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Impact of Fermentation Conditions and Purification Strategy on Bacterial Cellulose Properties

Choice of carbon source impacts crystallinity

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Bacterial cellulose (BC) has attracted much research interest, delivering a combination of exclusive properties, such as flexibility, hydrophilicity, crystallinity and a three-dimensional network. In this study, the effects of carbon source and cultivation conditions on BC production by the bacterium *Acetobacter xylinum* subsp. *sucrofermentans* DSM 15973 were assessed. Fructose was the most suitable carbon source and high BC concentrations up to 31 g l^{-1} were achieved in substrates with 60 g l^{-1} fructose under static culture conditions. Notably, BC production was equally high under the same fermentation conditions in agitated cultures (~30 g l^{-1}). Moreover, the effectiveness of sodium hydroxide and sodium hypochlorite solutions in BC purification and their potential impact on BC structure and properties were explored. The combination of weak NaOH and NaOCl proved an effective purification method, preserving the fibre structure and crystallinity of BC.

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 to microorganisms such as bacteria or algae (1–3). It is considered a renewable material and has the potential to replace fossil carbon in petroleum and coal in some applications (2). Additionally, cellulose has wide applications in the papermaking industry and as a reinforcing agent in polymers (4).

Some bacterial genera such as *Acetobacter* sp., *Rhizobium* sp., *Agrobacterium* sp., *Alcaligenes* sp., *Pseudomonas* sp. and *Sarcina* sp. can 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 synthesised cellulose as fibrils (3, 5). Among them, *Komagataeibacter xylinus* (also named *Gluconacetobacter xylinus* and *Acetobacter xylinum*) has been described as the most efficient BC producer (6, 7). 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 environmental conditions and to allow floating to the surface of growth media for bacteria to have better access to oxygen (8).

It has been previously reported in literature that the characteristics of BC 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 applications such

as skin tissue repair (9, 10) and artificial blood vessels (11). However, issues that constrain mass BC production in static cultures are associated with the need for a large working space, low BC productivity and increased labour costs, mainly due to prolonged cultivation time (12). To this end, improved culture systems have been suggested (for example, static cultures based on fed-batch strategy or horizontal lift bioreactors) aiming to increase BC productivity to a suitable level for commercial applications (13, 14). Compared with static cultures, smaller working space and less labour is required for agitated cultures, but the produced BC tends to get attached to the shaft of the reactor, rendering its collection difficult. Additionally, accumulation of non-BC-producing mutants have also been reported for agitated cultivation systems (15). Therefore, it is essential to evaluate the structural and chemical properties of BC in depth to assess the impact of the cultivation system and suggest possible BC applications.

Furthermore, the use of NaOH solution to remove contaminants from the cellulose network is the most used method, but it has been reported that high concentrations of NaOH 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 (16). However, the use of low concentrations of NaOH solutions seems to have a milder effect on the mechanical strength of BC (17). The oxidant NaOCl has also been used to achieve more efficient BC purification by oxidising 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 (18).

The aim of this study was to evaluate the ability *of A. xylinum* subsp. *sucrofermentans* to metabolise various carbon sources and their potential impact on BC production. To this end, various simple sugars (glucose, fructose, arabinose and xylose) were investigated as carbon sources for BC production. Additionally, purification strategies of BC were investigated. The aim was to assess their effectiveness as well as to provide insights on the impact on the BC fibre structure.

2. Materials and Methods

2.1 Materials

Bacteriological agar was purchased from Oxoid Ltd, UK (stock keeping unit (SKU): LP0013T); D-(+)-glucose ≥99.5% from Sigma-Aldrich, USA (SKU: G8270); D-(−)-fructose ≥99% from Sigma-Aldrich (SKU: F0127); L-(+)-arabinose from Sigma-Aldrich (A3256); peptone from casein, enzymatic digest from Sigma-Aldrich (SKU: 82303); yeast extract from Fisher BioReagents, USA (SKU: BP1422-500); sodium phosphate dibasic ≥99% from Sigma-Aldrich (SKU: 795410); citric acid monohydrate ≥99.5% from Fisher Chemical, USA (SKU: C/6160/53); NaOH from Fisher Chemical (SKU: S/4920/53).

2.2 Preparation of Bacterial Strain and Fermentation Media

The bacterial strain *A. 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 for 15 min) Hestrin-Schramm media (H&S agar) (7), containing agar (20 g l^{-1}), glucose (30 g l^{-1}), peptone (5 g l^{-1}), yeast extract (5 g l^{-1}), sodium phosphate dibasic (2.7 g l^{-1}) and citric acid (1.15 g l^{-1}). The strain was sub-cultured in fresh H&S agar every two weeks to preserve its viability.

H&S liquid media served as pre-culture medium, consisting of glucose (30 g l^{-1}), peptone (5 g l^{-1}), yeast extract (5 g l^{-1}), sodium phosphate dibasic (2.7 g l^{-1}) and citric acid (1.15 g l^{-1}), prepared and sterilised at 121°C for 15 min. 50 ml of the pre-culture medium was then inoculated with a full-loop of *A. xylinum* subsp. *sucrofermentans* DSM 15973 and incubated at 30°C, agitated at 180 rpm for 24 h (SciQuid Incu-Shake MIDI, UK). Pre-cultures were homogenised in a Stomacher® 400 circulator lab blender (Seward Ltd, UK) at 230 rpm for 2 min, and then 1 ml of the culture suspension was transferred into 250 ml Erlenmeyer flasks containing 50 ml sterilised media (121°C for 15 min) for BC production. The medium used was a modified H&S medium containing a single carbon source at two different concentrations, peptone (5 g l^{-1}), yeast extract (5 g l^{-1}), sodium phosphate dibasic (2.7 g l^{-1}) and citric acid (1.15 g l^{-1}). The carbon sources were glucose (30 g l^{-1} and 60 g l^{-1}), fructose (30 g l^{-1} and 60 g l^{-1}), arabinose (30 g l^{-1}) and xylose (30 g l^{-1}). All carbon sources were of analytical grade and procured from Sigma-Aldrich. For the glucose- and fructose-based media, two types of growth conditions were applied: static and agitated. All inoculated cultures were incubated at 30°C while

a speed control orbital shaker (IKA) was utilised to provide a rotatory speed of 180 rpm for the cultures under agitated conditions.

2.3 Analytical Methods

One flask (total volume 50 ml) was collected at different time intervals during BC production, serving as independent time point for each culture. The pH of the collected samples was measured by a calibrated pH meter (7Easy, Mettler-Toledo Ltd, UK) and then the pH of the main culture was manually and aseptically corrected between pH 6.0 and pH 6.2 by the addition of 6.0 M NaOH sterilised solution, if needed. Collected culture samples (50 ml) were homogenised in a Stomacher® 400 circulator lab blender (Seward Ltd, 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 Whatman® qualitative filter paper, Grade 1. The separated BC was washed twice with distilled water to remove any residual media and dried in an oven (UFP 500, Memmert, Germany) at 50°C for 24 h, until constant weight. The dried BC was placed in a Petri dish, sealed with parafilm and stored in a cabinet at room temperature until further analysis.

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

Residual carbon source concentration was determined by 3,5-dinitrosalicylic acid (DNS) method (19). Standard solutions of known concentrations of sugars were prepared and used to construct calibration curves and quantify sugar concentrations in culture samples.

2.4 Bacterial Cellulose Purification

Produced BC samples from fructose-based cultures were subjected into either alkali treatment (0.1 M NaOH, analytical grade, Sigma-Aldrich) for 30 min at 25°C (20) or a combination of alkali and NaOCl treatment (0.1 M NaOH solution at 25°C for 30 min, followed by immersion in NaOCl (analytical grade, Sigma-Aldrich) 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 (SciQuid Incu-Shake MIDI, UK) and the rotary speed was set as 180 rpm. After the treatment, samples were washed with distilled water, frozen at –20°C overnight and freeze dried (VirTis Scientific sentry 2.0) until further analysis.

2.5 X-Ray Diffractometry

The degree of crystallinity in BC samples was determined using a D8 Advance powder diffractometer with a copper source (Bruker, USA, wavelength 1.54 Å). The 2θ angle ranged from 5.0° to 64.0848° with 0.02106 mm increments for 1.2 s. The data was collected on a LYNXEYE detector (Bruker). The aperture slit was 6 mm, and the detector was equipped with a Nichol attenuator.

2.6 Fourier Transform Infrared Spectroscopy

Infrared spectra of BC samples were obtained using a Spectrum 100 FTIR spectrometer (PerkinElmer Inc, USA) 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.

2.7 Statistical Analysis

Statistical analysis was performed using Microsoft Excel (Microsoft Corporation, USA). Fermentations were performed in triplicates and data are expressed as mean value and standard deviation of three independent cultures. Analytical methods were also performed at least in triplicate and values are expressed as mean value and standard deviation of three independent measurements.

3. Results and Discussion

3.1 Effect of Carbon Source on Bacterial Cellulose Production in Agitated Cultures

Initially, the bacterial strain *A. xylinum* 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 BC production. **Table I** depicts the performance of the strain in cultures with 30 g l^{-1} of carbon source, under agitated culture conditions.

Among all sugars tested as carbon sources under agitation conditions at initial concentration of 30 g l^{-1}, fructose was the most suitable substrate for BC synthesis for the bacterial strain. In fructose-based media, BC reached a maximum of 9.4 g l^{-1},

Table I Bacterial Cellulose Production, Yield and Substrate Uptake Rate During Cultivation of *A. xylinum* in Different Carbon Sources under Agitated Conditions (S_i = 30 g l^{-1})

Carbon source	Cultivation time, h	Substrate uptake rate (r_S), g l^{-1} h^{-1}	BC production, g l^{-1}	BC yield, g g^{-1} of consumed substrate
Glucose	142	0.295	1.10 ± 0.02	0.04
Fructose	196	0.199	9.40 ± 0.01	0.39
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^{-1}; temperature 30°C; agitation speed 180 rpm. Data presented as mean values ± standard deviation (n = 3)

after 196 h 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 I**), it is evident that glucose was the most assimilable substrate for *A. xylinum*; however, this was not reflected in the respective BC production achieved in this medium where BC production reached only 1.1 g l^{-1}. In media supplemented with glucose, the pH of the culture dropped drastically during the first 50–60 h of the fermentation (from pH 6.2 to pH 4.2), indicating the formation of gluconic acid as a byproduct (20). On the contrary, in arabinose, xylose and fructose-based media, the pH of the culture dropped only slightly (from pH 6.2 to pH 5.8) within the first 24 h and remained unchanged until the end of the fermentation.

From a metabolic path point of view during BC synthesis, glucose is phosphorylated under the catalysis of a membrane-bound enzyme (glucokinase) to form glucose-6-phosphate (G6P). Subsequently, G6P enters the process of G6P isomerisation and uridine diphosphate glucose synthesis, which then 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 (21). Although glucose and fructose are isomers, and in most cases their catabolic pathway within the cell is the same, it has been reported that the activities of the enzymes involved in metabolism and BC synthesis differ depending on the bacterial species and carbon source (21, 22). In studies investigating the enzymatic activity of different carbon sources catabolism by *A. xylinum*, it has been shown that phosphoglucose isomerase activity in cells in the presence of fructose was 132-fold higher than in cells grown in glucose-supplemented media (21). 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 (which metabolically competes against BC synthesis), thereby achieving increased biopolymer production compared to glucose-based media. This could provide grounds to explore fructose-rich byproduct streams that could be used as substrates for BC production. Given that the cost of substrates is usually around 30% or higher of the total production costs (22, 23), 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 *A. 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 (24).

Based on this first set of results, glucose and fructose were used in higher initial concentrations (60 g l^{-1}) in agitated cultures, aiming to evaluate the performance of the microorganism in terms of BC production. **Table II** depicts data obtained in these experiments, at time points where BC was maximum.

Again, fructose-based media were proved as the most suitable for BC production, reaching 30.87 g l^{-1}. Glucose-based media performed poorly in terms of BC production (4.5 g l^{-1}), while a rapid drop in the medium pH during the first 80 h of fermentation (~pH 3.1), indicated the production of organic acids (gluconic acid) under these cultivation conditions. This was also further supported by the high substrate uptake rate at higher substrate concentrations of glucose (0.262 g l^{-1} h^{-1}). As expected, the substrate uptake rate dropped slightly in media with higher substrate

Table II Bacterial Cellulose Production, Yield and Substrate Uptake Rate During Cultivation of *A. xylinum* in Different Carbon Sources under Agitated Conditions (S_i = 60 g l^{-1})

Carbon source	Cultivation time, h	Substrate uptake rate (r_S), g l^{-1} h^{-1}	BC production, g l^{-1}	BC yield, g g^{-1} of consumed substrate
Glucose	382	0.262	4.53 ± 0.01	0.09
Fructose	196	0.122	30.87 ± 0.07	0.69

Cultivation conditions: initial carbon source concentration ~60 g l^{-1}; temperature 30°C; agitation speed 180 rpm. Data presented as mean values ± standard deviation (n = 3)

Table III Bacterial Cellulose Production, Yield and Substrate Uptake Rate During Cultivation of *A. xylinum* in Different Carbon Sources under Static Conditions

Carbon source	Initial concentration g l^{-1}	Cultivation time, h	Substrate uptake rate (r_S), g l^{-1} h^{-1}	BC production, g l^{-1}	BC yield, g g^{-1} 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)

concentrations, indicating a slower growth rate as a result of partial substrate inhibition.

3.2 Effect of Carbon Source on Bacterial Cellulose Production in Static Cultures

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

Compared with the agitated fermentation conditions, the performance of the two carbon sources in the static environment was better than that in the agitated environment, especially for glucose. BC in glucose-based media in static conditions increased eight-fold at 30 g l^{-1} of substrate and two-fold at 60 g l^{-1} (**Table I**). The maximum BC production was noted in fructose-based media (S_i = 60 g l^{-1}), reaching 31 g l^{-1}, accompanied by an improved yield of 0.67 g per gram of consumed substrate. Worth also mentioning was the fact that in both substrate concentrations, carbon source was not depleted from the medium (**Figure 1** and **Figure 2**).

Lu *et al*. (25) compared five different cellulose producing strains in H&S media under static and agitated conditions and showed that all tested strains performed better in static cultures. The vast majority of growth cultures are nutrient-rich but low in oxygen content, so aerobic bacteria can only achieve BC proliferation at the interface between air and culture medium (26). In a static fermentation environment, the BC membrane on the surface of the medium restricts bacteria from obtaining oxygen from the air (27), 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 BC productivity. However, agitation can induce the emergence of mutant cells, resulting in reduced cellulose production (28). The spontaneous selection of cellulose-negative (Cel–) variants also affects the spread of agitated culture. Under uniform aeration conditions in the agitated culture, extensive cell growth appeared, increasing the probability of cell mutation, leading to a decrease in cellulose production (29). By contrast, in static cultures the formed BC membrane floats on the surface of media which limits access of oxygen to the lower layer of the culture, prohibits intensive cell growth and lowers the probability of mutation into non-cellulose producing cells.

Different incubation conditions, such as culture medium, nutrition concentration and incubation time, have been reported to affect the crystallinity of BC (26, 30). The X-ray diffraction (XRD) patterns of BC produced by fructose and glucose under agitation condition are shown in **Figure 3**. Among these three samples, three peaks can be found near

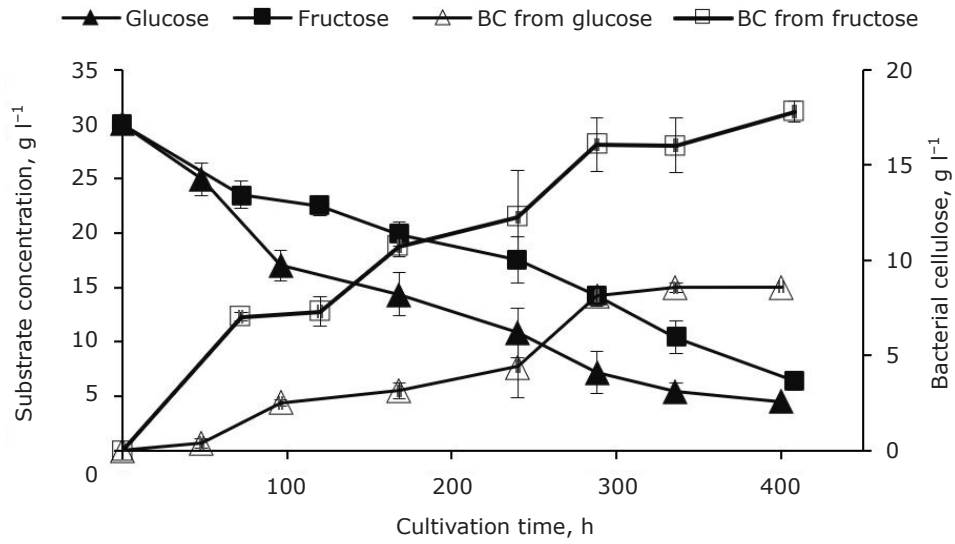

Fig. 1. Kinetics of substrate concentration and BC production in glucose-based and fructose-based cultures of *A. xylinum*, under static conditions (S_i = 30 g l^{-1})

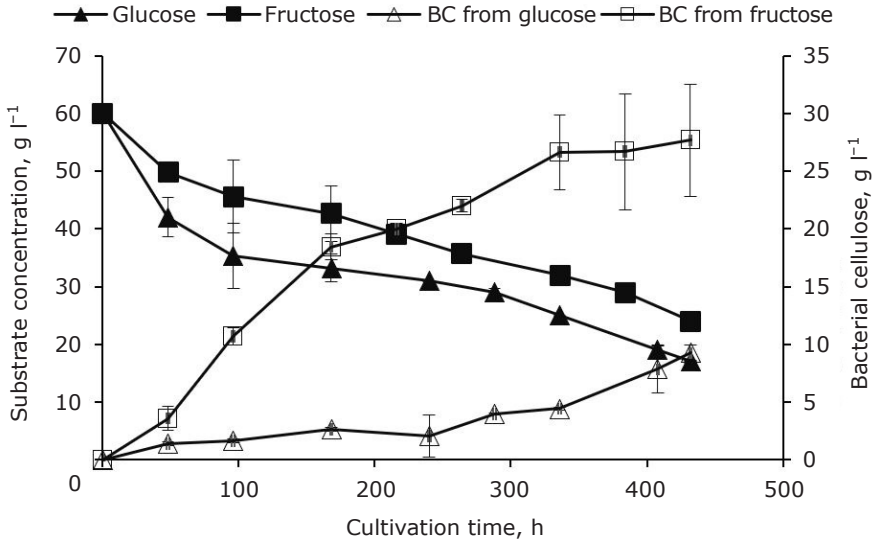

Fig. 2. Kinetics of substrate concentration and BC production in glucose-based and fructose-based cultures of *A. xylinum*, under static conditions (S_i = 60 g l^{-1})

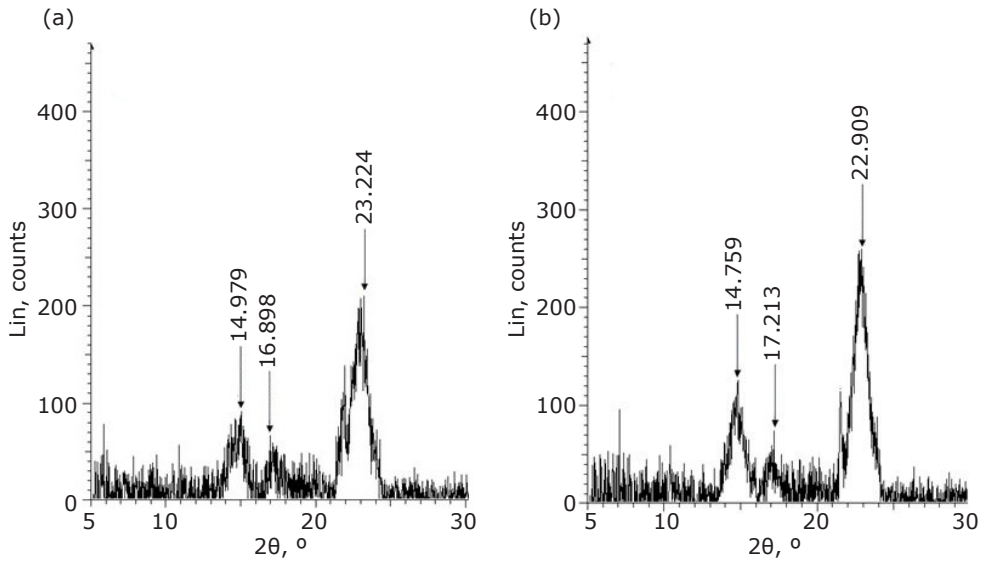

Fig. 3. XRD patterns of BC obtained from: (a) fructose-based media; (b) glucose-based media

2θ = 14.7, 16.8 and 22.8, corresponding to the crystal planes (1$\bar{1}$0), (110) and (200), respectively, proving the existence of cellulose I (30, 31). The absence of characteristic peaks at 2θ = 12.1 and 20.8 for cellulose II (29) means that cellulose II is not present in these samples.

The crystallinity index of BC produced in fructose-based media was the lowest, about 51.2%, whereas for glucose the value was 81.0%. Typically, BC exhibits a high degree of crystallinity. *A. xylinum* Y22 strain has been reported to achieve 90.5% under static conditions (32), while *G. xylinus* (CGMCC 2955) has been reported to produce BC with crystallinity index between 80.5–85.0% in glycerol and glucose-based media (33). Variations between samples tested in the current study may be due to changes in the hydrogen bonds between cellulose molecules (32), which may suggest that the presence of glucose is beneficial for the crystallisation process during BC synthesis and reduces relatively amorphous regions (34).

3.3 Impact of Purification Strategy on Bacterial Cellulose Structure

BC samples produced in fructose-based media in static cultures were purified by alkali and alkali/NaOCl solutions and their structural properties were evaluated.

Fourier transform infrared (FTIR) analysis is considered a reliable method to identify functional groups of BC as well as impurities. In the case of pure BC, the broad band at 3450 cm^{-1} is attributed to the O–H stretching vibration (35). The 2820 cm^{-1} band represents aliphatic C–H stretching vibrations (35). 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 (36). Peaks observed in the 1100–1073 cm^{-1} region are assigned as C–O–C stretching at the β-(1→4)-glycosidic bond or linkage in BC (37). Redundant absorption peaks are still found in the spectrum of the sample, in addition to the standard absorption peaks of functional groups of BC. These include 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}) (17). The presence of these peaks and the peak areas can be used to compare the effectiveness of different purification methods in removing impurities.

As depicted in **Figure 4(a)**, after purification with 0.1 M NaOH, the peaks of functional groups associated with impurities were still prominent. In BC samples purified by NaOH and NaOCl, the results show a similar chemical structure at both NaOCl concentrations (**Figures 4(b)** and **4(c)**); 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.

Based on XRD analysis, it was noted that samples treated with alkali exhibited relatively high crystallinity (**Table IV**). In samples treated with NaOCl, their crystallinity increased as the concentration of NaOCl increased (from 63.1% to 91.7%).

Generally, downstream processing involves separation of the BC from the culture medium by applying simple procedures such as filtration or centrifugation, whereas the purification of the biopolymer aims to remove impurities such as cells and nutrients. It has been reported 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 formed is dramatically reduced, which also leads to a reduction in the strength of the dried BC material (17). Based on the FTIR spectra, a two-step purification process, i.e. mild alkaline solution followed by a NaOCl solution at mild temperatures (25°C), is capable of removing non-cellulose materials such as protein and nucleic acids derived from bacterial cells and culture broth. NaOH is mainly responsible for entrapped cell lysis, whereas NaOCl serves as a bleaching agent to remove impurities which are not removed with NaOH alone. An effective purification method results in better BC fibril interaction and increases the intrinsic hydrogen bonding within BC while maintaining the cellulose I polymorph, thus preventing the formation of cellulose II which is associated with inferior mechanical properties of the biopolymer.

4. Conclusions

Fructose was found to be the most suitable carbon source for BC production by *A. xylinum*. The choice of carbon source was found to impact BC crystallinity which could denote differences

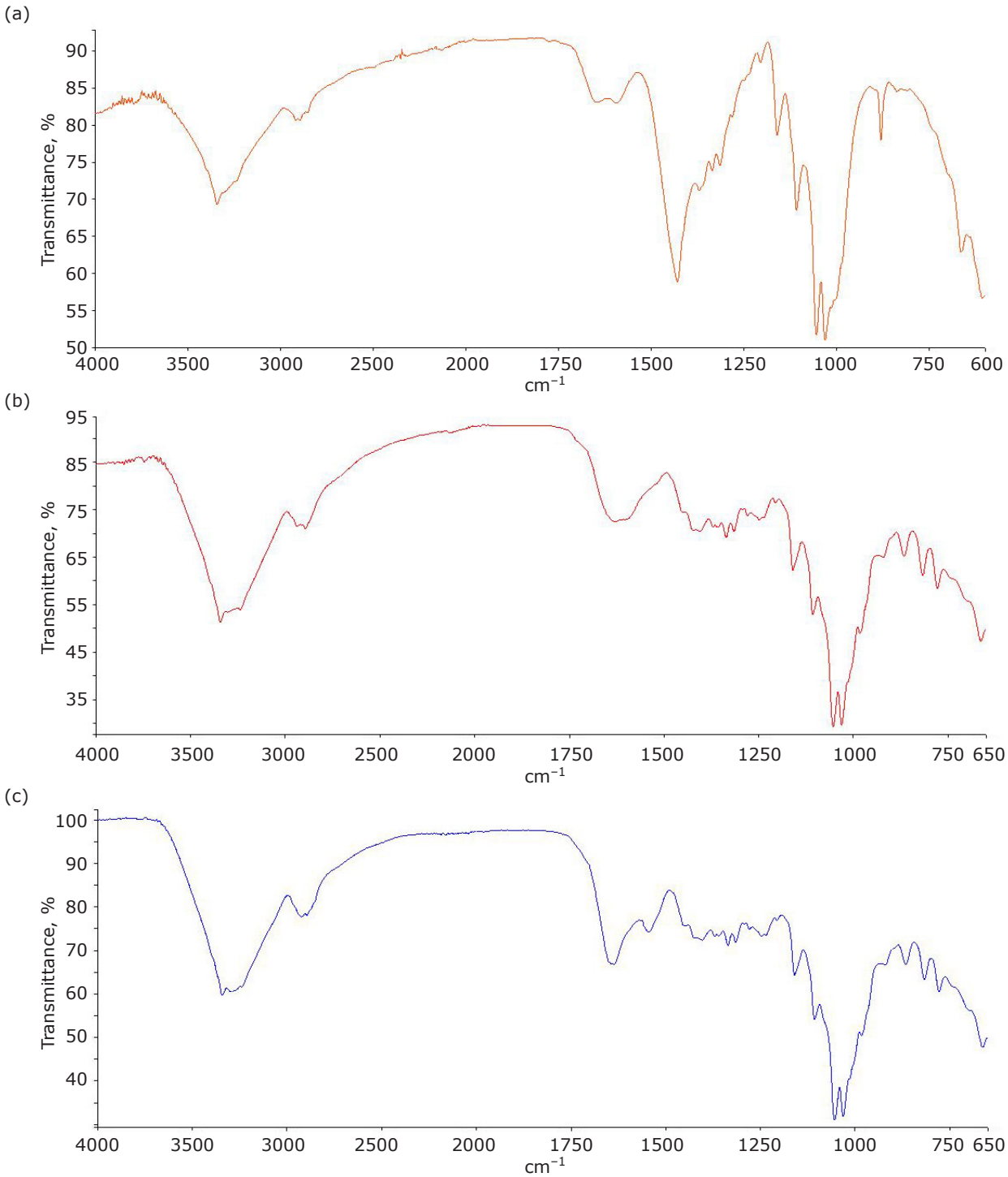

Fig. 4. FTIR spectra of BC samples treated with: (a) 0.1 M NaOH; (b) 0.1 M NaOH and 1.5% (w/w) NaOCl; (c) 0.1 M NaOH and 2.5% (w/w) NaOCl

Table IV Impact of Different Purification Methods on Bacterial Cellulose Crystallinity Index

Purification method	Concentration	Crystallinity index, %
Control	Not applicable	24.3 ± 0.5
Alkali treatment	0.1 M NaOH	70.7 ± 2.5
Two-step treatment	0.1 M NaOH and 1.5% (w/w) NaOCl	63.1 ± 1.1
	0.1 M NaOH and 2.5% (w/w) NaOCl	91.9 ± 2.3

in functional properties and would impact final biopolymer characteristics. The combination of mild concentrations of aqueous NaOH and NaOCl solutions effectively removed contaminants and led to a more rigid biopolymer.

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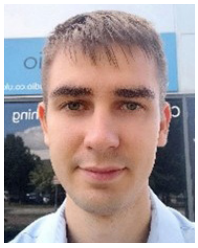

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