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Impact of Fermentation Conditions and Purification Strategy on Bacterial Cellulose Properties

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Abstract

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 *Komagataeibacter xylinus* subsp. *sucrofermentans* DSM 15973 were assessed. Fructose was the most suitable carbon source 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). Moreover, the effectiveness of NaOH and NaOCl solutions in BC purification, and their potential impact on BC structure and properties were explored. The combination of weak NaOH and sodium hypochlorite (NaOCl) was 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 paper-making industry, as reinforcing agent in polymers (4).

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 (3,5). Among them, *Komagataeibacter xylinus* (also named as *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 environment conditions, and to provide floating to the surface of growth media in order for bacteria to have better access to oxygen (8).

In 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 field applications such as skin tissue repairing (9,10) and artificial

blood vessel (11). However, issues that constrain mass BC production in static cultures are associated with the requirement of 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 (e.g. 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 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. Additionally, accumulation of non-BC-producing mutants have been also 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 commonly 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 seem 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 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 (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, aiming 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 (stock keeping unit [SKU]: LP0013T); D- (+)-Glucose ≥ 99.5% from Sigma-Aldrich (SKU: 795410); 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 (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).

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/ 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). 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), 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. 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 (IKA, UK). 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 a single carbon source at two different concentrations (see below), 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 sources, these were glucose (30 g/L and 60 g/L), fructose (30 g/L and 60 g/L), arabinose (30 g/L) and xylose (30 g/L) (all carbon sources were of analytical grade and procured from Sigma-Aldrich, UK). For the glucose and fructose-based media, two types of growth conditions were applied, namely static and agitated. All 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 conditions.

2.3 Analytical methods

One flask (total volume 50 mL) was collected in 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 (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 6.0 M NaOH sterilised solution, if needed. Collected culture samples (50 ml) 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 WhatmanTM 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.

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 dried at 105°C oven (Memmert, UK) for 24 hours and 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, UK) for 30 min at 25°C (21) or a combination of alkali and sodium hypochlorite treatment (0.1 M NaOH solution at 25°C for 30 min, followed by immersion in NaOCl (analytical grade, Sigma-Aldrich, UK) 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 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 (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 data was collected on a Lynxeye detector. The aperture slit was 6 mm, and the detector was equipped with a Nichol attenuator.

2.6 Fourier Transform Infrared Spectroscopy (FT-IR)

Infrared spectra of BC samples were 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.

2.7 Statistical Analysis

Statistical analysis was performed using Microsoft Excel Office 365. 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 BC 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 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 *A. 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
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 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. 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 (21). On the contrary, in arabinose, xylose and fructose-based media, the pH of the culture dropped only slightly (from 6.2 to 5.8) within the first 24 hours 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 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 (22). 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 (22,23). 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 (22). 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% or higher of the total production costs (23-24), 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 (25).

Based on this first set of results, glucose and fructose 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 *A. 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

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. 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.

3.2 Effect of carbon source on BC 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 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 *A. 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, 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). 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 1 and 2).

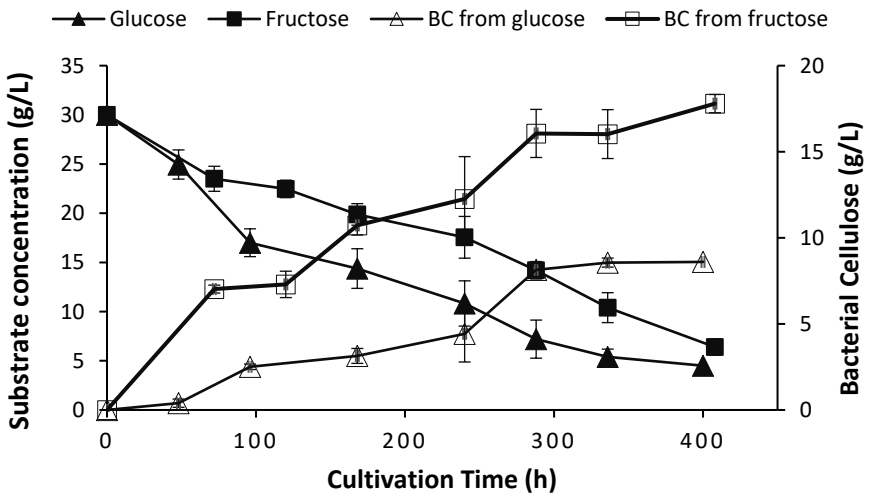

Fig 1: Kinetics of substrate concentration and BC production in glucose-based and fructose-based cultures of *A. xylinum*, under static conditions (S_i=30 g/L)

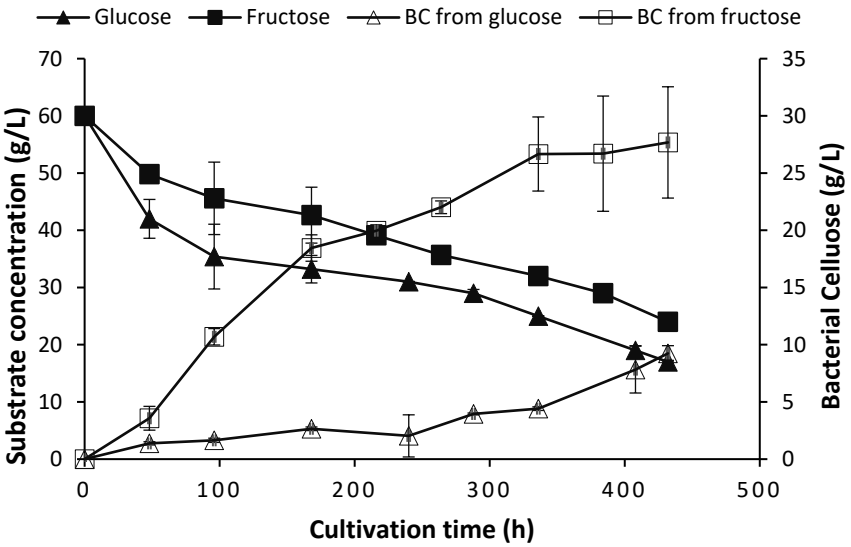

Fig 2: Kinetics of substrate concentration and bacterial cellulose production in glucose-based and fructose-based cultures of *A. xylinum*, under static conditions (S_i=60 g/L)

Lu et al. (27) compared five different cellulose producing strains in HS 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 (28). In a static fermentation environment, the BC membrane on the surface of the medium restricts bacteria from obtaining oxygen from the air (29), 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 (30). The spontaneous selection of the cellulose-negative (Cel-) variants is another factor affects the spread of agitated culture. Under a uniform aeration condition in the agitated culture, extensive cell

growth appeared increasing the probability of cell mutation, leading to a decrease in cellulose production (31). 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 but 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 (28,32). The 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 2θ = 14.7, 16.8 and 22.8, corresponding to the crystal planes (1ī 0), (1 1 0) and (2 0 0), respectively, proving the existence of Cellulose I (32,33). The absence of the characteristic peaks at 2θ = 12.1 and 20.8 for Cellulose II (31) means that Cellulose II is not present in these samples.

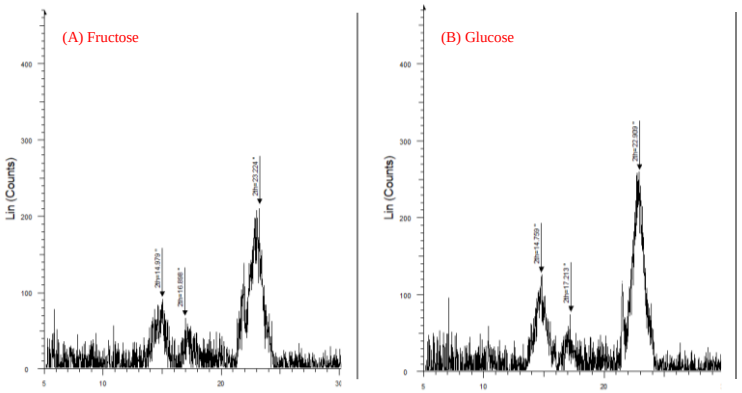

Fig 3. XRD patterns of BC obtained from: (A) Fructose and (B) Glucose based media.

The crystallinity index of BC produced in fructose-based media was the lowest, about

51.2%, whereas for glucose 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 (34), while *G. xylinus* (CGMCC no. 2955) has been reported to produce BC with crystallinity index between 80.5- 85.0% in glycerol and glucose-based media (35). Variations between samples tested in the current study may be due to changes in the hydrogen bonds between cellulose molecules (34), which may suggest that the presence of glucose is beneficial for the crystallization process during BC synthesis and the reduction of relatively amorphous regions in BC (39).

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/ sodium hypochlorite solutions and their structural properties were evaluated.

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 (36). The 2820 cm^{-1} band represents aliphatic C-H stretching vibrations (36). 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 (37). 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 (38). 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}) (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 4a, after the 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 4b and 4c), 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.

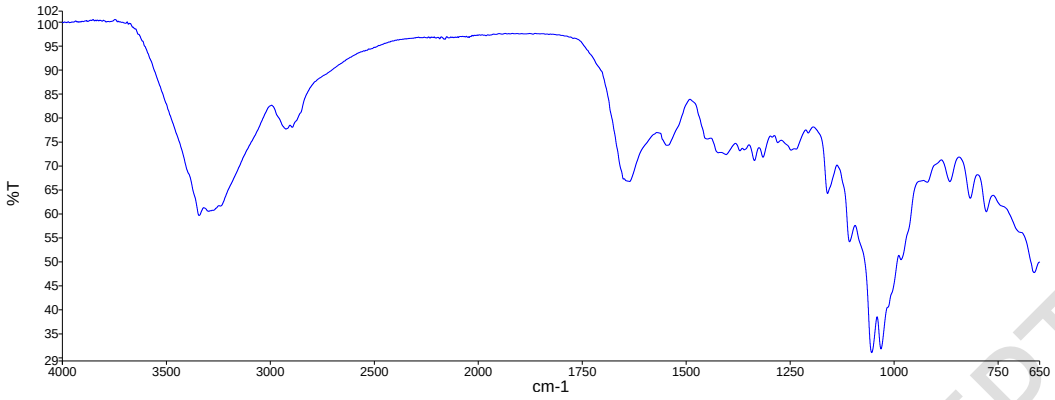

Fig 4 FT-IR spectra of BC samples treated with (a) 0.1 M NaOH; (b) 0.1 M NaOH and 1.5% (w/w) NaOCl and (c) 0.1 M NaOH and 2.5% (w/w) NaOCl.

Based on XRD analysis, it was noted that samples treated with alkali exhibited relatively high crystallinity (Table 4). In samples treated with NaOCl, their crystallinity increased as the concentration of sodium hypochlorite increased (from 63.1 to 91.7%).

Table 4: Impact of different purification methods on BC 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 0.1 M NaOH and 2.5% (w/w) NaOCl	63.1 ± 1.1 91.9 ± 2.3

Generally, the downstream processing involves separation of the BC from the culture medium by applying simple procedures (e.g., 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 that are formed is dramatically reduced, which also leads to a reduction in the strength of the dried BC material (17). Based on the FT-IR 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, in removing impurities which are not removed with NaOH alone. An effective purification method, 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).

4. Conclusions

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