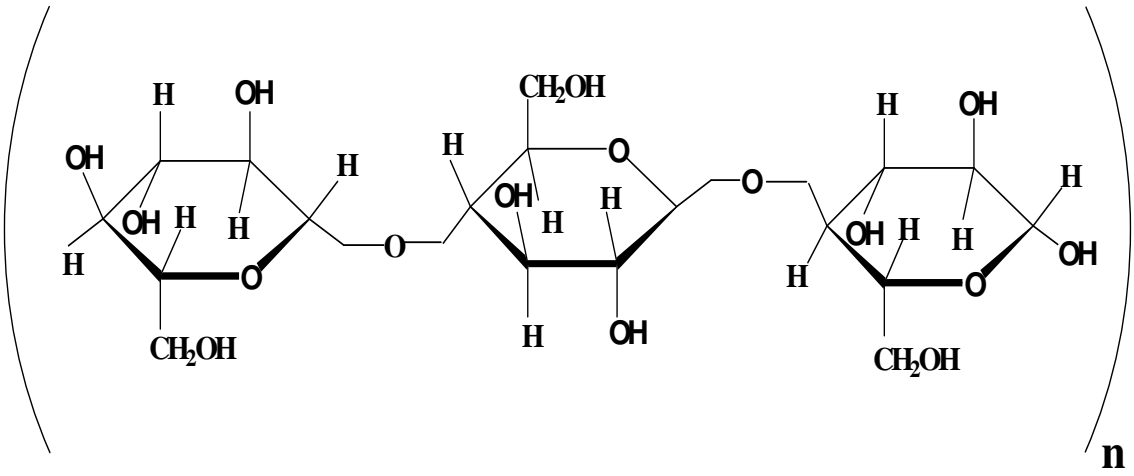

Figure 1. The structure of cellulose

Bacterial cellulose (BC) is synthesized by several bacterial genera, of which *Acetobacter* strains are best known. An overview of BC producers is presented in Table 1. BC belongs to specific products of primary metabolism. The polymer structure depends on the organism, although the pathway of biosynthesis and mechanism of its regulation are probably common for the majority of BC-producing bacteria (Ross *et al*., 1991; Jonas and Farah, 1998).

Table 1. Bacterial cellulose producers (Jonas and Farah, 1998, modified)

Genus	Cellulose structure
Acetobacter	Extra-cellular pellicle
Achromobacter	Fibrils
Aerobacter	Fibrils
Agrobacterium	Short fibrils
Alcaligenes	Fibrils
Pseudomonas	No distinct fibrils
Rhizobium	Short fibrils
Sarcina	Amorphous cellulose
Zoogloea	Not well defined

Acetobacter xylinum (*A. xylinum*) (synonyms *A. aceti* subsp. xylinum *A. xylinus*), which is the most efficient producer of cellulose, has been recently reclassified and included within the novel genus *Gluconacetobacter*, as *Gluconacetobacter xylinus* (Yamada *et al*., 1998, 2000) together with some other species (*G. hansenii, G. europaeus, G. oboediens* and *G. intermedius*).

A. xylinum is a simple Gram-negative bacterium, rod to oval shape, strictly aerobe bacteria, acetic acid bacteria, *A. xylinum* which has been applied as model microorganisms for basis and applied studies on cellulose production (Cannon and Anderson, 1991). It usually found on fruits and vegetables, in vinegar, fruit juices and alcoholic beverages. Although synthesis of an extracellular gelatinous mat by *A. xylinum* was reported for the first time in 1896 by A.J. Brown (Brown and Saxena, 2000), attracted more attention in the second half of the 20th century. Intensive studies

on BC synthesis using *A. xylinum* as a model bacteria were started by Hestrin *et al*., (1947, 1954), who proved that resting and lyophilized *Acetobacter* cells synthesized cellulose in the presence of glucose and oxygen. Later, Colvin (1957) detected cellulose synthesis in samples containing cell-free extract of *A. xylinum*, glucose and ATP.

BC has a very fine fiber structure with the average fiber diameter about 0.1 microns, with indeterminate fiber length. It is non-nutritive, non toxic on subject studied in extensive toxicological testing, neutral in sensory quality providing no taste and little tendency to mask other flavors. The fibers show high water binding and strong hydrogen bonding (Kent *et al*., 1991). Cellulose fiber is an effective thickener at low concentrations and can interact synergistically with other viscosity builders such as carboxymethylcellulose (CMC), xanthan, hydroxymethylcellulose to improve their efficiency and effectiveness (Valla *et al*., 1989). The cellulose synthesized by *A. xylinum* is identical to that made by plants in respect to molecular structure. However, the secreted polysaccharide is free from lignin, pectin and hemi-cellulose as well as other biogenic product, which are associated with plant cellulose. Additionally, extra-cellular synthesized BC microfibrils differ from plant cellulose with respect to its ultrafine network architecture, high crystallinity, high water absorption capacity, high, mechanism strength in the wet state and mouldability during formation (Klemm *et al*., 2001). Because of the unique properties, resulting from the ultrafine reticulated structure, BC has found a multitude of applications in paper, textile, in food industry and as biomaterials in cosmetics and medicine as a consequence of the special properties (Ring *et al*., 1986). Wider application of this polysaccharide is obviously dependent on the scale of production and its cost. Therefore, basic studies run

together with intensive research on strain improvement and production process development.

2. Physiological functions

In natural habitats, the majority of bacteria synthesized extracellular polysaccharides, which form envelopes around the cells (Costeron, 1999).

2.1 To hole the cell in aerobic environment

Cells of cellulose-producing bacteria are entrapped in the polymer network, frequently supporting the population at the liquid-air interface (Williams and Cannon, 1989).

2.2 To colonize on food and substrate

The polymer matrix takes part adhesion of the cells onto accessible surface and facilitates nutrient supply, since their concentration in the polymer lattice is markedly enhanced due to its adsorptive properties, in comparison to the surrounding aqueous environment (Jonas and Farah, 1998; Costeron, 1999). Some authors suppose that cellulose synthesized by *A. xylinum* also plays a storage role and can be utilized by the starving microorganisms (Okamoto *et al*., 1994).

2.3 Prevent potential competitor

Because of the viscosity and hydrophilic properties of the cellulose layer, the resistance of producing bacterial cells against unfavorable changes (a decrease in water content, variations in pH, appearance of toxic substances,

pathogenic organisms, etc.) in a habitat is increased and they can growth and develop inside the envelope (Ross *et al*., 1991).

2.4 Protect from ultraviolet radiation (UV)

BC can protect UV light because it is opacity and can protect *A. xylinum* from the killing effect of UV light (Koo *et al*., 1991). As much as 23% of the acetic acid bacteria cells covered with BC survived a 1 h treatment with UV. Removal of the protective polysaccharide brought about a drastic decrease in their viability (3% only) (Ross *et al*., 1991).

2.5 Help retain moisture

It may be used as moisturizer because it is very hydrophilic and aid in moisture retention to prevent dry of the nature substrates for *A. xylinum* while the bacterium growing on them (Ross *et al*., 1991).

3. Properties of bacterial cellulose

The physicological properties of cellulose such as water holding capacity, viscosity of disintegrated cellulose suspension and the Young's modulus of dried sheet are revealed (Watanabe *et al*., 1998; Igushi *et al*., 2000).

One of the most important features of BC is its chemical purity, which distinguishes this cellulose from plants, usually associated with hemicelluloses, lignocellulose (Fiedler, 1989) and waxy aromatic substance removal of which is inherently difficult (Ross *et al*., 1991). BC can be purified using less energy or chemical-intensive processes without hazardous by-products (Hong *et al*., 2005), high

water-binding capacity due to a large surface area can be hold large amount of water (up to 200 times of its dry mass). Majority of water is not bound with the polymer and can be squeezed out by gentle pressing (Czaja *et al*., 2005). High tensile strength, extremely insoluble and elasticity, durability and shape retention, high crystallinity index due to BC has a reticulated structure, in which numerous ribbon-shaped fibrils, are composed of highly crystalline and highly uniaxially oriented cellulose subfibrils. This three dimensional structure, not found in the plant originating cellulose, brings about higher crystallinity index (60-70%) of BC (Wananabe *et al*., 1998). The BC can be molded into any shape and size during it synthesized, depending on the fermentation technique and conditions used (Bielecki *et al*., 2002). Highly nano-porous material that allows for the potential transfer of antibiotics or other medicines into wound, while at the same time serving as an efficient physical barrier against any external infection (Czaja *et al*., 2005). Ougiya *et al*., (1997) showed that bacterial cellulose from *A. xylinum* subsp. *sucrofermentans* has the highest emulsion-stabilizing effect among the examined cellulose materials such as microcrystalline cellulose, microfibrinated cellulose, xanthan gum and sorbitan monolurate. Extensive toxicologic evaluations on BC from *A. aceti* subsp. *xylinum* have shown no adverse effects on the subjects studied. Schmitt *et al*., (1991) were tested for ocular irritant and not a dermal irritant in rabbit.

4. Structure of bacterial cellulose

Cellulose is an unbranched polymer of ß-1,4-linked glucopyranose residues. Extensive research on BC revealed that it is chemically identical to plant cellulose (PC), but its macromolecular structure and properties differ from the latter.

Nascent chains of BC aggregate to form subfibrils, which have a width of approximately 1.5 nm and belong to the thinnest naturally occurring fibers, comparable only to subelemental fibers of cellulose detected in the cambium of some plants and in quinee mucous (Kudlicka, 1989). BC subfibrils are crystallized into microfibrils (Jonas and Farah, 1998), these into bundles, and the latter into ribbons (Yamanaka *et al*., 2000). Dimensions of the ribbons are 3-4 (thickness) × 70-80 nm (width), according to Zaar, (1977) or 3.2 × 133 nm, according to Brown *et al*., (1976) or 4.1 × 117 nm, according to Yamanaka *et al*., (2000) whereas the width of cellulose fibers produced by pulping of birch or pine wood is two orders of magnitude larger ($1.4\text{-}4.0 \times 10^{-2}$ and $3.0\text{-}7.5 \times 10^{-2}$ mm, respectively). The ultrafine ribbons of microbial cellulose, the length of its ranges from 1-9 μm, form a dense reticulated structure, stabilized by extensive hydrogen bonding. The crystallinity index and crystalline size are calculated based on X-ray diffraction measurements (Watanabe *et al*., 1998). BC is also distinguished from its plant counterpart by a high crystallinity index (above 60%). The degree of polymerization (DP) of cellulose and the DP distribution are determined by high-performance gel permeation chromatography (Watanabe *et al*., 1998) and DP usually between 2,000 and 6,000 (Jonas and Farah, 1998), but in the some cases reaching even 16,000 or 20,000 (Watanabe *et al*., 1998), whereas the average DP of plant polymer varies from 13,000 to 14,000 (Teeri, 1997).

Macroscopic morphology of BC strictly depends on culture conditions. In static conditions, bacteria accumulate cellulose mats on the surface of the nutrient broth, at the oxygen-rich (air-liquid interface). The subfibrils of cellulose are continuously extruded from linearly ordered pores at the surface of the bacterial cell, crystallized into microfibrils and forced deeper into the growth medium. Therefore,

the leather-like pellicle, supporting the population of *A. xylinum* cells, consists of overlapping and intertwisted cellulose ribbons, forming parallel but disorganized planes (Jonas and Farah, 1998). The adjacent static-BC strands branch and interconnect less frequently than these in BC produced in agitated culture, in a form of irregular granules and fibrous strands, well-dispersed in culture both (Vandamme *et al*., 1998). The strands of reticulated agitated-BC interconnect to form a grid like pattern, and have both roughly perpendicular and roughly parallel orientations (Watanabe *et al*., 1998).

Differences in three-dimensional structure of agitated-BC and static-BC fibrils are noticeable in their scanning electron micrographs. The static-BC fibrils are more extended and piled above one another in a criss-crossing manner. Strands of agitated-BC are entangled and curved (Johnson and Neogi, 1989). Besides, they have a larger cross-sectional width (0.1-0.2 μm) than static-BC fibrils (usually 0.05-0.10 μm). Morphological differences between static-BC and agitated-BC contribute to varying degrees of crystallinity, different crystallite size and Iα cellulose content.

Two common crystalline forms of cellulose, designated as I and II, are distinguishable by X-ray, nuclear magnetic resonance (NMR), Raman spectroscopy, and infrared analysis (Johnson and Neogi, 1989). It is known that in the metastable cellulose I, which is synthesized by the majority of plants and also by *A. xylinum* in static culture, parallel ß-1,4-glucan chains are arranged uniaxially, whereas ß-1,4-glucan chains of cellulose II are arranged in a random manner. They are mostly anti-parallel and linked with a lager number of hydrogen bonds that result in higher thermodynamic stability of the cellulose II. Agitated-BC has a lower crystallinity index and a smaller crystallite size than static-BC (Watanabe *et al*., 1998). It was also

observed that a significant portion of cellulose II occurred in BC synthesized in agitated culture. In nature, cellulose II is synthesized by few organisms only (some algae, moulds and bacteria, such as *Sarcina ventriculi*) (Jonas and Farah, 1998).

5. Biochemistry of bacterial cellulose synthesis

5.1 Carbon metabolism in *Acetobacter xylinum*

Two main amphibolic pathways are operative in *A. xylinum* bacteria (Figure 2) the pentose phosphate cycle for oxidation of carbohydrates and the Krebs cycle for the oxidation of organic acids and related compounds (Ross *et al*., 1991). Phosphofructokinase is absent or weakly absent (De Ley *et al*., 1984), resulting in no or only a weak glycolytic activity, indicating that glucose cannot be metabolized anaerobically. However, Tonouchi *et al* (1996) detected phosphofructokinase activity in *A. xylinum* subsp. *sucrofermentans* BPR 2001. These researchers also investigated several specific enzyme activities involved in cellulose synthesis, and demonstrated the existence of a phosphotransferase system for fructose uptake in cellulose-producing *Acetobacter* cells. Gluconeogenesis can occur from oxaloacetate via pyruvate by means of the enzymes oxaloacetate decarboxylase and pyruvate phosphate dikinase.

5.2 Cellulose synthesis in *Acetobacter xylinum*

A. xylinum converts various carbon compounds, such as hexoses, glycerol, dihydroxyacetone, pyruvate and dicarboxylic acids into cellulose, usually with about 50% efficacy (Cannon and Anderson, 1991). The latter compounds enter

the Krebs cycle and due to oxalacetate decarboxylation to pyrovate undergo conversion to hexoses via gluconeogenesis, similarly to glycerol, dihydroxyacetone and intermediates of the pentose phosphate cycle as shown in Figure 3.

The direct cellulose precursor is uridine-diphosphoglucose (UDPGlc), which is a product of conventional pathway, common of many organisms, including plants and involving glucose phosphorylation to glucose-6-phosphate (Glc-6-P), catalyzed by glucokinase (GK), followed by isomerization of this intermediate to glucose-1-phosphate (Glc-1-P), catalyzed by phosphoglucomutase (PGM) and conversion of the latter metabolite to UDPGlc by uridine-diphosphoglucose pyrophosphorylase (UDPGlc pyrophosphorylase) which catalyzing the synthesis of UDPGlc is the key enzyme in the cellulose biosynthesis (Tonouchi, 1996). They display cellulose synthase (CS) activity that was confirmed *in vitro* by mean of observation of cellulose synthesis, catalyzed by cell-free extracts of Cel^- strains (Saxena *et al*., 1989). Some *Acetobacter* strains produce extracellular cellulose fibrils as a part of its normal metabolic activity. UDPGlc which produced from UTP and glucose-1-phosphate is the direct precursor in the synthetic pathway of cellulose (Ishikawa, 1997). Furthermore, the pyrophosphorylase activity varies between different *A. xylinum* strains and the highest activity was detected in the most effective cellulose producers, such as *A. xylinum* subsp. *sucrofermentants* BPR 2001. The latter strain prefers fructose as a carbon source, displays high activity of phosphoglucoisomerase and processes a system of phosphotransferases, dependent on phosphoenolpyruvate. The system catalyses conversion of fructose (Fru) to fructose-1-phosphate (Fru-1-P) and further to fructose-1,6-biphosphate (Fru-1,6-bisP) as shown in Figure 3.

5.3 Cellulose synthase (CS)

Cellulose synthase of *A. xylinum* is a typical anchored membrane protein, having molecular mass of 400-500 kDa and CS is localized on the cytoplasmic side of the cell membrane. Because of this localization, purification of CS was extremely difficult, and isolation of the membrane fraction, before CS solubilization and purification was necessary (Lin and Brown, 1989). Furthermore, *A. xylinum* CS appeared to be a very unstable protein (Lin and Brown, 1989). CS isolation from membranes was carried out using digitonin (Mayer *et al*., 1989), or detergents (Triton X-100) and treatment with trypsin (Saxena *et al*., 1989), followed by CS entrapment on cellulose. Mayer *et al*., (1989), the purified CS preparation contained 3 different types of subunits, having molecular mass of 90, 67 and 54 kDa. Saxena *et al*., (1989) found only 2 types of polypeptides (83 and 93 kDa).

5.4 Mechanism of bacterial cellulose biosynthesis

Formation of BC is catalyzed by the cellulose synthase complexes aligned linearly in the *A. xylinum* cytoplasmic membrane. Synthesis of the metastable cellulose in *A. xylinum* and other cellulose-producing organisms, including at least two steps such as

- polymerization of glucose molecules to the linear 1,4-ß-glucan,
- assembly and crystallization of individual nascent polymer chains into supramolecular structures, characteristic for each cellulose-producing organism.

5.4.1 Mechanism of ß-1,4-glucan polymerization

Han and Robyt (1998) proposed that BC biosynthesis involved 3 enzymes embedded in the cytoplasmic membrane: cellulose synthase (CS), lipid pyrophosphate (LP: UDPGlc-PT) and lipid pyrophosphate phosphohydrolase (LPP). The reaction mechanism, called by the authors the insertion reaction, is presented in Figure 4.

Reaction 1: The first enzyme transfers Glc-1-P from UDPGlc onto the lipidmonophosphate (Lip-P), thus giving the lipid pyrophosphate-α–D-Glc (LipPP-α-Glc). The α configuration on the anomeric carbon, involved in the Glc phosphoester bond, remains the same as in the substrate molecule. The product second product of this reaction is UMP (according to the Brown's model, UDP is released).

Reaction 2: To catalyze by CS, the glucose residue is transferred from one LipPP-α–Glc molecule onto another one, and the ß-1,4-glycosidic linkage between the two glucose residues is formed, due to the attack of the C-4 hydroxyl group of one of them onto C-1 hydroxyl group of the second Glc (from the second LipPP-α-Glc).

Reaction 3: The hydrolysis of the lipid pyrophosphate formed in the previous step occurs, and another Glc-α-1P from UDPGlc can be attached to the LipP, released in this reaction. The cycle of these three reactions (as shown in Figure 3) is continued to give the ß-1,4-glucans chain of an appropriate length.

5.4.2 Assembly and crystallization of cellulose chains

A. xylinum is a simple Gram-negative bacterium which has an ability to synthesize a large quantity of high-quality cellulose organized as twisting ribbons of

microfibrillar bundles (Brown *et al*., 1976). During the process of actual bacteria, then polymerized into single, linear ß-1,4-glucan chains and finally secreted outside the cells through a linear row of pores located on their outer membrane (Haigler, 1980). The subsequence assembly of the ß-1,4-glucan chains outside of the cell is a precise, hierarchical process. Initially, they form subfibrils (consisting of 10-15 nascent ß-1,4-glucan chains), then latter microfibrils, and finally bundles of microfibrils consisting of a loosely wound ribbon, which is comprised of about 1,000 individual glucan chains (Figure 5) (Ross *et al*., 1991).

Synthesis of BC is a precisely and specifically regulated multi-step process, involving a large number of both individual enzymes and complexes of catalytic and regulatory proteins, whose supramolecular structure has not yet been well defined. The process includes the synthesis of uridinediphosphoglucose (UDPGlc), which is the cellulose precursor, followed by glucose polymerization into ß-1,4-glucan chains, and nascent chain association into characteristic ribbon-like structure, formed by hundreds or even thousands of individual cellulose chains. Pathways and mechanisms of UDPGlc synthesis are relatively well known, whereas molecular mechanisms of glucose polymerization into long and unbranched chains, their extrusion outside the cell, and self-assembly into fibrils (Brown *et al*., 1976).

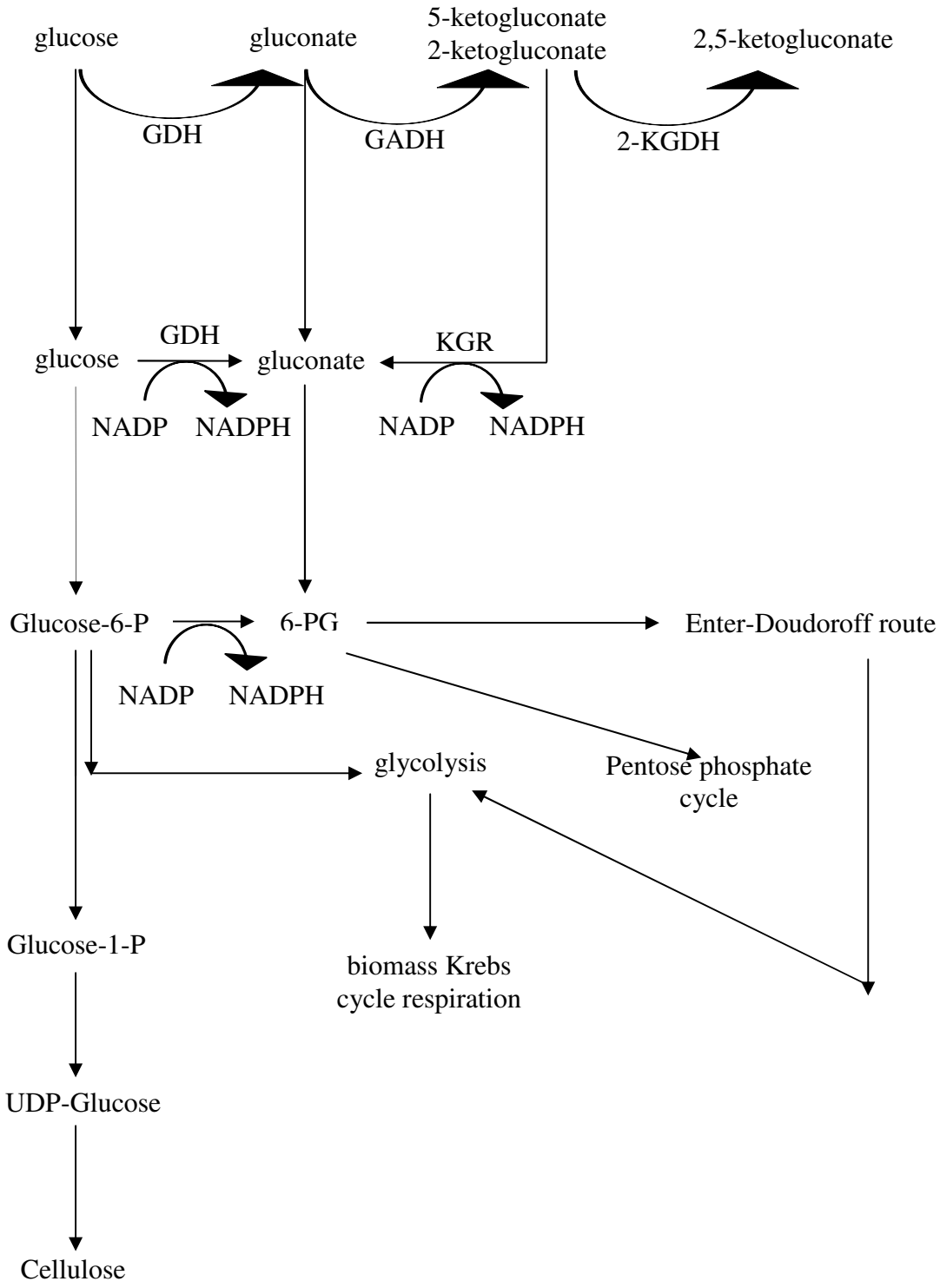
glucose
gluconate
5-ketogluconate
2-ketogluconate
2,5-ketogluconate
GDH
GADH
2-KGDH
glucose
GDH
gluconate
KGR
NADP
NADPH
NADP
NADPH
Glucose-6-P
6-PG
Enter-Doudoroff route
NADP
NADPH
glycolysis
Pentose phosphate
cycle
Glucose-1-P
biomass Krebs
cycle respiration
UDP-Glucose
Cellulose

Figure 2. Pathways of carbon metabolism in *Acetobacter xylinum* (De Wulf *et al*., 1996)

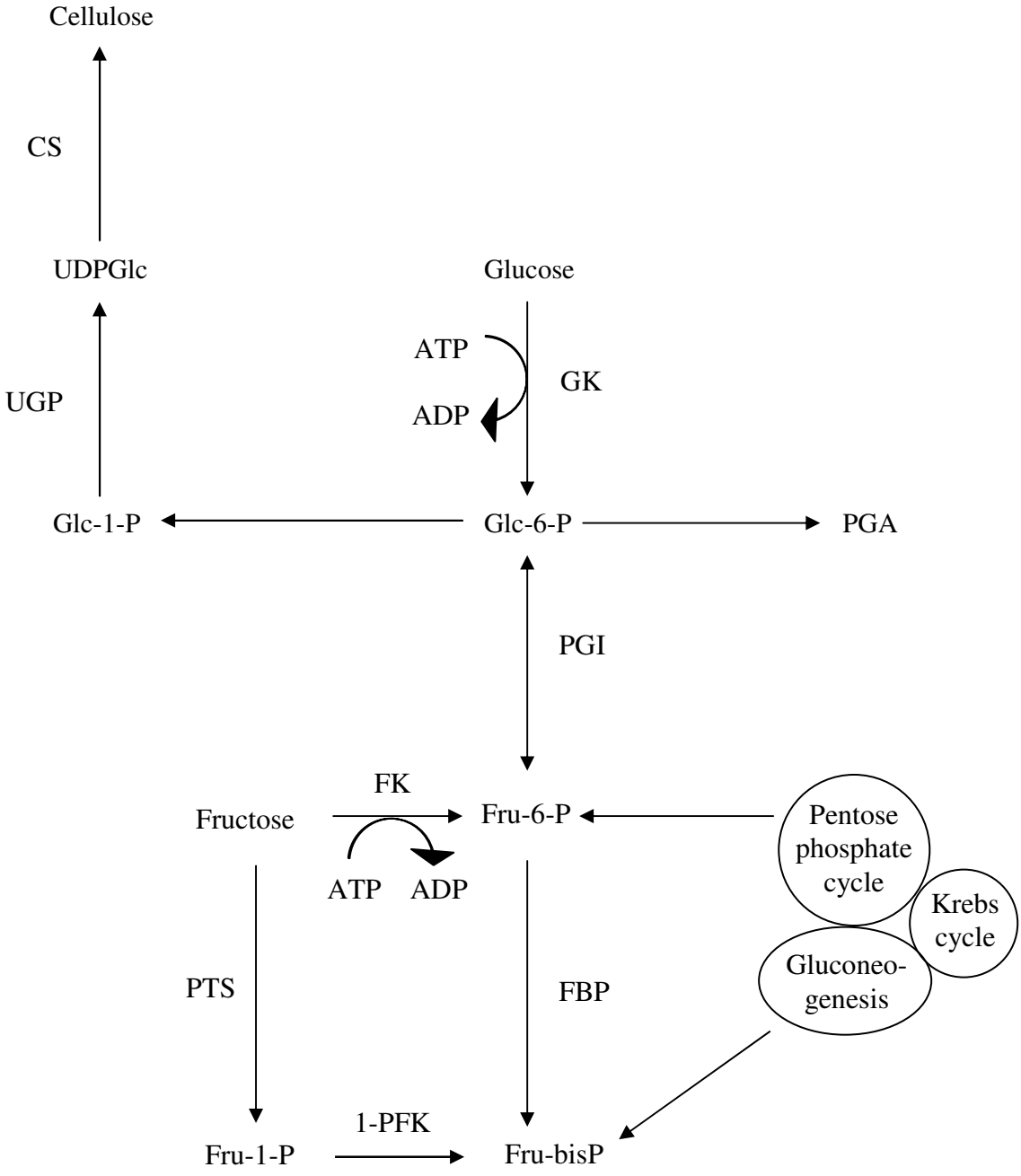

Figure 3. Pathways of carbon metabolism in *Acetobacter xylinum* (Ross *et al*., 1991; Tonouchi *et al*., 1996)

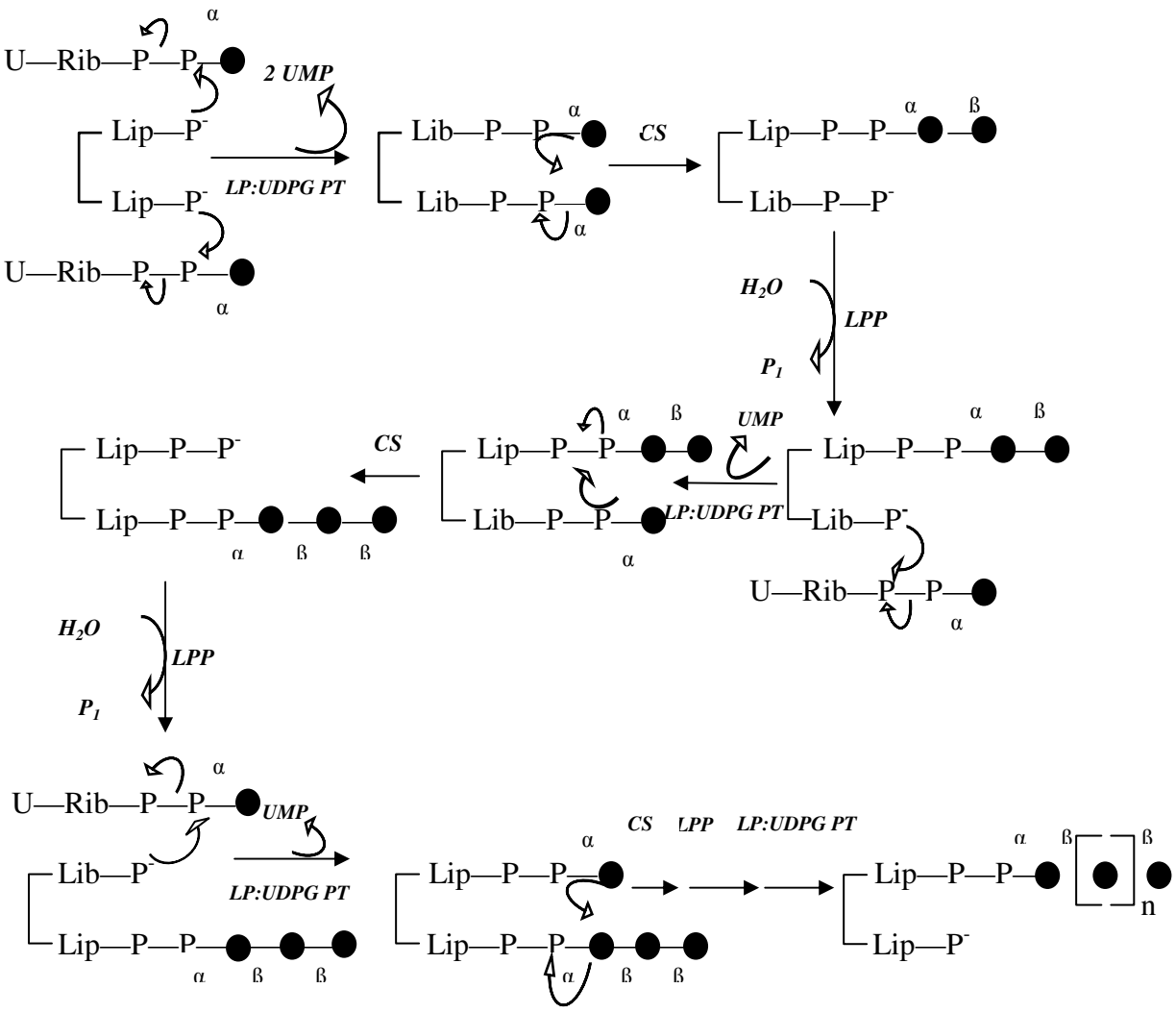

Figure 4. Mechanism of bacterial cellulose biosynthesis involving lipid intermediated (Han and Robyt, 1998)

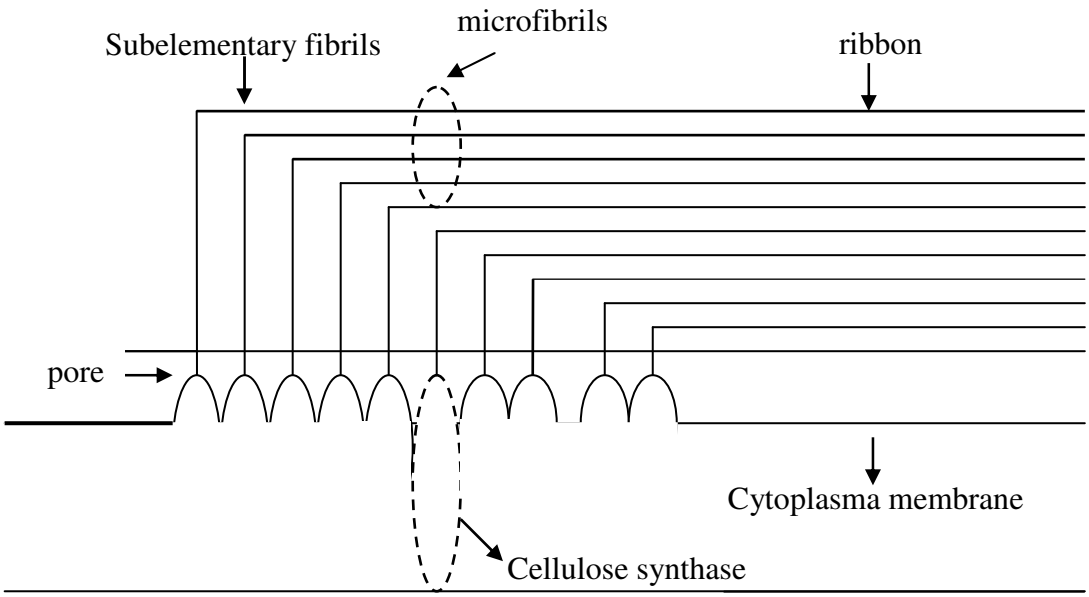

Figure 5. Scheme for the formation of bacterial cellulose (Vandamme *et al*., 1997)

6. Bacterial cellulose productions

The choice of a cultivation technique is dependent on further biopolymer commercial destination, considering that cellulose ultrastructure and its physical and mechanical properties are strictly influenced by the culture method (Galas *et al*., 1999). Cellulose can be produced in the several techniques for BC production have been reported, some of which demonstrate a potential tool for economic and commercial BC production.

6.1 Stationary culture conditions

In stationary culture method, gelatinous membrane of BC is accumulated on the surface of a culture medium and BC is produced and forms a pellicle. The productivity of BC is dependent on the surface area of the medium. Therefore a system in which a pellicle is formed on an oxygen-permeable membrane was developed to increase the surface area of the pellicle (Yoshino *et al*., 1996). BC yield in static cultures is mostly dependent on the surface: volume ratio (s/v). Optimum s/v ratio protects from either too high (unnecessary) or too low aeration (cell growth and BC synthesis termination). Krystynowicz *et al*., (1997) reported values of s/v ratio vary from 2.2 cm^{-1} to 0.7 cm^{-1}. However, the stationary culture method has disadvantages such as high labor cost and lower productivity (Zuo *et al*., 2006), synthesis of the polymer only in the form of a sheet and relatively low productivity, contributed to the development of new fermentation process (Masaoka *et al*., 1993). The conventional method of BC production by static cultivation is not applicable to large-scale industrial production; thus, the establishment of an economical production process is urgently required (Toyosaki *et al*., 1995). The

control of BC synthesis in static culture is very difficult since the pellicle limits an access to the liquid medium. The particularly important parameter, which requires continuous control is pH. Accumulation of keto-gluconic acids in culture broth brings about a decrease in pH much below its value that is optimum for growth of bacteria and the polysaccharide synthesis because conventional methods of pH adjustment can not be used in static cultures. Vandamme *et al*., (1998) applied an *in situ* pH control *via* an optimized fermentation medium design, based on introducing acetic acid as an additional substrate for *Acetobacter* sp. LMG 1518. Products of acetic acid catabolism counteracted the pH decrease caused by keto-gluconic formation and provided constant pH of the growth medium, equal to 5.5, throughout the whole process.

6.2 Agitated culture conditions

Cellulose can be produced in the form of a fibrous suspension, irregular masses, pellets or spheres. Screening of strains suitable and the influence of the culture medium components on the BC productivity have been reported (Dudman, 1960). Since the productivity of BC in agitated culture is dependent on the growth of the BC-producing organisms increasing the cell density is essential for increasing BC productivity. Since the growth of *Acetobacter* (a typical aerobic BC producer) is dependent on oxygen supply, oxygen supply is directly associated with BC productivity in an aerated and agitated culture of *Acetobacter*. However, excessive oxygen supply is reported to result in a decrease in BC productivity because of a loss of substrate by direct oxidation (Yamanaka *et al*., 1989). Agitated culture is considered more suitable for the commercial cellulose production of BC mainly due to the higher production rates that potential can be achieved (Yamanaka *et al*., 1989).

BC production in agitation and aeration encounters many problems such as spontaneous appearance of *Cel*$^-$ mutans (cellulose non-producers), which contributes to a decline in polymer synthesis (Ross *et al*., 1991). Optimized agitation and aeration prevent turbulence, which negatively effects cellulose polymerization and crystallization, thus reducing the polysaccharide yield (Laboureur, 1988). Laboureur (1988), who applied the rate of agitation equal to 60 rpm and aeration of 0.6 vvm were optimum for *A. aceti* subsp. *xylinum* ATCC 2178 strain cultured in 300 litre fermentor for 45 h at 30°C and 10 g of BC per litre a day was obtained. Recently investigations showed that in agitated culture, high oxygen supply and high volumetric agitated power are required for increased of BC productivity (Kouda *et al*., 1998). Other factors such as agitator configuration, effects of oxygen and carbon dioxide pressure on BC productivity have been investigated (Ring *et al*., 1989). Agitated culture method often leads to morphological and structural abnormalities of bacterial cellulose due to high shear stress induced by agitation (Zuo *et al*., 2006).

6.3 Horizontal fermentors

Cellulose production in horizontal fermentor is the combination of stationary and submerged cultures. The polymer is deposited on the surface of rollers or discs, rotating around the long axis. A part of their surface temporarily dips in the liquid medium or is above its surface (in the air). The advantages of this method include a large polymer surface, synthesis of cellulose in a form of hollow fibers, different in diameter, as well as good process control, easy scale enlargement, appropriate accessible surface for adhesion of bacteria and product deposition, higher rate of cellulose production (Sattler and Fiedler, 1990).

7. Factor effecting growth and cellulose production

The rate of cellulose production depended proportionally on the surface-area of the culture medium and was unaffected by the depth and volume of the medium. The optimum pH for cellulose production was 4.0 to 6.0. Glucose, fructose and glycerol were preferred carbon sources for cellulose production. Embuscado *et al*., (1994) selected that the strain of *A. xylinum* for optimum cellulose production. The *A. xylinum* will be grown on shaker and statically. Optimal fermentation of non-agitated production of cellulose by selected strains will be determined. Four factors such as carbon-sources concentration, pH, temperature of incubation and their relationship on cellulose yield will be determined.

7.1 Isolation from natural sources and improvement of bacterial cellulose producing strains

The genetic analysis of cellulose biosynthesis in *A. xylinum* has included the isolation of mutants that effect cellulose production, characterization of indigenous plasmid species, and the cloning of gene in the process. A number of researchers, beginning with Schramm and Hestrin (Schramm and Hestrin, 1954), have described the isolation of cellulose-negative (*cel*$^-$) mutants. Both spontaneous and mutagen-induced variants have been isolated. Many reports have indicated that apparent spontaneous celluloseless mutants arise at a high rate when wild-type cells are grown in aerated liquid culture (Smith, 1990). The frequency with which these mutants occur also increases with the age of the culture. The spontaneous mutants have a mucoid appearance on solid agar like true *cel*$^-$ mutants, but the majority reverts back to wild-type when grown statically in broth culture (Cannon and Anderson,

1991). A variety of mutagens have been used in an effort to induce mutation in *A. xylinum* i.e., nitrosoguanidine, nitrous acid are very effective whereas hydroxylamine and ultraviolet light are relatively ineffective (Valla *et al*., 1989; Williams and Cannon, 1989). The characteristics of *A. xylinum* strains are shown in Table 2.

One of the methods enabling selection of proper *A. xylinum* strain, is the screening for strains, which cannot oxidize glucose via gluconic acid (Winkelman and Clark, 1984; Johnson and Neogi; 1989; De Wulf *et al*., 1996; Vandamme, 1998) to 2,-5-or 2,5-ketogluconate.

Table 2. Characteristics of *Acetobacter xylinum* strains (Cannon and Anderson, 1991)

Characteristic	Wild type	Cel-negative	Overproducer
- Colony morphology	Small-rough	Large, mucoid	Small-rough
- Fluorescence with Tinopal	Bright	Dull	Bright
- Pellicle production	Pellicle	No pellicle	Thick pellicle
- Relative cellulose production (wet weight)	1.0	0.0-0.1[a]	5.0-6.0

a The trace amounts of cellulose produced in these pellicle-deficient cultures are thought to be cellulose II.

7.2 Carbon and nitrogen sources

The factors affecting the BC production yield, many attentions was paid to carbon sources. Numerous mono-, di- and polysaccharides, alcohols, organic

acids and other compounds were compared by Jonas and Farah (1998), who found out that the preferred carbon sources were D-arabitol and D-mannitol, which presence resulted in 6.2 or 3.8 higher cellulose production, respectively, in comparison to glucose. Both sugars alcohols provided stabilization of pH throughout the culture, since were not converted to gluconic acids. Tonouchi *et al*., (1996) used a strain of *A. xylinum* to obtain cellulose from glucose and fructose, found out that fructose stimulated the activity phosphoglucose isomerase and UDPGlc pyrophosphorylase, thus enhancing cellulose yield. Matsuoka *et al*., (1996) investigated BC synthesis by *A. xylinum* subsp. *sucrofermentous* BPR 2001 in agitated culture and found out that the presence of lactate in the growth medium stimulated bacterial growth and enhanced 4-5 times of cellulose yield. The preferred nitrogen sources are yeast extract and peptone, which are basic components of the model medium developed by Hestrin and Schramm (1954). It was also found out that significant part of the expensive medium components, i.e. yeast extract and bactopeptone, can be placed with corn steep liquor. Waste plant materials such as sugar beet molasses, spent liquors after glucose separation from starch hydrolysates, as well as whey and some pharmaceutical industry wastes (e.g. spent liquors after dextran precipitation with ethanol) were appropriate medium components (Krystynowicz *et al*., 2000).

The satisfying microbial cellulose production can be achieved using optimum growth medium composition, designed by mathematical methods and computer analysis (Joris *et al*., 1990, Embuscado *et al*., 1994, Galas *et al*., 1999). Some other compounds, strongly stimulating cellulose production by *A. xylinum* strains, like derivatives of choline, betaine and fatty acids (salts and esters), were also selected (Hikawa *et al*., 1996).

7.3 Effect of pH and temperature

Analysis of the influence of pH on *A. xylinum* cellulose yield and properties, indicates that optimum pH depends on a strain, and usually varies between 4.0-7.0 (Johnson and Neogi, 1989; Galas *et al*., 1999). For instance, Ishikawa *et al*., (1995) and Tahara *et al*., (1997) who applied for studies two different *A. xylinum* strains, observed the highest polymer yield at pH 5.0. Except pH of a nutrient broth, also temperature influenced BC yield and properties. Krystynowicz *et al*., (1997) reported that the temperature range from 28 to 30°C and its variations caused changes of cellulose degree of polymerization and water-binding capacity. Tahara *et al*., (1997) revealed that pH 5.0, optimum pH for the strain growth and BC synthesis, the activity of both cellulases is several times higher than at pH 4.0, at which the BC production is only slightly declined.

7.4 Fermentor types

Synthesis of BC is run either in static culture or in submerged conditions, providing proper agitation and aeration, necessary for medium homogeneity and effective mass transfer. The choice of culture conditions strictly depends on polymer destinstion.

8. Bacterial cellulose purifications

BC obtained through stationary or agitated culture is not pure and contains some impurities such as culture broth components and *A. xylinum* cells. Prior to use in medicine, food production or paper industry, all these impurities must be removed. One of the most widely used purification methods is based on treatment of

BC with solutions of hydroxides (mainly sodium and potassium), sodium chlorate and hypochlorate, H_2O_2, diluted acids, organic solvent or hot water. The reagents can be used alone or in combinations (Yamanaka *et al*., 1990).

BC was immersed in their solutions for 14-18 h, in some cases up to 24 h at elevated temperature (55-65°C), markedly reduces the number of cells and coloration degree. BC was boiled in 2% NaOH solution after preliminary running tap water treatment (Yamanaka *et al*., 1989). Above, it was immersed in 0.1% NaOH at 80°C for 20 minutes, and next washed it with distilled water (Watanabe *et al*., 1998). The processes of purification of BC was developed, crude BC was washed in running tap water for overnight, followed by boiling in 1% NaOH solution for 2 h, washed its in tap water to accomplish NaOH removal for 24 h, neutralized with 5% acetic acid (Krystynowicz *et al*., 1997). In medical application of BC requires special processes to remove bacterial cells and toxins, which can cause pyrogenic reaction. One of the most effective protocols begins with gentle pressing of BC pellicle and immersed in 3% NaOH for 12 h (repeat 3 times) and after that incubated in 3% HCl solution, pressed and thoroughly washed in distilled water. The purified pellicle is sterilized in autoclave or by cobalt-60 radiation (Ring *et al*., 1986). Dilute alkaline solutions are capable of hydrolyzing and removing impurities present in the cellulose pellicle. After alkaline treatment and washing in distilled water, the cellulose mass can be dried and processed into pure cellulose membranes (Supaphol and Spruviell, 2000). Cellulose consists of amorphous and crystalline regions existing together. The degree of crystallinity is known to vary depending on the origin and mode of chemical treatment of the material. Attalla and Vanderhart (1984) showed that native cellulose is a composite of two or more distinct crystalline forms mainly comprising 1α and 1ß

phases. Sugiyama *et al*., (1991) confirmed the existence of two different crystalline forms co-existing within single cellulose micro fibrils through electron microscopy and diffraction studies. The hydrothermal annealing treatment using alkaline solutions can cause transformation of metastable 1α phase, which occurs predominantly in bacterial cellulose, to more thermally stable 1ß phase (Yamamoto *et al*., 1989).

9. Applications of bacterial cellulose

Commercial application of this polymer results from its unique properties and developments in effective technology of production, based on growth of improved microbes on cheap waste materials. The advantage of BC is its chemical purity and the absence of substance usually assisting the plant polysaccharide, which requires laborious purification. Besides, the shape of BC sheet, its area and thickness can be tailored by means of culture conditions. Relatively easy BC modification during its biosynthesis, enables regulation of such properties as molecular mass, elasticity, resilience, water holding capacity, crystallinity index, etc.

9.1 Filter membranes

Filter membranes are categorized as symmetric, asymmetric, porous or non-porous, depending on their filtration behavior, materials used and manufacturing processes. Normally, they are manufactured from polymers composite materials between two polymers, or between polymer and ceramic. Related theories, membrane type and application can be studies in the handbook provided by Ho and Sirkar (1992). It has been well known that bacteria, such as *Acetobacter, Rhizobium*, *Argrobacterium* and *Sarcina*, synthesize bio-polymers. Among these, gram-negative

A. xylinum is claimed to be an effective cellulose-producing bacterium and is widely used (Jonas and Farah, 1998; Yang *et al*., 1998). It can be simply grown in a shallow tray with a culture medium such as coconut juice, sugarcane juice, vinegar, and fermented beverage, which are plentiful locally. Cellulose network formed as a sheet floating on the medium surface has been proved to have high tensile strength, elasticity, resilience, durability, shape-retention, high water binding capacity, non-toxic and non-allergen (Schmitt *et al*., 1991).

9.2 Paper industries

BC is an excellent component of papers, providing better mechanical properties. Microfibrils of the bacterial polymer from a great number of hydrogen bonds when the paper is subjected to drying, thus giving improved chemical adhesion and tensile strength (higher value of the Young's modulus). BC containing paper show better retention of solid additive such as filler and pigments, but are also more elastic, air-permeable, resistant to tearing and bursting forces, and bind more water (Iguchi *et al*., 2000). A good effect of BC containing paper such as improved ageing resistance, was achieved by adding of BC to cotton fibers to obtain handmade paper, use as information and document paper, pressboard, paperboard and bookbinding, use as old documents repairing, appropriate ink receptivity and specific snap (Krystynowicz *et al*., 1997). BC was added to improve the surface coating for specific paper such as to improve gross, brightness, smoothness, ink receptivity and tensile strength. Substitution of BC for paper and organic polymers could greatly reduce pollution of the environment (Jenelten, 1998).

9.3 Food industries

Chemically pure and metabolically inert, BC has been applied as non caloric bulking and stabilizing agent in processes food. The first successful commercial application of BC in food production is nata de coco (Sutheland, 1998). It is a traditional dessert from Philippines, prepared from coconut milk and coconut water with sucrose, which serve as a growth medium for BC producing bacteria. Consumption of the pellicle is believed to protect against bowel cancer, artheriosclerosis and coronary thrombosis and prevent sudden rise of glucose in the urine. BC-containing food product is Chinese Kombucha (Teakvass or tea-fungus), obtained by growing yeast and acetic acid bacteria on tea and sugar extract. The pellicle formed on the surface contains both cellulose and enzymes healthy for humans. Their abiotic activity is especially stimulating for large bowel and the whole alimentary tract. Kombucha is believed to protect from some cancers (Iguchi *et al*., 2000). The preparations of bioactive anthocyanin enriched in dietary fiber are excellent for functional food production. BC also appeared to be an attractive component of bakery products, since it plays a role of dietary fiber, is taste and odorless and prolongs the shelf-life.

9.4 Miscellaneous uses

BC can be applied as a carrier for immobilization of biocatalysts because the large surface area, high durability and superior adsorptive properties as well as possibility of modification by mean of physical or chemical methods. Cellulose gel containing immobilized animal cells was used for their breeding, in

production of interferon, interleukin-1, cytostatics and monoclonal antibodies (Iguchi *et al*., 2000).

9.5 Medical applications

9.5.1 Bacterial cellulose as a potential scaffold for tissue engineering of cartilage

The main purposes of articular cartilage, which contains a small number of cells (chondrocytes) in an extra cellular matrix (ECM) mainly composed of water, collagen type II and proteoglycans, are to cover the ends in bones in joins to provide frictionless movement and to distribute loads (Wilkins *et al*., 2000). Ostheoarthritis is a disease of synovial joints resulting in pain and loss of function for the patient (Freeman, 1973). Damaged cartilage has limited regenerative capacity and therefore over 1 million patients in the United States require treatment for cartilage defects each year. However, presently these treatments result in limited pain relief and restorative tissue function.

Thus, tissue engineering has potential to provide a supply of functional cartilage for the repair and regeneration of compromised native soft tissues (Schreiber *et al*., 1999). The use of scaffolds in the tissue engineering of cartilage is essential in order to support cell proliferation and maintain their differentiate function in addition to definition of the shape of new growing tissue. A variety of scaffold materials have been evaluated including natural polymers like collagens, alginate, hyaluronic acid, fibringlue and chitosan and synthetic polymers including polyglycolic acid (PGA), polylactic acid (PLA), polyvinyl alcohol (PVA), polyhydroxyethylmethacrylate

(pHEMA) and poly *N*-isopropylacrylamide (pNIPAA). However, tissue constructs with native mechanical properties have not yet been described in the literature. It is also desirable to utilize a scaffold material that has the porosity necessary to support cell in growth and effective mass transport while also supplementing the mechanical properties of engineered tissue. This scaffold must also be biocompatible and support native ECM and biopolymer production, with degradation rates commensurate with the rate of the new tissue formation.

BC is secreted by *A. xylinum* which has unique properties including high water holding capacity, high crystallinity, a fine fiber network, and high tensile strength (Wilkins and Browning, 2000). BC has potential to be used as a substrate for tissue engineering of cartilage due to its high strength in the wet state as well as its moldability in situ, biocompatibility and relatively simple, cost-efficient production (Schreiber *et al*., 1999).

9.5.2 Bacterial cellulose as a potential wound dressing

Biofilm from modified bacterial cellulose displays several advantages such as biological dressing when applied on exudation or bloody tissue. It is valuable as a temporary skin substitute in the treatment of skin wounds such as burns, ulcers, grafts and as an adjuvant in all abrasions (Fontana *et al*., 1990).

The cellulose biomembrane originating from bacterial fulfils a function similar that of the epidermis in relation to the dermis due of its physicochemical and electrostatic properties. The cellulose biomembrane establishes a suitable microenvironment, creating optimum physiological conditions necessary for correct wound healing, which indeed reduces the cost of treatment especially in cases of

burns and venous leg ulceration (Slezak *et al*., 2004). Selective permeability with regard to liquids and gases allows transpiration and permits gas exchange and thermoregulation. Moreover, it improves the wound's granulation, which allows rapid and complete wound healing. Due to its electrostatic properties, the membrane adheres firmly to the wound site and protects the wound and nerve endings from mechanical stimulation and reduces pain. One type of bacterial alkali-cellulose biomembrane was obtained through biosynthesis from the cells of *A. xylinum*. In contrast to cellulose obtained from wood, bacterial cellulose has a microfibrous structure and its hypo-allergic, non-toxic, non-irritant, biodegradable, non-pyrogenic, highly hydrophilic and biocompatible (Ulmer *et al*., 2002).

The small size of BC fibrils seems to be a key factor that determines its remarkable performance as a wound healing system. Furthermore, the never dried cellulose membrane is a highly nano-porous material that allows for the potential transfer of antibiotics or other medicine into the wound, while at the same time serving as an efficient physical barrier against any external infection. BC produced in the form of gelatinous membrane can be molded into any shape and size during its synthesis, depending on the fermentation technique and conditions used (Bielecki *et al*., 2002). Unlike celluloses of plant origin, BC is entirely free from lignin and hemicelluloses. A vigorous treatment with strong bases at high temperatures allows the removal of cells embedded in the cellulose net and it is possible to achieve a non-pyrogenic, non-toxic and fully biocompatible biomaterial dressing material (Alvarez *et al*., 2004). Cellulose pad from static culture is a ready-to-use, usually wound dressing material that meets standards for modern wound dressing. It is sterilizable, biocompatible, porous, elastic, easy to handle and store, adsorbs exudation, provide

optimum humidity, which is essential for fast wound healing, protects from secondary infection and mechanical injury, does not stick to the newly regenerated tissue and alleviates a pain by heat adsorption from burns. BC sheets are also excellent carrier for immobilization of medicine preparations, which speed up the healing process.

Wound dressing produced from bacterial cellulose

There have been several publications and reports on the successful use of BC as a medical product such as Biofill® is product of BC with wide applications. It has been used for several skin injury treatments such as basal cell carcinoma/skin graft, severe body burns, facial peeling, sutures, dermabrasion, skin lesions, chronic ulcer, and both donor and receptor sites in skin grafts (Fontana *et al*., 1990). Cases of second and third degree burns, ulcers and others could be treated successfully with BioFill® as tempolary substitute for human skin. The authors documented the following advantages for Biofill® in more than 300 treatments such as immediate pain relief, close adhesion to the wound bed, diminished postsurgery discomfort, reduced infection rate, easiness of wound inspection (transparency), faster healing, improved exudates retention, spontaneous detachment following reepithelization and reduced treatment time and costs. Only one disadvantage was mentioned limited elasticity in areas of great mobility (Klemm *et al*., 2001). Gengiflex® was developed to recover periodontal tissues. Novaes (1992) who described a complete restoration of an osseus defect around and IMZ implant in association with a Gengiflex® therapy. Gengiflex® is biocompatible, inert and hypoallergenic (Gottlow *et al*., 1994).

10. Topical antimicrobial agents used in burn wounds

A cutaneous burn denudes the skin of keratin layer and a number of layers of essential skin cells, as well as inducing localized reduction of blood supply, thus resulting in tissue hypoxia (Hettich, 1986). Loss of this protective integuum results in bacterial penetration into the underlying tissue, which can result in fatal burn sepsis. Prevention of burn wound sepsis is dependent on optimum wound management, including the use of topical antimicrobial and prompt closure of the wound defect. The advent of topical antimicrobials in the 1960s reduced the mortality from burns wound sepsis from 60% to 28% (Pruitt *et al*., 1968). However, burn wound sepsis remains and important and potentially remediable cause of significant mortality and morbidity (Pruitt *et al*., 1998). The advent of multiresistant bacteria has hastened the almost exclusive use of silver-base topical antimicrobial agents (TAAs) for the prevention of burn wound sepsis (Klasen, 2000).

During the late 1960s, silver sulphadiazine was the gold standard topical anti-microbial used in burns patients around the world. An outbreak of resistant *Staphylococcus aureus* in the burns unit in Royal Melborne Hospital in 1971 led Dr. M. Clarke to introduce 0.2% chlorhexidine digluconate to 1% silver sulphadiazine for attempt to control this outbreak. The trial was successful and the resultant dressing was introduced as Silvazine^{TM} (1% silver sulphadiazine and 0.2% chlorhexidine digluconate). The transition from standard 1% silver sulphadiazine to 1% silver sulphadiazine with 0.2% chlorhexidine digluconate resulted in a 50% reduction of burn wound sepsis (Georage *et al*., 1997).

Silver sulphadiazine is an excellent of activity, low toxicity and ease of application with minimal pain, it is still the most frequently used topical agent. Silver

sulphadiazine is thought to act via inhibition of DNA replication and modifications of the cell membrane and cell wall. This drug is bactericidal against species of both Gram-negative and Gram-positive organisms, but resistance has occasionally been reported (Fox *et al*., 1968).

11. Chlorhexidine

Chlorhexidine is a biguanide compound, widely used in clinical practice as a skin and mucous membrane antiseptic and disinfectant. Chlorhexidine is a disinfectant which is effective against a wide range of vegetative Gram-positive and Gram-negative bacteria; it is ineffective against acid fast bacteria, bacterial spores, fungi and viruses (Bondar, 2000). It disrupts the plasma membrane of the bacterial cell and cellular contents are lost and it is more effective against Gram-positive than Gram-negative bacteria, some species of *Pseudomonas* and *Proteus* being relatively less susceptible (Kuyyakanond *et al*., 1992). Chlorhexidine is most active at a neutral or slightly alkaline pH, but its activity is reduced by blood and other organic matter (Sawada, 1985). As local chlorhexidine treatment has proved to be effective in the prevention of nosocomial respiratory tract infections, urinary tract infections in transurethral surgery, reducing material and neonatal infections morbidity during childbirth, and decreasing mortality in experimental intra-abdominal sepsis (Bondar *et al*., 2000). As a handwash or surgical scrub, 4% chlorhexidine causes a greater initial decrease in the number of cutaneous bacteria than does either 7.5% povidone iodine or 3% hexachlorophene and it has a persistent effect equal to or greater than that of hexachlorophene (Smylie *et al*., 1973). 0.5% solution in 95% ethanol experts a greater effect than 4% chlorhexidine emulsion, 3% hexachlorophene solution (Lowbury *et*

al., 1971). A 1% aqueous solution is more erratic and has a less persistent action than do the other preparations. In experiments in which gloved hands were inoculated with large number of *Providencia, Serratia*, *Pseudomonas* and *Escherichia*, 4% chlorhexidine appeared to be somewhat less effective than 7.5% povidone-iodine (Dineen, 1978).

11.1 Chlorhexidine gluconate

Chlorhexidine gluconate is used in disinfectant solutions, creams and gels. A 0.5% solution in alcohol (70%) is used for the pre-operative disinfection of the skin and 0.05% aqueous solution is used as a wound disinfectant. A 0.05% solution in glycerol is used for urethral disinfection and catheter lubrication, and a 0.02% solution for bladder irrigation. A 1% cream is used in obstetrics. A 0.02% solution containing sodium nitrite 0.1% is used for the storage of sterile instrument (Martindale, 1993).

11.2 Chlorhexidine acetate

Chlorhexidine acetate is used for skin disinfection and in concentration of 0.01% as a preservative for eye-drops (Martindale, 1993).

11.3 Chlorhexidine hydrochloride

Chlorhexidine hydrochloride has been used in creams and dusting powders; a cream containing 0.1% of chlorhexidine hydrochloride and 0.5% of neomycin sulphate is used in the prophylaxis and treatment of nasal carriers of *Staphylococci* (Martindale, 1993).

11.4 Chlorhexidine digluconate

Chlorhexidine digluconate is commercially available as a 20% w/v aqueous solution since the substance cannot be isolated as a solid. Its molecular structure is shown in Figure 6. It is soluble in water to at least 50% w/v but high viscosity makes such concentrated solutions inconvenient to use. Chlorhexidine digluconate solution has been used as a topical antiseptic and disinfectant effective against a wide range of bacteria, some fungi and some viruses (Martindale, 1993). The addition of chlorhexidine hydrochloride or digluconate 0.2% w/v of silver sulphadiazine cream used in the treatment of burns reduced the isolation rate of *Staphylococcus aureus* in 51 patients to 9.2% compared with 34% in 91% patients treated with silver sulphadiazine alone (Clarke, 1975).

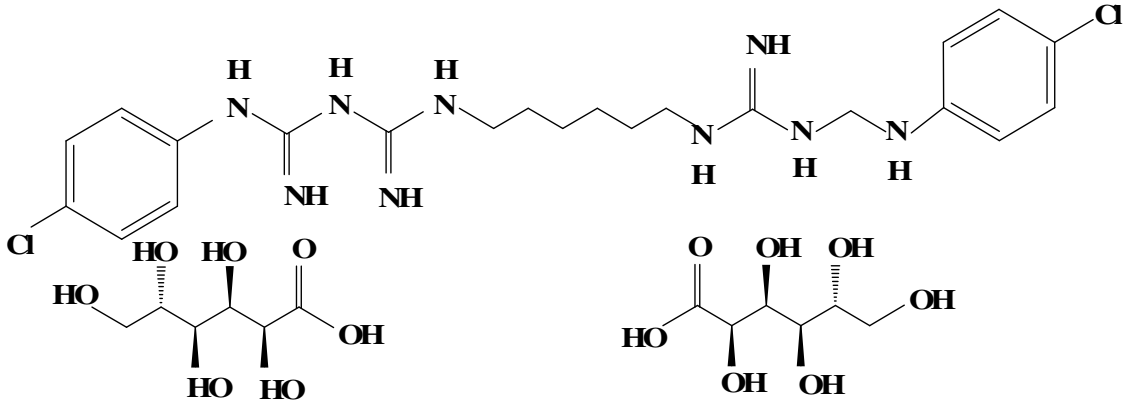

Figure 6. Molecular structure of chlorhexidine digluconate

12. Aims and scope of this thesis

The aims of this study were to evaluate the properties of BC produced from different carbon sources and to prepare the film of BC from different carbon sources containing chlorhexidine digluconate for studying the release of chlorhexidine digluconate.

Scope of this thesis;

1. To evaluate the properties of BC produced from different carbon sources
2. To prepare the film of BC containing chlorhexidine digluconate for studying the release of chlorhexidine digluconate from BC