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Biotechnological production of bacterial cellulose

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Contents

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Abstract

Bacterial cellulose (BC) is highly pure and has unique properties such as flexibility, hydrophilicity, crystallinity, and a 3D network. This study investigated the effects of carbon source and cultivation conditions on BC production and structure. The most suitable carbon source was found to be fructose, and high BC concentrations were achieved under static and agitated cultures. NaOH and NaOCl solutions were effective in removing trapped cells, but high alkali concentrations led to structural damage of BC. A combination of weak NaOH and Nicoll was the most effective purification method that preserved the fibre structure and crystallinity of BC. In vitro gastrointestinal simulation showed that BC increased viscosity in the stomach but did not have prebiotic effects. Overall, BC has potential as a satiety food formulation.

Chapter 1: Introduction

Cellulose is one of the most abundant biopolymers on Earth, and is mainly of plant, wood and bacterial origin. Cellulose originating from bacteria exhibits the highest purity and has thus attracted the interest of many researchers and industrial sectors (Huang et al., 2013). From a structural point of view, bacterial cellulose (BC) consists of randomly assembled ribbon-shaped fibrils, composed of 7-8 mm wide elementary nanofibrils aggregated in bundles (Gorgieva and Trček, 2019). Unlike plant cellulose, BC is an ultrafine material, does not contain lignin, hemicellulosic components and other impurities, (Conner 1995). In addition, BC is characterised by high degree of polymerization, high crystallinity, high capacity in tension or compression (Czaja et al., 2004), properties that render BC an attractive material in diverse areas.

Media used for BC production mainly require carbon and nitrogen sources and salts to buffer pH (Molina-Ramírez et al., 2017), and it has been reported that in microbial fermentation techniques to produce BC, the cost of the substrate is often more than half of the total process cost (Vazquez et al., 2012). However, microorganisms have metabolic preferences even if they are the same genus between different species (Vazquez et al., 2012). In addition, the mode of oxygen supply during cultivation (either static or agitated culture) also has an effect on the production and physicochemical properties of BC (Singhsa et al., 2018).

BC already has a long history of use in culinary cultures across the world, such as in the case of the Philippine dessert Nata, a bacterial cellulose gel (Phisalaphong et al., 2012). Moreover, multiple toxicology experiments in recent years have proved that BC is safe and non-toxic to rats and mice (Dourado et al., 2017), whereas as early as 1992, the US Food and Drug Administration included BC as a material with "generally recognized as safe" status (GRAS) (Shi, Zhang, Phillips and Yang, 2014). However, the impact of BC on the digestive system is still under reported. Literature evidence suggests that increasing the viscosity of food can reduce food intake or suppress appetite (Zhu et al., 2013), and high viscosity glucomannan has been used commercially as a satiety food ingredient. As such, the effect of high viscosity BC hydrocolloid solution on digestion is still largely unknown. In addition, dietary fibre has been shown to have a series of health benefits, they can assist in reducing the risk of chronic diseases such as diabetes, obesity, cardiovascular disease, and diverticulitis (Cho & Almeida, 2012). Established dietary fibres derive mainly from plant-based materials and include β-glucan, arabinoxylans and fructans, among others. Dietary fibres that can survive hydrolysis in the upper gastrointestinal tract and are utilized by gut bacteria, producing organic acids and other metabolites, imparting thus beneficial effects on the host, these fibres are also known as prebiotics (Zhu et al., 2013). So far, there has been scarce information in the international literature in terms of BC digestion and whether this polymer has potential as a prebiotic.

1.1 Overall aims and objectives of the study

The overall aim of the present study was to explore the production and downstream processing of BC, by evaluating the impacts of fermentation parameters and purification on production yield, BC structure and digestion. As such, the main objectives of the study were as follows:

1. Evaluate the effect of carbon source on BC synthesis and structure.
2. Establish a purification protocol for synthesised BC and assess the impact on its structural properties.
3. Assess the performance of BC during *in-vitro* gastrointestinal digestion simulation and its prebiotic potential.

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Chapter 2: Literature review

2.1Background information

Cellulose, produced by plants or bacteria, is an almost inexhaustible resource of organic polymers on Earth and with global economic importance (Kim et al. 2006). Due to the good biocompatibility, high rigidity, fibrous morphology and low cost of fibre (Huang et al., 2013), the production of manmade cellulosic fibres amounted to 6.5 million metric tons in 2020 (Fernández, 2021) and widely used in various fields such as strengthening polymers and paper (Miao and Hamad, 2013; Zimmermann et al., 2010), food stabilizers and thickeners (Shi, Zhang, Phillips and Yang, 2014), and food packaging (Spence et al., 2010).

Bacterial cellulose is a homopolymer consisting of hundreds to thousands of β (1→4) linked D-glucose units (As shown in Fig. 1 below). The repeating unit of the polymer consists of two glucose molecules bound together such that one molecule is rotated 180° relative to the other (Mohite and Patil, 2014). Cellulose has abundant surface hydroxyl groups, forms a large number of intermolecular and intramolecular hydrogen bonds, and has the characteristics of hydrophilicity, chirality, biodegradability and a wide range of chemical modification capabilities (Klemm et al., 2005). The origin and treatment of cellulose affects the specific assembly and supramolecular order of cellulose, which determine the properties of cellulose (Eichhorn et al., 2010).

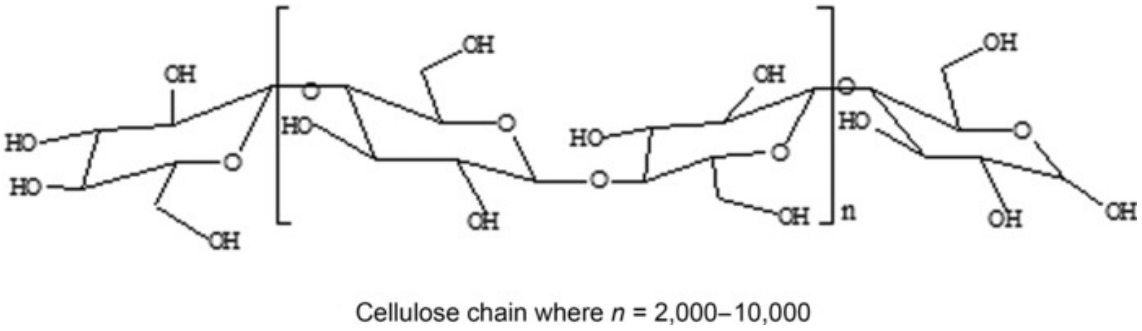

Figure. 1: chemical structure of cellulose

In 1886, Brown (1886) discovered a jelly-like membrane on the surface of vinegar fermentation broth and proposed the concept of bacterial cellulose (BC), which was also described as "a sort of moist skin, swollen, gelatinous and slippery..." (Ring, 1982). One of the most well-known applications of bacterial cellulose is in making nata-de-coco, a native Filipino dessert. The locals ferment coconut water to produce gel sheets about 1 cm thick, which are then cut into squares of 1 cm × 1 cm. Washed cubes are often served with flavoured syrups, jellies, or other fruit cocktails (Nadia, 2012). Similar foods can also be prepared using other fruit juices, such as Nata-de-pina produced from pineapple (Iguchi et al., 2000). Nowadays, Nata-de-coco is mass-produced and exported to other countries, such as Malaysia and Japan (Nadia, 2012).

Bacterial cellulose (BC) has the same molecular formula as plant cellulose, but it has a unique and complex three-dimensional porous network structure (Hu et al., 2014). Most importantly, BC does not contain impurities such as lignin and hemicellulose, making it a high-purity cellulose source (Mohite and Patil, 2014). The differences between BC

and plant cellulose (PC) are also reflected in high crystallinity (70-80%) and different degrees of polymerization (DP) (Barud et al., 2011); the DP of BC is between 2000 and 6000 (Jonas and Farah, 1998), while the average DP of PC varies from 13000 to 14000 (Teeri, 1997). BC also has high tensile strength, moldability, and is insoluble in most solvents (White and Brown, 1981; George et al., 2005a; George et al., 2005b), it is even 100 times thinner than cellulose fibrils obtained from plants and has good shape retention (Mohite and Patil, 2014). The typical Young's modulus of BC is in the range of 15 to 35 GPa and tensile strength is in the range of 200 to 300 MPa. The water holding capacity can reach up to 100 times its own weight (Mohite and Patil, 2014), while BC also exhibits excellent biodegradability and biocompatibility (Dahman, 2009). These amazing properties have aroused great interest and explored the wide application of BC in various fields such as medical, food, advanced acoustic diaphragms (Hu et al., 2014), stimulating more research in the potential application possibilities of BC.

HPMC is a semi-synthetic, inert viscoelastic polymeric cellulose, it is widely used in commercial products such as eye drops, adhesives, and film-forming agents (Cano-Barrita & León-Martínez, 2016; de Silva & Olver, 2005; Williams et al., 2001; Kaur et al., 2018). The molecule of MHPC has a cellulose backbone consisting of β-d-glucose units with (1→4) linkages, but the three free-hydroxyl groups are partially etherified with methyl groups (Cano-Barrita & León-Martínez, 2016). However, the properties of hydroxypropyl methylcellulose films depend largely on their linear structure and molecular weight (Ghadermazi et al., 2019), which leads to HMPC with molecular

weights between 1.3 × 104 and 2 × 105 g mol-1, with a DP of 70 to 1100 relative to glucose monomers (Cano-Barrita & León-Martínez, 2016). However, HPMC needs to be heated to 70-90°C to form hydrogel solution, which limits the application of HPMC in the food field (Noval et al., 2020).

2.2Bacterial cellulose origin, biosynthesis and structure

2.2.1 Strains of BC production

Research has shown that BC can be produced by different bacteria. *Rhizobium* and *Agrobacterium* can synthesize cellulose microfibrils to adsorption to plant hosts, but the yield is extremely low. Bacteria with high BC yields that can form significant amounts of BC membranes are mainly *Gluconacetobacter* (formerly *Acetobacter*), *Gluconobacter*, and *Asaia* in *Acetobacteriaceae* (Li et al., 2021; Wang et al., 2019). Some popular BC-producing *Acetobacteriaceae* strains are listed in Table. 2, along with their reported BC production. *A. xylinum* (Brown, 1886; Gromet-Elhanan and Hestrin, 1963; Geyer, Klemm and Schmauder, 1994), *A. hansenii* (Jung et al., 2005; Park, Jung and Park, 2003) and *A. pasteurianus* (Yoshino, Asakura and Toda, 1996) are considered the most effective BC producing bacteria, while *A. xylinum* has been successfully applied in the commercial production of BC due to its high productivity (Wang et al., 2019).

Table. 2: Bacterial cellulose production by bacterial strains.

Genus	**Specie**	**Strain**	**Strain origin**	**BC production (g/L)**	**Carbon source**	**Properties of BC**
Komagataeibacter	*Xylinus*	ATCC53524	-	3.83	Glucose (20 g/L)	Purity, high crystallinity, and densely packed network
		ATCC700178	-	5.65	Fructose (40 g/L)	
		ATCC23770	-	6.23	Sulfate (SAFS) hydrolysate (Glucose 14.1 g/L, xylose 3.3 g/L)	High crystallinity, stable mechanical property and nanoscale polymer (the fibres were in the range 15 to 70 nm, with an average width of 35 to 40 nm)
		ATCC23767	-	2.93	Glucose	Nanoscale dimensions (length of several μm, width between 20 and 70 nm) and nanostructured network
		BPR 2001	Black cherry	4.4	Corn steep liquid (20 ml/L) & Fructose (40g/L)	
		BPR2002	Grape	-	-	
		BPR2003	Melon	-	-	
		BRC5	-	15.3	Corn steep liquid (8% v/v) & Glucose (2 w/v)	

	CGMCC2955	Fermentation substrates of vinegar	1.58	Glucose (25 g/L)	
	FC01			Glucose (2% w/v)	Dense fibril structure
	K3	Kombucha culture	0.28	Glucose (20g/L)	Purity and high crystallinity
Hansenii	P2A	Vinegar fermentation waste, vinegar samples, and fresh	3.25	Glucose (2%)	Under static conditions: an ordered and dense network of fibrils with diameters as thin as 8–10 nm; under agitated cultivation conditions: much looser clump of disordered short and thin fibrils, relative crystallinity index at 72.6–78.7% (78.7, 72.6, and 77.3% for static, shaken, and agitated conditions, respectively) and with a single degradation step (starting at 280–300 °C)
	PJK	Rotten apple		-	High purity

Some non-*Acetobacteriaceae* bacteria, including *Bacillus sp.*, *Leifsonia sp.*, *Salmonella sp.*, *Erwinia sp.*, *Enterobacter sp.*, *Pseudomonas sp.*, and *Shewanella sp.*, have also been shown to have the ability to synthesize BC. For example, *Bacillus amyloliquefaciens*, can synthesize substances with a structure similar to that of BC under static conditions and suspend on the surface of the solution (Li et al., 2021). The Gram-positive bacteria *Leifsonia sp.* CBNU-EW3 was screened from earthworms, it can also produce BC membranes in static acidic conditions, but it takes longer to form membranes that the initial formation of BC membranes can be seen on top of the medium until day 5. Additionally, the thickness of the BC membranes is not uniform (Velmurugan et al., 2015). *Salmonella enterica* is a short, rod-shaped strain that produces BC with a honeycomb-like microstructure, with the bacteria at the centre of the honeycomb. This is thought to be a different gene sequence for the production of BC (Jahn et al., 2011). The cellulose synthesized by *Dickeya dadantii* 3937 has a fibre network structure similar to that of the *Acetobacteriaceae*, but its fibres are beaded, possibly is caused by its unique third type of secretory system (T3SS) (Jahn et al., 2011).

BC produced by *Enterobacter* sp. CJF-002 is similar in cellulose network structure to the one produced by *G. xylinum* ATCC23769, as their membrane production genes are very similar. Moreover, the process of BC membrane producing in *Enterobacter* sp. CJF-002 belongs to growth coupling type, which means that the synthesis of BC has no obvious lag period. Its membrane formation speed is faster than that of G. *xylinum* ATCC23769 and the network structure of cellulose is also denser. In general, *Enterobacter* sp. CJF-

002, as the *Enterobacter* sp, with the highest BC production, has shown great potential for industrial applications (Sunagawa et al., 2012). Furthermore, *Enterobacter* sp. is a facultative anaerobic microorganism that can generate sufficient energy for fibre synthesis under both anaerobic and aerobic conditions (Ji et al., 2016). *Shewanella oneidensis* MR-1 is also a facultative anaerobe. During aerobic growth, its bacterial metabolism is active, and a pronounced biofilm is produced on the surface of the medium (Liang et al., 2012). In addition, *Pseudomonas* spp. RV14, a Gram-negative bacterium, can also produce bacterial cellulose, but its membrane-producing capacity is slightly lower than that of *Gluconacetobacter* spp. RV28 (Rangaswamy et al., 2015).

Escherichia coli has developed into a potential cell factory for bacterial cellulose production due to its advantages of fast growth, clear genetic background, mature genetic engineering tools, and stable plasmid system (Li et al., 2021). In addition to utilizing its natural synthetic pathway to produce BC, there are many studies using this strain as a chassis cell for heterologous bacterial cellulose production. A functional and stable BC production system was established in *E. coli* by recombinant expression of the BC synthase operon (bcsABCD) and upstream operons (cmcax, ccpAx). Compared with BC produced by *Acetobacter hansenii*, the length and diameter of BC were effectively increased (Buldum et al., 2017). Wild-type *E. coli* Nissle 1917 heterologously expressed the cellulose synthase subunit D (bcsD) gene of *Gluconacetobacter xylinus* BPR2001, improving the crystal structure of bacterial cellulose by 17% compared to wild type (Sajadi et al., 2017). Furthermore, *E. coli* Nissle 1917 has been reported to achieve

increased BC production without affecting its crystallization index, by heterologous expression of bcsA and B genes from *Gluconacetobacter xylinus* (Sajadi et al., 2019). Imai et al. (2014) indicated that *Gluconacetobacter xylinus* of CesA and CesB were heterologous in *E. coli* and synthesized very fine cellulose with unnatural crystalline structure. The results suggest that *E. coli* could be used as a platform strain for functional analysis of cellulose synthase and synthesis of new nanomaterials.

2.2.2 Benefit of BC for the producing microbe

Plant cellulose in the cell wall can have a protective effect on the cell, and BC seems to have a similar effect. Within natural habitats, BC plays a structural role through its mechanical, chemical, and/or biological protection, as in the case of *A. xylinum* and *Sarcina ventriculi*. It can also assist in cell adhesion processes necessary for symbiotic or infectious interactions, such as *Rhizobium* sp. and *Agrobacterium* sp. (Ross, Mayer and Benziman, 1991). It has also been suggested that the BC membrane produced by *A. xylinum* can reduce the competition of other organisms for limited resources. In addition, microorganisms can also synthesize BC to obtain oxygen for metabolic activities (Quintana-Quirino et al., 2019). Strictly speaking, BC cannot be regarded as a biofilm or an adhesive extracellular polysaccharide (Mohite and Patil, 2014), but it can protect organisms to a certain extent from UV radiation and retain moisture (Quintana-Quirino et al., 2019), promote colonization and increase the competitiveness for looting nutrients (Mohite and Patil, 2014).

2.2.3 Mechanism of biosynthesis of BC

The biosynthesis of bacterial cellulose can be divided into three steps. Firstly, uridine diphosphate glucose is synthesized. Then the cellulose molecular chain is synthesized. Finally, cellulose molecular chains are crystallized and polymerized to form BC.

2.2.3.A Synthesis of Uridine Diphosphate Glucose

In microbial cells, the carbon source from the medium is catalysed by a series of enzymes to uridine diphosphate glucose (UDP-glucose), an important precursor for BC synthesis. In *G. xylinum*, for example (Fig. 3), the glucose molecule produces energy and intermediate metabolites for bacterial growth through the pentose phosphate cycle (HMP) and the tricarboxylic acid (TCA) cycle. At the same time, glucose is catalysed by glucokinase, phosphoglucomutase and UDP-glucose pyrophosphorylase to form UPD-glucose, a precursor for cellulose synthesis. In the above process, glucose phosphate dehydrogenase in HMP determines whether bacteria use glucose for growth or cellulose synthesis (Karlstaedt et al., 2020; Li et al., 2021). In addition, UDP-glucose pyrophosphorylase located in the cytoplasm, is a key enzyme in UDP-glucose synthesis. In the absence of this enzyme, cellulose cannot be synthesized, due to the lack of precursors. Studies have shown that UDP-glucose pyrophosphorylase activity is higher in high cellulose-producing strains, but there are significant differences between strains (Vuong et al., 2005).

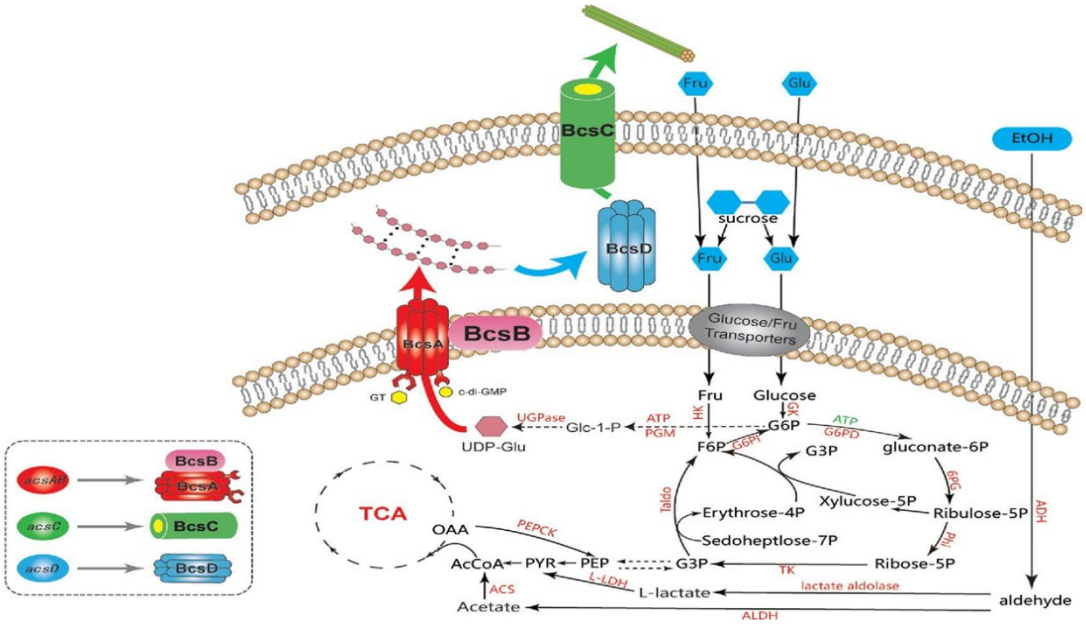

Figure. 3: A detailed biosynthesis pathway of various biomolecules involved in bacterial cellulose synthesis of *G. xylinum*. BC is synthesised via cellulose synthase, which is comprised the subunits of BcsAB, BcsC, and BcsD. First of all, carbon sources are catalysed by various of enzymes to form UPD-glucose, a precursor for cellulose synthesis. Then, the synthesis of β-1,4-glucan chains (red line) is produced with the catalysing of the BscAB subunit, which is expressed by the gene acsAB. Subsequently, the BscD subunit secreted by the gene acsD is involved in the crystallisation of β–1,4-glucan chains, to produce sub fibrils with a diameter of 1.5 nm (blue line). After that, the sub fibrils are discharged from the adventitia via the BscC subunit and finally aggregate to form a single fibre (green line). PGM, phosphoglucomutase; UGPase, UDP-glucose pyrophosphorylase; GK, glucokinase; HK, hexokinase; ACS, Acyl-CoA synthetase; PEPCK, phosphoenolpyruvate carboxykinase; G6PD, glucose-6-phosphate dehydrogenase; 6-PG, 6-phosphogluconate dehydrogenase; Taldo, transaldolase; TK, transketolase; Phi, ribosephosphate isomerase; ALDH, acetaldehyde dehydrogenase; ADH, alcohol dehydrogenase; L-LDH, L-lactate dehydrogenase; G6PI, glucose-6-phosphate isomerase; GT, glycosyltransferase. (Li et al., 2021)

Similar glucose metabolism and UDP-glucose synthesis pathways exist in other types of cellulose-producing microorganisms under anaerobic conditions, such as the facultative anaerobic *Enterobacter* sp. FY07 (Ji et al., 2016). Other non-glucose carbon sources, such as sucrose and fructose, can be involved in cellular metabolism and BC synthesis through various pathways such as TCA and gluconeogenesis (Matsushita et al., 2016; Velasco-Bedrán and López-Isunza, 2007). According to Tonouchi et al. (1996) (Fig. 4), fructose can be phosphorylated to fructose-6-phosphate (F6P). The phosphoglucose isomerase (PGI) existing in the cytoplasm of *A. xylinum* can be used to promote the interconversion of F6P and G6P; the subsequent metabolic process is the same as for glucose (Zhong et al., 2013). It is also reported that the activities of the enzymes involved in metabolism and BC synthesis differ depending on the bacterial species and carbon source (Tonouchi et al., 1996; Ross, Mayer and Benziman, 1991). *A. xylinum subsp. sucrofermentans* BPR2001 was used to study changes in enzymatic activity in different carbon sources, and data showed that PGI activity in cells in the presence of fructose was 132-fold higher than in cells grown in glucose-supplemented media (Tonouchi et al., 1996). Sucrose cannot be transported across the cell membrane, so it needs to be hydrolysed to fructose and glucose in the periplasm before being involved in metabolism and BC production (Velasco-Bedrán and López- Isunza, 2007).

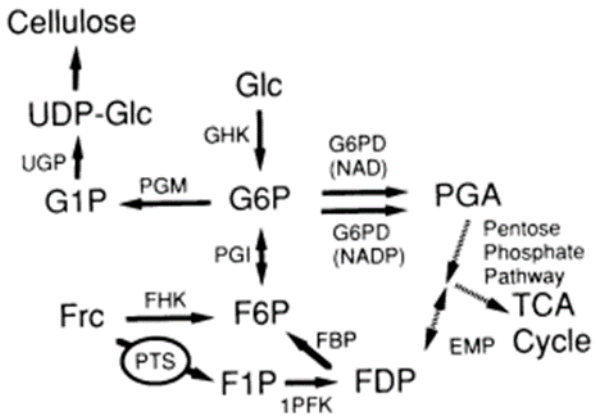

Figure. 4: biosynthesis pathway in *G. xylinum* with glucose and fructose (Tonouchi et al., 1996)

2.2.3.B Synthesis of Cellulose Molecular Chains

Under the action of cellulose synthase, UDP-glucose is catalysed to form β-1,4 glucan chains, namely cellulose microfibrils. Previous studies suggested that cellulose synthase is a glycosyltransferase, located on the cell membrane and plays a key role in the polymerization of cellulose molecular chains. In *G. xylinum*, the optimum temperature of the enzyme is 30°C, and the optimum reaction pH is 7.5-8.5 (Lin & Brown Jr, 1989). Recently, it was found that this enzyme is a protein complex containing multiple subunits, consisting of at least 4 types of cellulose synthase operon encoded proteins, BscA, BscB, BscC and BscD, respectively (Yoshinaga et al., 1997; Römling and Galperin, 2015). In most bacteria, BscA and BscB are functionally identical, which were used for the synthesis of cellulose *in vitro* alone. Other subunits vary between strains and regulate cellulose synthase activity and cellulose production by affecting cellulose synthesis, regulation and secretion (Römling and Galperin, 2015)

Cellulose synthase is expressed from an operon gene set. Taking *G. hansenii* ATCC 53582

as an example, its operon contains acsAB, acsC, acsD, *cmcax*, *ccpAx* and *bglxA*. Among them, acsAB, acsC and acsD are thought to be involved in the catalytic secretion of cellulose. *cmcax* encodes an endo-beta-1,4-glucanase, while *bglxA* encodes a beta-glucosidase, both of which are cellulases. The function of *ccpAx* is not clear, but it is presumed to be related to the distribution and arrangement of the subunits of cellulose synthase. But the missing of *ccpAx* results in a disorganized of cellulose synthase structure, leading to the reducing of BC production (Florea et al., 2016; McManus et al., 2016). Cellulose synthases comprising subunits like BcsA, BcsB, BcsC, BcsD and BcsZ, are known as type I cellulose synthases and are mainly found in *G. xylinum*. The complex containing the BcsA, BcsB, BcsC, BcsE and BcsZ subunits is called type II cellulose synthase and is usually represented by *Escherichia coli*. Type III cellulose synthase contains four subunits, namely BcsA, BcsB, BcsK and BcsZ, which are typically represented by *Agrobacterium tumefaciens* (Römling and Galperin, 2015). Also, more than one cellulose synthase complex may be contained in one bacterium; *Enterobacter* sp. FY-07 contains the above three cellulose synthase operons, expressing type I and type II cellulose synthases, but only type I cellulose synthase is secreted, which is required for cellulose production (Florea et al., 2016). In *G. hansenii* ATCC 53582, two different type II cellulose synthase operons appeared, in addition to the type I cellulose synthase operon, it is thought that two newly discovered operons may be related to the high production of cellulose (Florea et al., 2016).

BcsA-BcsB is the most important part for catalysing cellulose synthesis. All cellulose-

producing microorganisms include these two subunits. Taking the crystal structure of BcsA-BcsB obtained in *Rhodobacter sphaeroides* as an example (Morgan et al., 2012), the BcsA subunit contains eight trans-membrane components and two cytoplasmic binding moieties in this complex, while BcsB is in the periplasm space and anchored to the cell membrane by trans-membrane helix. Structurally, the complex is a channel extending from the glycosyltransferase across the membrane to the periplasmic space. The size of this channel allows passage of several glycoside units, suggesting that the synthesis of extracellular cellulose may take place here (Römling & Galperin, 2015). Experimental attempts to isolate this complex to achieve BC synthesis *in vitro* (Basu et al., 2016; Basu et al., 2017), and genes encoding the enzymatic complex have also been recombinantly expressed in *E. coli* to synthesize BC (Imai et al., 2014). Located in the periplasmic space, BcsC is hypothesized to be associated with peptidoglycan and other cellulose synthase complexes, contributing to the extracellular secretion of glucan. BscC has been found in many bacteria, such as *G. xylinus*, *Dickeya dadantii*, *Burkholderia phymatum*, *Salmonella enterica*, *Pseudomonas putida*, *Burkholderia mallei*, and *Chromobacterium violaceum* (Römling & Galperin, 2015). BscD is a cylindrical polymer with a diameter of approximately 90 Å, formed from protein proteins, can simultaneously synthesize four different dextran molecular chains (Hu et al., 2010).

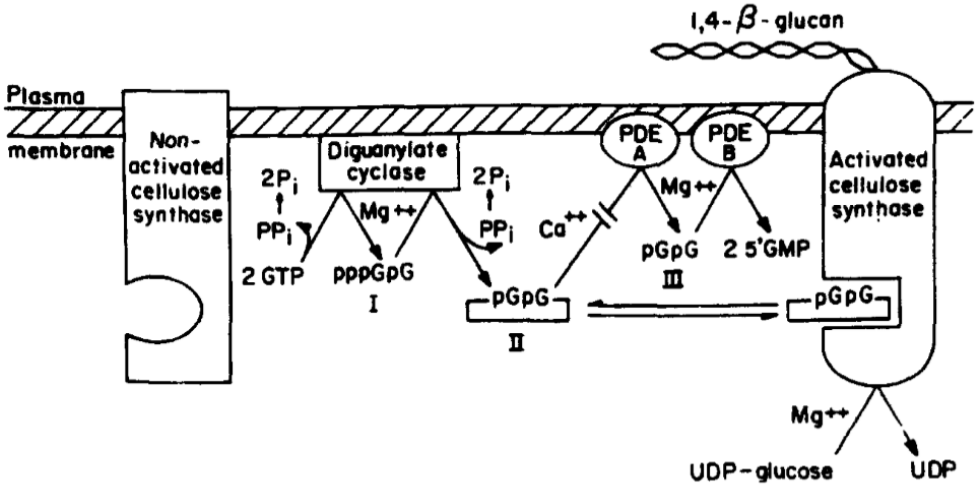

Fig. 5: Model for regulation of cellulose biosynthesis in A. xylinum (Ross et al., 1987)

Additionally, the activity of cellulose synthase varies widely between cellulose and non-cellulose producers, a co-factor named cyclic di-GMP (c-di-GMP) is deemed as the key factor. As shown in Fig. 5, it works as an allosteric activator of the membrane-bound cellulose synthase by binding to the enzyme directly in a reversible way at a regular site, totally different from the catalytic or the substrate-binding site. Diaguanylate catalyses two molecules of GTP via the linear intermediate pppGpG to c-di-GMP while the process of c-di-GMP inactivation needs two enzymes, that of c-di-GMP phosphodiesterases A and B (PDE-A and PDE-B). During the inactivation process, c-di-GMP is cleaved and forms pGpG by PDE-A, then both of them are degraded to two molecules of 5'-GMP rapidly. Experiments show that calcium ions (Ca^{2+}) can inhibit the activity of PDE-A to some extent (Vandamme et al., 1998; Ross et al., 1987; Ross et al., 1991).

In general, in the case of type I cellulose synthase, the Bcs A and Bcs B subunits,

expressed by the acsA and acsB genes, are responsible for the synthesis of cellulose microfibrils, while the BscD subunit is involved in the crystallization of sub-fibrils and is finally excreted by the BscC subunit. At the same time, other cofactors, such as c-di-GMP, ATP, Ca^{2+} and Mg^{2+}, are involved in the regulation of cellulose synthase activity. c-di-GMP is essential for cellulose synthesis and activates the BcsA and BcsB subunit to initiate the extension of the glucan chain (Florea et al., 2016). And the synthesis of c-di-GMP requires energy from the cell, making cellulose production organically linked to bacterial energy metabolism (Ji et al., 2016). Furthermore, the presence of Mg^{2+} is necessary for cellulose synthesis. When ethylenediamine tetra-acetic acid is added to chelate metal ions, catalysis is also terminated due to the dependence of BcsA and BscB on Mg^{2+} (Basu et al., 2016).

2.2.3.C Crystallization and Polymerization of Cellulose

BC is excreted from the cell membrane after intracellular glucan chain synthesis, polymerisation and crystallisation. In *G. xylinum*, cellulose synthase is distributed along the long axis of the cell. Approximately 50 micropore sites in each cell that secrete and excrete cellulose, at a cellulose synthesis rate of approximately 2 $\mu m \cdot min^{-1}$ (Ullah et al., 2016a). According to current studies, the BcsD subunit of the complex is primarily responsible for the crystallization of the dextran chains, while the BcsC subunit is responsible for the efflux of crystalline cellulose.

Overall, the secretion and assembly of BC mainly proceed through the following steps:

First, UDP-glucose forms β-1,4-glucan chains under the catalysis of BscA and BscB subunits. Subsequently, 10-15 β-1,4-glucan chains combine under the action of van der Waals forces to form sub-fibrils with a diameter of about 1.5 nm, which is mainly regulated by the BcsD subunit. These synthetic sub-fibrils are then discharged from the cell through the BcsC subunit (micropore) (Nicolas et al., 2021). Relying on hydrogen bonds between molecular chains, the sub-fibrils aggregate into microfibrils with a diameter of 5-8 nm. The microfibrils formed single bacterial cellulose fibres with a diameter of 75 nm through hydrogen bonding again.

2.2.4 Structural Properties of BC

A. xylinum can produce two forms of BC, namely the ribbon like polymer Cellulose I, and the thermodynamically more stable amorphous polymer, Cellulose II (Structure is shown in Fig. 6 below) (Yu and Atalla, 1996). Cellulose I consists of parallel β-1,4 glucan chains arranged uniaxially, while the β-1,4 glucan chains of Cellulose II are arranged in a random manner (Mohite and Patil, 2014). After regeneration or mercerization (alkali treatment), Cellulose I irreversibly changes its structure to Cellulose II with antiparallel oriented polymer chains rather than parallel as in Cellulose I crystals (Bergenstråhle et al., 2010).

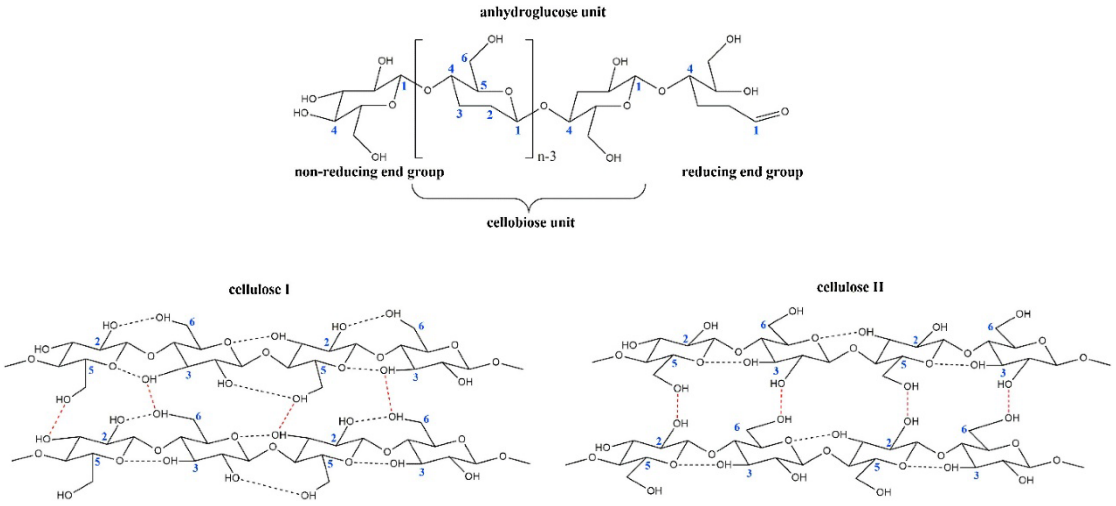

Figure. 6: The molecular structure of cellulose and the different supramolecular structures of cellulose I and II (Credou and Berthelot, 2014)

There are two polymorphs of Cellulose I, Cellulose Iβ and Cellulose Iα, which are both representative crystalline forms of natural cellulose. In nature, Cellulose Iβ and Cellulose Iα occur simultaneously in different proportions, and the source of cellulose is a crucial factor. Cellulose Iα presents a single chain triclinic unit, and it is the main component of bacterial cellulose, while Cellulose Iβ presents a double chain monoclinic basic structure and is mainly found in higher plants such as cotton and wood (Bergenstråhle et al., 2010; Oprea and Voicu, 2020).

As mentioned earlier, β-1,4-glucan chains are linked together by β-ether bonds to form sub-fibrils. Each anhydro glucose has one primary and two secondary hydroxyl functional groups, which makes BC hydrophilic (McNamara et al., 2015). These microfibrils are 100 times smaller than vegetal counterpart (Swingler et al., 2021). The excreted sub-fibrils are randomly intertwined with each other to form a dense matrix of cellulose fibres

that are built up through numerous inter- and chemical bonds; mainly hydrogen bonds, which occur between successive cellulose sheets. This high level of hydrogen bonding also allows cavities to form within the cellulose and possesses an ionic charge, allowing materials such as silver ions to be embedded. This property of BC will allow the loading of cellulose with specific antimicrobial agents with ionic charges that can target pathogenic microorganisms that cause infection (Volova et al., 2018). The increase in the number volume of cavities in BC also makes the water holding capacity of BC higher (UI-Islam, Khan and Park, 2012). The average fibre diameter of BC is only 1.5 nm, much lower than that of PC, allowing higher surface area, flexibility, elasticity and tensile strength (Prosvirnikov et al., 2018)

2.3 Biotechnological production of bacterial cellulose

2.3.1 Cost effective media used for BC production

The choice of bacterial source is not the only factor affecting BC synthesis, and the medium is also critical. Although a variety of media can be used for BC production, selection of the appropriate media and conditions to achieve the highest BC yield is critical (Desai et al., 2006). Through degradation or polymerization, microorganisms can utilize the components in the medium to achieve the synthesis of specified metabolites, and the nutrient availability of microorganisms affects the productivity and concentration of specific metabolites (Hussain et al., 2019). A variety of nutrients, including carbon, hydrogen, oxygen, nitrogen, vitamins, hormones and trace elements, are required to synthesize designated metabolites (Pirt, 1975). Zhang and Greasham (1999) classified

culture media into two categories, chemically defined or synthetic media and undefined or complex media. Chemically defined media consist of known concentrations of chemicals, while undefined media consist of natural ingredients at unknown concentrations. The HS medium developed by Hestrin and Schramm (1954) is the most commonly used chemically defined medium for BC production. Nowadays, various new media have been tried for bacterial cellulose production, such as HS-ascorbic acid (HSA) medium (Keshk, 2014), Zhou's medium (Zhou et al., 2007), and Son medium (Son et al., 2003). However, the chemical components in most chemically defined media are very expensive, which limits the production of BC on an industrial level (Son et al., 2003). It has been reported that the cost of media accounts for 50-65% of the total expenditure of BC production (Hussain, Sajjad, Khan and Wahid, 2019). Therefore, the development of low-cost but high BC-yielding media is an important consideration in the fermentation process (Vazquez et al., 2012; Velásquez-Riaño and Bojacá, 2017)

Recently, various cellulosic wastes from renewable agroforestry residues or industrial by-products have been used as carbon sources to enhance BC production and achieve economic cost reduction. Agricultural waste is a potential source of renewable energy, it has attracted attention because of its easy access, low cost and sustainability, and only 10% of agricultural waste is used in bio-composites, biomedicine and automobiles components and other industries (Dungani et al., 2015). There have been attempts to reduce the cost of BC production using agricultural waste. Cheng et al. (2017) used acetic acid pre-hydrolysis liquor of agricultural corn stalk as the carbon source for BC synthesis and

found that corn stalk hydrolysate contained glucose (3.87 g/L), xylose (29.61 g/L), mannose (1.84 g/L)), furfural (2.95 g/L), lignin (4.01 g/L) and acetic acid (18.73 g/L). Finally, 2.86 g/L of BC was obtained using this medium, and the structural analysis showed that the diameter of the BC fibres was between 20-70 nm and the length was between 300 nm and several um. In another study, wheat straw hydrolysate treated with calcium hydroxide contained 40 mg/ml of total reducing sugars (Hong et al., 2011). 15.4 g/L BC was achieved when wheat straw hydrolysates were used as a growth medium, 60% higher than that obtained from glucose, sucrose and mannitol as sole carbon sources in defined media (Hong et al., 2011). Inedible peels are rich in reducing sugars, vitamins, proteins, and various acids, and banana peels have been shown capable of replacing part of traditional carbon and nitrogen sources for BC synthesis (Adnan 2015). Dried orange peel waste from the fruit juice processing industry has a moisture content of 10% and includes sugar (30-40%), pectin (15-25%), cellulose (8-10%) and hemicellulose (5-7%) %). Pre-treated dried orange peels with cellulase and pectinase to increase the concentration of fermentable sugars, led to 4.2-6.32 times higher BC production than that obtained in HS medium (Kuo et al., 2017). Furthermore, no significant differences were observed in the FTIR spectra (Kuo et al., 2017). Further examples of agricultural wastes used in BC production are listed in Table 7.

Table. 7: Using agricultural waste as a feedstock for the production of BC

Agriculture waste	**Microbe**	**Maximum BC productivity**	**References**
Citrus peels (lemon, mandarin, orange and grapefruit)	*Komagataeibacter hansenii* GA2016	3.92 BC/100 g	(Güzel and Akpınar, 2018)
Sugar cane juice and pineapple residues	*Gluconacetobacter medellinensis*	3.24 g/L	(Algar et al., 2014)
Enzymatic hydrolysate of wheat straw	*Acetobacter xylinus* ATCC 23770	8.3 g/L	(Chen, Hong, Yang and Han, 2013)
Coffee cherry husk	*Gluconacetobacter hansenii* UAC09	8.2 g/L	(Rani and Appaiah, 2011)
Cashew tree exudates	*Komagataeibacter rhaeticus*	6.0 g/L	(Pacheco et al., 2017)

Extracted date syrup	*Acetobacter xylinum* 0416 MARDI	5.8 g/L	(Lotfiman et al., 2016)
Cheap agricultural product konjac powder	*Acetobacter aceti* ATCC 23770	2.12 g/L	(Hong and Qiu, 2008)
Sago by-product	*Beijerinkia fluminensis* WAUPM53 and *Gluconacetobacter xylinus* 0416 (Reference strain)	0.47 g/L and 1.55 g/L for reference strain	(Voon et al., 2018)
Pineapple and watermelon peels	*Komagataeibacter hansenii* MCM B-967	125 g/L (on a wet weight basis)	(Kumbhar, Rajwade and Paknikar, 2015)

The beer and beverage industry generates a large number of by-products every day, these wastes are rich in various nutrients, therefore, these can be treated biologically for cost-effective and efficient waste management. Traditional rice wine distilleries produce a large amount of makgeolli sludge, containing total nitrogen (0.81 g/L), glucose (10.24 g/L), organic acids (1.15 g/L), alcohol (0.93% v/v) and metal ions, and can be used to

support microbial growth. BC produced from makgeolli sludge displays the unique fibrous network structure of BC, and a polymorphic form of Cellulose I (Hyun et al., 2014). After several series of physical and chemical treatments, Waste beer yeasts (WBY) hydrolysate was used for BC synthesis by *G. hansenii* CGMCC 3917 as carbon and nitrogen sources. The water absorption, retention and release capacities of the produced BC were similar to those of the BC produced from HS medium. Surface characterization showed that the microfibrils of the produced BC membranes were loose and randomly arranged with a lot of space (Lin et al., 2014). In addition, molasses, a major by-product of the sugar industry, was also used in BC production as a carbon source in HS media. The results showed the increased production of BC as compared to HS medium. Additionally, BC produced in both media had similar physicochemical properties and IR spectra, although the viscosity of BC produced from molasses media was lower (Keshk and Sameshima, 2006). Textile industry wastes were also hydrolysed and detoxified for the production of value-added products, such as BC. After a series of treatments, textile waste hydrolysate was used as the medium, which yielded 1.88 g/L of discoloured hydrolysate and 1.59 g/L of coloured hydrolysate (Kuo et al., 2010). Some cases of BC production using industrial waste, and the BC production they achieved, are listed in Table 8.

Table. 8: Using industry waste as a feedstock for the production of BC

Industrial wastes	**Microbe**	**Maximum BC productivity**	**References**
Waste from beer fermentation broth	*Gluconacetobacter hansenii* PJK KCTC 10505BP	8.46 g/L	(Ha et al., 2008)
Black strap molasses and brewery molasses	*Gluconoacetobacter xylinum* ATCC 23768	3.05 g/L	(Khattak et al., 2014)
Sweet lime pulp waste	*Komagataeibacter europaeus* SGP37	6.30 g/L	(Dubey, Singh and Singh, 2018)
Water-soluble fraction from pulping waste liquor	*Acetobacter xylinum* ATCC 10245	High yield	(Uraki et al., 2002)
Sugar cane molasses	*Acetobacter xylinum* ATCC 10245	223% as compared to	(Premjet, Premjet and Ohtani, 2007)

		100% in HS medium	
Waste fiber sludge sulfate and sulfite fiber sludges	*Gluconacetobacter xylinus* ATCC 23770	11 g/L	(Cavka et al., 2013)
Molasses	*Acetobacter xylinum* BPR2001	5.30 g/L	(Bae and Shoda, 2004)
Sugarcane molasses	*Komagataeibacter rhaeticus*	4.01 g/L	(Machado et al., 2018)
Crude glycerol, flour-rich waste streams and sunflower meal hydrolysates	*Komagataeibacter sucrofermentans* DSM 15973	13.3 g/L	(Tsouko et al., 2015)
Carob and haricot bean	*Gluconacetobacter xylinus* ATCC 700178	3.2 g/L	(Bilgi et al., 2016)

medium			
Waste yeast biomass	*Gluconacetobacter xylinus* CH001	2.9 g/L	(Luo et al., 2017)
Wastewater after pullulan polysaccharide fermentation	*Gluconacetobacter xylinum* BC-11	1.177 g/L	(Zhao et al., 2018)

2.3.2 Culture systems for the production of BC

There are three main methods for the preparation of BC, static, agitated/shaking, and bioreactor cultures. The macroscopic morphology, microstructure and properties of BC produced by different methods are also different (Wang et al., 2019). For example, in static fermentation cultures, BC will accumulate on the surface of the nutrient solution to form a gel-like BC film (Rani and Appaiah, 2011), while in agitated/shaking cultures, BC will appear as asterisk-like, sphere-like, or irregular masses (Watanabe et al., 1998). The final application of BC and the desired physicochemical, morphological and mechanical properties determine the most suitable fermentation method.

2.3.2. Static culture method

Static fermentation is the most common method for BC production, that is, incubating the

bacteria in a container filled with fresh growth medium for a period of time to obtain BC; the appropriate temperature and pH (28-30 °C and 4 < pH < 7) are also set (Wang et al., 2019). Membranous BC will form at the air-liquid interface and have a denser surface on the side exposed to air, and the thickness of the BC film also increases with the incubation time (Luo et al., 2014; Lin et al., 2013; Song et al., 2009). The technique of static culture is relatively simple, and the produced BC has 3-D network structure with high porosity (Wang et al., 2019), giving BC some advantages, such as extremely high water holding capacity (WHC) resulted by a wider internal surface area (Huang et al., 2013). In addition, BC produced in static culture is smooth and uniform, which renders it suitable in medical field applications such as in skin tissue repairing (Fu, Zhang & Yang, 2013; Fu, Zhou, Zhang and Yang, 2013) and artificial blood vessels (Klemm et al., 2001). However, the most serious problems restricting large-scale production are the working space and fermentation time (Shi et al., 2014); usually the static fermentation method takes 5-10 days, and the production of BC is directly related to the surface area of the gas-liquid interface (Wang et al., 2019), which leads to higher costs.

2.3.2.B Agitated/shaking culture method

The agitated/shaking culture method is proposed to reduce the cost of BC production and increase the efficiency of BC production. The basic principle is to increase or optimize the oxygen delivery to the bacteria during the fermentation, but the effect seems to be unsatisfactory. Multiple experiments found that agitation/shaking and static produced the same amount of BC for the same duration (Czaja et al., 2004; Toyosaki et al., 1995).

Cellulose-nonproducing cells (Cel^-) were discovered in agitated culture by Schramm and Hestrin in 1954 (Schramm and Hestrin, 1954). Culture conditions are considered to be the main reason for the conversion from cellulose producing cells to cellulose nonproducing cells (Cel^-) (Leisinger et al., 1966). Under a uniform aeration condition in the agitated culture, extensive cell growth is likely to increase the probability of cell mutation, leading to a reduction in cellulose production (Joris and Vandamme, 1993). Actions include controlling the dissolved oxygen concentration below 30% saturation and select mutant strains which are insensitive to high aeration or agitation rate may help to reduce this phenomenon (Joris and Vandamme, 1993).

Overall, the instability of bacterial strains in high shear forces are some of the disadvantages of the agitated/shaking culture method. The degree of polymerization and crystallinity of BC produced by the agitated/shaking culture method is usually low, and the mechanical properties also deteriorate (Kouda et al., 1997; Kouda, Yano and Yoshinaga, 1997; Kouda et al., 1998; Kouda et al., 1996).

2.3.2.C Bioreactor culture method

In attempts to establish conditions and processes for BC production on an industrial scale, different bioreactors have been designed and tested. The airlift bioreactor is an energy efficient reactor that has been tested for BC production (Chao et al., 1997); the strengths of an airlift bioreactor are the provision of sufficient oxygen for aerobic fermentation and the production of less shear stress. Chao and colleagues improved the airlift reactor's

efficiency via an internal loop, and the highest BC yield reached 10.4 g/l, but the BC produced under this condition was spherical, similar to BC obtained in agitated/shaking culture method and was accompanied by low mechanical strength (Chao et al., 2000). An internal-loop airlift reactor was used to study the effect of fructose concentration on BC yield. Under oxygen-enriched air conditions, BC production rate increased to 0.093 g/L/h and the BC yield was enhanced from 11% to 18%. The highest BC yield of 35% with the highest production rate of 0.22 g/ L/h was observed at 60–70 g/L of initial fructose concentration media (Chao et al., 2001). Figure 9a shows the schematic diagrams of an internal-loop airlift reactor and a modified airlift bioreactor (Chao et al., 2000; Wu and Li, 2015). However, all of these airlift reactor types produced BC particles with elliptical pellet morphology (Chao et al., 2000). Wu and Li (2015) improved the airlift bioreactor by replacing the rectangular wire-mesh draft tube by using a series of simple net plates, and harvested BC membranes with larger water holding capacity.

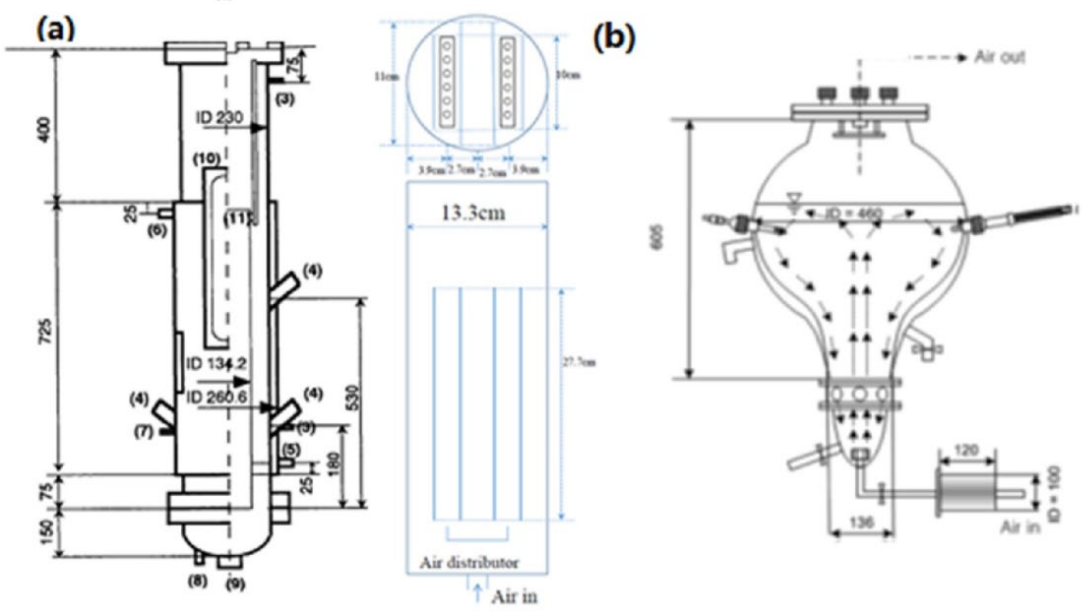

Figure. 9: (a) Schematic diagram of a 50 L internal-loop airlift reactor and (b) a modified airlift bioreactor. (Chao et al., 2000; Wu and Li, 2015)

Figure 9b shows a modified airlift-type bubble column bioreactor, which produces lower shear forces but a high oxygen rate, with the highest BC production rate of 2.27 g/L/day. The BC production reached 5.6-6.8 g/l under pure oxygen conditions, demonstrating the possibility of large-scale production of BC, but the BC produced in the modified airlift-type bubble column bioreactor has low mechanical properties, low crystallinity, low molecular weight and low polymerization (Choi et al., 2009; Song et al., 2009).

In rotating disc bioreactors, several circular disks are fitted on a rotating central shaft with an inlet for inoculation, as shown in Figure 10. The rotating discs allow their surfaces to interact alternately with air and growth medium to produce uniformly structured BC, ultimately increasing the productivity of BC at the expense of reduced mechanical properties (Kim et al., 2007; Krystynowicz et al., 2002), and the time required for

production is reduced (Lin et al, 2013b). Modification of rotating disc bioreactors using plastic composites (PC) made from agricultural waste to increase yields of BC and thus reduce costs. Typically, rotating disc bioreactors are designed so that half of the disk area is submerged in the medium and the other half is exposed to the air. Therefore, they need to be re-inoculated after each BC harvest (Wang et al., 2019). However, PC supporting a rotating disk bioreactor (PCS-RDB) can be fully immersed into the culture media and provide a rough surface for bacteria attachment, resulting in a high BC yield. This means that PCS-RDB can produce BC without re-inoculation, thus maintaining its productivity for at least five cycles, and the production of BC in this semi-continuous operation has been satisfactorily attempted (Lin et al., 2013b). The effect of different additives on the efficiency of PCS-RDB was also evaluated, and it was found that the presence of CMC and avicel could significantly improve the BC production. With the addition of 0.8% avicel to the bioreactor, the BC yield could reach 0.64 g/slice, and the BC in the final pellet form had similar water holding capacity but lower mechanical properties than the BC obtained without the addition of the bioreactor (Lin et al., 2016).

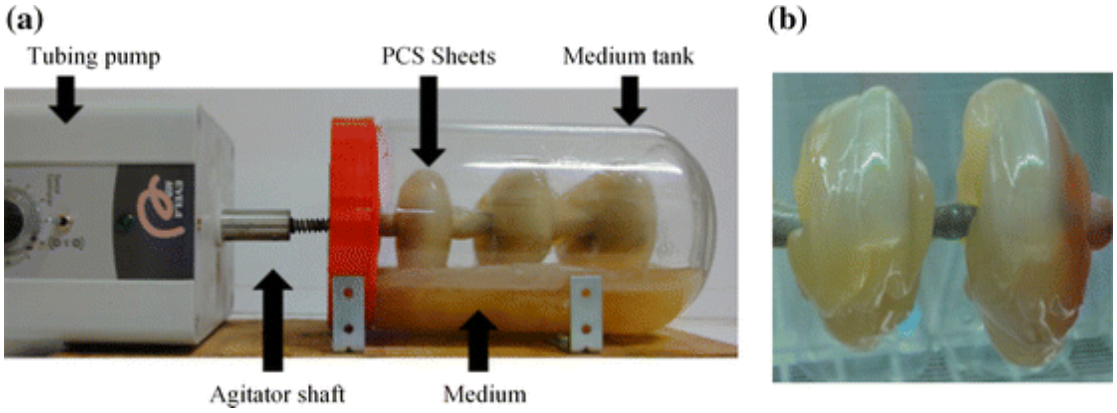

Figure 10: (a) PCS-RDB reactor and (b) bacterial cellulose attached to the rotating disks (Lin et al., 2013).

Some other reactors have also been explored for their ability to reduce the cost of BC fermentation. The trickling bed reactor used for vinegar production was also used for BC production. Compared to the static culture method, the trickling bed reactor has a larger surface area to volume ratio and produces lower shear forces than the agitated/static culture method. The final BC has various excellent properties, such as high degree of −OH association, as well as high polymerization, purity, water holding capacity, porosity, and thermal stability (Lu and Jiang, 2014). BC production can be increased by using technologies such as immobilized-cell reactors, cell-recycle reactors, and hollow fibre reactors (Lin et al., 2013). Biofilm reactor, one of the immobilized-cell reactors, is a good example of using a high biomass density system to reduce the cost of BC production. Cheng et al. (2009) successfully increased BC production from 2.82 g/L to 7.05 g/L using a plastic composite support (PCS) biofilm reactor, and produced BC, similar to pellicle form, with a tensile strength similar to the observed BC pellicle form (Cheng et al., 2009).

2.4 Downstream process of BC production

2.4.1 Purification of BC

Purified BC is non-toxic, transparent, biocompatible, hydrophilic, porous, gas and water permeable, with high surface area and water absorption capacity. All these properties, along with high mechanical strength, are essential in a wide range of applications in technology and biomedicine. As an extracellular substance, BC membranes are harvested from the culture medium, contain a large number of BC-producing cells, as well as nutrient solution residues. Before BC can be put into application, bacteria inside need to

be removed and purified from bacterial lipopolysaccharides. (Pigaleva et al., 2019). In medical applications, for example, Gram-negative BC-producing bacteria are highly dangerous because they contain lipopolysaccharides (LPS) (endotoxins) that are toxic to animals. Before BC membranes can be used for medical applications, the amount of endotoxin should be reduced to below 0.5 endotoxin units (EU)/ml (Martínez Ávila et al., 2014; Pigaleva et al., 2019). Pigaleva et al. (2019) reported purification of BC using supercritical carbon dioxide (S-CO_2). The data show that S-CO_2-treated BC is no longer a network structure composed of individual interwoven fibres but becomes a monolithic porous matrix containing isolated individual voids without significant penetration. Furthermore, its Young's modulus increased by two times, and its tensile strength increased by about 5 times, compared with the untreated BC control group, but it is not effective enough at removing endotoxins to achieve medical grade results. Gea et al. (2011) soaked two pieces of BC membranes in 2.5 wt.% NaOH overnight, and then continued to soak one of them in 2.5 wt.% NaOCl overnight. After rinsing the samples with distilled water, the effects of the two purification methods on the structure, mechanical properties, and morphology of BC membranes were compared. Compared with the untreated BC control group, the Young's modulus of the samples treated with only alkali soaking increased by two times, but after the secondary treatment with NaOCl, the Young's modulus of the samples was further enhanced, and the thermogravimetric properties was also enhanced. Furthermore, the two-step treatment did not promote the conversion of native cellulose from cellulose type I to cellulose type II, which is also undesirable.

The BC films were purified with different concentrations of alkali (NaOH), and the results showed that the tensile strength of the samples treated with 10% NaOH decreased significantly, from 208 MPa to 165 MPa, and part of the BC component had been dissolved by NaOH, resulting in a bulk fractured layer. The BC membrane swelled after alkali treatment, resulting in the damage of the network and layer in the BC film (Suryanto et al., 2019). Shibazaki et al. (1997) found that crystal conversion to cellulose II occurred if the concentrations of NaOH were above 12% (w/w). The levelling-off degree of polymerization (LODP) of the BC membranes decreased from 150 to 50 after being treated with 18% (w/w) NaOH. The vast majority of BC membrane purification experiments are performed on 0.3-3 mm thick films, but thicker BC membranes are often required for the use as a matrix for implants. In the case of thick films, traditional purification protocols can be challenged by low solvent availability and slow removal of residual material (Martínez Ávila et al., 2014). Since lipopolysaccharide molecules are heat-resistant, the problem of dehydrogenation is even more serious (Pigaleva et al., 2019).

2.4.2 Modification of BC

BC is an important biopolymer that is preferred in industry because of its simple production process (Rastogi and Banerjee, 2020). The structure of the BC is formed by the inter and intramolecular bonds of hydroxyl groups with supramolecular interactions, resulting in the packing of the cellulose fibrils in fibrillar and semi-crystalline form

(Machado et al., 2018). Due to the functional hydroxyl and the high surface area, BC can be used as both matrix and reinforcements in biotechnology (Avcioglu, 2022). However, the lack of antibacterial and antioxidant capacity of BC limits its application in food, medical and other fields (Esa et al., 2014; Rastogi and Banerjee, 2020). It has been experimentally demonstrated that BC can adsorb different liquids and combine strongly with other biological materials (Machado et al., 2018; Rastogi and Banerjee, 2020), and there are two basic composite synthesis approaches, *in situ* and *ex situ*. In *ex situ* synthesis, reinforcement materials are added to the polymer matrix during polymer synthesis and become part of the composite (Saibuatong and Phisalaphong, 2010, Serafica et al., 2002, Ul-Islam, Khan and Park, 2012). *Ex situ* synthesis is characterized by ease of processing and can be synthesized with both solids and liquids (Serafica et al., 2002). However, there are difficulties in composites synthesis due to quick penetration of suspended particles which lead to the disturbances in the basic structure of BC (Ul-Islam et al., 2012; Shah et al., 2013), whereas synthetic antimicrobial-BC composites are also limited (Serafica et al., 2002). In *ex-situ* synthesis, the polymer matrix is impregnated with reinforcing materials to produce composites. Using this method for composite production, the basic structure of BC is preserved, but only submicron to nano sized particles can penetrate in BC, and it is difficult to achieve uniform distribution of particles (Shi et al., 2012; Yano et al., 2007; Ul-Islam, Khan and Park, 2012)

2.4.2.A *In situ* BC composites synthesis

The method that adding reinforcing materials to the growth medium during BC

fermentation is called in situ BC composite synthesis. With prolonged fermentation time, the formed BC network could capture a variety of added materials, and the encaged materials were considered to be part of the BC fiber network (Horii et al., 1997; Tang et al., 2009; Ul-Islam, Khan and Park, 2012). Aloe vera was added to the growth medium in a static environment to form a BC-Aloe vera composite films that would envelop the aloe vera, while obtained composite shows excellent physical and mechanical properties (Saibuatong and Phisalaphong, 2010). Similarly, adding different concentrations of PBH solution to the fermentation medium not only affected the BC yield, but also changed the morphology and crystallinity of BC (Ruka, Simon and Dean, 2013).

In situ compounding in static culture methods is difficult because BC synthesis occurs at the nutrient solution and air interface, and particles cannot mix well with BC (Shah et al., 2013). Agitated/shaking culture method and rotating disc bioreactors have been used to solve this problem that continuous stirring can help the added material to be entrapped in the BC fibrils and prevent the particles from settling (Cheng et al., 2009; Yan et al., 2008). In addition, the in situ composite synthesis also has limitations, such as the inability to directly add bioactive (antibacterial) agents (Ag, ZnO, TiO_2, etc.) to the culture medium because they have toxic effects on microorganisms, and the synthesized BC composites are cultured by stirring Moreover, BC composite synthesized through agitated/shaking culture cannot be applied as a gel or sheet in biomedical applications, while gels/sheets formed in static culture cannot entrap reinforcement materials for composite synthesis. (Shah et al., 2013).

2.4.2.B Ex situ BC composites synthesis

Problems that occur with in situ synthesis can be solved by ex situ synthesis. The link between BC and reinforcement materials may be physical or through hydrogen bonding. Liquid substances and tiny solid particles can easily penetrate and become engrossed inside the porous BC matrix. In addition to this physical adsorption, the -OH moieties in the BC cellulose chains can form hydrogen bonds with the invading reinforcement material. The most important advantage of using this method for composite synthesis is that the original structure of BC remains almost completely unchanged. Ex situ synthesis has been applied to the synthesis of many BC composites, while reinforcing materials include polymers, inorganic materials, metals, and metal oxides (Evans et al., 2003; Maneerung et al., 2008; Nakayama et al., 2004; Shi et al., 2012; Ul-Islam, Khan and Park, 2012; Ul-Islam et al., 2012; Yano et al., 2007; Yoon et al., 2006). Some composite materials have been successfully synthesized, such as BC-Pd, BC-CNT, and BC-MMT (Evans et al., 2003; Yoon et al., 2006)

The size and properties of the reinforcing materials are the main obstacles to limit the ex situ BC composites synthesis. Specifically, only submicron to nanometre-sized materials can be impregnated into the BC matrix. This is because larger particles cannot enter the BC pores. Hydrophobic materials also cannot bind to BC. Furthermore, the structural arrangement of BC fibrils is not always uniform, resulting in reinforcements that may not be uniformly distributed within the BC matrix (Shah et al., 2013)

2.5 Applications of BC in food

2.5.1 Toxicity and nutritional functionality of BC

Toxicological assessment of food and food substances is critical and necessary. To assess whether the use of BC is beneficial to humans, toxicology testing of BC should be performed before human consumption (Dourado et al., 2017). Extensive toxicology experiments including acute, sub-acute and subchronic oral toxicity assays using mice, and the results showed that BC consumption has no reproductive toxicity, embryotoxicity and teratogenicity in mice, and no inflammatory response (Okiyama et al., 1993b; Pakpeankitvatana et al., 2007; Hagiwara et al., 2010; Fu, Zhang and Yang, 2013; Andrade et al., 2011; Cullen et al., 2000; Xu et al., 2014). As a traditional Asian food, BC has been eaten as Nata by humans for a long time, proving the safety of BC in the food industry (Panerari et al., 2008; Dourado et al., 2017; Phisalaphong et al., 2012), and BC has been listed by the US Food and Drug Administration as generally recognized as safe (GRAS) in 1992 (Chau et al., 2008).

Dietary fiber (DF) from grains, fruits, and vegetables has a variety of nutritional benefits for the small and large intestines of humans. It has been reported that feeding both plant cellulose and BC to rats enhanced the excretion of total lipids (144-182%), cholesterol (136-203%) and bile acids (259-479%) in faeces, and BC can lower blood lipids and cholesterol in hamsters more effectively than plant cellulose. This is because dietary fibre with high cation-exchange capacity (CEC) can capture, destroy and break down lipid

emulsions, resulting in reduced diffusion and absorption of cholesterol and lipids, while the CEC of BC is much higher than that of plant cellulose This means that BC can be used as a low-calorie bulking ingredient and participate in the development of new fibre-rich functional foods in different forms (Chau et al., 2008). As a dietary fibre supplement, BC has been shown to inhibit obesity induced by high fat diet in mice by attenuating insulin resistance, liver injury and inflammation, enhancing antioxidant defence system and regulating the secreting of adipocytokines and adipogenesis-associated proteins (Zhai et al., 2018a). In addition, BC supplementation effectively increased the length of villi cells and the thickness of colonic mucosa and muscle, as well as the thickness of colonic mucosa and muscle in rats, thereby protecting colonic smooth muscle cells from apoptosis. These findings suggest that BC has the potential to act as dietary fibre for alleviating constipation and regulating of short-chain fatty acids and gut microbiota (Zhai, Lin, Zhao and Yang, 2018a).

2.5.2 BC as food ingredient

BC has been explored for its potential application in the food industry because of its suspending, water retention, thickening and emulsifying stability. Okiyama et al. (1993a) used BC as a filler material to reinforce fragile food hydrogels. Adding BC to paste-like foods can improve the food texture by reducing stickiness, e.g., adding BC to tofu to provide firmness and better texture (Huang et al., 2013). The addition of BC to Chinese meatballs does not affect the sensory and shelf stability of the product. In addition, it provides an acceptable juiciness and chewiness to the meatballs as it retains its moisture

content for a minimum storage period of 1 month (Lin and Lin, 2006). BC has also been used in vegetarian meat preparation, in combination with Monascus extract obtained from a natural red pigmented mould. The colour and shape of the vegetarian meat is stable and has similar the flavour of natural meat (Purwadaria et al., 2010; Ng and Shyu, 2004) and it can be considered as a potential substitute for animal products. In conclusion, BC has the unique advantage over other polysaccharides of being thermally stable and unaffected by external conditions such as pH and ionic strength (Petersen and Gatenholm, 2011; Cacicedo et al., 2016). These findings further demonstrate the power and potential of BC as a food additive to improve its quality and shelf life and to limit calories in its blend products.

2.5.3 BC in delivery of bioactive agents

Due to the gel behaviour and high surface area, BC has great potential for the transport of bioactive agents. There have been several reports describing successful cases of loading of bioactive agents by BC or modified BC. For example, L-ascorbic acid was loaded into BC aerogel, which were modified to possess as much as 100% of their initial water content using surface tension of solvent rewetting. This rehydration property with an associated high pore volume further affirms the suitability of BC aerogels as a sustained release matrix (Ullah et al., 2016). BC can also be used as an effective delivery system for proteins from serum albumin, whereas freeze-drying reduced the protein loading capacity of BC (Müller et al., 2013). Other researchers have investigated the development of BC as a delivery system for antimicrobial nanoparticles, including TiO_2,

Ag and ZnO (Dobre and Stoica-Guzun, 2013; Khan et al., 2014). Zhai et al. (2018b) have demonstrated that emulsions stably developed from low concentrations of BC formed from nanofibers can be used as new food-grade Pickering emulsions and have great potential for the delivery of lipophilic bioactive substances in the food industry.

2.5.4 BC in enzymes and cell immobilization

The immobilization of enzymes and cells is one of the key technologies required for food production. Some functional parameters of the fermentation industry have been greatly aided by immobilized enzyme technology (Fernandes, 2010, Kilara et al., 1979). For example, wet-form BC beads with a diameter of 500-1500 μm were used to immobilize glucoamylase. The relative activity of immobilized glucoamylase remained above 77% at pH 2.0, the highest value in the literature. Even in the low temperature region of 20°C, the relative activity exceeds 68% (Wu and Lia, 2008). Kirdponpattara and Phisalaphong (2013) used a composite of BC and calcium alginate (CA) to immobilize yeast, and the yeast immobilized on the composite was more efficient in terms of ethanol production. Fijałkowski et al. (2016) immobilized probiotic strains of *Lactobacillus* spp. by impregnating BC membranes with probiotics or adding probiotics in the medium for BC production and demonstrated that whichever method is used, BC can be used as immobilized support cells for *Lactobacillus*, protecting them from gastric juices (Fijałkowski et al., 2016).

2.6 Conclusion

Due to its unusual properties, BC has huge potential of applications in food as well as other fields. BC is degradable, non-toxic, and can be regenerated by agricultural and industrial wastes. These characteristics are in line with the requirements of modern society for environmental protection and food safety. However, the yield and cost of BC are still the main issues limiting the large-scale production of BC. While improving fermentation techniques and finding excellent strains, advanced biotechnologies, such as genetic engineering, can be tried. In addition to being a natural food, BC still has great application potential in the food industry. The application of BC in food packaging materials, delivery of bioactivities, enzymes and cell immobilization still needs extensive and in-depth research. Surface modification of BC is particularly promising, due to the large number of chemically reactive sites in the BC structure, allowing modification to provide tailored surface functionality while maintaining its core properties.

Although low-cost commercial production is still a challenge, and the impact of BC as a functional food on human health also needs to be further verified, it is believed that BC will have a promising future as a unique ingredient for differentiated novel food products with sensory and health appeals.

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Chapter 3: The impact of carbon sources on the production and properties of bacterial cellulose in static and agitated conditions

Abstract

This study aimed to assess the effect of carbon source type and concentration on the production and structural properties of bacterial cellulose (BC). BC was synthesized using bacterial strain *Acetobacter xylinum* subsp. *sucrofermentans* DSM 15973, in static and agitated batch-flask cultures. Hestrin-Schramm medium was modified with glucose, fructose, sucrose, xylose and arabinose as carbon sources (30 and 60 g/L). Parameters such as BC yield, substrate consumption and structural properties of produced BC were assessed. The results showed that fructose was the most suitable carbon source for the bacterium used, and high BC concentrations up to 31 g/L were achieved in substrates with 60 g/L fructose under static culture conditions. Notably, BC production was equally high under the same fermentation conditions in agitated cultures (~30 g/L). Xylose and arabinose were not suitable for BC synthesis for the particular strain, as low substrate uptake and subsequently poor growth were observed. SEM showed that cellulose synthesized from fructose was denser, and it also possessed higher crystallinity index and water holding capacity value. FTIR spectra demonstrated that carbon sources had no effect on the chemical structure of bacterial cellulose.

3.1 Introduction

Cellulose is one of the most abundant polymers composed of β- (1→4) glucose units; it

can be obtained from a variety of sources, ranging from plants, and prokaryotic organisms to algae (Brown 1886; Ross et al. 1991; Nobles et al. 2001; Jung et al., 2005; Islam et al., 2017). It is considered a renewable material and has the potential to replace fossil carbon in petroleum and coal in some applications (Jung et al, 2005). Additionally, cellulose has wide applications in paper-making industry, as reinforcing agent in polymers (Grishkewich et al., 2017, Ng et al., 2015).

Most of the cellulose used in paper and textile industries is plant cellulose, representing the main structural component of plant cell walls (Son et al., 2003) and it is also an important participant in the global carbon cycle, whereby microorganisms obtain carbon from plant cellulose through biodegradation (Weimer et al., 1991). However, plant cellulose contains a large number of impurities, such as pectin, lignin and hemicellulose (Jonas & Farah, 1998; Vandamme et al., 1998) and the presence of these impurities limits to some extend the application of plant cellulose in certain fields, such as food and medicine. In contrast, the cellulose of bacterial origin exhibits the highest purity and has thus attracted much research interest, delivering a combination of exclusive properties, such as flexibility, high water holding capacity, hydrophilicity, crystallinity, mouldability, absence of lignin and hemicellulose and biomimetic three-dimensional (3D) network (Gorgieva and Trček, 2019).

Some bacterial genera such as *Acetobacter* sp., *Rhizobium* sp., *Agrobacterium* sp., *Alcaligenes* sp., *Pseudomonas* sp., and *Sarcina* sp. have the ability to produce BC, but it

is noted that only a few bacterial species, taxonomically related to the genus *Acetobacter* sp. (acetic acid bacteria) can extracellularly secrete the synthesized cellulose as fibrils (Vandamme et al., 1998; Islam et al., 2017). Among them, *Acetobacter xylinum* (also named as *Gluconacetobacter xylinum* and *Acetobacter xylinum*) has been described as the most efficient BC producer (Delmer, 1999; Mikkelsen et al., 2009). Although the specific biochemical events of bacterial secretion of BC are still not very clear, there is a conjecture that BC is produced to avoid ultraviolet damage to the cells, to protect from harsh chemical environment conditions, and the BC can also provide buoyancy for the bacteria that the bacteria can float to the surface of the growth medium, so as to obtain oxygen more efficiently (Huang et al., 2013).

In has been previously reported in literature, that the characteristics of bacterial cellulose may be affected by external culture conditions. For example, both static and agitated conditions are widely applied in BC production. Static cultures are excellent in keeping the regular shape of BC and maintaining its good morphology. Generally, BC produced in static cultures is smooth and uniform, and is regarded as a suitable material in medical field applications such as skin tissue repairing (Fu, Zhang & Yang, 2013; Fu, Zhou, Zhang & Yang, 2013) and artificial blood vessel (Klemm et al., 2001). However, issues that constrain mass BC production in static cultures are associated with the requirement of a large working space and high manual labour costs, (Shi et al., 2014). Comparing with static culture, smaller working space and less labour is required in the case of agitated cultures, but the produced BC tends to get attached on the shaft of the reactor, rendering

its collection often difficult. When agitated cultures are applied, the produced BC can form multi-shaped pulps, filaments and spheres (Shi et al., 2014), that are caused by sheer stress arising from the agitation of the medium, which limits the formation of smooth gelatinous membrane and results in the accumulation of small aggregates of bacterial cellulose. These granular or fibrous aggregates have a disordered structure compared to BC from static cultures, although their fibril networks are similar. Due to the presence of this disordered structure, the BC produced in agitated cultures has a higher retention aid function and higher surface accessibility than that in static culture, because the filler granules can be captured by ultrafine BC fibril and retained in the BC-based products. Based on this feature, BC produced in agitated culture is widely used in paper making, or as active packing material by carrying antimicrobial agents to increase the shelf or safety of products (Tomé et al., 2010). Therefore, it is essential to evaluate the structural and chemical properties of BC in depth before investigating potential applications. Similarly, for *Acetobacter xylinum* subsp. *sucrofermentans*, the effects of different carbon sources on the BC properties are still not well documented.

The aim of this study was to evaluate the ability of *Acetobacter xylinum* subsp. *Sucrofermentans* to metabolise various carbon sources and their potential impact on BC production. To this end, various simple sugars (glucose, fructose, sucrose, arabinose, and xylose) were investigated as carbon sources for BC production. The physical and chemical characteristics of the produced BC were determined and discussed in depth.

3.2 Materials and methods

3.2.1 Materials

Bacteriological agar was purchased from Oxoid Ltd (stock keeping unit [SKU]: LP0013T); D- (+)-Glucose ≥ 99.5% from Sigma-Aldrich (SKU: 795410); D-(–)-Fructose ≥ 99% from Sigma-Aldrich (SKU: F0127); Sucrose from Sigma-Aldrich (84097-250G); D-(+)-Xylose from Sigma-Aldrich (W360600-1KG); L-(+)-arabinose from Sigma-Aldrich (A3256); Peptone from casein; enzymatic digest from Sigma-Aldrich (SKU: 82303); Yeast extract from Fisher Bioreagents (SKU: BP1422-500); Sodium phosphate dibasic ≥ 99% from Sigma-Aldrich (SKU: 795410); Citric acid monohydrate ≥ 99.5% from Fisher Chemical (SKU: CI6160I53); Sodium hydroxide from Fisher Chemical (SKU: SI4920I53).

3.2.2 Preparation of bacterial strains

The bacterial strain *Acetobacter xylinum* subsp. *sucrofermentans* DSM 15973, originating from *Leibniz Institute* DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, was used in the study. The bacterial strain was grown and maintained on sterilised (121°C/ 15 min) Hestrin and Schramm media (H&S agar) (Mikkelsen et al., 2009), containing agar (20 g/L), glucose (30 g/L), peptone (5 g/L), yeast extract (5 g/L), sodium phosphate dibasic (2.7 g/L), and citric acid 1.15 g/L). To keep the viability, strains were sub-cultured in fresh H&S agar every two weeks.

3.2.3 Pre-culture preparation

H&S liquid substrate (H&S media) served as pre-culture medium consisting of glucose (30 g/L), peptone (5 g/L), yeast extract (5 g/L), sodium phosphate dibasic (2.7 g/L), and citric acid (1.15 g/L), prepared and sterilized at 121 °C for 15 min. A full loop of Acetobacter xylinum subsp. Sucrofermentans DSM 15973 was inoculated into 50mL of the pre-culture medium and then incubated at 30°C, agitated at 180 rpm for 24 h (IKA, UK).

3.2.4 Bacterial cellulose (BC) production

Pre-cultures were homogenized in a stomacher (Seward 400) at 230 rpm for 2 min, and then 1 ml of the culture suspension was transferred into 250 mL Erlenmeyer flasks containing 50 ml sterilized media (121°C for 15 min) for BC production. The medium used was a modified H&S medium, containing single carbon source at two different concentrations, peptone (5 g/L), yeast extract (5 g/L), sodium phosphate dibasic (2.7 g/L), and citric acid (1.15 g/L). In terms of carbon source concentrations, these were glucose (30 g/L and 60 g/L), fructose (30 g/L and 60 g/L), sucrose (30 g/L and 60 g/L), arabinose (30 g/L) and xylose (30 g/L). For the glucose and fructose-based media, two kinds of growth conditions were applied, namely static and agitation. All of the inoculated cultures were incubated at 30°C while a speed control orbital shaker (IKA, UK) was utilised to provide a rotatory speed of 180 rpm for the cultures under agitated condition.

Two flasks from each culture (acting as repeats) were collected as sampling points at different time intervals during BC production. Collected culture samples were

homogenised in a stomacher (Seward 400 circulator, P/4/518) at 230 rpm for 2 min to release the wrapped cells from BC, and vacuum filtration followed to separate BC and culture media, using 70 mm Qualitative 1 Whatman^{TM} filter paper. The separated BC was washed twice with distilled water to remove any residual media and dried in an oven (Memmert, UK) at 50°C for 24 h, until constant weight. The dried bacterial cellulose was placed in a petri dish, sealed with parafilm and stored in a cabinet at room temperature until further analysis.

3.2.5 Analytical methods

The pH of the collected samples was measured by a calibrated pH meter (Mettler-Toledo) and then the pH of the main culture was manually and aseptically corrected between 6.0 and 6.2 by the addition of 6 M NaOH sterilised solution, if needed.

Culture media filtrates were centrifuged (Thermo Scientific Heraeus Multifuge X3R) at 10,000 *x* g at 4°C for 30 min twice and the resulting biomass pellet was transferred into a pre-weighted glass vial, and then dried in an oven (Memmert, UK) at 105°C for 24 hours and until constant weight measurement.

Residual carbon source concentration was determined by 3,5-dinitrosalicylic acid (DNS) method (Lindsay, 1973). Briefly, 0.1 ml of the supernatant was mixed with 0.1 ml DNS reagent (3, 5-dinitrosalicylic acid), treated at 100°C for 5 min and then mixed immediately with 1 ml of distilled water. The mixture absorbance was measured at 540

nm using a spectrophotometer (Thermo Electron Corporation BioMate3). Standard solution of known concentration of sugars (0.1 g/L, 0.2 g/L, 0.5 g/L, 1 g/L, 1.5 g/L and 2 g/L) were prepared and used in the DNS protocol as described above to construct calibration curves and quantify sugar concentrations in the culture samples.

3.2.6 Scanning Electron Microscopy (SEM)

BC samples were coated with gold nanoparticles at 11 mA using an Edwards Sputter Coater S150B. The BC surface analysis was carried out using the SEM microscope (FEI Quanta 600 FEG) with accelerating voltage of 2 kV and at a working distance of 10.1-10.4 mm. Magnifications of 5,000 x and 10,000 x were used. ImageJ (ImageJ 1.53t) was used to measure the diameters of 50 randomly selected BC fibres and then the average diameter was calculated.

3.2.7 X-Ray diffractometry (XRD)

The degree of crystallinity was determined using a Bruker D8 Advance powder diffractometer with a copper source (wavelength 1.54 angstroms). The 2-theta angle ranged from 5.0 to 64.0848 degrees with 0.02106 mm increments for 1.2 seconds. The data was collected on a Lynxeye detector. The aperture slit was 6 mm, and the detector was equipped with a Nichol attenuator. The obtained XRD curves were analysed by TOPAS (Bruker AXS, version 4.2), to calculate the Crystallinity Index (CI).

3.2.8 Fourier Transform Infrared Spectroscopy (FT-IR)

The infrared spectrum was obtained using a Perkin Elmer precisely spectrum 100 ATR FT-IR Spectrometer, dried BC flakes were analysed in the spectrometer region of 4000-650 cm^{-1} and with a resolution of 4 cm^{-1}. The result for each sample was the average of 32 scans.

3.2.9 Water holding capacity (WHC)

The water holding capacity of the samples was measured according to the method described by Schrecker and Gostomski (2005). Briefly, 0.2 g ± 0.005 g of dried BC samples were immersed into distilled water for 24 h, to allow the dried BC samples to swell up completely, until the weight of bacterial cellulose remains constant. A tweezer was used to remove the BC sample out from the water and gently shake it to remove any excess water from the surface of the sample. Rehydrated samples were weighed in an analytical balance and marked as wet weight. Subsequently, samples were dried in the oven (Memmert, UK) at 60°C for 24 h so until constant weight. The water holding capacity (WHC) was calculated according to the following equation (Eq. 1):

$$\text{WHC} = \frac{mass\ of\ water\ removed\ (g)}{dry\ weight\ of\ BC\ membrane\ (g)} \qquad \text{Eq. 1}$$

3.2.10 Statistical Analysis

Statistical analysis was performed using Microsoft Excel Office 365. Fermentations were performed in triplicates and data are expressed as mean value +/- standard deviation of three independent cultures. Analytical methods were also performed at least in triplicate

and values are expressed as mean value +/- standard deviation of three independent measurements. SPSS Statistics 27 (IBM, USA) was used to carry out the statistical analysis. One-way analysis of variance (ANOVA) was used to evaluate the significant difference between 3 carbon sources in WHC at the significant level 0.05. Duncan test was selected for multiple comparisons if equal variances were assumed, otherwise, Tamhane's T2 test was used.

3.3 Results and discussion

3.3.1 Effect of carbon source on BC production in agitated cultures

Initially, the bacterial strain *Acetobacter xylinum* subsp. *sucrofermentans* was cultivated in batch-flask cultures containing modified H&S media, which incorporated different types of sugars as carbon source. The objective of these initial experiments was to assess the impact of carbon source on bacterial cellulose production. Table 1 depicts the performance of the strain in cultures with 30 g/L of carbon source, under agitated culture conditions.

Table 1: Bacterial cellulose production, yield and substrate uptake rate during cultivation of *Acetobacter xylinum* in different carbon sources under agitated conditions (S_i=30 g/L)

Carbon source	Cultivation time (h)	Substrate uptake rate (r_S, g/L/h)	BC production (g/L)	BC yield (g/g of consumed substrate)
Glucose	142	0.295	1.10±0.02	0.04
Fructose	196	0.199	9.40±0.01	0.39
Sucrose	262	0.214	4.19±0.51	0.18
Xylose	186	0.088	0.36±0.02	0.04
Arabinose	186	0.070	0.25±0.02	0.02

Cultivation conditions: Initial carbon source concentration ~30 g/L; temperature 30°C, agitation speed: 180 rotations per min. Data presented as mean values ± standard deviation (n=3).

Among all sugars tested as carbon sources under agitation conditions at initial concentration of 30 g/L, fructose was the most suitable substrate for BC synthesis for the particular bacterial strain. In-fructose based media, BC reached a maximum of 9.4 g/L, after 196 hours of fermentation. On the contrary, xylose and arabinose-based media exhibited poor growth and low BC production. Literature suggests that pentoses enter the tricarboxylic acid cycle (TCA) in the form of acetyl Co-A, providing energy for bacterial growth, instead of channelling the carbon flux towards BC production.

Based on the substrate uptake rate (r_S) for each carbon source (Table 1), it is evident that glucose and sucrose were the most assimilable substrates for *Acetobacter xylinum*; however, this was not reflected in the respective BC production achieved in these media and especially in glucose-based ones, where BC production reached only 1.1 g/L. In media supplemented with glucose, the pH of the culture dropped drastically during the first 50-60 h of the fermentation (from 6.2 to 4.2), indicating the formation of gluconic

acid as a by-product (Toda et al. 1997). Specifically, during the synthesis of bacterial cellulose, glucose is phosphorylated under the catalysis of a membrane-bound enzyme, glucokinase, to form an important intermediate glucose-6-phosphate (G6P). Subsequently, G6P enters the process of glucose-6-phosphate isomerization and UDP-glucose synthesis, which then latter forms BC. At the same time, G6P can also be dehydrogenated to generate phosphogluconic acid, and enters the pentose phosphate pathway and TCA cycle, providing essential energy for bacterial growth and metabolism (Ross et al., 1991; Wang et al., 2018; Yoshinaga et al., 1997; Tonouchi et al., 1996). Zhong et al. (2013) compared the metabolic flux of the bacterium *G. xylinum* (CGMCC no. 2955) in different carbon sources and indicated that 19% of the available glucose concentration in the medium was incorporated into BC, while 40% of glucose was fluxed into the by-product gluconic acid. In comparison, about 25% of fructose was used for BC synthesis. It has also been reported that in some BC-producing strains, such as *G. xylinum*, acidic compounds in the growth medium are utilised through tricarboxylic acid cycle (TCA) when cultivated in fructose-based media. The TCA cycle aims to generate energy and promote cell growth (Jung et al. 2010). According to Tonouchi et al. (1996), fructose can be phosphorylated to fructose-6-phosphate (F6P). The phosphoglucose isomerase (PGI) existing in the cytoplasm of *A. xylinum* can be used to promote the interconversion of F6P and G6P, the subsequent metabolic process is the same as for glucose (Zhong et al., 2013). It is also reported that the activities of the enzymes involved in metabolism and BC synthesis differ depending on the bacterial species and carbon source (Tonouchi et al., 1996; Ross et al., 1991). *A. xylinum* subsp. s*ucrofermentans* BPR2001 was used to study changes in enzymatic

activity in different carbon sources, and data showed that PGI activity in cells in the presence of fructose was 132-fold higher than in cells grown in glucose-supplemented media (Tonouchi et al., 1996). This means that fructose can be efficiently converted into G6P, which is involved in cell metabolism and BC synthesis, avoiding the formation of gluconic acid, thereby achieving an increase in BC production. All these are in agreement with the findings of the current study and explain the higher BC production yields obtained in fructose-based media.

With regards to sucrose-based media, BC production was also notable (~4 g/L), whereas the substrate uptake rate was also satisfactory (r_S=0.214 g/ L/ h). Sucrose is a disaccharide composed of glucose and fructose subunits. According to Velasco-Bedrán and López-Isunza (2007), sucrose cannot be transported through the cell membrane, but is firstly hydrolysed to glucose and fructose in the periplasm, through the extracellular secretion of invertase enzyme. This is also linked with reduced efficiency of carbohydrate-to-cellulose conversion, according to Embuscado et al. (1994), which was also the case in the current study.

Finally, in terms of biomass production, this varied across substrates and was in the range between 0.1to 0.54 g/L. The highest biomass production (0.54 g/L) was noted in sucrose-based media, further supporting the evidence of more carbon being fluxed towards cell growth, rather than BC production. Generally, biomass production in BC-producing strains is widely under-reported in literature, due to the nature of the bacterial cells being

trapped in the BC polymer. As such, biomass production in such fermentations is usually underestimated.

Based on this first set of results, it was decided that xylose and arabinose will not be used in further experiments. On the contrary, glucose, fructose and sucrose were used in higher initial concentrations (60 g/L) in agitated cultures, aiming to evaluate the performance of the microorganism in terms of BC production. The following Table 2 depicts data obtained in these experiments, at time points where BC was maximum.

Table 2: Bacterial cellulose production, yield and substrate uptake rate during cultivation of *Acetobacter xylinum* in different carbon sources under agitated conditions (S_i=60 g/L)

Carbon source	**Cultivation time (h)**	**Substrate uptake rate (r_S, g/L/h)**	**BC production (g/L)**	**BC yield (g/g of consumed substrate)**
Glucose	382	0.262	4.53±0.01	0.09
Fructose	196	0.122	30.87±0.07	0.69
Sucrose	386	0.171	30.90±0.01	0.61

Cultivation conditions: Initial carbon source concentration ~60 g/L; temperature 30°C, agitation speed: 180 rotations per min. Data presented as mean values ± standard deviation (n=3).

Again, fructose-based media were proved as the most suitable for BC production, reaching 30.87 g/L, followed by a matching performance in sucrose-based media (30.9 g/L). Glucose-based media performed poorly in terms of BC production (4.5 g/L), while a rapid drop in the medium pH during the first 80 h of fermentation (~3.1), indicated the production of organic acids (gluconic acid) under these cultivation conditions. This was also further supported by the high substate uptake rate at higher substrate concentrations

of glucose (0.262 g/L/h). As expected, the substrate uptake rate dropped slightly in media with higher substrate concentrations, indicating a slower growth rate as a result of partial substrate inhibition.

3.3.2 Effect of carbon source on BC production in static cultures

The next step of the experimental design involved the evaluation of static culture conditions on BC production. For this purpose, two carbon sources were chosen, glucose and fructose, which has shown contrasting biochemical behaviour in the agitated culture experiments. The results are depicted in Table 3 below.

Table 3. Bacterial cellulose production, yield and substrate uptake rate during cultivation of *Acetobacter xylinum* in different carbon sources under static conditions.

Carbon source	Initial concentration (g/L)	Cultivation time (h)	Substrate uptake rate (r_S, g/L/h)	BC production (g/L)	BC yield (g/g consumed substrate)
Glucose	30	336	0.129	8.60±0.26	0.34
Fructose		408	0.056	17.79±0.53	0.75
Glucose	60	456	0.195	9.71±0.67	0.21
Fructose		432	0.098	31.12±4.86	0.67

Cultivation conditions: temperature 30°C; static conditions. Data presented as mean values ± standard deviation (n=3).

Compared with the agitated fermentation conditions, data showed that the performance of the two carbon sources in the static environment was better than that in the agitated environment, especially for the glucose. BC in glucose -based media in static conditions increased 8-fold at 30 g/L of substrate and 2-fold at 60 g/L (Table 1). In terms of kinetics,

the fermentation time was prolonged (Figure 3.1), a fact that was also underlined by the generally slower substrate uptake rates (0.056-0.195 g/L/h)

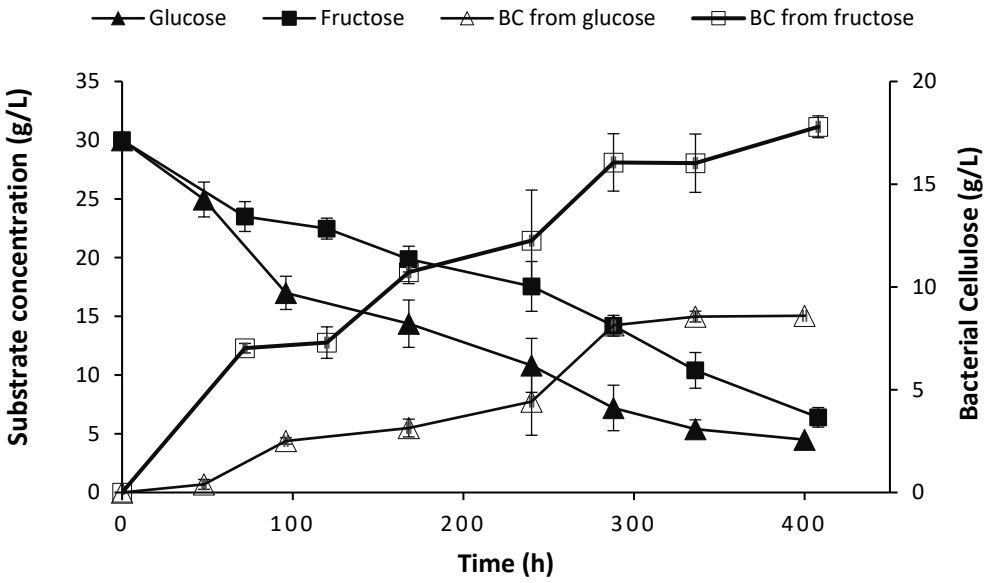

Figure 3.1: Kinetics of substrate concentration and bacterial cellulose production in glucose-based and fructose-based cultures of *Acetobacter xylinum*, under static conditions (S_i=30 g/L)

The maximum BC production was noted in fructose-based media (S_i=60 g/L), reaching 31 g/L, accompanied by an improved yield of 0.67 g per g of consumed substrate. Worth also mentioning was the fact that in both substrate concentrations, carbon source was not depleted from the medium (Figures 3.1 and 3.2).

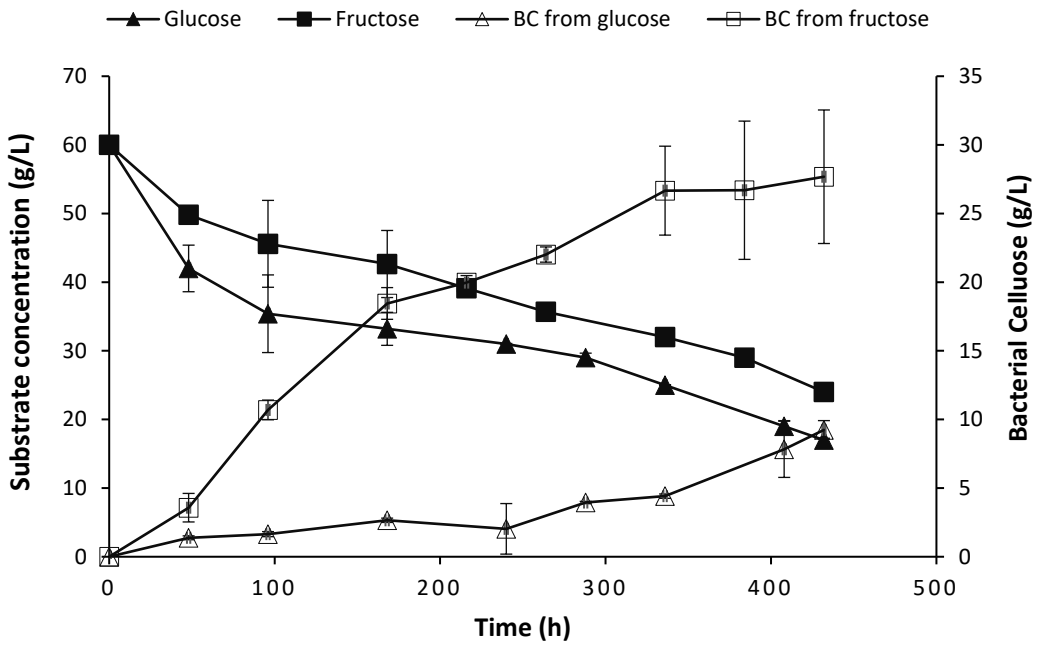

Figure 3.2 Kinetics of substrate concentration and bacterial cellulose production in glucose-based and fructose-based cultures of *Acetobacter xylinum*, under static conditions (S_i=60 g/L)

Lu et al. (2020) compared five different cellulose producing strains (*Komataeibacter xylinus* ATCC 53263, *K. xylinus* DSM 2004, *K. xylinus* DSM 46603 and *K. intermedius* DSM 11804) in HS medium under static and agitated conditions. The results showed that all five selected strains performed better in static cultures than in agitated cultures. The vast majority of growth cultures are nutrient-rich but low in oxygen content (Singhsa et al., 2018), so aerobic bacteria can only achieve the BC proliferation at the interface between air and culture medium (Iguchi et al., 2000; Lee and Zhao, 1999). In a static fermentation environment, the BC membrane on the surface of the medium restricts bacteria from obtaining oxygen from the air (Rani and Appaiah, 2011), which is considered to be a major disadvantage of static culture. Agitation conditions can increase the diffusion of oxygen into the medium, resulting in an increase in bacterial cellulose productivity. However, it has been reported that if wild-type *A. xylinum* is repeatedly

grown and transferred in shake flask cultures, it spontaneously converts to a *A. xylinum* Cel-mutant that does not synthesize bacterial cellulose (Valla & Kjosbakken, 1982; Hestrin & Schramm, 1954). Singhsa et al. (2018) also believe that agitated culture conditions can induce the emergence of mutant cells, resulting in reduced cellulose production. Under a uniform aeration condition in the agitated culture, extensive cell growth appeared increasing the probability of cell mutation, which leading to a deduction of cellulose production (Joris, and Vandamme, 1993). By contrast, formed BC membrane floats on the surface of media in static culture which limit access of oxygen to the lower layer of the culture, prohibits intensive cell growth but lowers the feasibility of mutation into non-cellulose producing cells. This may be the reason why bacterial cellulose yield were higher in static fermentation conditions in the current study.

3.3.3 Structural properties of bacterial cellulose samples

The next step of the experimental design involved the assessment of the type of carbon source on the structural properties of bacterial cellulose. To this end, bacterial cellulose samples obtained from cultures in media with 30 g/L, under agitated conditions were investigated. It is noted that BC samples were freeze-dried for this assessment.

3.3.3.1 Scanning electron microscopy (SEM)

The morphology of BC synthesized in diffirent conditions was analyzed by Scanning Electron Microscopy (SEM). Additionally, it was also possible to observe any presence of residual bacteria inside the bacterial cellulose fibres by SEM. The SEM images of all

samples are depicted in Figure 3.3 (A,B,C,D,E), including a control (fresh BC sample, as obtained from the batch-flask) (Figure 3.3 F). Worth mentioning is the fact that upon harvest, BC samples were subjected to mechanical homogenisation, in order to remove attached cells in the polymer and allow for a reliable quantification of produced biomass. This approach was validated by SEM, as no bacterial cells were found in the treated samples (Figure 3.3 A-E). On the contrary, in the control sample (Figure 3.3 F), cells of *A. xylinum* were found entaged within the BC fibre.

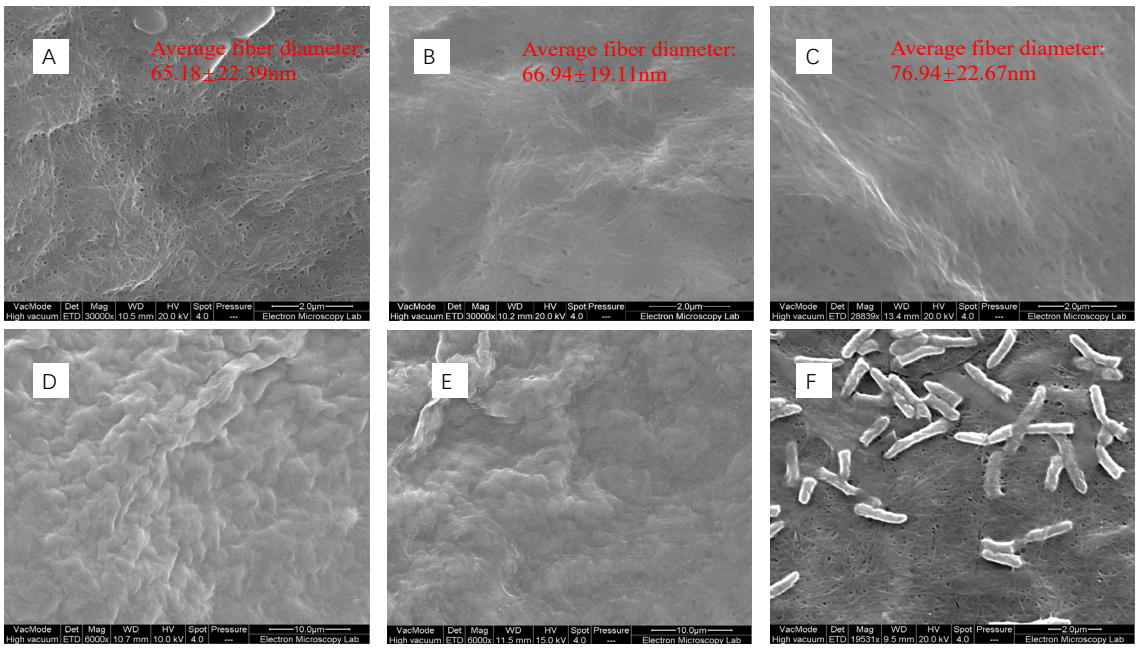

Figure 3.3 SEM images of produced BC samples: (A), from glucose-based media; (B), from fructose-based media; (C): from sucrose-based media; (D): from arabinose-based media; (E): from xylose-based media; (F): fresh BC control sample without pre-treatment

With regards to the morphology of the BC samples, sub-elementary fibres have been reported to be approximately 2-4 nm in diameter and then form microfibrils. These microfibrils bundle to form ribbon-like fibres with an average width of 22-80 nm (Iguchi

et al. 2000; Tokoh et al. 1998; Mohammadkazemi et al., 2015; Vasconcelos et al., 2017). This is generally consistent with the results observed in the current experiment, demonstrating that the carbon source has no effect on the fibril synthesis of bacterial cellulose. However, bacterial cellulose is a highly porous multilayer structure with a high aspect ratio (Zhijiang et al. 2012; Lai et al., 2013; Vasconcelos et al., 2017). It was found that the fibres of BC samples from glucose were irregularly arranged, and pores formed by multiple fibres could be seen. The samples produced from fructose and sucrose, on the other hand, had more densely packed fibre and fewer pores. The fibrous structure of the bacterial cellulose produced in media supplemented with pentoses (xylose and arabinose) was also very fine, but they were so sensitive to the light beam emitted by the SEM instrument and as such, scans at the 2 μm level could not be obtained. This may be due to the poor performance of the strain in pentose-based media, resulting in the accumulation of insufficiently thick bacterial fibrils.

3.3.3.2 X-ray diffractometry (XRD)

Different incubation conditions, such as culture medium, nutrition concentration and incubation time, have been reported to affect the crystallinity of BC (Yamamoto and Horn, 1994; Tokoh et al., 1998; Vazquez et al., 2012; Ruka et al., 2012; Dórame-Miranda et al., 2019). The XRD patterns of bacterial cellulose produced by fructose, sucrose and glucose under agitation condition are shown in Figure 3.4. Among these three samples, three peaks can be found near $2\theta = 14.7$, 16.8 and 22.8, corresponding to the crystal planes ($1\bar{1}$ 0), (1 1 0) and (2 0 0), respectively, proving the existence of Cellulose I (Castro et al.,

2011; Dórame-Miranda et al., 2019). The absence of the characteristic peaks at 2θ = 12.1 and 20.8 for Cellulose II (Oh et al., 2005; Vazquezet al., 2012) means that Cellulose II is not present in these samples.

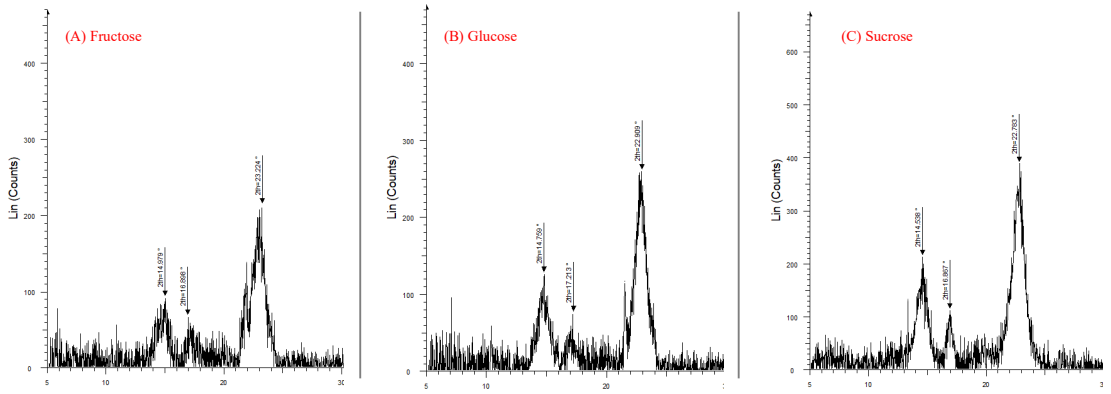

Figure 3.4: XRD patterns of BC obtained from: (A) Fructose; (B) Glucose and (C) Sucrose.

The Crystallinity Index of BC produced in fructose-based media was the lowest, about 51.2%, whereas for glucose and sucrose were 81.0 % and 87.4%, respectively. Typically, bacterial cellulose exhibits a high degree of crystallinity. *A. xylinum* Y22 strain has been reported to achieve 90.5% Crystallinity Index under static conditions (Lu and Jiang, 2014), while *G. xylinus* (CGMCC no. 2955) can utilize glycerol and glucose to produce BC with Crystallinity Index between 80.5- 85.0% (Zhong et al., 2013).

The Crystallinity Index of all samples are within this interval. Under the conditions that were explored in this study, the effect of different carbon sources on the Crystallinity Index of BC may be due to changes in the hydrogen bonds between cellulose molecules (Lu and Jiang, 2014), which may suggest that the presence of glucose is beneficial for the

crystallization process during bacterial cellulose synthesis and the reduction of relatively amorphous regions in bacterial cellulose (Zhong et al., 2013).

3.3.3.3 Fourier Transform Infrared Spectroscopy (FT-IR)

Figures 3.5 depicts the FTIR spectra of the BC produced under hexose-based media. It was found that BC obtained from fructose and sucrose media had a similar spectrum to that of glucose. Representative absorption peaks of BC were present in all three samples. The band at 3350 cm^{-1} is attributed to the presence of hydroxyl (-OH) stretching vibrations, and the absorption band at approximately 2895 cm^{-1} corresponds to CH bond in typical cellulose type I. The band at 1050 - 1065cm^{-1} is considered to be related to the vibration of the C-O-C and C-O-H sugar rings. There is a weak absorption band in the range of 892 cm^{-1}, designated as C-O-C stretch at the beta (1-4) bond, and represents an amorphous cellulose absorption band. The absorption band at 1430 cm^{-1} belongs to the CH_2 symmetric bending vibration and is related to the crystallinity, also known as the cellulose crystallization band, the peak detected around 1650 cm^{-1} corresponds to the H-OH bending, which comes from the water absorbed by BC. The peak near 1360 cm^{-1} is caused by CH bending, and the peaks appearing at 1280 cm^{-1} and 1204 cm^{-1} are also caused by the same characteristic group (Nelson and O'Connor, 1964; Ciolacu et al., 2011; Dórame-Miranda et al., 2019; Gomes et al., 2013; Trovatti et al., 2011; Oh et al. 2005; Jung et al. 2010; Shi et al., 2014; Goh et al., 2012).

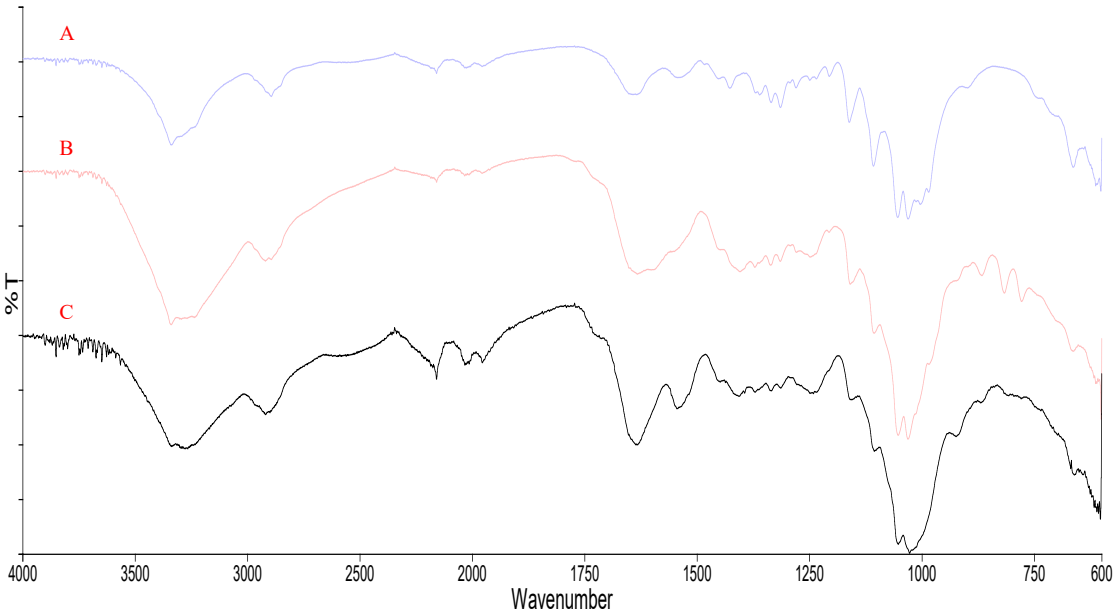

Figure 3.5: FT-IR analysis of BC sample produced in: (A) glucose-based media; (B): Fructose-based media; and (C): sucrose-based media.

Generally, it can be observed that all FT-IR spectra were similar, indicating that the obtained polymers had similar chemical structures, but the BC from fructose condition is more amorphous as also verified by XRD analysis, as described in Section 3.3.4.

3.3.3.4 Water Holding Capacity (WHC)

Water holding capacity (WHC) is considered one of the most important physical characteristics for BC, especially when considering applications in the biomedical industry, for example as a dressing material. Appropriate moisture content in the dressing material can accelerate the wound healing process and protect it from contamination (Cienchanska, 2004; Ul-Islam et al., 2011), promote the penetration of active substances into the wound, and make the dressing simple and painless (Islam et al., 2012), and relieve pain during the formation of new skin (Shezad et al., 2010). Table 4 presents the water holding capacity values of BC samples obtained from media supplemented with different

carbon sources (carbon source concentration at 30g/L).

Table 4: Water holding capacity values (g/g) of BC samples produced in different carbon sources

Substrate	**WHC (g/g)**
Glucose	57.72 ± 1.10^{b}
Fructose	68.32 ± 6.94^{ab}
Sucrose	69.26 ± 3.31^{a}

Note: Averages within the same column followed by the same letters for each carbon source did not show any significant difference ($P > 0.05$).

BC samples obtained by sucrose-based media exhibited the highest holding capacity (69.26 g/g), followed by fructose (68.32 g/g), while glucose-derived one had the lowest value. The BC samples produced by Szymańska et al. (2022) had a WHC as high as 93.43 g/g, The WHC of the modified BC was studied by Islam et al. (2012), and the WHC of the BC sample without any treatment was 106.34 g/g, and the WHC of the BC sample of Liu et al. (2019) was also about 93.63 g/g.

The water holding capacity of BC can be affected by a variety of factors. It has been reported that the increase in the amorphous part can affect the hydration capacity of the BC, and the amorphous part in the sample can even absorb more water from the atmosphere. This means that BC with low crystallinity tends to have higher WHC (Vazquez et al., 2012; Park et al., 2010), a fact that was also verified in this current experiment, as fructose-derived BC exhibited the lowest crystallinity among the samples. In addition, water molecules are physically trapped on the surface and within the BC

matrix composed of reticulated fibrils, which means that the large amount of empty space between the BC fibrils causes more water to be permeated and adsorbed onto the material. Therefore, the 3D network structure features of BC have a special contribution in increasing the water holding capacity; the larger the surface area, the larger the WHC of BC samples. However, tightly arranged microfibrils bind water molecules more efficiently than loosely arranged microfibrils because they have stronger hydrogen bonding interactions and are more effective in protecting water evaporation (Islam et al., 2012; Ougiya et al., 1998; Shah et al., 2010).

3.4 Conclusion

The production of bacterial cellulose and the investigation of its physicochemical properties in different cultivation conditions was assessed. Based on the obtained data, it can be suggested that fructose is the most suitable carbon source for BC production by *A. xylinum*. It was also shown that the choice of the carbon source does not seem to influence the structure and spatial arrangement of bacterial cellulose, although it can impact its crystallinity and subsequently its water holding capacity properties. Therefore, depending on the intended applications of BC, the information provided in this chapter can lay the foundation for the production of BC with desired structural and functional properties.

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Chapter 4: Purification strategies and their impact on bacterial cellulose structural properties

Abstract

During bacterial cellulose production, cell metabolites, growth medium, and cells are trapped within its fibre network. These impurities can affect the performance of BC and limit its application. In this study, the effectiveness of NaOH and NaOCl solutions in BC purifications, and their effect on BC structure and properties were explored. All methods were effective in removing trapped cells, but homogenisation could not remove growth medium residues effectively. High alkali concentrations (1.0 M) combined with 40°C led to structural damages of BC. Lower NaOH concentrations (0.25 and 0.5 M) at mild temperature (25°C), resulted in densely arranged fibre network according to SEM images, but evidence of conversion of Cellulose I to Cellulose II was noted. The combination of weak NaOH (0.1 M) and NaOCl was proved an effective purification method, that preserved the fibre structure and crystallinity of BC.

4.1 Introduction

In contrast to plant cellulose, bacterial cellulose (BC) is characterised by superior high purity and high crystallinity. These attributes have attracted much research attention in the last decade. Bacterial cellulose possesses similar chemical composition with that of plant cellulose, whilst its structure and physical properties are different (Cannon and Anderson, 1991). Previous studies have elucidated that the diameter of BC fibrils is about

one hundredth to one thousandth of that of plant cellulose fibrils. This ultrafine BC fibril forms an ultrafine network, allowing BC to have a unique large surface area, higher water holding capacity, strong tensile strength, and higher degree of polymerization (Dahman, 2009; Meftahi et al., 2009; Ul-Islam et al., 2012; Klemm et al., 2001; Yoshinaga et al., 1997). Additionally, BC production is not subjected to regional and climatic conditions. The fibril size, surface area and some other desired properties can also be tailored by artificial induction, by adding additives and other measures. For example, multiple reactors and carbon sources have been invested for the production of BC of different shapes and fibre size (Shi et al., 2014; Mikkelsen et al., 2009); Carboxymethylcellulose (CMC) introduced media have been reported as suitable for the production of BC (Cheng et al., 2009); composites of hydrolysed gelatine peptides and CMC have been shown to significantly improve the rehydration ratio and rate of BC (Chen et al., 2013); the tensile modulus and strength were increased by more than 2.5 times after BC was epoxy laminated while maintaining its desirable magnetic and flexibility properties (Mashkour, Moradabadi and Khazaeian, 2017).

During cell growth and bacterial cellulose production, bacterial cells are entrapped into the BC membrane gradually. These cells, together with residual nutrients may affect the physicochemical properties and mechanical strength of BC sheet/membrane, thus, BC purification is often required to obtain high quality of bacterial cellulose and expand the range of its potential applications.

The use of NaOH solutions to remove contaminants from the cellulose network is the most commonly used method, but it has been reported that high concentrations of NaOH solution can disrupt the hydrogen bonds between chains, resulting in an irreversible transformation of cellulose I to cellulose II, which significantly affects the mechanical strength and elastic modulus of BC, thus limiting its applications (Nishi et al., 1990). However, the use of low concentrations of NaOH solutions seem to have a milder effect on the mechanical strength of BC (McKenna et al., 2009). The oxidant NaOCl has also been used to achieve more efficient BC purification by oxidizing pollutants in cellulose, and the two-step purification method of NaOH-NaOCl has been reported to offer BC samples of higher purity, which can be more efficiently used in the apparel and paper industries (Gea et al., 2011).

In this study, the purification of bacterial cellulose was investigated under a variety of conditions, including the combinations of NaOH and NaOCl solutions of varying concentrations, soaking temperature, as well as mechanical purification methods (homogenisation). The overall aim of this work was to assess the effectiveness of each purification strategy, as well as to provide insights on the impact on the BC fibre structure.

4.2 Materials and Methods

4.2.1 Materials

Bacteriological Agar from Oxoid Ltd (stock keeping unit [SKU]: LP0013T); D-(–)-Fructose ≥ 99% from Sigma-Aldrich (SKU: F0127); Peptone from casein; enzymatic

digest from Sigma-Aldrich (SKU: 82303); Yeast extract from Fisher Bioreagents (SKU: BP1422-500); Sodium phosphate dibasic ≥ 99% from Sigma-Aldrich (SKU: 795410); Citric acid monohydrate ≥ 99.5% from Fisher Chemical (SKU: CI6160I53); Sodium hydroxide from Fisher Chemical (SKU: SI4920I53); Solium hypochlorite solution (NaOCl) from Fisher Bioreagents (S/5040/21)

4.2.2. Preparation of bacterial strains

The bacterium *Acetobacter xylinum subsp. sucrofermentans* DSM 15973, derived from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, was used in the study. The bacterial strain was grown and maintained on sterilised Hestrin and Schramm Agar (H&S agar) (Mikkelsen et al., 2009), with agar (20 g/L), glucose (30 g/L), peptone (5 g/L), yeast extract (5 g/L), sodium phosphate dibasic (2.7 g/L), and citric acid 1.15 g/L). In order to preserve the strain viability, the microorganism was sub-inoculated on freshly prepared H&S agar every two weeks.

4.2.3 Pre-culture preparation

Pre-culture medium was prepared with glucose (30 g/L), peptone (5 g/L), yeast extract (5 g/L), sodium phosphate dibasic (2.7 g/L), and citric acid (1.15 g/L) and then sterilized at 121°C for 15 minutes. *Acetobacter xylinum subsp. sucrofermentans* DSM 15973 was inoculated into 50 ml of pre-medium and incubated for 24 hours at 30°C at 180 rpm (IKA Labortechnik speed control orbital shaker KS501 digital)

4.2.4 Bacterial cellulose production

Modified H&S media, containing fructose (30 g/L), peptone (5 g/L), yeast extract (5 g/L), sodium phosphate dibasic (2.7 g/L), and citric acid (1.15 g/L), were prepared and sterilized (121°C for 15 min) for batch-flask cultures. The pre-culture samples were homogenized by a stomacher (Seward 400 circulator, P/4/518) at 230 rpm for 2 min, and then 1 mL of cell suspension was added into 250 mL Erlenmeyer flasks containing 50 mL modified H&S media. Batch-flask fermentations were carried out under static conditions at 30°C for 200 h, for the production of fresh BC samples. The produced BC samples were separated from the culture by vacuum filtration and used for further treatments.

4.2.5 Bacterial cellulose purification

The produced fresh BC samples were subjected into four different purification methods, as described below:

i) *Mechanical Treatment*. BC samples were homogenized at 230 rpm for 2 min by a stomacher (Seward 400 circulator, P/4/518). This pre-treatment served as the control.

ii) *Alkali treatment*. Alkali treatment was modified based on Shibazaki et al., (1997), BC samples were immersed into 60 ml of NaOH solution at different concentrations (0.1 M, 0.25 M, 0.5 M and 1 M) for 30 min at 25°C and 40°C

iii) *Two-stage method*. Fresh BC samples were homogenized at 230 rpm for 2 min by a stomacher (Seward 400 circulator, P/4/518), and then immersed into 60 ml of NaOH solution at different concentrations (0.1 M, 0.2 5M, and 0.5 M) for 30 min at 25°C.

iv) *Two-step chemical method* (Gea et al., 2011). The first step of BC purification was achieved by immersing BC samples into 60 ml of 0.1 M NaOH solution at 25°C for 30 min. Subsequently, BC samples were transferred into 60 ml of NaOCl solution at concentrations of 1.5% and 2.5% (w/w), at 25°C for 30 min.

All immersion steps were performed in an orbital shaker (IKA Labortechnik, UK) and the rotary speed was set as 180rpm. After the treatment, samples were washed by distilled water, frozen at -20°C overnight and freeze dried (VirTis Scientific sentry 2.0), until further analysis.

4.2.6 Scanning Electron Microscopy (SEM)

Dehydrated BC samples were coated with gold nanoparticles at 11 mA using an Edwards Sputter Coater S150B. The BC surface analysis was carried out using the FEI Quanta 600 FEG SEM microscope, with accelerating voltage of 2 kV and at a working distance of 10.1-10.4 mm. Magnifications of 5,000 x and 10,000 x were applied.

4.2.7 X-ray Diffractometry (XRD)

The degree of crystallinity in BC samples was determined using a Bruker D8 Advance powder diffractometer with a copper source (wavelength 1.54 angstroms). The 2-theta angle ranged from 5.0 to 64.0848 degrees with 0.02106 mm increments for 1.2 seconds. The aperture slit was 6 mm, and the detector was equipped with a Nichol attenuator. The obtained XRD curves were analysed by TOPAS (Bruker AXS, version 4.2), to calculate

the Crystallinity Index (CI).

4.2.8 Fourier Transform Infrared Spectroscopy (FT-IR)

The infrared spectrum was obtained using a Perkin Elmer precisely spectrum 100 ATR FT-IR Spectrometer. Dried BC flakes were analysed in the spectrometer region of 4000-650 cm^{-1} and with a resolution of 4 $cm^{-1.}$ The result for each sample was the average of 32 scans.

4.3 Results and discussion

4.3.1 Morphological analysis of pre-treated BC samples

Morphological analysis, through the use of SEM, was applied to investigate the surface of BC samples and detect any changes incurred after pre-treatment. The SEM images in the following Figure 4.1 depict differences in surface morphology of BC pre-treated with different concentrations of NaOH solutions at 25 °C. Homogenised-only samples were also included as control.

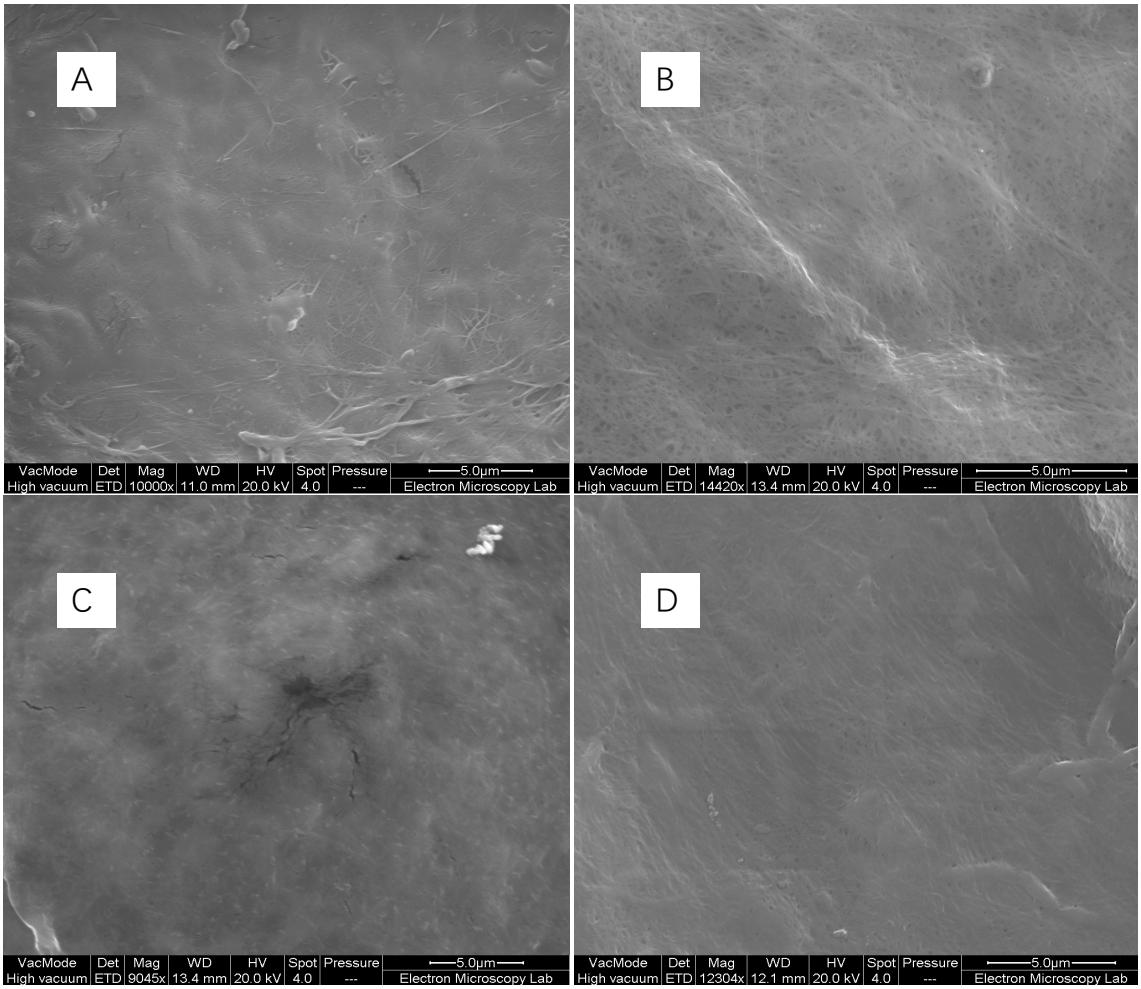

Figure 4.1 SEM images of BC treated by: (A) 0.1 M NaOH; (B) 0.25 M NaOH; (C) 0.5 M NaOH; (D) homogenised sample (control). Conditions: 25°C; 30 min; agitation speed 180 rpm

With regards to BC samples treated with low-concentration NaOH solution (0.1 M), a part of the fibre network structure was visible, but at the same time, debris and media impurities were also seen (Fig. 4.1A). Increasing the concentration of NaOH solution to 0.25 M led to a densely arranged cellulose network (Fig. 4.1B), with pores between fibres clearly visible, demonstrating relatively high purity of the sample. However, BC samples treated with 0.5 M NaOH solution became more fragile and as a result, the electron beam from the SEM caused severe damage in the centre of the sample (Fig. 4.1C). It is noteworthy that when BC samples were treated with 1.0 M NaOH, they became extremely

fragile to the extent that SEM images could not be obtained. The samples were completely damaged during the treatment process. The use of 1.0 M NaOH appears to have had a detrimental effect on the structural integrity of the BC samples, rendering them unsuitable for SEM imaging. Also, with regards to NaOH treatment at 40°C, all BC samples became very fragile, and the SEM images revealed a destroyed fibril network (indicative image in Figure 4.2 below). As a result of this observation, subsequent pre-treatments with NaOH were carried out only at 25°C.

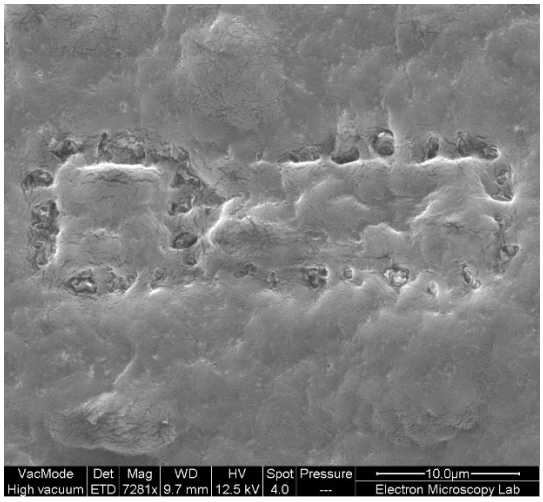

Figure 4.2 SEM image of BC showing a destroyed fibril network, after treatment with 0.25 M NaOH at 40°C. Conditions: 40°C; 30 min; agitation speed 180 rpm

The two-stage purification method (homogenisation and chemical pre-treatment with NaOH), lead to less visible BC microstructures at 0.1 M NaOH (Fig. 4.3 A). By increasing the NaOH concentration to 0.25 M (Fig. 4.3 B), the fibre network structure was visible in places, but the spaces between fibres were still indistinct, indicating the presence of impurities, whereas at 0.5 M NaOH, the BC samples became brittle and more susceptible

to damage (Fig. 4.3 C).

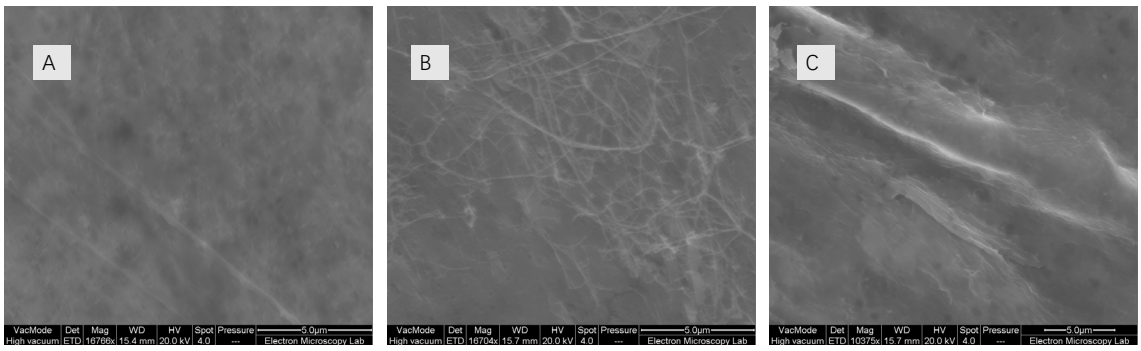

Figure 4.3 SEM images of BC treated by homogenisation and (A) 0.1 M NaOH; (B) 0.25 M NaOH; (C) 0.5 M NaOH.

Conditions: homogenisation at 230 rpm for 2 min; 25°C immersion in NaOH for 30 min; agitation speed 180 rpm

The last approach tested on the purification of BC was the combination of NaOH and NaOCl solutions. As depicted in Figure 4.4A, in 1.5% (w/w) NaOCl, transparent areas were visible in the treated sample, indicating absence of any residual growth medium, whereas the fibrils were clearly inter-connected, indicating the preservation of the 3D structure of the polymer.

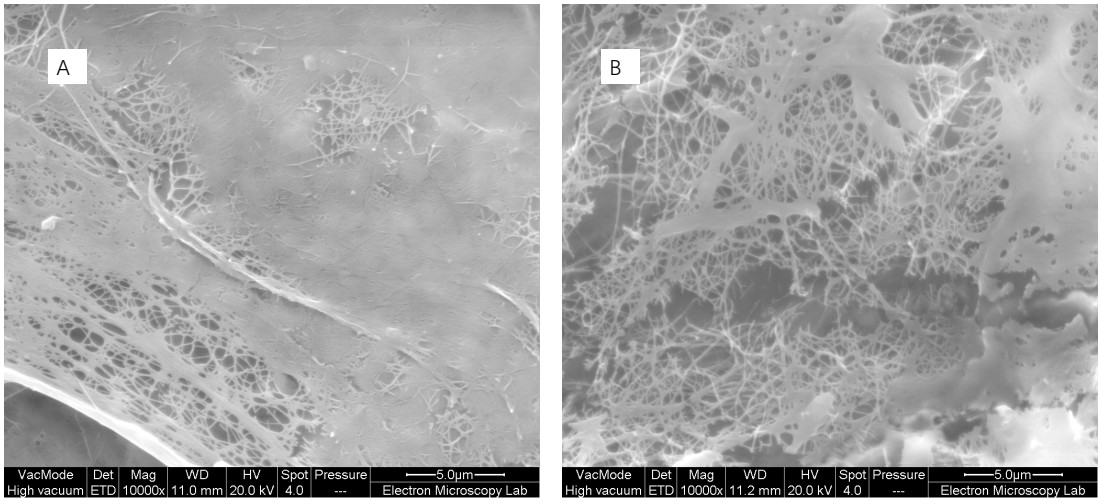

Figure 4.4: SEM images of BC treated by two-step purification with 0.1 M NaOH and: (A) 1.5% (w/w). NaOCl; (B) 2.5% (w/w) NaOCl. Conditions: 25°C; immersion in NaOH and NaOCl for 30 min each; agitation speed 180 rpm.

At higher NaOCl concentrations (2.5%, w/w.), the porous structure of bacterial cellulose remained intact and with larger pores (Fig. 4.4 B), demonstrating the effectiveness and efficiency of the method.

During bacterial cellulose fermentation, the ultra-fine sub-basic cellulose fibrils are synthesized and extruded from the enzyme complex into the growth medium. This cellulose is initially amorphous and then crystallized to form Cellulose I (Cai and Kim, 2009), and assembled into microfibrils, which in turn are bundled into fibrils (Cai and Kim, 2009). However, both bacterial cells and growth medium can be trapped inside the fibrous structure of BC during its formation, limiting the possibility of forming hydrogen bonds. In all the bacterial cellulose purification methods mentioned above, homogenisation and water washing alone was found not to be enough for efficient impurities' removal. NaOH has the ability to dissolve some non-fibrous impurities in BC,

so that the bacterial cellulose can be purified to a certain extent. The increase in the concentration of the NaOH solution could improve the purification efficiency, but the concentration needs to be controlled below 0.5 M, because high concentrations of NaOH tend to destroy the fibre structure of BC (Han et al., 2018). Specifically, Suryanto et al. (2019) also demonstrated that purification treatments with higher concentrations of NaOH altered the OH bonds and reduced the mechanical strength of BC films. At the same time, the high concentration of alkaline solution will also cause the bacterial cellulose to shrink and bend significantly. These changes may also be accompanied by changes in the crystal structure of BC, and more importantly the irreversible conversion from Cellulose I to Cellulose II, which is not desirable (Gea et al., 2011; Nishi et al., 1990). In the current study, the combination of homogenisation and NaOH treatment did not offer any distinctive advantage over the NaOH treatment alone. This may be due to the formation of cellulose pulp after the block-shaped bacterial cellulose sample was homogenised, which made it difficult for the NaOH solution to penetrate through the deep layer of the pulp. On the other hand, BC samples purified with NaOCl solution revealed the internal structure of BC more clearly, demonstrating the effectiveness of NaOCl solution in removing impurities which cannot be removed by NaOH solution alone. This is because NaOCl can react with water to form moderately strong bases and weak acids (Gea et al., 2011), and the hypochlorous acid formed in this process is an efficient active bleaching agent, which can achieve the purpose of purifying BC. However, the strong oxidative properties of high concentrations of NaOCl can lead to the breaking of hydrogen bonds between cellulose fibres and even trigger the degradation of cellulose

(Amarasekara, Wang and Grady, 2020).

4.3.2 Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR analysis is considered a reliable method in identifying functional groups as well as impurities of BC. In the case of pure BC, the broadband at 3450 cm^{-1} is attributed to the O-H stretching vibration (Zhijiang, Chengwei and Guang, 2012). The 2820 cm^{-1} band represents aliphatic C-H stretching vibrations (Zhijiang, Chengwei and Guang, 2012). The peaks at 1650 cm^{-1}, 1370 cm^{-1} and 1032 cm^{-1} are due to residual water bending vibrations, CH_2 symmetry bending vibrations and C-O stretching vibrations (Wang et al., 2016). Peaks observed in the 1100-1073cm^{-1} region are assigned as C-O-C stretching at the β-(1→4)-glycosidic bond/linkage in BC (Kamal, Misnon and Fadil, 2020). In the spectrum of the sample, in addition to the standard absorption peaks of functional groups of BC, there are still redundant absorption peaks found, such as peaks related to carbonyl group (1339 cm^{-1}), NH stretching of peptides (1595-1607 cm^{-1}), OH stretching of alcohols (976-979 cm^{-1}), phosphate groups (976-979 cm^{-1}) and aromatic rings (1595-1607 cm^{-1}; 815-817 cm^{-1}) (Gea et al., 2011). The presence of these peaks and the peak areas can be used to compare the effectiveness of different purification methods in removing impurities. For example, carbonyl groups can indicate the presence of aldehydes and ketones produced by bacteria. Alcohols and aromatic rings can also be produced as metabolites. The NH extension indicates the presence of the peptide, which may come from cell debris in the BC, or residues of the growth medium, whereas the phosphate group may come from disodium hydrogen phosphate (Na_2HPO_4), which is a component

of the H&S modified growth medium used in this study.

According to the FT-IR spectra in Figure 4.5, after the purification with different NaOH concentrations at 25°C (Fig. 4.5A- C), the peaks of functional groups associated with impurities were more prominent at low NaOH concentrations and faded in higher NaOH concentrations.

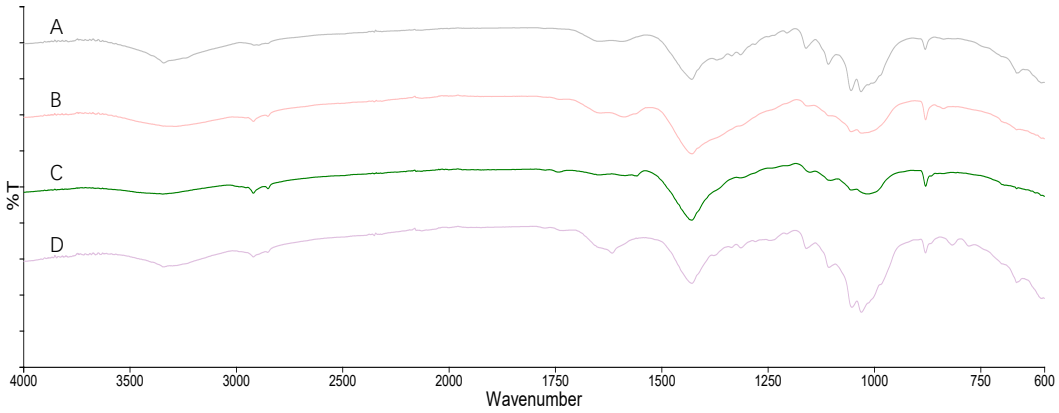

Figure 4.5 FT-IR spectra of pre-treated BC samples by: A: 0.1 M NaOH; B: 0.25 M NaOH; C: 0.5 M NaOH; D: homogenised only (control). Conditions: 25°C; 30 min; agitation speed 180 rpm

In terms of cellulose, the stretching of O-H in Cellulose II is reflected in the peaks around 3495 cm^{-1} and 3433 cm^{-1}, and the peaks around 3345 cm^{-1} and 3278 cm^{-1} are caused by the stretching of OH in Cellulose I (Gea et al., 2011). Increasing the NaOH concentration to 0.25 M and 0.5 M, the peaks at these positions become rounded and more prominent (Figures 4.5 B and C). This means that the high concentration of NaOH solution may promote Cellulose I conversion to amorphous or very poorly crystalline Cellulose II (Shibazaki, Kuga and Okano, 1997). The combination of homogenisation and NaOH

treatment did not seem to offer any distinctive advantage, as peaks corresponding to alkaline, nitrogen and phosphate functional groups (1400 cm^{-1}-1000 cm^{-1}) were still prominent in the FT-IR spectra (Figure 4.6).

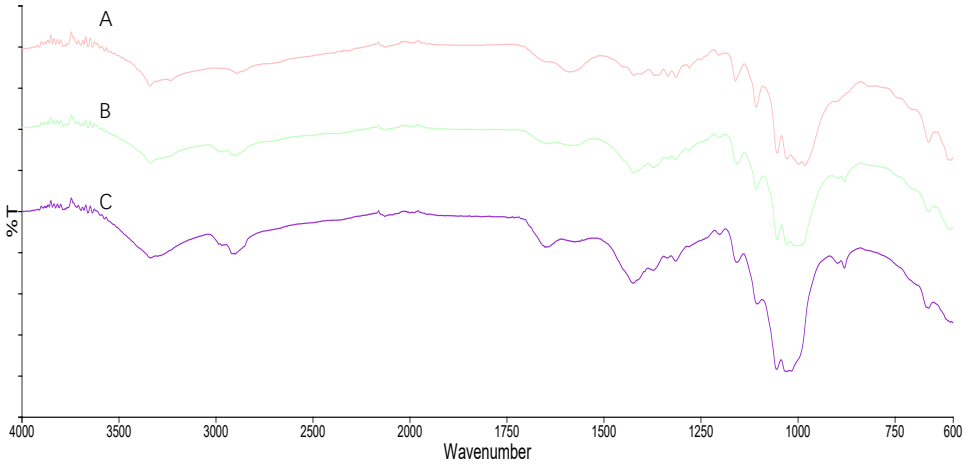

Figure 4.6 FT-IR spectra of BC samples treated by homogenisation and various NaOH concentrations: A: 0.1M; B: 0.25M; C: 0.5MConditions: 25°C; 30 min; agitation speed 180 rpm

In BC samples purified by NaOH and NaOCl (two-stage method), the results show a similar chemical structure at both NaOCl concentrations (Figure 4.7), however, the peaks corresponding to the aromatic rings and N-H stretching of peptides were smaller for 1.5% (w/w) NaOCl which may indicate a higher efficiency of the lower NaOCl concentration for removing these compounds.

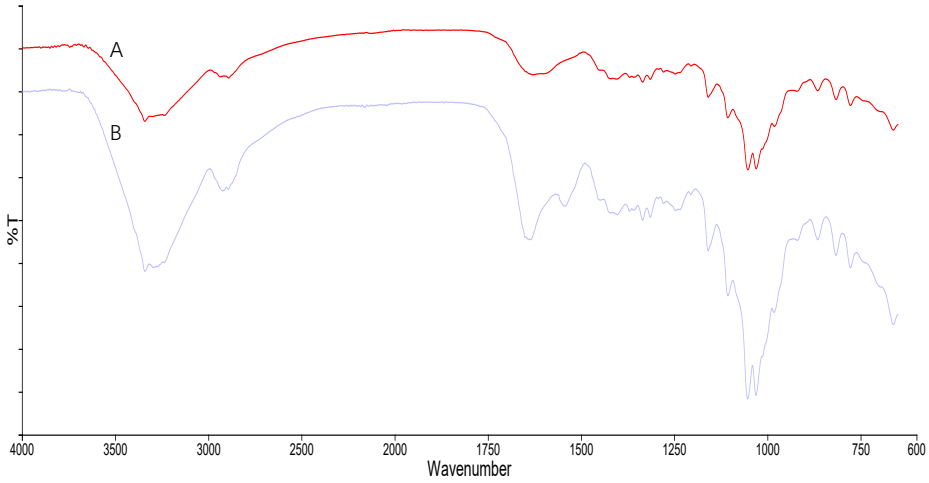

Figure 4.7. FT-IR spectra of BC after two-step purification with 0.1 M NaOH and: (A) 1.5 % (w/w) NaOCl; (B) 02.5% (w/w) NaOCl. Conditions: 25°C; immersion in NaOH and NaOCl for 30 min each; agitation speed 180 rpm.

4.3.3 X-ray diffractometry (XRD)

The crystallinity index (CI) of BC samples treated with different purification methods were evaluated by XRD patterns. As shown in Figure 4.8D, three peaks can be found at 2θ=14.6, 16.5 and 22.8 for the homogenized BC (control), which correspond to the typical Cellulose I structure (Castro et al., 2011; Dórame-Miranda et al., 2019). The broad diffraction peaks around 14° and 22°, correspond to characteristic interplanar spacings of Cellulose Iα and Cellulose Iβ phases (Kamal, Misnon and Fadil, 2020). In samples treated with alkali, (Fig 4.8 A-C), the characteristic peaks of Cellulose I were prominent, but there was a narrow but sharp peak appearing at 2θ=21.5, which is close to the characteristic peak of Cellulose II (Kafle et al., 2014). It has been reported that the peaks appearing around 12, 19.9 and 21.8 correspond to the (1$\bar{1}$ 0), (1 1 0) and (020) planes,

respectively and are associated with Cellulose II (Gong et al., 2017; Kafle et al., 2014). The increase in NaOH concentration to 0.5 M (Fig. 4.8C), lead to peaks at 2θ=14 and 22.8 disappearing, giving place to a large number of small peaks after 30, indicating the conversion of Cellulose I to Cellulose II and ultimately a less crystalline BC (Gong et al. 2017).

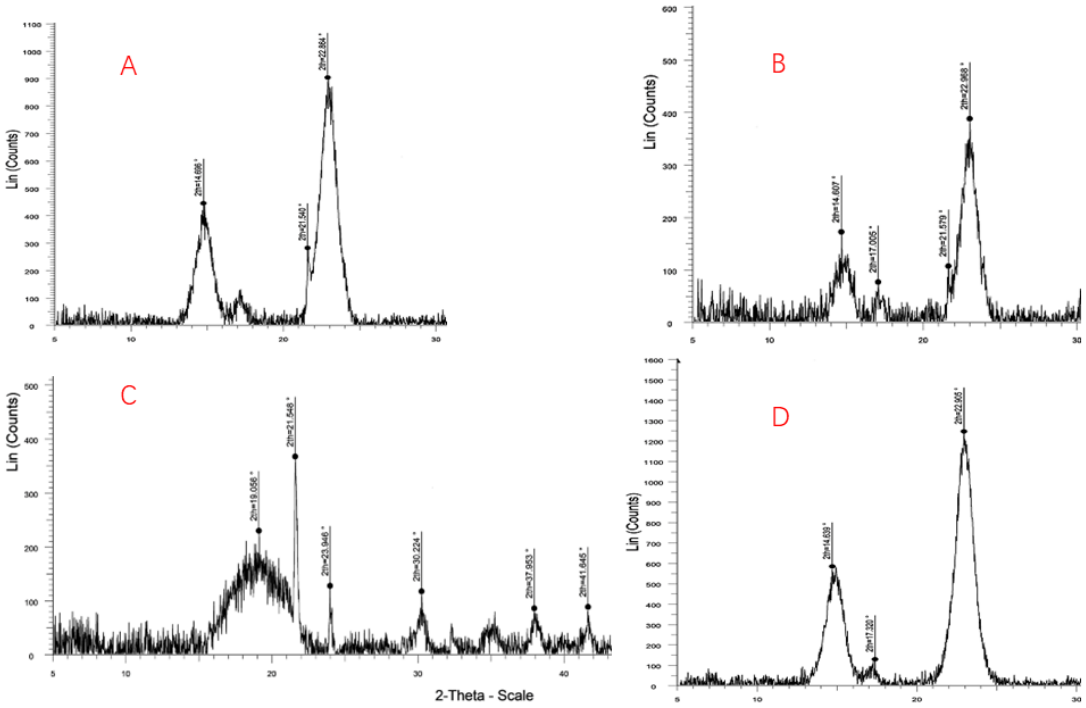

Figure 4.8 XRD patterns of BC treated by: (A) 0.1 M NaOH; (B) 0.25 M NaOH; (C) 0.5 M NaOH, (D) homogenised sample (control). Conditions: 25°C; 30 min; agitation speed 180 rpm

With regards to the XRD patterns of BC samples treated by homogenisation and increasing alkali concentrations, only the samples treated with 0.1M NaOH had three complete characteristic peaks of Cellulose I (Fig. 4.9A), which is an indication that the crystallinity of BC was preserved. For the other two sets of samples (Fig. 4.9 B and C),

the peak at 2θ = 16.5 disappeared, while only a broad peak spanning 15 to 25 and a peak at 21.5 were present, meaning that highly crystalline Cellulose I was partially hydrolysed and formed amorphous regions. This is undesirable in most cases, as an increase in the amorphous region leaded to a decrease in the crystallinity index of the BC, which in turn leaded to a decrease in the mechanical and barrier properties of the BC (Shanshan, Jianqing and Zhengwei, 2012). The three characteristic peaks of cellulose I were intact in Figure 4.10 A and B, demonstrating the effectiveness of the two-step chemical method to remove impurities and maintain the integrity of the BC crystalline structure.

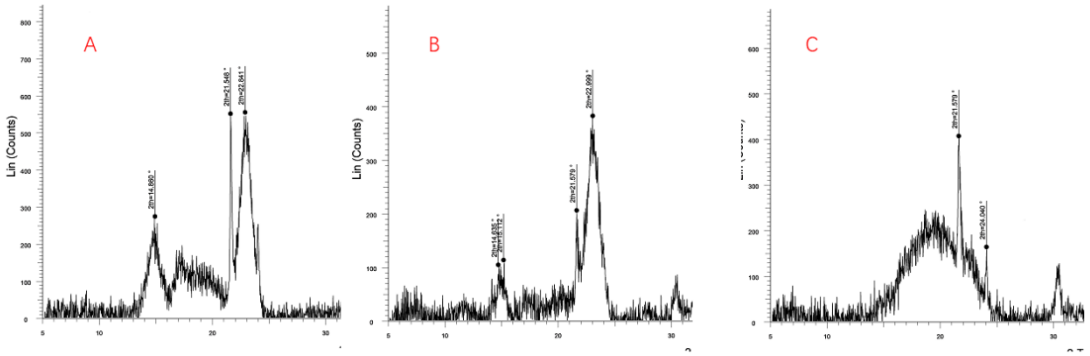

Figure 4.9: XRD patterns of BC treated by homogenisation and (A) 0.1 M NaOH; (B) 0.25 M NaOH; (C) 0.5 M NaOH. Conditions: homogenisation at 230 rpm for 2 min; 25°C immersion in NaOH for 30 min; agitation speed 180 rpm

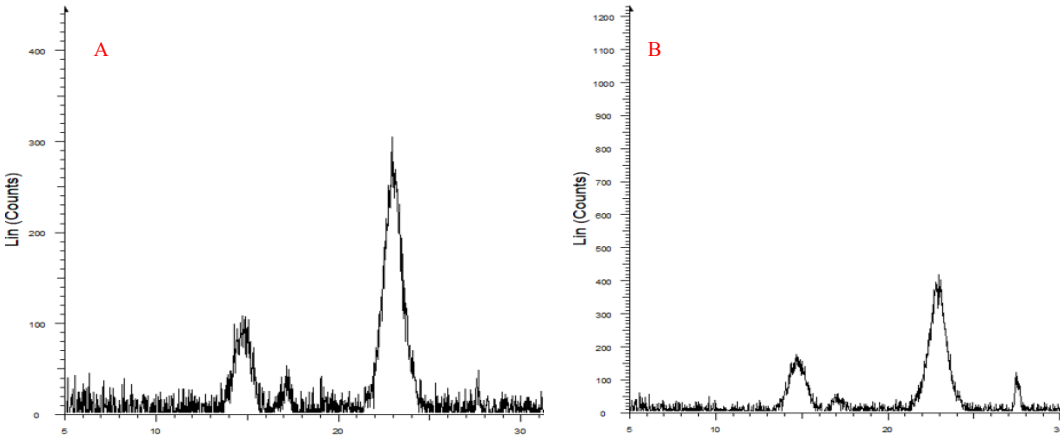

Figure 4.10: XRD patterns of BC treated by Two -stage purification with 0.1 M NaOH and: (A) 1.5 % (w/w) NaOCl; (B) 02.5% (w/w) NaOCl. Conditions: 25°C; immersion in NaOH and NaOCl for 30 min each; agitation speed 180 rpm.

The crystallinity index (CI) of all BC samples is shown in Table 4.1. Samples treated with alkali exhibited decreasing crystallinity as alkali concentration increased (from 70.1% to 31.6%). Similar values were obtained for samples that were first homogenised and then treated with alkali (Table 4.1). The only exception was in samples treated with NaOCl, where their crystallinity increased as the concentration of sodium hypochloride increased (from 63.1 to 91.7). This result is in agreement with the morphological structure observed for those samples in SEM images.

Table 4.1: Impact of different purification methods on BC crystallinity Index

Purification method	**Concentration**	**BC crystallinity Index (%)**
Homogenization	Not applicable	24.3
Alkali treatment	0.1 M NaOH	70.7
	0.25 M NaOH	56.0
	0.5 M NaOH	31.6
Two-stage treatment (Homogenization and alkali)	0.1M NaOH	69.3
	0.25M NaOH	30.3
	0.5M NaOH	30.2
Two-step treatment	0.1M NaOH + 1.5%wt. NaOCl	63.1
	0.1M NaOH + 2.5%wt. NaOCl	91.9

The data showed that BC samples treated with mild NaOH concentrations (0.1 M) had a higher proportion of crystalline regions. Kamal et al. (2020) obtained similar CI percentages for NaOH purified BC samples, noting that the removal of impurities by NaOH incurred changes in the orientation of the microcrystalline BC fibre structure, providing highly ordered polymer, thereby increasing its relative crystallinity. However, this was not confirmed in this study when greater NaOH concentrations were used. The discrepancy of the results in chemically treated BC indicates its high structural heterogeneity, which may be the result of insufficient penetration of the cellulose network by alkaline solutions. Sghaier et al. (2012) treated cellulose with 20% NaOCl, resulting

in a 6% increase in crystallinity index. Several studies suggested that the amorphous regions of NaOCl-treated cellulose are reduced, that is, if the amorphous regions of the cellulose are attacked during processing, chain scission and peeling reactions can occur, which reducing the total amount of amorphous regions of low crystallinity (Poletto et al., 2011; Sghaier et al., 2012).

4.4 Conclusion

This study evaluated the effectiveness of homogenization and chemical treatment for purifying BC, as well as the integrity of the unique fibrous structure after purification. SEM visualisation indicated that impurities in BC can be effectively removed using homogenisation and alkali treatment. Soaking in high concentrations of NaOH solution and high soaking temperature were not favourable as they damage the fibril network structure. FTIR spectra confirmed the effectiveness of NaOH in removing contaminants, but combined with the XRD pattern and CI data, it can be concluded that high alkaline concentrations lead to more amorphous BC. Overall, the purification method has a significant impact on the structure and properties of BC but should be chosen according to its desired application.

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Chapter 5: Evaluation of in-vitro digestion of bacterial cellulose

Abstract

Freeze-dried bacterial cellulose powder was digested using an *in vitro* gastrointestinal (GI) simulation model, including oral, small intestine and distal colon steps. Bacterial enumeration was monitored using fluorescent *in situ* hybridization (FISH), and the fermentation end products, namely short chain fatty acids (SCFA), were analysed using HPLC. The viscosity of BC was higher than glucomannan (control for upper GI simulation) throughout the digestion in the upper gastrointestinal tract, while XRD and FTIR results showed no significant structural changes in BC after the digestion of the upper gastrointestinal tract. FISH showed that BC did not seem to significantly stimulate a rise in the total bacterial population. In *in vitro* batch trials with 1% BC as substrate, acetic acid seemed to be promoted, but no significant effect was noted on the accumulation of other SCFAs. Overall, BC has potential as a satiety food formulation, by increasing viscosity in the stomach but does not exert any prebiotic effects.

5.1 Introduction

Overconsumption of energy dense food products and unbalanced nutritional diet are examples of reasons for increasing prevalence of obese and overweight people in the world (Drewnowski and Darmon, 2005). Therefore, formulated foods with increased satiety ability become particularly attractive as they help consumers feel full for an extended period of time, instead of relying on a strong will to endure hunger (Fiszman

and Varela, 2013).

Fibres can reduce the feeling of hunger by a number of mechanisms during digestion. According to Slavin and Green (2007), chewing time is increased in fibre-rich products, which limits intake by promoting the secretion of saliva and gastric juice and results in an expansion of the stomach. During the gastric phase, fibre bulk expansion promotes gastric distension during meal ingestion, activating vagal afferents, which send signals from the stomach to the brain and result in the perception of fullness and satiety (Fiszman and Varela, 2013; Wang et al., 2008). Additionally, it has been hypothesized that those viscous mixtures and/or gels decrease hunger due to distension of the gastric antrum and/or altered transport of nutrients to the small intestine in lumps, prolonging the intestinal phase of nutrient digestion and absorption, and the time course of post-absorptive signals (Fiszman and Varela, 2013; Nilsson et al., 2008). In general, the gastric emptying properties of food are critical for satiety because the gastric signals associated with satiety are only physical in nature (Rebello et al., 2014). Moreover, the priority of the stomach in the digestion process makes the feeling of satiety derived from the stomach take precedence over the feeling of fullness derived from the intestine (Shang et al., 2020). Therefore, there is a merit to design and develop satiety foods based on their gastric emptying properties.

Viscous soluble dietary fiber has been shown to occupy gastric space and delay gastric emptying (Guo et al., 2021), which has attracted interest in the study of dietary fiber.

Konjac glucomannan is of interest because of its ultrahigh viscosity after full hydration, compared to other dietary fibres with appetite modulating effects. Glucomannan is well tolerated, virtually unaffected by the gastrointestinal tract, and remains stable at different pH (McCarty, 2002; Lu, Zheng and Miao, 2018; Zhao et al, 2015). Bacterial cellulose (BC) also has similar properties. It is a fibrous nanomaterial synthesized by some bacteria in the form of extracellular polysaccharide (Azeredo et al., 2019). In addition, small amounts of BC can make aqueous solutions viscous (Tsalagkas et al., 2016). These properties appear to be suitable for screening satiety food formulations. Glucomannan has been successfully used as a satiety food formulation, but limited research has been done on BC as a satiety-promoting food ingredient.

The concept of functional food has been proposed, that is, food that is beneficial to improve the health of the host. Prebiotics is a particular example (Bajury et al., 2017), as they actively control the composition of the microbial population in the human colon. Gut microbiota is one of the most complex microbial ecosystems and it has a crucial role in human health, such as the extraction of energy from food, alterations in the appetite signaling pathway (Krajmalnik-Brown et al., 2012; Tidjani Alou, Lagier and Raoult, 2016), as well as participating in host metabolism (Wang et al., 2019).

Gut microbiota can ferment and decompose prebiotics, producing large amounts of short-chain fatty acids (SCFA) (den Besten et al., 2013), including acetate, propionate, butyrate, and some others. These microbial metabolites have many beneficial effects on host health,

such as lowering pH to control sensitive pathogens, increasing mineral absorption, and promoting intestinal motility (den Besten et al., 2013; Takagi et al., 2016). Propionate can limit lipogenesis and lower the cholesterol (Hosseini, Grootaert, Verstraete and Van de Wiele, 2011). Butyrate is a major energy source for intestinal epithelial cells, and it also regulates intestinal cell growth and differentiation of intestinal cells, it also has strong anti-inflammatory activity, and achieves anticancer properties by stimulating apoptosis (Hamer et al., 2012; Zhang et al., 2018). Prebiotic intake helps restore normal gut microbiota (Lai et al., 2014), while inulin is one of the most recognized prebiotics (Pham et al., 2018). The complex polysaccharides resist the digestive process in the upper gastrointestinal tract and are eventually fermented by the gut microbiota to some extent (Roberfroid et al., 2010). As a polysaccharide, the ability of BC to act as a prebiotic can be explored.

In this study, the viscosity change of BC solution in a simulated digestion model (SGD) was determined and compared with a control group, namely a solution made of glucomannan, to explore potential of BC as a satiety food formulation. Subsequently, the potential of BC as a prebiotic was investigated by *in vitro* gut batch culture fermentation. Bacterial population was enumerated with Fluorescent in situ hybridization (FISH) and HPLC was used to analyse the production of SCFAs. The chemical composition and crystallinity of BC substrates were analysed using FTIR and XRD.

5.2 Materials and methods

5.2.1 Materials

Bacteriological agar from Oxoid Ltd (stock keeping unit [SKU]: LP0013T); D-(–)-Fructose ≥ 99% from Sigma-Aldrich (SKU: F0127); Peptone from casein; enzymatic digest from Sigma-Aldrich (SKU: 82303); Yeast extract from Fisher Bioreagents (SKU: BP1422-500); Sodium phosphate dibasic ≥ 99% from Sigma-Aldrich (SKU: 795410); Citric acid monohydrate ≥ 99.5% from Fisher Chemical (SKU: CI6160I53); Sodium hydroxide from Fisher Chemical (SKU: SI4920I53); Glucomannan (Konjac; High Viscosity) from Megazyme (Product Code: P-GLCMH); Glucomannan (Konjac; Low Viscosity) from Megazyme (Product Code: P-GLCML); α-Amylase from Bacillus licheniformis from Sigma-Aldrich (SKU: A4551-100MG); Pepsin from porcine gastric mucosa from Sigma-Aldrich (SKU: P7000-100G); Pancreatin from porcine pancreas from Sigma-Aldrich (SKU: P1750-25G); Bile extract porcine Sigma-Aldrich (SKU: B8631-100G); peptone water from Oxoid Ltd (CM0009); yeast extract from Oxoid Ltd (LP0021); NaCl FROM Scientific Laboratory Supplies; K_2HPO_4 from Sigma-Aldrich (P8281); KH_2PO_4 from Sigma-Aldrich (P5655); $NaHCO_3$ from Sigma-Aldrich (S5761); $MgSO_4 \cdot 7H_2O$ from Fisher Chemical (M/1050/53); $CaCl_2 \cdot 6H_2O$ from Sigma-Aldrich (21108); tween 80, hemin, vitamin K_1, L-cysteine, bile salts, resazurin were obtained from Oxoid Ltd; inulin (Orafti1 ST, Beneo, Tienen, Belgium); paraformaldehyde solution (PFA solution) from Sigma-Aldrich (P6148)

5.2.2 Preparation of Bacterial cellulose (BC)

The bacterial strain *Acetobacter xylinum subsp. sucrofermentans* DSM 15973 was

cultivated under static conditions at 30°C in modified H &S medium, containing fructose (30 g/L), peptone (5 g/L), yeast extract (5 g/L), sodium phosphate dibasic (2.7 g/L), and citric acid (1.15 g/L). The pH of the cultivation culture was controlled between 6.0 and 6.2 by the addition of 6 M NaOH solution. After 200 h of cultivation, collected BC was immersed into 0.25 M NaOH solution at 25°C for 30 min and then washed to neutral pH. The purified BC samples were freeze dried (VirTis Scientific sentry 2.0) and stored in the freezer at -20°C before further analysis.

5.2.3 *In vitro* Simulated Gastrointestinal Digestion (SGD) of BC

5.2.3.1 Preparation of BC and glucomannan substrates

Freeze dried BC was grinded to powder and sieved through an 850-micron sieve to ensure that all sample powders have a similar particle size distribution. Samples of bacterial cellulose powder (BC), low-viscosity glucomannan (LG) and high-viscosity glucomannan (HG), were mixed with 500 ml distilled water to prepare 0.5% and 1.0% (w/v) substrates, stirred by a magnetic stirrer, at 37°C. In the case of BC samples, they were additionally homogenized (1.6 x 10^4 rpm for 3) min using an Ultra Turrax Homogenizer (Ika, model) before stirring.

5.2.3.2 *In vitro* Simulated Gastrointestinal Digestion (SGD)

Substrates containing 0.5% and 1.0 % (w/v) of either BC or glucomannan were mixed with 0.52 mL of 0.32 % α-amylase: $CaCl_2$ (0.001mol L^{-1}, pH 7) (w/v) in a glass screw topped Duran bottle, incubated at 30°C in an orbital shaker for 30 min to mimic the

process of digestion in the mouth. Then the pH of the mixture was dropped to 2 with 6 mol/L HCl, 2.085 ml of 10.8% pepsin: HCl (0.1mol L^{-1}) (w/v) was added, and incubation continued at 30°C for 2 h in an orbital shaker (model) to simulate the digestion phase of stomach. Subsequently, the pH was corrected to 7.0 with 6 mol/L HCl and 6 mol/L NaOH. 11.7 mg pancreatin and 0.2925g bile dissolved in 10.4 mL sodium bicarbonate ($NaHCO_3$, 0.5mol L^{-1}) were added to the mixture, and incubation was carried out at 30°C for 3 h to mimic the digestion process carried out in the small intestine (Mills et al., 2008).

5.2.3.3 Viscosity determination

A rotational viscometer (IKA ROTAVISC me-vi Complete) with a cylindrical spindle (IKA SP-1) was applied for the viscosity determination of original samples as well as those deriving from each digestion phase. Aliquots of each sample were transferred to a 600 mL beaker and a water bath was used to maintain the temperature at 30°C. The rotational speed was set at the range of 0.01 rpm to 200 rpm and the viscosity was recorded.

All samples and operations were two times repeated in order to minimum errors. After the SGD, samples using BC as substrate were freeze-dried and stored at -20°C freezer, until further analysis

5.2.4 Faecal batch culture fermentation

5.2.4.1 Faecal sample and preparation

Faecal samples were collected from three individuals (18-45 years of age). All volunteers were in good health, did not smoke and had not taken antibiotics for at least 6 months prior to the study. Faecal samples were collected on the day of the experiment and used immediately. Post collection, samples were diluted 1 to 10 (w/v) with anaerobic phosphate buffered saline (PBS; 0.1 M; pH 7.4) and homogenised in a stomacher at 230 rpm for 2 min, marked as the faecal slurry.

5.2.4.2 Faecal batch culture fermentations

Normal size batch culture fermentation vessels (300 mL) were autoclaved and aseptically filled with 135 mL of basal nutrient medium, containing peptone water (2 g/L), yeast extract (2 g/L), NaCl (0.1 g/L), K_2HPO_4 (0.04 g/L), KH_2PO_4 (0.04 g/L), $NaHCO_3$ (2 g/L), $MgSO_4 \cdot 7H_2O$ (0.01 g/L), $CaCl_2 \cdot 6H_2O$ (0.01 g/L), tween 80 (2 mL/L), hemin (50 mg/L), vitamin K_1 (10 mL/L), L-cysteine (0.5 g/L), bile salts (0.5 g/L), resazurin (1 mg/L). To simulate the condition of the terminal region of the human large intestine, O_2-free N_2 was sparged overnight to establish anaerobic conditions, vessels were kept at 37 °C using a circulating water bath and pH was controlled between 6.7 and 6.9 using an automated pH controller (Fermac 260; Electrolab, UK). 15 mL of faecal slurry was inoculated into the vessels. Pre-digested BC powders (1.5 g), marked as BC 0.5 and BC 1, were added to the vessels as the treatment groups. Additionally, vessels with standard inulin (1.5 g) and without any substrate were used as positive control (PC) and negative control (NC), respectively. During 48 hours of fermentation, 5 mL of the samples were collected at 5 time points (0 h, 6 h, 24 h, 36 h, and 48 h). Fluorescent *in situ* hybridisation (FISH) was

applied for bacterial enumeration and HPLC was used to detect the concentration changes of organic acids.

5.2.4.3 Batch culture sample processing

For the preparation of FISH analysis, 375 μL batch culture sample were mixed with 1125 μL 4% (w/w) paraformaldehyde solution (4% PFA solution) at 4°C, for 4 hours. After that, the mixtures were centrifuged for 5 min at 11337 *x* g (Eppendorf centrifuge minispin, Eppendorf, UK) at room temperature (20˚C). The supernatant was carefully removed by a pipette while the solid residual was suspended twice in 1mL PBS solution, centrifuged at 11337 x g for 5 min and the resulting supernatants were discarded. Finally, the solid residuals were re-suspended in 150 μL PBS solution and 150 μL ethanol, vortexed and stored in the freezer at -20°C.

For the preparation of HPLC analysis, 1 mL of the batch culture sample was centrifuged at 1133 x g for 3 minutes. The supernatant was collected and stored at -20°C.

For the FT-IR and XRD analysis, batch culture samples were freeze dried and stored at 20°C.

5.2.4.4 Bacterial enumeration

Fluorescent *in situ* hybridisation (FISH) was used to enumerate the population of selected groups of bacterial. Specific regions of 16S rRNA were labelled with commercially

synthesized oligonucleotide probes, which are coated with the fluorescent dye Cy3, and then the samples were hybridized as described by Daims et al. (2005). Probes used were listed as follows: Non Eub for control probe complementary to EUB338 (Wallner et al., 1993); Eub338I for most Bacteria (Daims et al., 1999); Eub338II for *Planctomycetales* (Daims et al., 1999); Eub338III for *Verrucomicrobiales* (Daims et al., 1999); Bif164 for *Bifidobacterium* spp. (Bif) (Langendijk et al., 1995); Lab158 for *Lactobacillus* and *Enterococcus* (Lab) (Harmsen et al., 1999); Bac303 for most *Bacteroides genus - Prevotella*, and some *Porphyromonadaceae* (Bac) (Manz et al., 1996); Erec482 for most of the *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa and XIAb) (Erec) (Franks et al., 1998); Rrec584 for *Roseburia* genus (Rrec) (Walker et al., 2005); Ato291 for *Atopobium* cluster (Ato) (Harmsen et al., 1999); Prop853 for *Clostridium* cluster IX (Prop) (Walker et al., 2005); Fprau655 for *Feacalibacterium prausnitzii* and relatives (Fprau) (Hold et al., 2003); DSV687 for *Desulfovibrio* genus (DSV) (Devereux et al., 1992); Chis150 for Most of the *Clostridium histolyticum* group (*Clostridium* cluster I and II) (Chis) (Franks et al., 1998).EUB338, EUB338II and EUB338III were mixed together, named as EUB mix to determine the total bacterial (Total) (Daims et al., 1999). Hybridization and wash conditions for various probes are listed in Table 5.1.

Table 5.1 Hybridisation and washing conditions for oligonucleotide probes.

Probe Name	Sequence (5' to 3')	Hybridisation Pre-treatment	Formamide (%) in Hybridisation buffer	Hybridisation and Washing Temperature (°C)
Non Enb	ACTCCTSCGGGAGGCAGG	None	30	35-37
Eub 338I	GCTGCCTCCCGTAGGAGT	None	30	35-37
Eub 338II	GCAGCCACCCGTAGGTGT	None	30	35-37
Eub 338III	GCTGCCACCCGTAGGTGT	None	30	35-37
Bif 164	CATCCGGCATTACCACCC	Lysozyme	30	35-37
Lab 158	GGTATTAGCAYCTGTTTCCA	Lysozyme	30	35-37
Bac 303	CCAATGTGGGGGACCTT	Lysozyme	30	35-37
Erec 482	GCTTCTTAGTCARGTACCG	Lysozyme	30	35-37
Rrec 584	TCAGACTTGCCGYACCGC	None	30	35-37
Ato 291	GGTCGGTCTCTCAACCC	Lysozyme	30	35-37
Prop 853	ATTGCGTTAACTCCGGCAC	Lysozyme	30	35-37
Fprau 655	CGCCTACCTCTGCACTAC	Lysozyme	30	35-37
DSV 687	TACGGATTTCACTCCT	None	30	35-37
Chis 150	ACTCCTSCGGGAGGCAGC	Lysozyme	30	35-37

5.2.4.5 Short chain fatty acid analysis

Short chain fatty acids (SCFA) were determined by HPLC. Batch culture fermentation samples were filtered by 0.22µm filter and then analysed with a HPLC (Agilent Infinity 1260 series) equipment with an Aminex HPX-87H column (Bio-rad, Hercules, CA) and DAD and RID detectors in series. The mobile phase was 5 mM H_2SO4 at 0.6 mL/min flow rate. The temperature of the column was set at 65 °C. External calibration curves of known standards were used to quantify the detected metabolites.

5.2.5. Bacterial cellulose Characterisation

To understand the chemical composition and crystal structure of BC treated by SGD and gut batch culture fermentation, FT-IR and XRD were applied. Untreated BC was labelled as BCA, SGD-treated sample was labelled as BCB, and the BC sample after both SDG

treatment and gut batch culture fermentation was labelled as BCC.

5.2.5.1 FT-IR

The infrared spectrum was obtained using a Perkin Elmer precisely spectrum 100 FT-IR Spectrometer in the region of 4000-650 cm^{-1} and with a resolution of 4 cm^{-1}. The result for each sample was the average of 32 scans.

5.2.5.2 X-RD

The degree of crystallinity was determined using a Bruker D8 Advance powder diffractometer with a copper source (wavelength 1.54 angstroms). The 2-theta angle ranged from 5.0 to 64.0848 degrees with 0.02106 mm increments for 1.2 seconds. The data was collected on a Lynxeye detector. The aperture slit was 6 mm, and the detector was equipped with a Nichol attenuator.

5.2.6 Statistical analysis

Mean values and standard deviations were calculated using Microsoft Excel Office 365. Data from FISH and HPLC were analysed with IBM SPSS Statistics 25. Changes in specific bacterial groups and SCFA production were assessed using a one-way analysis of variance (ANOVA).

5.3 Results and discussion

5.3.1 Viscosity changes of glucomannan and BC solutions during *in-vitro* upper GI

digestion simulation

The viscosity properties of ingested foods may also have ability to modulate digestion in the gastrointestinal tract and impart the feeling of satiety. As such, samples from each stage of the *in-vitro* upper gastrointestinal tract were collected and their viscosity was measured.

Viscosity curves of bacterial cellulose and glucomannan hydrocolloid solutions were obtained under different rotational speeds. The solutions exhibited a shear-thinning behaviour as the rotational speed increased (Annex 1). Bi et al. (2018) suggested that shear thinning properties are related to molecular rearrangements in the shear direction caused by molecular structure. In the low shear rate region, the shear force cannot cause significant changes in the molecular structure, because an inner-molecular forces was provided to resist the change of the molecular structure by external forces. Therefore, the viscosity of the sample was higher at lower shear rates. As the shear rate increased, the inner-molecular force was insufficient to resist the external force, eventually causing the intact molecule to be unfolded, which greatly reduces the shear resistance behaviour of the molecule, leaded the decrease of the apparent viscosity (Drummond et al., 2004).

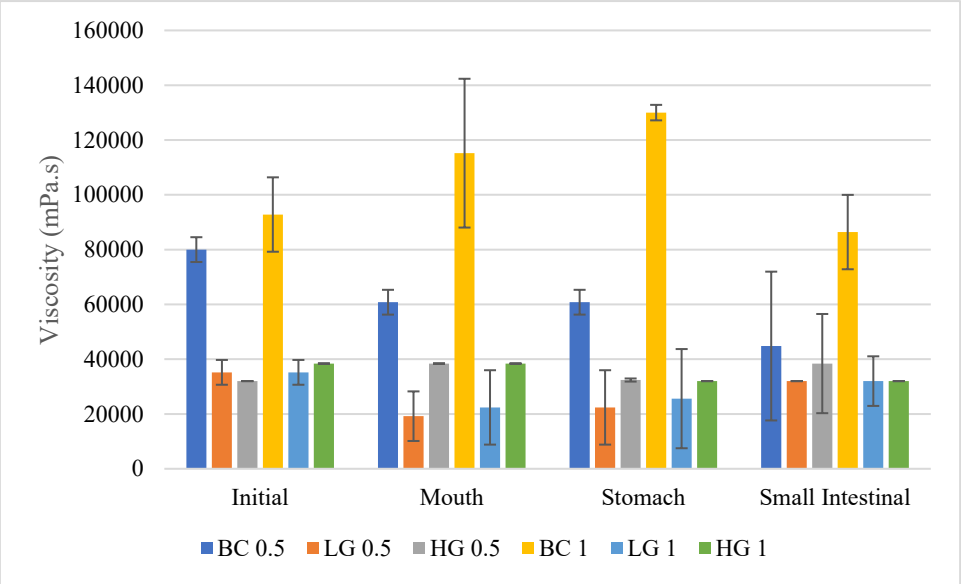

Figure. 5.1: Viscosities of samples after each stage of the in-vitro upper gastrointestinal simulation at the rotational speed of 0.01 rpm. BC 0.5: 0.5% w/v Bacterial cellulose substrate. BC1: 1% w/v Bacterial cellulose substrate. LG0.5: 0.5% low viscosity glucomannan substrate. LG1: 1% low viscosity glucomannan substrate. HG0.5: 0.5% high viscosity glucomannan substrate. HG1: 1% high viscosity glucomannan substrate

Since the viscosities of all samples at high speeds were almost the same, a comparison was not possible; therefore, the viscosity values obtained at low speeds (0.01 rpm) were chosen for comparison. Based on Figure 5.1, it was noted that the viscosity of some of the hydrocolloid solutions changed slightly through each stage of the SGD. Specifically, the viscosity of the 0.5% BC hydrocolloid solution was highest at the start of the in-vitro simulation, decreased after the oral phase and remained relatively stable after the gastric phase, and was further decreased during the small intestine phase. The 0.5% LG hydrocolloid solution exhibited a different trend, decreasing in the oral stage and then gradually increased in the next two stages. Progressive dilution with the various digestive juices might be a factor in this phenomenon as a decrease in the substrate concentration

is known to cause the reduction of the hydrocolloid solution viscosity (Mao and McClements, 2012). On the other hand, the presence of biopolymers (e.g., mucin in the mouth) or increased aggregation (e.g., in the stomach) can counteract the dilution effect caused by the addition of digestive juices, and the viscosity of the sample increases instead (Mao and McClements, 2012). This is consistent with the results of this experiment.

With regards to BC samples with 1.0 % (w/v) concentration, their viscosities were higher than those of 0.5% BC hydrocolloid solution in all stages in SGD; on the other hand, low- and high- viscosity glucomannans had similar viscosities in all stages (Figure 5.1). BC fibrils are long and have a high aspect ratio. In the BC hydrocolloid solutions, such structures can more easily maintain their elongated shape and contact each other, ultimately combined into long and stacked fibrils with higher aspect ratios (Vinogradov et al., 2020). In other words, the rheological behaviour of BC hydrocolloid solution was dictated by the fine geometry of the highly ordered strips. Ma et al. (2020) compared the effect of glucomannan concentration on the viscosity, pointing out that the viscosity of glucomannan solution increased significantly with increasing glucomannan concentration. However, this is inconsistent with the data from this trial. The differences of glucomannan sources and types, as well as the viscosity determination methods may make these studies difficult to compared.

Shang et al. (2020) used 3D printed human stomachs to study the effect of breakfast

viscosity on gastric emptying rate, demonstrating that the retention time of high viscosity glucomannan samples in the stomach was almost twice that of samples without glucomannan added. This is due to the high flow resistance caused by high viscosity samples, which may work against the mechanical forces exerted by the gastric lumen when gastroduodenal pressure and mechanical forces pass through the pylorus (Wu et al., 2017). Some consumer tests have shown that sticky meals improve subjective satiety and reduce appetite for food (Shang et al., 2020). Research by Zhu (2013) also showed that by reducing food intake and gastric emptying rates, participants' satiety increased, and hunger was suppressed after eating a high-viscosity meal. There were also clinical trial data demonstrating that beverages with alginate significantly reduced hunger responses, and a clear dose response was observed (Peters et al., 2011). In this experiment, the viscosity of BC hydrocolloid solution was generally higher than that of LG and LG, demonstrating the ability of BC to delay gastric emptying and increase satiety.

FTIR was used to identify functional groups in BC samples to assess the effect of upper GI simulation on the structure of bacterial cellulose (Figure 5.2.)

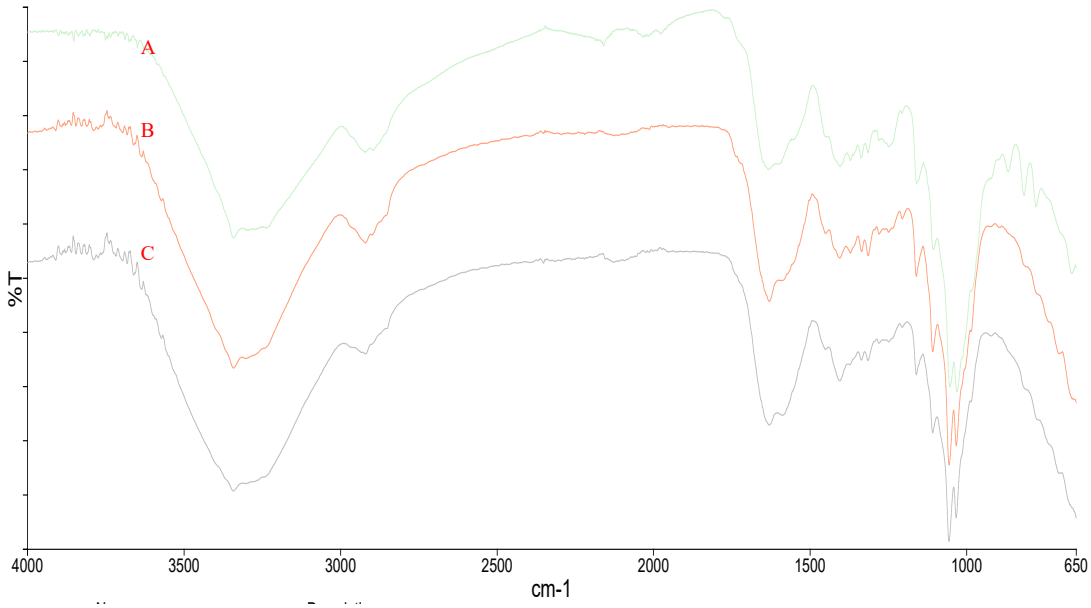

Figure 5.2: FTI-R spectra of BC samples: (A) original BC sample (prior to digestion); (B) 0.5% BC sample after upper GI digestion; (C) 1.0 % BC sample after upper GI digestion.

All diffraction curves were typical BC structures. For example, a typical absorption peak appears around 3300 cm^{-1}, which corresponds to the stretching vibration of O-H of Cellulose I. For the weak peak around 2900 cm^{-1}, the C-H stretching groups of CH_2 and CH_3, whereas the peak detected around 1650 cm^{-1} corresponds to the H-O-H bending, which comes from the water absorbed by the BC. The peaks around 1360 cm^{-1}, 1280 cm^{-1} and 1204 cm^{-1} are caused by C-H bending. Since β-glucan (1→4) linked linear nanofibrils is the basic structure of the bacterial cellulose (Shi et al., 2014), the C-O-C antisymmetric bridge stretching of (1, 4)-β-D-glucoside appearing near 1160 cm^{-1} is regarded as a basic indicator of the presence of BC in the analysed sample (Wang et al., 2017). These results suggest that the structure of BC did not change during *in-vitro* simulation of the upper GI digestion.

5.3.2 *In vitro* faecal batch fermentations of pre-digested BC samples

5.3.2.1 Enumeration of bacterial populations by FISH

BC samples deriving from the simulation of the upper gastrointestinal tract, were subsequently subjected to *in vitro* faecal batch cultures, in order to evaluate their digestion behaviour in the colon.

The effect of pre-digested BCs on the bacterial population that constitutes the human gut microbiota was assessed by FISH. Positive control (inulin) and negative control (basal medium) were also included for comparison purposes. Changes in bacterial populations during faecal batch cultures are shown in Figure 5.3.

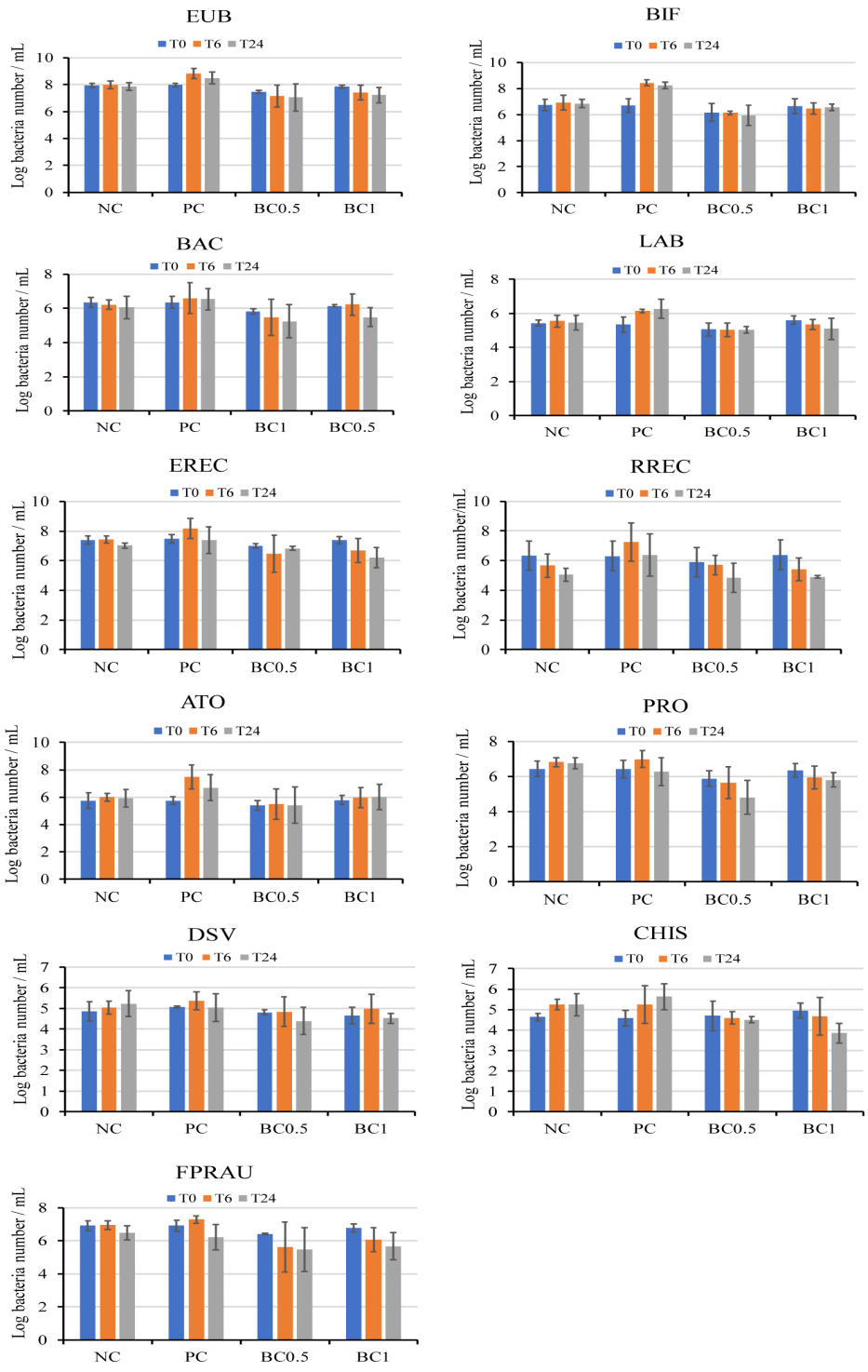

Figure 5.3: Influence of Inulin, BC0.5 and BC1 on the gut microflora in a pH-controlled batch culture. Samples collected at 0, 6 and 24 hours were labelled as T0, T6 and T24

Compared with the negative control group, inulin significantly stimulated the growth of

total bacteria during the fermentation process (P<0.05) as expected, whereas *Lactobacillus/Enterococcus*, *Atopobium* cluster and *Bifidobacterium* spp. were also significantly stimulated (P<0.05). In cultures with 0.5 % (w/v) and 1.0% (w/v) pre-digested BC, the growth of *Bifidobacterium* spp. and *Clostridium* cluster IX was inhibited (P<0.05), compared with the negative control, indicating no apparent prebiotic potential for BC.

5.3.2.2 SCFA analysis

Total SCFA concentrations during faecal batch culture fermentations are shown in Figure 5.4 A.

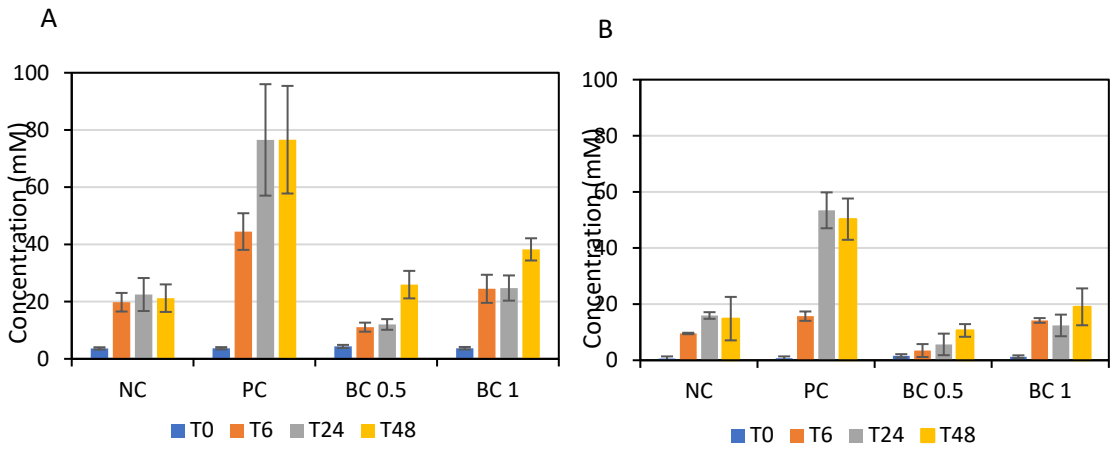

Figure 5.4: (A) Total SCFA and (B) acetic acid production, in faecal batch fermentations over the course of time. NC: Negative control (Basal media); PC: Positive control (Inulin); BC 0.5: Bacterial cellulose as substrate at 0.5% (w/v); BC 1: Bacterial cellulose as substrate at 1.0% (w/w)

Total SCFAs peaked at 24 h, with the positive control (inulin) exhibiting the higest accumulation (~80 mM). Cultures with bacterial cellulose (0.5% and 1.0%, w/v) showed

a slower fermentation rate, and SCFA peaked at 48 h, remaining in any case lower than that of the positive control and in similar levels to the negative control. Acetate was predominantly produced among all SCFA (Figure 5.5 B), whereas lower concentrations of lactate (intermediatte metabolite), butyrate and propionic acid were also seen (Figure 5.5).

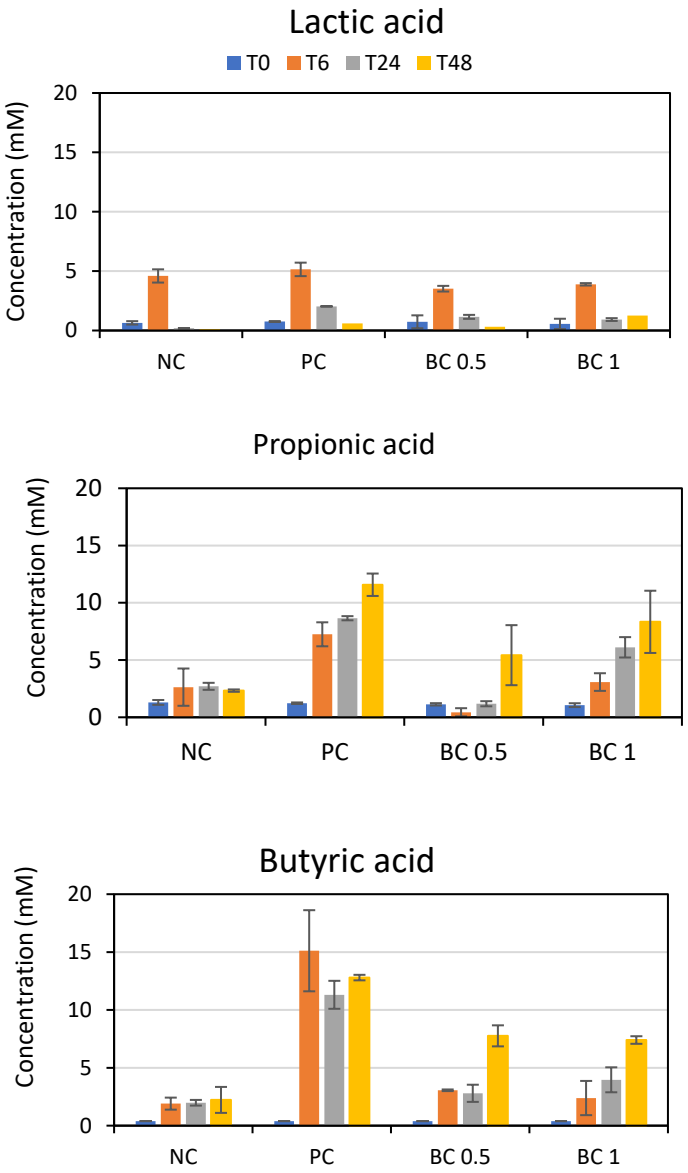

Figure 5.5: Evolution of lactic, propionic and butyric acids production, in faecal batch fermentations over the course of time. NC: Negative control (Basal media); PC: Positive control (Inulin); BC 0.5: Bacterial cellulose as substrate at 0.5% (w/v); BC 1: Bacterial cellulose as substrate at 1.0% (w/w)

It has been suggested that the intestinal microflora can be regarded as a human organ with bacterial cells, as they can help the host perform different functions (Vulevic et al., 2008). Specific microbial groups, such as *Bifidobacterium* and *Lactobacillus*, have been involved in beneficial effects on the host, which include the promotion of gut integrity, resistance against pathogens, and immunomodulation (Blum et al., 2002; Meydani and Ha, 2000; Gibson and Roberfroid, 1995). Inulin is found in many foods, such as wheat, onions, bananas, garlic and leeks (Van Loo et al., 1995), and it has been reported to have *in vivo* and *in vitro* microbial modifying abilities (Salminen et al., 1998; Vulevic et al., 2008). Inulin is a classical prebiotic oligosaccharide, it cannot be digested and absorbed in the upper gastrointestinal tract and passes through the large intestine in its original form (Zaporozhets et al., 2014). However, in the colon, inulin is mostly fermented by *Bifidobacterium* being saccharolytic bacteria and has a positive impact on human health (Slavin 2013). An increase in *Bifidobacterium* is considered a marker of intestinal health and many studies have highlighted their beneficial effects on the prevention of colorectal cancer, colon regularity and acute diarrhoea (Fehlbaum et al., 2018), *Bifidobacterium* can also exert immunomodulatory abilities and promote immune attack against malignant cells (Gibson and Roberfroid, 1995). Notably, short-chain structured inulin (oligofructose) would be more advantageous than long-chain inulin because many isolated *Bifidobacterium* are unable to utilize long-chain inulin (Chung et al., 2016; Rossi et al., 2005; Koutsos et al., 2017). Meanwhile, the population of *Lactobacillus/Enterococcus*, *Bacteroides genus – Prevotella* in PC vessels increased in response to inulin stimulation,

which were consistent with many *in vivo* and *in vitro* studies (Kleessen et al., 1997; Liu, Gibson and Walton, 2016)

After 6 h of fermentation, BC0.5 and BC1 did not significantly stimulate the growth of *Bifidobacterium* ($P = 0.052$; $P = 0.71$), and they also showed no significant different in the growth of *Lactobacillus/Enterococcus* from 0 h until 24 h. A study showed that lower degree of polymerization (DP) oligodextran was a better substrate for *Bifidobacterium* and *Lactobacillus/Enterococcus* (Olano-Martin et al., 2000). Alfilasari et al. (2021) share the same view that short-chain oligosaccharides were more bifidogenic than resistant starch, which has a more complex structure and is more slowly degraded by the gut microbiota. Biosynthetic BC has a DP of up to 14,000 (Tahara et al., 1997), while inulin has a DP in the range of 3 to 60 (van de Wiele et al., 2007), which may also explain the reason why pre-digested BC seemed to be more slowly fermented. After 24 h of fermentation, BC0.5 and BC1 still did not stimulate *Lactobacillus/Enterococcus* and *Bifidobacterium* significantly, but they significantly limited the growth of *Clostridium cluster* IX, reducing their numbers from 5.88 $\log^{10}$ cells/mL to 4.81 $\log^{10}$ cells/mL and from 6.34 $\log^{10}$ cells/mL to 5.81 $\log^{10}$ cells/mL, respectively, compared to 0 h.

SCFAs are mainly produced through the saccharolytic fermentation of carbohydrate and have been shown to make a significant contribution to host health (Bajury et al., 2017). In most cases, SCFAs in the colon are mainly acetate, propionate and butyrate. They can achieve alterations in gut microbiology and growth inhibition of pH-sensitive pathogens,

increase mineral absorption and affect intestinal motility by lowering pH in the gut (den Besten et al., 2013). It has been demonstrated that the fermentation of prebiotic substrates leads to increased production of SCFAs (Poeker et al., 2018). In all vessels (except the negative control one), the concentration of lactate increased during the first 6 hours and then decreased. This is because lactate may be further metabolised by some cross-feeding bacteria to acetate, propionate and butyrate, such as *Bifidobacterium* and *Bacteroides* (Bendiks et al., 2020; Alfilasari, Sirivongpaisal and Wichienchot, 2021). Besides, lactate does not accumulate in healthy individuals, except under some conditions of intestinal disease, such as lactate concentrations that can be as high as 100 mM in patients with ulcerative colitis (Vernia et al., 1988; Sarbini et al., 2011; Bajury et al., 2017).

Acetate is the most abundant SCFA produced by colonic microbiota (Connolly, Lovegrove and Tuohy, 2010), and the highest acetate concentration is obtained after 24 hours of fermentation using inulin as a supplement. Normally, *Bifidobacterium* break down carbohydrates, producing strong acids (acetate and lactate) as the end product of metabolism and achieving a lowering of luminal pH (Bajury et al., 2017). It is reported that the production of acetic and propionic acids by intestinal bacteria can have the opposite effect on hyperlipidaemia (Connolly, Lovegrove and Tuohy, 2010). Some animal studies have shown that produced propionate in the colon inhibits cholesterol synthesis in the colon and liver (Demigné et al., 1995; Wolever, Spadafora and Eshuis, 1991; Alvaro et al., 2008) Propionic acid accumulation was associated with several bacterial groups, including the *Clostridium histolyticum* group and *Bacteroides genus - Prevotella* (Pompei

et al., 2008). In this experiment, the propionic acid concentrations of PC and NC reached the maximum after 6 h of fermentation, at which point the propionic acid concentrations were PC > BC1 > NC > BC0.5. Continuing into the 24th hour of fermentation, BC1 had the highest propionic acid concentration, followed by BC0.5, and NC had the lowest propionic acid concentration. However, in the present study, no corresponding increase in the number of *Bacteroides genus - Prevotella* occurred, and the number of *Clostridium histolyticum* group in BC1 at 24 h even showed a significant decrease compared to 0 h. This suggests that the increase in propionate may be due to cross-feeding between acetate/lactate producers such as *Bifidobacterium* (Walker et al., 2005). Butyrate has received much attention not only because it is the main source of energy for colon cells (Alfilasari, Sirivongpaisal and Wichienchot, 2021), but also because of its antitumour properties (Cummings, 1984; Forest et al., 2003). *Feacalibacterium prausnitzii* and *E. rectale/Roseburia spp.* are the two most prominent butyrate producers (Zhang et al., 2018), but butyrate accumulation is extremely sensitive to the dietary structure of volunteers, a high protein and low carbohydrate diet leads to a reduction in the number of *E. rectale/Roseburia spp.* and a decrease in the concentration of butyrate in faeces (Flint et al., 2012). The addition of inulin, BC0.5 and BC1 did not significantly ($P>0.05$) affect the change in numbers of *Feacalibacterium prausnitzii* and *E. rectale/Roseburia spp.*, with a consistent downward trend in bacterial population. However, the concentration of butyric acid in the PC reached a maximum at 6h, the subsequent decrease in concentration may be due to the absorption of butyric acid as an energy source by the small intestine (Pryde et al., 2002).

With regards to the structure of BC samples following faecal batch fermentations, FT-IR results revealed that the polymer was digested to a large extent by gut microbiota (Figure 5.6).

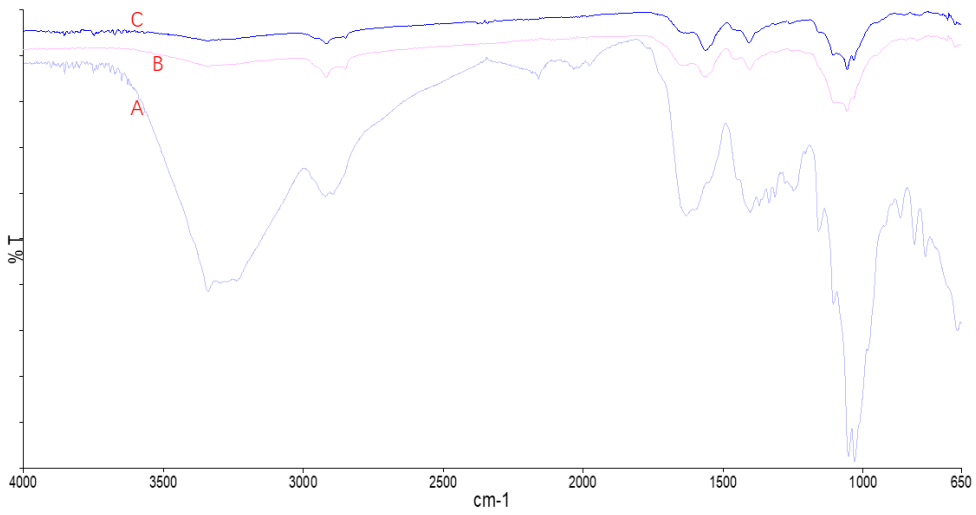

Figure 5.6: FTI-R spectra of BC samples: (A) original BC sample (prior to digestion); (B) 0.5% BC sample after faecal batch fermentation; (C) 1.0 % BC sample after faecal batch fermentation

Compared with the original BC sample (Fig. 5.6 A), the characteristic Cellulose I and II peaks of 0.5% and 1.0% (w/v) samples decreased significantly. Cellulose I was hydrolysed, and the FT-IR patterns resembled to that of BC treated with cellulase (Auta et al., 2017). This observation was also confirmed by the X-RD patterns of BC after upper GI simulation and faecal batch culture fermentation respectively (Figure 5.7A-B and C-D).

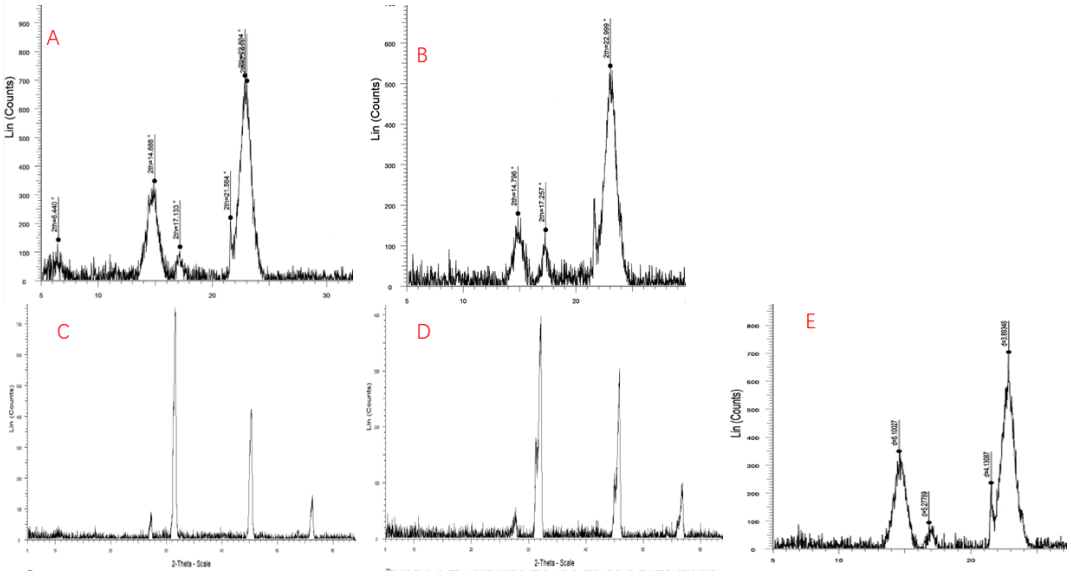

Figure 5.7: XRD patterns of: (A): 0.5% BC after upper GI digestion; (B): 1% BC after upper GI digestion ; (C): 0.5% BC after faecal batch culture fermentation; (D): 1% BC after faecal batch culture fermentation; (E): original BC (not digested)

Specifically, in the pattern of original BC sample (Fig. 5.7E), three peaks can be found around 2θ=14.6, 16.5 and 22.8, which are related to the crystal plane of (1$\bar{1}$ 0), (1 1 0) and (2 0 0), respectively, corresponding to typical cellulose I structures (Castro et al., 2011; Dórame-Miranda et al., 2019). There was also a small but sharp peak at 23.9, which is close to the characteristic peak of cellulose II (Kafle, Greeson, Lee and Kim, 2014). The absorption peaks at the same position were also found in the patterns of BCB 0.5% and BCB 1%, after upper GI digestion, indicating that the BC structure retained at the end of upper GI digestion simulation. However, BC samples collected after faecal batch cultures demonstrated absence of Cellulose I and II characteristic peaks and exhibited degradation

peaks only (2θ=27, 31, 45 and 56). This was also further confirmed by the crystallinity index of the samples: the original BC sample exhibited an index of 52% and both BC samples after upper GI simulation had an index of ~45%. However, the same samples after faecal batch cultures had a crystallinity index of only 28%, indicating the degradation of the polymer by gut microbiota.

5.4 Conclusion

The evaluation of BC during simulation of the GI digestion revealed that the polymer can retain its structure during upper GI digestion and possibly providing a satiety effect, due to its high-water holding capacity properties and the conferred changes in the viscosity of the stomach simulation. However, it was shown that BC is degradable in the colon, without stimulating beneficial bacteria (nor pathogenic bacteria); this study showed that BC does not hold any prebiotic potential. The relatively show production of SCFA could denote that BC is slowly fermented in the gut, thus it is likely to confer desired benefits to the host, such as bowel movement, which is also linked with overall gut health.

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Chapter 6: General Discussion and Future Recommendations

Bacterial cellulose (BC) is a biopolymer produced predominantly by acetic acid–producing bacteria and has received great attention in the last two decades due to its impressive physical, mechanical, and biological features. Its chemical structure is identical to plant cellulose; however, BC possesses superior properties than the plant cellulose in terms of purity, crystallinity, biocompatibility, and mouldability.e.

The main aim of Chapter 3 was to screen *Acetobacter xylinum subsp. sucrofermentans* DSM 15973 for its BC-producing properties, on a variety of simple sugars and assess the effect (if any) of the carbon source on the structure of BC. It was found that fructose was the best-performing substrate for BC production, whereas glucose did not promote polymer production. Although glucose and fructose are isomers, and in most cases their catabolic pathway within the cell is the same, it has been reported that that the activities of the enzymes involved in metabolism and BC synthesis differ depending on the bacterial species and carbon source (Tonouchi et al., 1996; Ross et al., 1991). In studies investigating the enzymatic activity of different carbon sources catabolism by *A. xylinum*, it has been showed that phosphoglucose isomerase (PGI) activity in cells in the presence of fructose was 132-fold higher than in cells grown in glucose-supplemented media (Tonouchi et al., 1996). This means that fructose can be efficiently converted into glucose-6-phosphate (G6P), which is involved in cell metabolism and BC synthesis, avoiding the formation of gluconic acid (which metabolically competes against BC synthesis), thereby

achieving increased biopolymer production compared to glucose-based media. This could provide the ground for the exploration of fructose-rich by-product streams that could be used as substrates for BC production. Given that the cost of substrates is usually around 30% of the total production costs, the use of fructose-rich wastewaters such as expired beverages, and fruit juices would represent sustainable carbon and fermentation water sources for BC production by *Acetobacter xylinum subsp. sucrofermentans* DSM 15973. Moreover, these wastewaters usually contain vitamins, such as ascorbic acid, which have been shown to act as inducers for BC production (Keshk, 2014).

In terms of carbon source impact on the structure of BC, it was found that BC produced in fructose-based media had lower crystallinity (~52%) as opposed to sucrose- and glucose-based media, that lead to BC polymers with high level of crystallization (>80%). BC polymers with low crystallinity are expected to exhibit reduced tensile strength, dimensional stability, and density, but at the same time increased chemical reactivity and swelling (Lupaşcu et al., 2022). The latter could render fructose-based BC ideal for applications as epidermal membrane in wound healing and also as drug delivery material, whereby drug release rates are linked with biopolymer swelling properties (Ullah et al., 2016).

Another aspect investigated in Chapter 3 was the effect of aeration, mainly delivered by agitated and static cultures, and it was seen that the bacterial strain performed better in static cultures, especially in glucose- and sucrose-based media. Although widely adopted

in BC production, static cultures have certain limitations, mainly because of their extensive cultivation time and their usually low productivity. In addition, bacteria exposed to unequal conditions during cultivation (both in terms of nutrients, oxygen, and population distribution) produce BC layers that may exhibit uneven thickness. To date, both static and agitated fermentations have been successfully used for the industrial production of BC; the choice between the methods for BC production depends on the application scenarios as the morphologies and properties of BC obtained by the two methods are very different. Static fermentations lead to the production of a gelatinous pellicle that is formed at the air-liquid interface of the culture media, whereas agitated fermentations end up with the production of small irregular pellets, fully suspended in the culture media, observations that were also seen in the current study. Worth also mentioning is the fact that BC obtained by agitated cultures has a lower degree of polymerization and a lower crystallinity in comparison with that produced in static cultures; however, in both scenarios, the same microstructure of 3D reticulated network is maintained. As such, static fermentation is preferred for the production of BC requiring fixed geometries and high-water holding capacity, such as nata de coco, wound dressing, and face mask. On the other hand, BC produced by agitated fermentation represents superior suspending stability, which is predominately used for particulate suspension in beverages (Hussain et al., 2019).

In Chapter 4, different purification strategies were investigated for BC polymers, employing alkali and sodium hypochlorite solutions, in various combinations. Generally,

the downstream processing involves the separation of the BC produced from the culture medium and the purification of the biopolymer. The BC can be removed from the culture medium applying simple procedures (e.g., filtration or centrifugation), regardless of the cultivation system used. However, the recovered BC from the medium is not pure, since it contains some impurities such as cells and nutrients, thus it needs to be purified before subsequent application. It has been reported in literature that organic impurities deriving from the medium which are used in the culture process, as well as the rod-shaped *A. xylinum* cells, cover the pores of the BC surface, preventing contact between fibrils within the network. As a result, the number of hydrogen bonds that can form is dramatically reduced, which also leads to a reduction in the strength of the dried BC material (Gea et al., 2011). In the current thesis, it was shown that by using a two-step purification process, i.e., mild alkaline solution, followed by a NaOCl solution at mild temperatures (25°C), non-cellulose materials such as protein and nucleic acids derived from bacterial cells and the culture broth were removed from the polymer. NaOH is mainly responsible for entrapped cell lysis, whereas NaOCl serves as a bleaching agent, in removing impurities which are not removed with NaOH alone. An effective purification method, as the one described in the current study, results in better BC fibril interaction, increases the intrinsic hydrogen bonding within BC, while maintaining the Cellulose I polymorph, thus prevent the formation of cellulose II (the latter being associated with lower mechanical properties of the biopolymer). Moreover, the application of strong alkali solutions (1.0 M), and especially at elevated temperature (40°C) led to structural transformation and damage of the BC, indicating the overall sensitivity of the polymer to strong alkali penetration and

polymorphic transformation from cellulose I to cellulose II, and eventually to disruption of the fibril network. Worth noting is that the downstream processing for BC is usually easier and cheaper when compared to the procedures required to purify plant-derived cellulose. Nevertheless, the choice of BC downstream processing can impact the final characteristics, and therefore should be selected based on its desired application.

Finally, in Chapter 5, BC behaviour was evaluated during simulation of gastrointestinal digestion. BC is considered a dietary fibre, approved as a "generally recognized as safe" (GRAS) food by the US Food and Drug Administration (FDA). Even though BC has been long consumed within Asian culinary cultures, there is scarce information in the international literature with regards to its fate upon consumption and its impact in the gut. Moreover, the water-holding capacity of BC has led to the hypothesis that it could be used as food ingredient in formulations that increase satiety upon consumption, this becomes a useful material in tackling non-communal diseases such as obesity. Initial trials investigating the behaviour of purified, dehydrated BC samples in the upper gastrointestinal tract showed that BC remained largely intact structurally, whereas it also increased the viscosity of the gastric solution. This could indicate that BC could be used as a satiety-inducing agent. low-calorie / low cholesterol. The latter would be an added benefit to existing evidence that BC can be successfully incorporated to low-calorie/ low-cholesterol food formulations (Zhijun et al., 2014). However, further work is required to establish the food model/ matrix that could deliver such properties upon digestion, as well as any possible ingredient interactions that might alter this effect.

BC samples deriving from the simulation of upper gastrointestinal digestion, were further submitted into faecal batch culture fermentations, in order to evaluate its indigestibility and prebiotic potential. It was shown that BC did not stimulate the growth of beneficial gut bacteria, such as Bifidobacteria, whereas XRD analysis on the BC substrates upon digestion completion revealed a reduced crystallinity and complete modification of the BC structure. This led to the conclusion that BC was partially fermented by microorganisms in the gut, which contradicts with literature arguments on BC being completely indigestible (although *in vitro* and *in vivo* studies are practically absent in the international literature). However, it is likely that BC could contribute to intestinal transit and bowel movement. It should be highlighted that no dose response effects were evaluated during faecal batch fermentations, therefore further investigation is required to establish how BC is fermented by gut microflora. First, the interaction between probiotics and BC needs to be investigated. Examples include conducting *in vitro* studies to assess the direct interaction between bacterial cellulose and probiotic strains such as Bifidobacteria, investigating the growth kinetics, adhesion and survival of probiotic colonies in the presence of bacterial cellulose; Investigate the potential synergistic effects of BC in combination with prebiotics or other dietary components on probiotic growth and activity. Animal *in vivo* simulations can then be used to assess the effect of BC on the composition and diversity of the gut microbiota. The mechanisms underlying the breakdown of BC crystal structures in the gut are also worthy of investigation, such as exploring the enzymatic activities involved in the degradation of BC by gut microbes and

identifying the specific microbial taxa and their enzymatic repertoires responsible for BC degradation. By carrying out these research activities, we can further elucidate the interaction between BC and gut microbiota. This research will provide valuable insights into the potential use of BC as a prebiotic or gut health-promoting ingredient, helping to develop evidence-based dietary recommendations and innovative foods that support human health.

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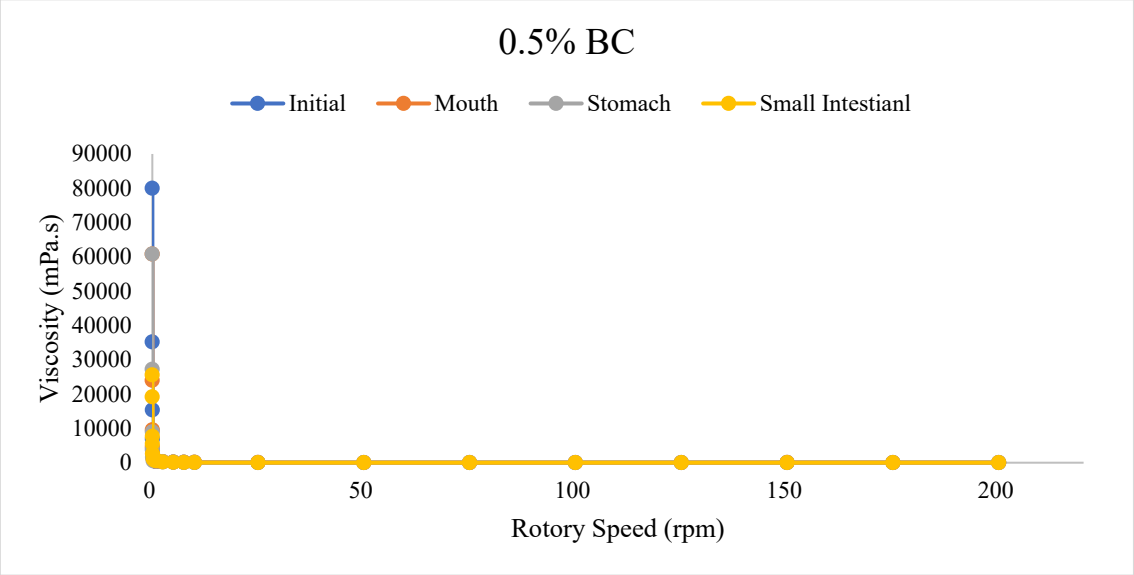
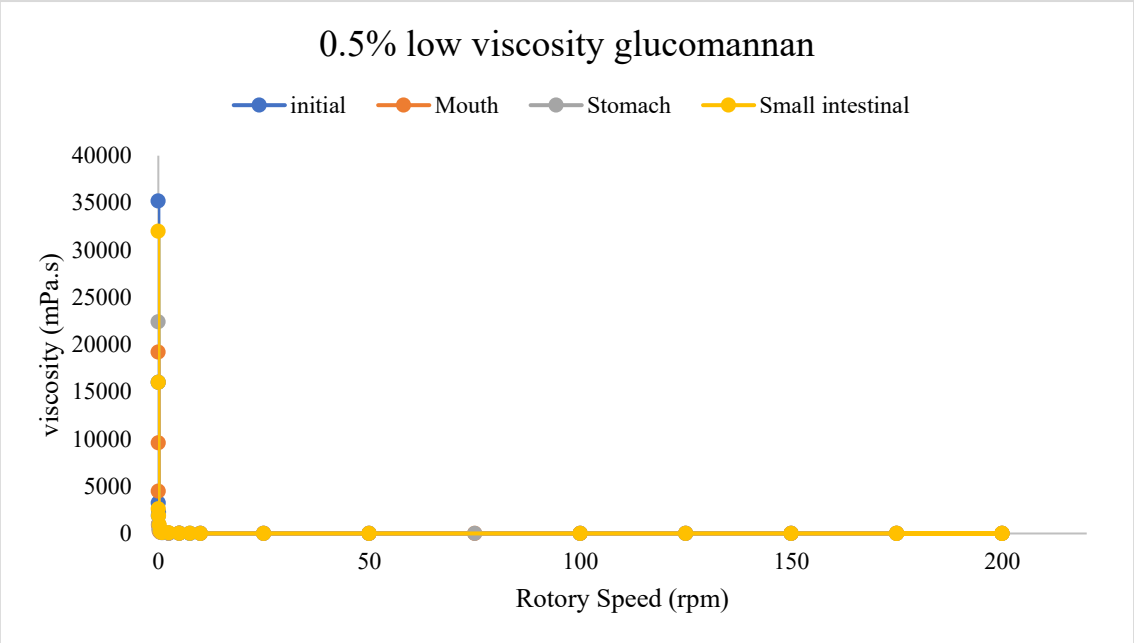
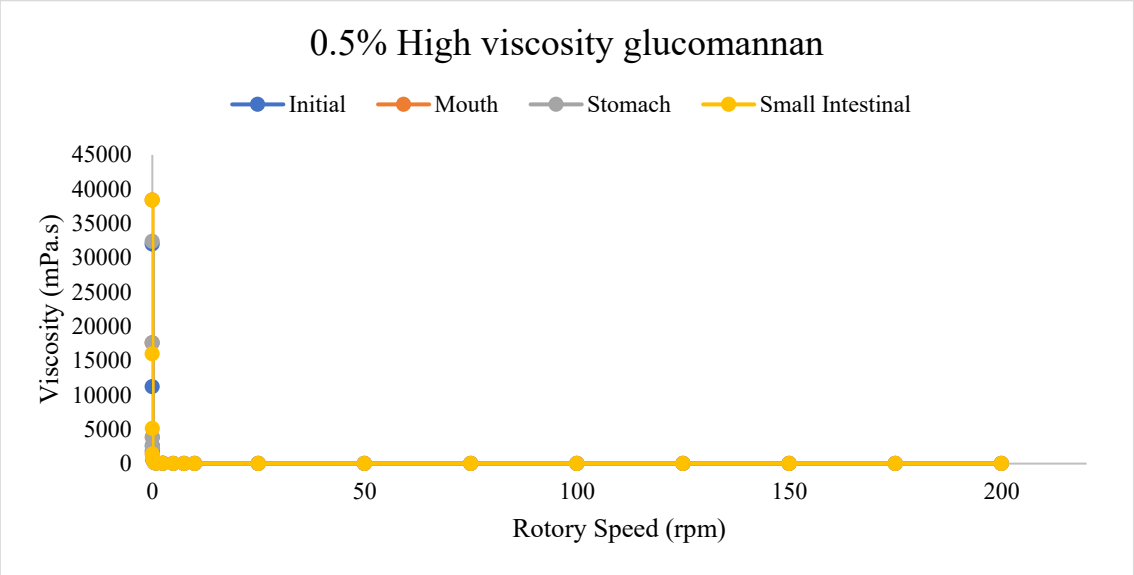
Appendix for Chapter 5:

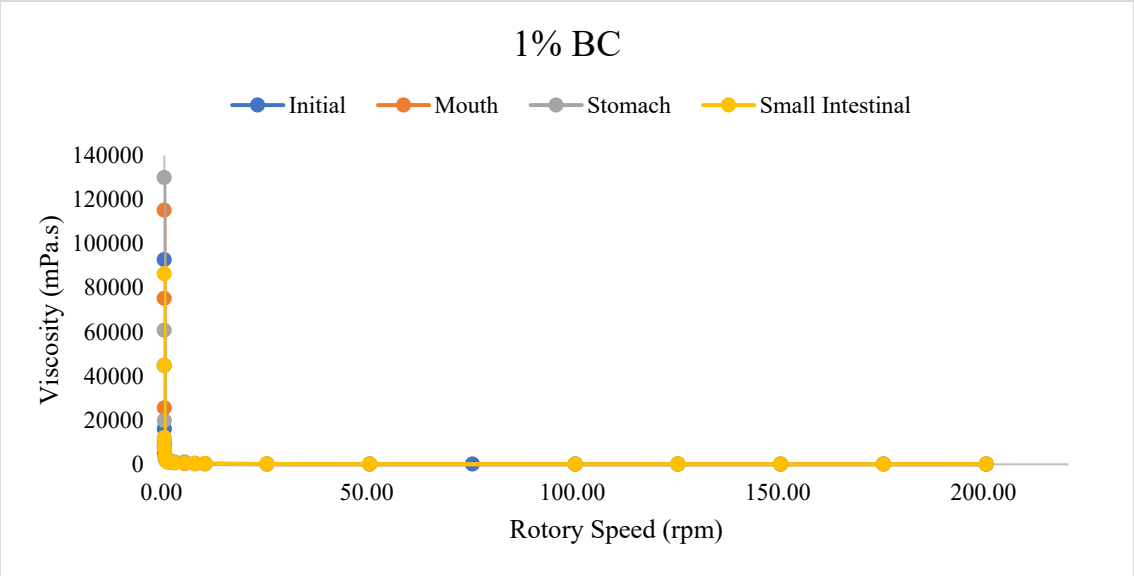
Viscosity versus rotational speed curves of samples at different stages in SGD.

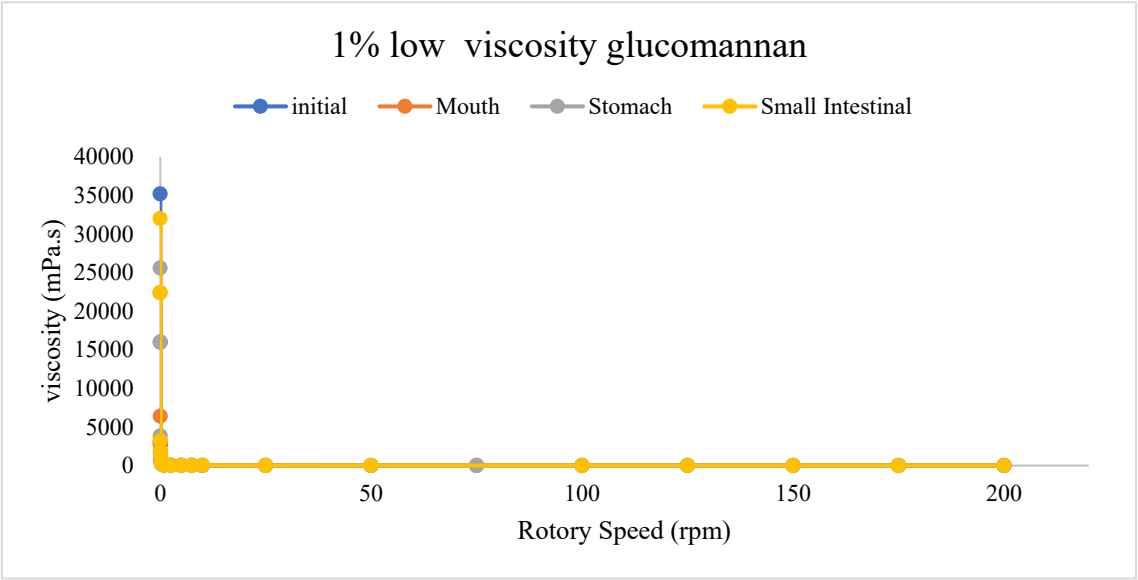


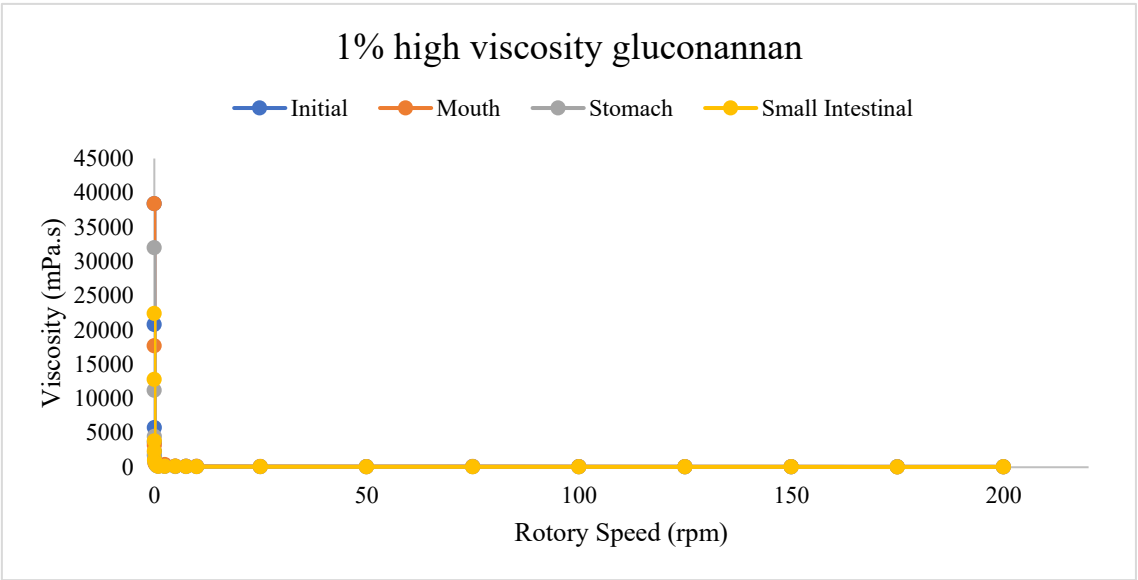




Viscosity versus rotational speed curves of samples at different stages in SGD.




1% low viscosity glucomannan
initial
Mouth
Stomach
Small Intestinal
viscosity (mPa.s)
40000
35000
30000
25000
20000
15000
10000
5000
0
0
50
100
150
200
Rotory Speed (rpm)


1% high viscosity gluconannan
Initial
Mouth
Stomach
Small Intestinal
Viscosity (mPa.s)
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
0
50
100
150
200
Rotory Speed (rpm)