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Altering the growth conditions of *Gluconacetobacter xylinus* to maximize the yield of bacterial cellulose

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ABSTRACT

An extensive matrix of different growth conditions including media, incubation time, inoculum volume, surface area and media volume were investigated in order to maximize the yield of bacterial cellulose produced by *Gluconacetobacter xylinus*, which will be used as reinforcement material to produce fully biodegradable composites. Crystallinity was shown to be controllable depending on the media and conditions employed. Samples with significant difference in crystallinity in a range from 50% to 95% were produced. Through experimental design, the yield of cellulose was maximized; primarily this involved reactor surface area design, optimized media and the use of mannitol being the highest cellulose-producing carbon source. Increasing the volume of the media did achieve a higher cellulose yield, however this increase was not found to be cost or time effective.

1. Introduction

Cellulose is the most abundant polymer on earth and is increasingly of considerable interest in materials science as it has strong potential as a reinforcement material in composites since it is biodegradable, sustainable and renewable. Cellulose has long been produced from plant sources, however the use of bacterial cellulose is appealing for use due to its purity and highly crystalline nanostructure. *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) is a species of bacteria that produces high amounts of cellulose. When this species is grown in a laboratory under static conditions, cellulose forms as a thick mat called a pellicle at the air/surface interface. There have been several reports of different media used in the literature, as well as different carbon sources (El-Saied, El-Diwany, Basta, Atwa, & El-Ghwas, 2008; Hutchens, Leon, O'Neill, & Evans, 2007; Jung, Jeong, et al., 2010; Keshk & Sameshima, 2005; Keshk & Sameshima, 2006; Kim, Kim, Wee, Park, & Ryu, 2006; Masaoka, Ohe, & Sakota, 1993; Mikkelsen, Flanagan, Dykes, & Gidley, 2009; Nguyen, Flanagan, Gidley, & Dykes, 2008; Oikawa, Morino, & Ameyama, 1995; Oikawa, Ohtori, & Ameyama, 1995; Pourramezan, Roayaei, & Qezelbash, 2009; Ramana, Tomar, & Singh, 2000). Determining an optimal medium and an appropriate set of growth conditions that allows high levels of cellulose would aid in the viability of this technology to in an industrial situation. Additionally, determining growth conditions that produce high amounts of cellulose is necessary in order to complete further research using bacterial cellulose as reinforcement for biodegradable polymers as well as understanding any effects such conditions have on the basic materials' morphology and properties.

Tarr and Hibbert (1931) published a study in which they investigated pellicle growth with 25 different carbon sources. They reported high amounts of cellulose were produced when fructose, glucose and mannitol were used as carbon sources, a result confirmed by many more recent studies. Recent studies have also investigated different components of media by substituting carbon and nitrogen sources, often in Hestrin–Schramm (Schramm & Hestrin, 1954) media. Carbon sources including glucose, arabinose, arabitol, citric acid, ethanol, ethylene glycol, diethylene glycol, fructose, galactose, glucono lactone, glycerol, inositol, lactose, malic acid, maltose, mannitol, mannose, methanol, rhamnose, ribose, sorbose, starch, succharide, succinic acid, sucrose, trehalose, and xylose have been investigated (El-Saied et al., 2008; Hutchens et al., 2007; Jung, Jeong, et al., 2010; Keshk & Sameshima, 2005; Keshk & Sameshima, 2006; Kim et al., 2006; Masaoka et al., 1993; Mikkelsen et al., 2009; Nguyen et al., 2008; Oikawa, Morino, et al., 1995; Oikawa, Ohtori, et al., 1995; Pourramezan et al., 2009; Ramana et al., 2000) with various strains of *G. xylinus*.

In addition to the reports of cellulose yields from different media and different carbon and nitrogen sources, there have been mixed reports about alterations to the structure of cellulose

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in different media and with different growth conditions. Some authors have reported that the structure of cellulose is not affected by changing the carbon or nitrogen source (Keshk & Sameshima, 2006; Mikkelsen et al., 2009), whereas others have reported differences. El-Saied et al. (2008) reported a corn steep liquor and molasses medium resulted in a higher degree of crystallization over carbon and nitrogen sources such as glucose, mannitol, yeast extract and peptone, whereas Jung, Jeong, et al. (2010) and Jung, Lee, et al. (2010) reported a decrease in crystallinity in molasses medium compared to a complex medium control. In another study that examined the use of glycerol as the carbon source, cellulose was observed with 9% higher crystallinity compared to a glucose medium, whereas water-holding capacity and viscosity were lower in the glycerol medium (Jung, Lee, et al., 2010). When comparing cellulose produced under static and agitated conditions, cellulose from agitated culture resulted in a loss of mechanical strength with a decreased degree of polymerization, lower crystallinity index, lower cellulose I_α content, lower Young's modulus, higher water holding capacity and higher suspension viscosity in disintegrated form (Cheng, Catchmark, & Demirci, 2009; Czaja, Romanovicz, & Brown, 2004; Krystynowicz et al., 2002; Watanabe, Tabuchi, Morinaga, & Yoshinaga, 1998). Quero et al. (2010) used bacterial cellulose to produce composites by compressing strips of bacterial cellulose between two strips of polylactic acid films and determined that composites with bacterial cellulose cultivated for six days had improved mechanical properties over those with cellulose cultivated for three days, however the three-day cellulose composites showed enhanced interaction with the polylactic acid.

It is important to be able to grow sufficient cellulose of the appropriate morphology for nano-reinforcement under optimal conditions. An extensive study is presented here examining a variety of media, carbon source, incubation time, vessel size (surface area), inoculum volume and media volume, in order to determine protocols to achieve high yields of bacterial cellulose, while minimizing variation. To the authors' knowledge, the effect of these carbon sources on cellulose production has not been investigated in Zhou, Sun, Hu, Li, &Yang (2007), CSL (Toyosaki et al., 1995), or Son et al. (2003) media. Media have also been modified.

2. Experimental

2.1. Bacterial strain

A culture of cellulose-producing *G. xylinus* ATCC 53524 was kindly provided by Gary Dykes from the School of Science, Monash University, Malaysia.

2.2. Media

Several different types of media that have been previously reported to have optimized concentrations and are used to cultivate *G. xylinus* were selected and modified from the literature. Media used were Hestrin–Schramm (HS) (1954), Yamanaka et al. (1989), Zhou et al. (2007), CSL (Toyosaki et al., 1995), modified to exclude environmentally damaging and harmful components zinc sulfate hepahydrate and copper sulfate pentahydrate, and Son et al. (2003), modified to include 2% (v/v) corn steep liquor. The exact composition of the media is described below. All media were adjusted to pH 5.0 with HCl or NaOH and autoclaved at 121 °C for 20 min. The carbon sources glucose, mannitol, sucrose, fructose and glycerol were substituted in these media.

2.3. Growth conditions

Seed cultures were prepared by selecting a single colony from a working plate of Hestrin–Schramm agar (Schramm & Hestrin, 1954) and inoculating 10 mL of HS broth. These cultures were incubated for seven days at 28 °C under static conditions. Following growth, seed cultures were shaken vigorously to remove the bacterial cells from the cellulose pellicle. Pellicles were removed and the resulting cell suspension was used for inoculations. Cultures were grown in 200 mL conical flasks containing 50 mL of media and were inoculated at a concentration of 1% (v/v) of the cell suspension unless otherwise stated. Cultures were incubated for seven days at 28 °C under static conditions unless otherwise stated. Shaking conditions were investigated with cultures shaking at 100 rpm.

When investigating surface area, beakers of different sizes were used in order to achieve different surface areas. It was found that 50 mL of media in large beakers often dried out or only produced a very thin layer of cellulose as there was not enough depth for the cellulose to move into the media when the media was spread so thinly. To counteract this, volumes of 100 mL and 200 mL of media were also used in the different sized beakers. The 100 mL and 200 mL cultures were inoculated with 0.5% and 0.25% (v/v) inoculum volumes in addition to the typical 1% (v/v) inoculum, respectively.

2.4. Treatment of cellulose and yield determination

Following incubation periods, cultures were shaken vigorously to remove the attached bacterial cells. Pellicles were removed from cultures and rinsed to remove any residual media. Pellicles were washed with 0.1 M NaOH at 80 °C for 1 h, and then washed repeatedly until a neutral pH was obtained and air dried at room temperature. Pellicles were weighed once dry.

2.5. X-ray diffractometry

X-ray diffraction (XRD) was used to monitor the d_{1-10} spacing corresponding to the interlayer spacing of the crystalline structure of the bacterial celluloses, which fits the monoclinic I_β phase of bacterial cellulose. The XRD measurements were performed on the cellulose sheet samples using a Bruker D8 Diffractometer operating at 40 kV, 40 mA, Cu Kα radiation monochromatized with a graphite sample monochromator. A diffractogram was recorded between 2θ angles of 2° and 40°. Crystallite size was calculated using TOPAS™. The FWHM (full width at half maximum height) for the (1 −1 0) and (2 0 0) diffraction peaks was used for this calculation, as the third peak (1 1 0) could not provide reliable FWHM values due to the lower intensity at this peak. Calculations were conducted using the Scherrer equation with a shape factor constant of 1, and an instrument FWHM of 0.068° 2θ. Crystallinity was also calculated using TOPAS™ based on the method of Hindeleh and Johnson (1971). The amorphous area was determined using ICDD PDF card 00-060-1501, amorphous cellulose. The crystalline peak positions were selected based on positions given in Czaja et al. (2004). A pseudo Voigt Function was used to profile the peak shape and area for both the amorphous and crystalline components.

2.6. Fourier-transform infra-red

Fourier transform infra red (FTIR) spectroscopy was completed using Perkin-Elmer Spectrum 100 Spectrometer. Scans were completed between 4000 and 450 cm^{-1} with 16 convolutions. Baselines for each sample spectrum were normalized using the Spectrum software. I_α content was calculated using the peak heights at 750 and 710 cm^{-1} by the equation determined by Yamamoto, Horii, and Hirai (1996).

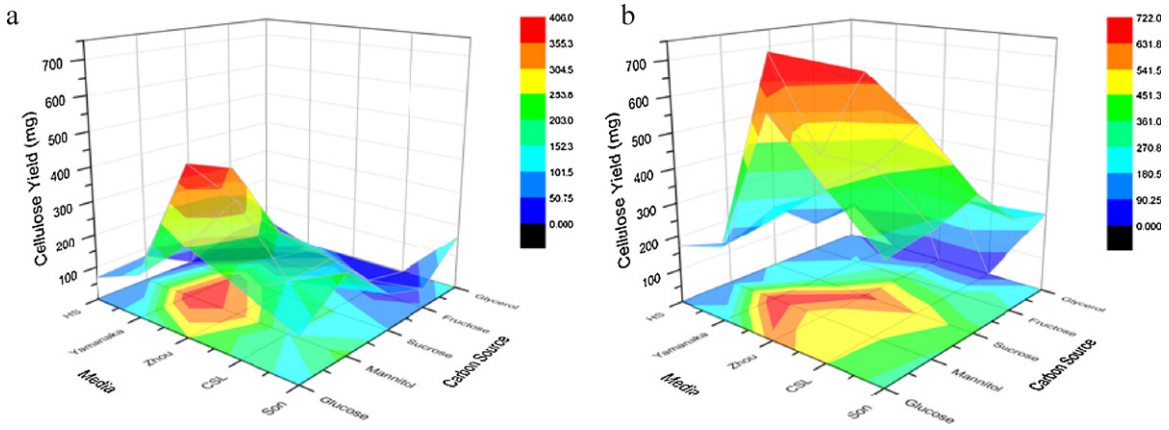

Fig. 1. Cellulose yields produced in different media with a variety of carbon sources after 4 days (a) and 7 days (b) of incubation.

2.7. Scanning electron microscopy

The samples were mounted and gold-coated in preparation for scanning electron microscopy (SEM) imaging. SEM was performed using the field-emission SEM JEOL 7001F operating at 5 kV.

3. Results and discussion

3.1. Different carbon sources

Several different carbon sources were compared, as well as the different media in order to determine which carbon sources produced the highest yields of cellulose and the productivity of the different media. The bacteria were incubated for four and seven days before cellulose was extracted in order to examine yields at different times (Fig. 1).

Of the media used here, the Yamanaka and Zhou media produced very high levels of cellulose, as can be seen with the peaks in Fig. 1, with Yamanaka-mannitol producing the highest yield. CSL and Son media also produced higher levels than HS media, particularly with mannitol as a carbon source. Glucose, mannitol and sucrose were the sugars here that produced consistently high yields of cellulose, regardless of the composition of the media, indicating that they should be used when attempting to achieve high amounts of bacterial cellulose. The exact composition of the modified media used here is presented in Table 1.

Yamanaka and Zhou media produced very high levels of cellulose, but also contained very high concentrations of the carbon source. It is surprising that Zhou media was more effective than CSL as their chemical compositions are so similar except for the trace elements. It appears that the trace elements included in the CSL media have no benefit. Son media was very effective in terms of its cellulose production considering its low concentration of carbon

Table 1
All the components and concentrations (%, w/v) of the different media.

Component	Media				
	Hestrin–Schramm	Yamanaka	Zhou	CSL	Son
Carbon source	2	5	4	4	1.5
Corn steep liquor	–	–	2	2	2
Yeast extract	0.5	0.5	–	–	–
Peptone	0.5	–	–	–	–
Na_2HPO_4	0.27	–	–	–	–
Citric acid·H_2O	0.115	–	–	–	–
$(NH_4)_2SO_4$	–	0.5	0.4	0.33	0.2
KH_2PO_4	–	0.3	0.2	0.1	0.3
$Na_2HPO_4·12H_2O$	–	–	–	–	0.3
$MgSO_4·7H_2O$	–	0.005	0.04	0.025	0.08
$CaCl_2·2H_2O$	–	–	–	0.00147	–
NaCl	–	–	–	–	–
$FeSO_4·7H_2O$	–	–	–	0.00036	0.0005
$ZnSO_4·7H_2O$	–	–	–	0.000173	–
$MnSO_4·H_2O$	–	–	–	0.000097	–
$CuSO_4·5H_2O$	–	–	–	0.0000005	–
$Na_2MoO_4·2H_2O$	–	–	–	0.000242	–
$NiCl_2·6H_2O$	–	–	–	–	–
$CoCl_2·6H_2O$	–	–	–	–	–
H_3BO_3	–	–	–	–	0.0003
p-Aminobenzoic acid	–	–	–	0.00002	–
Biotin	–	–	–	0.0000002	–
Calcium pantothenate	–	–	–	0.00002	–
Folic acid	–	–	–	0.0000002	–
Inositol	–	–	–	0.0002	–
Nicotinamide	–	–	–	0.00004	0.00005
Pyridoxine–HCl	–	–	–	0.00004	–
Riboflavin	–	–	–	0.00004	–
Thiamine–HCl	–	–	–	0.00004	–

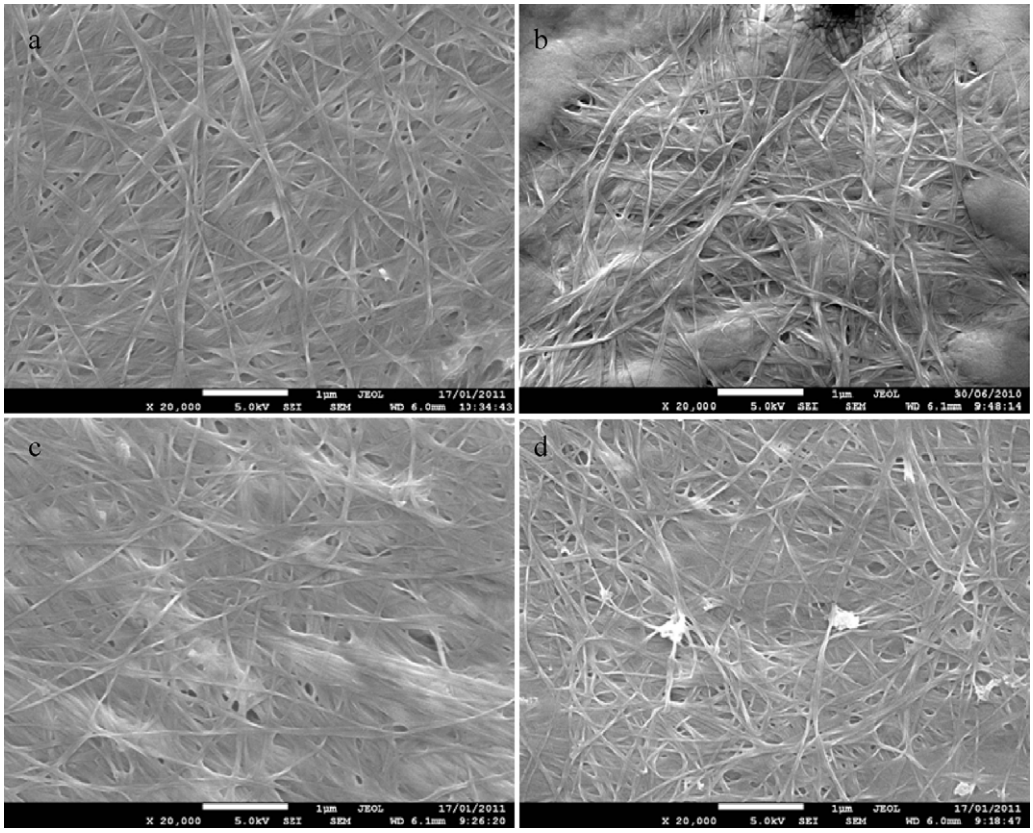

Fig. 2. SEM images of cellulose pellicles produced in various media. HS-glucose (a), Yamanaka-mannitol (b), Zhou-sucrose (c), and Zhou-mannitol (d).

source being that it is even lower than in HS media. For this reason, modified Son media may be particularly cost effective at producing high amounts of cellulose.

Cellulose is produced from hexose phosphate obtained by phosphorylated exogenous hexoses, or indirectly via the pentose cycle and gluconeogenic pathway (Ross, Mayer, & Benziman, 1991; Schramm, Gromet, & Hestrin, 1957). Three sugars were used here, glucose, mannitol and fructose, all of which are hexose sugars and produced moderate to high cellulose yields in all media. However, glucose and fructose, although structurally very similar, gave surprisingly different yields. Glucose consistently produced high yields, whereas fructose often gave much lower yields. As cellulose production is roughly proportional to cell growth (Ross et al., 1991), it may be that fructose cannot be utilized for cell growth as efficiently as glucose, as both are utilized for cellulose production by the same pathway (Schramm et al., 1957). Mannitol, unlike the other two hexose sugars, has no double bonds and does not exist as a ring structure, and has often been found to provide the very high yields amongst the carbon sources in HS and Yamanaka media (Hutchens et al., 2007; Mikkelsen et al., 2009; Nguyen et al., 2008), however to the authors' knowledge, it has not been investigated in the other media examined here. It thus seems that is more beneficial for cell growth and/or cellulose production.

Many of the sucrose media consistently produced low levels of cellulose after four days, but high levels after seven days, indicating that it had an increased lag period for cellulose production. This result is similar to that reported by Mikkelsen et al. (2009) who described sucrose as producing very low bacterial cellulose levels after 48 h of incubation, but very high levels after 96 h, however an increased lag period was observed here. This may be due variations in seed culture techniques. Sucrose is a disaccharide made up of two hexose sugars (glucose and fructose). We hypothesize that synthesis using this sucrose requires an additional metabolic step may be to catalyze the sucrose into glucose and fructose in order to achieve cellulose production. However, despite the typically observed lag period, high cellulose levels have been observed in a Zhou-sucrose medium after four days in an additional experiment and are presented below. It was surprising that glycerol, which has been reported as producing the highest cellulose yield in HS media by some authors (Jung, Jeong, et al., 2010), only produced cellulose in the HS and Son media. Glycerol, a three carbon sugar, is required to be converted by the pentose pathway in order to make it a potential precursor for cellulose synthesis. It is unknown why this process is ineffective in the Yamanaka, Zhou and CSL media. Both HS and Son media contained disodium hydrogen phosphate (Table 1), so it is likely that this component is the reason for cellulose production when glycerol is the carbon source. However, the two media do contain other components that differ from the other media, such as peptone and citric acid in HS medium, and thiamine hydrochloride in Son medium. Yamanaka-mannitol, Zhou-sucrose and Zhou-mannitol media were selected based on observations made here regarding their ability to produce high cellulose levels at four and seven days. HS-glucose medium was used as a baseline. These media were also examined for cellulose yield over time to compare the levels across a four-week period, as described below.

SEM of the pellicles produced revealed no apparent difference in the appearance and fibril diameter, as the cellulose produced under all conditions retained its interwoven, nano-sized structure. A sample of SEM micrographs of cellulose produced in the high achieving media is presented in Fig. 2. These media were used for further study.

Fibril width did not appear to be affected by the use of different media. The fibril widths were 40 nm ± 6 nm, a variation that was seen in all conditions, and has been previously observed.

Samples of cellulose from the selected media were analyzed by XRD and FTIR (Fig. 3). Cellulose I is the form of cellulose found in

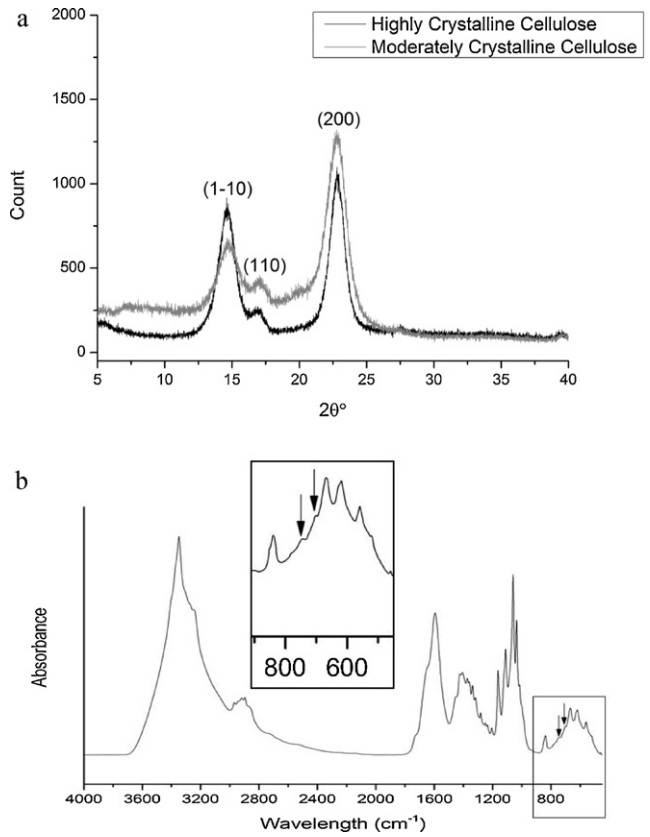

Fig. 3. Structural tests used to characterize bacterial cellulose. (a) XRD diffractograms with three peaks from cellulose produced with high and moderate crystallinity and (b) FTIR scan with I_α mass fraction determined from peaks at 750 and 710 cm^{-1}, as indicated.

nature composed of parallel chains (Delmer, 1987), and exists in two distinct allomorphs, I_α and I_β (Atalla & Vanderhart, 1984). The ratio of cellulose I_α and I_β produced in nature depends on the organism producing it. Changing the media composition has been shown to affect the amount of cellulose I_α produced by *G. xylinus* (Klemm et al., 2006). Variations between the cellulose produced in different media here were usually small (Table 2).

Bacterial cellulose has small crystallite sizes and high crystallinity. It has also been found that there is a strong correlation between crystallite size and I_α contents (Yamamoto et al., 1996), as seen here. All media resulted in cellulose with similar I_α contents, ranging from 68% to 79%. Cellulose I_α content is known to be high in bacterial cellulose, whereas plant cellulose is rich in cellulose I_β, the more stable of the two allomorphs (Atalla & Vanderhart, 1984). While the crystallite size and I_α content data did not differ greatly between media, there was variation in the crystallinity of the cellulose produced in the different media. Cellulose produced in the Yamanaka media showed a lower crystallinity than cellulose produced in the other media, with values of 69% and 50% for media with sucrose and mannitol, respectively. Fig. 3a gives examples of XRD diffractograms with high and moderate crystallinities. It can be observed that the moderately crystalline cellulose gives higher intensities in areas outside the peaks, indicating a greater amorphous region. Crystallite sizes were small (less than 8.0 nm) in the Yamanaka media but were slightly higher than in other media. Crystallite sizes have been reported in the literature as being calculated from the (1 −1 0) peak alone (Watanabe et al., 1998), from the (2 0 0) peak alone (Yamamoto et al., 1996), and from the three peaks (1 −1 0), (1 1 0) and (2 0 0) (Czaja et al., 2004). Here, we calculated crystallite sizes from an average of the (1 −1 0) and (2 0 0) peaks, obtaining good consistency from cellulose produced in the different media. It has previously been reported that never-dried cellulose and cellulose that has been air-dried exhibit differences in crystallite sizes (Fink, Purz, Bohn, & Kunze, 1997). This was not considered here as all cellulose sheets were air-dried under the same conditions and demonstrated similar crystallite sizes, indicating that the growth media does not impact this factor.

Table 2
Structural values for cellulose produced in different media.

Media	Crystallite size (nm)	Crystallinity (%)	Cellulose I_α content (%)	Cellulose I_β content (%)
HS-glucose	7.0	79	79	21
HS-mannitol	6.5	95	68	32
Yamanaka-sucrose	7.9	69	69	31
Yamanaka-mannitol	7.4	50	73	27
Zhou-mannitol	7.2	77	77	23
Son-mannitol	7.0	84	76	24
CSL-glucose	6.5	86	75	25

Bacterial cellulose is formed via a multistep process involving production and crystallization (Ross et al., 1991). Microfibrils are extruded through pores in the bacterial cell membrane, where they entwine and form ribbon structures (Cannon & Anderson, 1991). Additives have been included in the media for the production of bacterial cellulose and have been shown to interfere with aggregation of microfibrils (Benziman, Haigler, Brown, White, & Cooper, 1980) leading to lower crystallinity, however it is unlikely that this is the cause of the low crystallinity in the Yamanaka media seen here, as this media does not contain any components that should do this. It is more likely that the increased rate at which the Yamanaka media produces the bacterial cellulose causes a less perfect crystallization process, and thus whilst it is desirable to use a media in order to achieve high levels of cellulose, lowering the crystallinity of the product may nullify the Yamanaka media usefulness. Zhou-mannitol medium, a high cellulose producing medium, also gave slightly lower crystallinity, however this does not explain the extremely high crystallinity obtained from HS-mannitol medium. In considering these variations, it is important to select a medium for production of cellulose and to provide consistency for composites.

3.2. Cellulose production under shaking conditions

When *G. xylinus* is produced under agitated conditions, the cellulose has been found not to form as a pellicle but instead accumulates as irregular spherical pellets within the medium (Schramm & Hestrin, 1954). This was confirmed in this work, and it supported the findings that under agitated conditions, a significant decrease is also observed in cellulose yield (Schramm & Hestrin, 1954). When *G. xylinus* was grown under static and agitated conditions in the high cellulose-producing media Yamanaka-mannitol, Zhou-sucrose and Zhou-mannitol media, the difference in yield between the two conditions was not observed. It appeared that cellulose initially grew as spherical pellets in agitated culture, however once sufficient cellulose had been produced, an uneven pellicle developed with the pellets connected to it. It was also noted that the cellulose produced under agitated conditions provided a comparable yield to that produced under static conditions as can be seen in Fig. 4. The apparent difference in the yields between the two conditions in the Yamanaka-mannitol medium was due to a single agitated culture in which cellulose production was slightly slower than in the other cultures, and as a result the cellulose had not fully formed a pellicle from the pellets in the incubation period allowed. Regardless of this, the difference in yields is not statistically significant.

Previous studies have reported that growing *G. xylinus* under agitated conditions results in increased cell growth but decreased

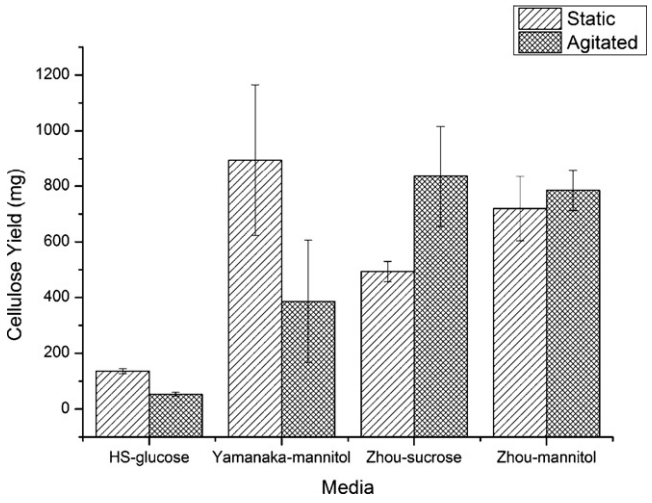

Fig. 4. Cellulose yields produced in different media under static and agitated conditions.

cellulose production over static cultures, and have hypothesized that this is due to increased aeration in the cultures that allows the cells to thrive, but decreases the need for cellulose to anchor the cells at the top of the media in order to be exposed to sufficient levels of oxygen (Czaja et al., 2004; Schramm & Hestrin, 1954). Based on the observation that pellicles did form in the agitated media once sufficient pellets were produced, we hypothesize that it is not the access to oxygen that limit the pellets formation, but rather the agitated nature of the cultures that does not allow the binding of cellulose to the edge of the flask. When cellulose is formed in static culture, its formation begins as a biofilm around the edge of the flask and spreads across the surface toward the centre. In agitated cultures, it may be that this biofilm cannot form due to the shaking, but once enough pellets are produced, binding of cellulose can occur on top of the pellets, and a pellicle is produced. Further consideration of access to oxygen as a limiting factor is discussed below.

SEM images of the cellulose produced under agitated conditions revealed different characteristics from the standard fibrillar structures usually produced in the Zhou media (Fig. 5).

The width of the fibrils in HS-glucose media were approximately 24 nm, indicating that the agitated conditions resulted in thinner fibrils. This is consistent with previous results (Czaja et al., 2004; Krystynowicz et al., 2002), however the cellulose produced under agitated conditions in Yamanaka-mannitol medium maintained fibrils widths of approximately 38 nm, similar to fibrils produced under static conditions. Zhou-sucrose and Zhou-mannitol media cellulose also did not show a decrease in fibril width, but rather an increase – with fibrils ranging from 38 to 55 nm. These widths are probably due to the higher production of cellulose and the resulting ability to bind and produce pellicle structures. It appears that bacterial cellulose produced in Zhou media under agitated conditions, whilst retaining some fibrillar structure, has a differing morphology from the cellulose produced in static culture or in HS-glucose and Yamanaka-mannitol media. It is likely that this morphology is caused by the corn steep liquor included in this media as a cheap, nutrient-rich alternative to yeast extract.

In order to use this cellulose material as a reinforcing agent and to maximize its fibrillar surface area, it is likely that the Zhou media under agitated conditions be avoided, in favor of Yamanaka-mannitol media, produced under either static or agitated conditions. Agitated conditions are also likely less favorable due to the reported loss of mechanical strength in bacterial cellulose of this nature (Czaja et al., 2004; Krystynowicz et al., 2002),

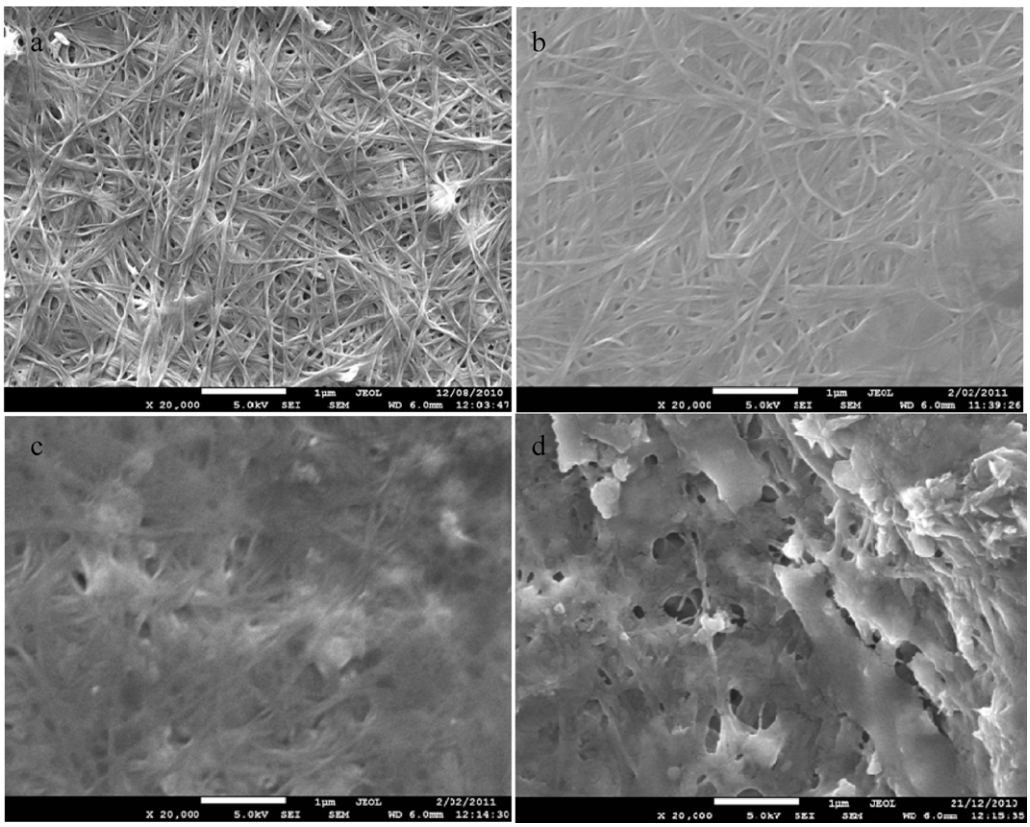

Fig. 5. SEM micrographs of cellulose produced in HS-glucose (a), Yamanaka-mannitol (b), Zhou-sucrose (c) and Zhou-mannitol (d) medium under agitated conditions.

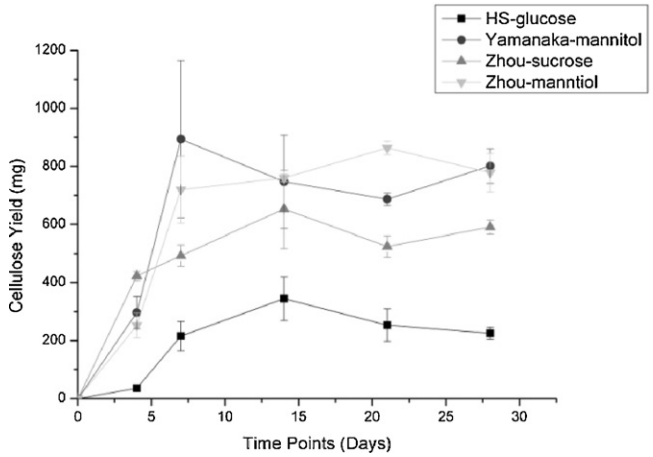

Fig. 6. Cellulose yields produced over time in high cellulose producing media.

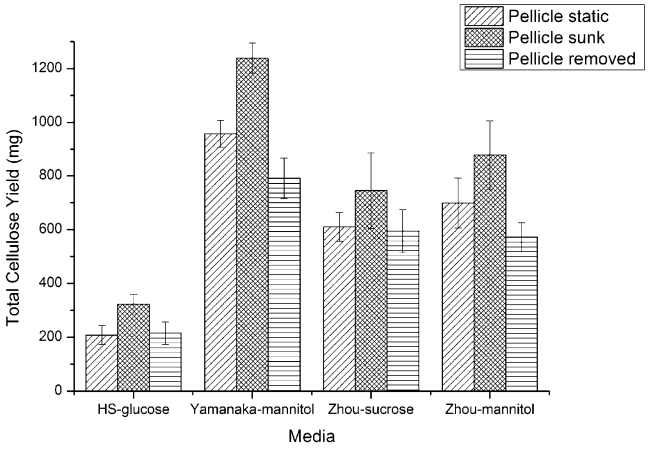

Fig. 7. Cellulose yields obtained from cultures in various media when flasks were shaken or had pellicles removed every five days.

as mechanical strength is one of the main reasons why bacterial cellulose has so much potential, though it may be necessary to use agitation if the production of bacterial cellulose is to be increased to an industrial scale. Mechanical strength of cellulose produced in Yamanaka-mannitol medium under both static and agitated conditions is to be further investigated with the formation of biocomposites.

3.3. Temporal aspects of cellulose production

Together with Hestrin–Schramm-glucose, cultures in the high producing cellulose media, Yamanaka-mannitol, Zhou-sucrose and Zhou-mannitol were grown over a range of time points to examine when the most cellulose is produced. It was found that the cellulose yield typically increased up to 14 days incubation, and then reached a plateau, with much of this growth occurring in the first seven days (Fig. 6). The yield was affected by the media, with both Yamanaka and Zhou media which contain a higher concentration of carbon source than HS medium, achieving greater cellulose yields. All media show similar cellulose production curves, but as the yield reaches a maximal level at approximately 14 days, the rate of cellulose production is invariant to the concentration or composition of the media. The Yamanaka medium resulted in approximately three to four fold increase in cellulose yield than HS, but contains only two and a half times more carbon source, indicating it is a good source of nutrients in order to achieve high levels of cellulose.

There were some differences in the amounts of cellulose produced between the cultures presented here, and those completed as part of the carbon source experiments described above. These differences may have been due to other subtle variations, such as the make-up of the media, the temperature of the incubator or other extraneous variables. Regardless, these cultures still produced extremely high amounts of cellulose compared to the traditionally used HS-glucose medium, and should be further considered in order to maximize bacterial cellulose yield.

3.4. Limitations to cellulose growth

As described above, no significant cellulose formation occurred after 14 days of incubation. The current wisdom is that the pellicle forms across the surface of the media in static culture in order to anchor the bacterial cells to the surface to allow for sufficient oxygen exposure (Cook & Colvin, 1980; Valla & Kjosbakken, 1982). However, based on the observed lack of cellulose production after fourteen days, we propose that after this time, the cellulose pellicle may be sufficiently thick that the bacterial cells are starved of oxygen, and thus are unable to actively grow after this time, resulting in the apparent plateau that is observed in Fig. 6. In order to test this theory, bacterial cellulose was produced in HS-glucose, Yamanaka-mannitol, Zhou-sucrose and Zhou-mannitol media under different conditions. The first condition involved the pellicle being produced statically, as normal. The second condition had the flasks gently shaken every five days in order to sink the pellicle to allow the cells more access to oxygen. Finally, the cultures were shaken every five days and the pellicles were removed. Cultures were grown for a total of 14 days. This was to allow the static cultures to reach the previously observed plateau.

It was found that removing the pellicles from the cultures every five days did not increase the cellulose yield over the pellicles that were produced statically over 14 days, and similar yields were obtained from these two conditions in all media examined (Fig. 7). However in all media, a slightly greater yield of cellulose was obtained when the flasks were gently shaken in order to dislodge and sink the pellicle, however this was not statistically significant in all media.

There have been conflicting reports when it comes to oxygenation and cellulose production, however it has been reported that oxygen is a limiting factor when it comes to bacterial cellulose (Krystynowicz et al., 2002), but this was not found to be the case here. Allowing access to more oxygen did not result in higher cellulose yields. As there was little or no cellulose production after 14 days of cell growth, it is likely that the plateau observed is a result of limitations in nutrients. It is interesting to note that despite the Yamanaka medium containing a higher concentration of carbon source and producing high levels of cellulose, cellulose is not produced for a longer period of time, but instead the rate of production is increased indicating that the nutrients are consumed faster, and the yield plateau is reached at approximately the same time as in the other media.

3.5. Surface area

As seen previously, when grown under static conditions, bacterial cellulose forms as a thick pellicle at the air–surface interface. Therefore, it seems likely that the greater the surface area of the media for the cellulose to spread across, the greater the amount of cellulose should be produced. A range of different sized beakers were used as the containers for this experiment with HS-glucose medium in order to achieve varying surface areas, and cultured were incubated for 14 days in order to maximize the cellulose yield. Different volumes of media were investigated and different inoculum volumes were used for the large cultures. However it was found

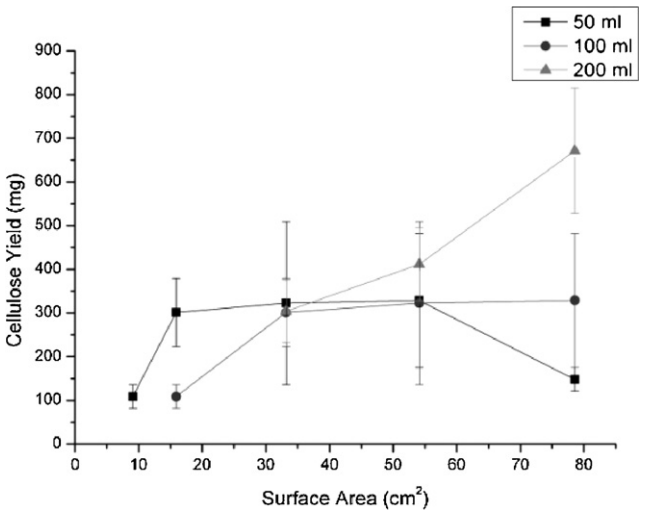

Fig. 8. Cellulose yield produced from varying volumes of media in beakers with different surface areas.

that cellulose production was not affected by inoculum volume as differences in cellulose yield between cultures with different inoculum volumes were not statistically significant (data not shown). This indicates that the number of cells introduced into the culture has little or no impact on the amount of cellulose produced. When a small volume of media was used (50 mL), the depth in the larger beakers was so shallow that the cellulose was only produced as a very thin layer and was therefore limited in its yield. Greater volumes of 100 mL and 200 mL allowed a relationship to be established between surface area and cellulose yield (Fig. 8). Provided that enough media was used to allow sufficient depth, the highest yields of cellulose were obtained from the largest containers (with the largest surface area).

Previous studies have examined the relationship between the ratio of surface area to media volume in terms of optimizing the yield of bacterial cellulose making the depth of the media for focus for obtaining high amounts of cellulose. It has been reported that the optimal surface area/volume ratio was 2.2 cm^{-1}, whereas Krystynowicz et al. (2002) found that a ratio of 0.71 cm^{-1} gave the highest yield. Using the largest surface area and 200 mL of media from this experiment, the highest yield of cellulose was obtained with a surface area/volume ratio of 0.39 cm^{-1}. This is a much smaller ratio than those previously reported, but at smaller volumes we found there was insufficient depth in the media for the cellulose to move down as it was produced. A volume of 110 mL would have given the ratio of approximately 0.71 cm^{-1}, but this was not investigated here. This suggests that the depth provided by 100 mL of media was only slightly too small and that this is a very complex relationship.

It was unexpected, however, that no significant difference was seen between the yields obtained in the smaller containers between the three different volumes of media. In the 200 mL and 600 mL beakers (with surface areas of 33 and 54 cm^2, respectively) the yield did not vary greatly with 50 mL, 100 mL and 200 mL of media, however the 100 mL beaker with a surface area of 16 cm^2, actually achieved a greater cellulose yield in 50 mL of media, compared to 100 mL of media. It appears that the greatest surface area had an increase in cellulose yield when the media volume increased, however it is likely that this was due to the limitations in growth caused by the shallow media. Therefore, if no increase is seen in cellulose yield when the volume of the media is increased and the volume of media used to obtain the cellulose is taken into account for determination of the cost effectiveness of the method, then more cellulose is produced per liter with lower volumes of media, provided that the media has sufficient depth for the cellulose to be produced in it.

3.6. Different volumes of media

In order to confirm the previous observation that there was no increase in cellulose yield with larger volumes of media in containers with the same surface area, a variety of media volumes were tested while maintaining a constant surface area. Cultures of HS-glucose medium of 100, 200 and 400 mL in 600 mL beakers were incubated for 14 days before the cellulose was removed, extracted and quantified. Cultures were allowed to grow after this time point to determine if any more cellulose would be produced, and produced cellulose was removed every seven days. The results are shown in Fig. 9a and b.

No difference was observed in the cellulose yield using different volumes of media in the previous surface area experiments, but a difference was observed here between the 100 mL cultures, and the 200 and 400 mL cultures after 14 days with more cellulose being obtained in the 200 mL and 400 mL cultures than in 100 mL (but no statistical significance between the 200 and 400 mL cultures). Despite twice (and four times) as much media being used in the larger cultures, the yield obtained was less than a two fold increase over a 14-day period. However, the 200 and 400 mL cultures were observed to continue to produce cellulose after this time.

From the cellulose yields obtained after 42 days of incubation, more cellulose was produced in the media with the greater volume, and therefore the greater amount of nutrients. It is also clear that increasing the volume of the media increased the time that cellulose could be produced, supporting the previous observations that it is the nutrients that limit the production of cellulose rather than the availability of oxygen. However, even after an extended incubation

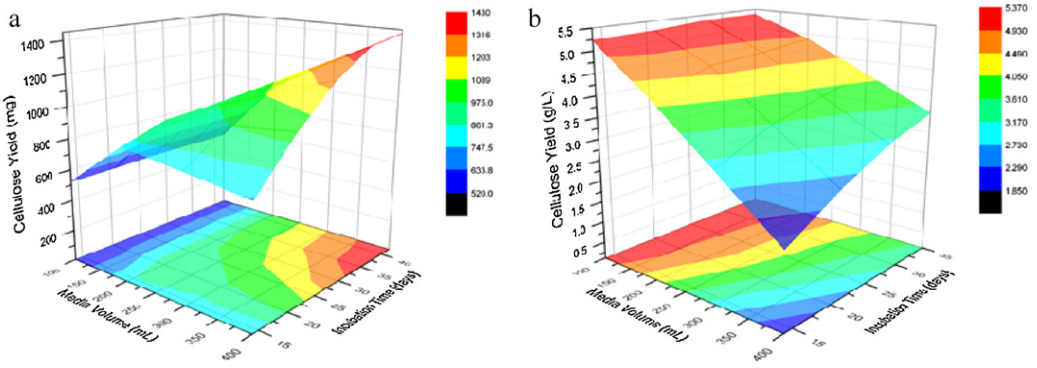

Fig. 9. Cellulose yields produced in different volumes of media with cellulose yield presented in mg (a) and g/L (b).

period, the increase in the cellulose yield in the larger cultures was not as productive once the actual volume of the media is taken into consideration by converting the yield into g/L. Fig. 9b shows that despite the amount of cellulose obtained increasing in the larger cultures, using larger volumes of media is not cost-effective as more cellulose is produced per liter when smaller volumes of media are used.

The surface area/volume ratio of the 100 mL culture was 0.57 cm^{-1}, larger than the ratio in the surface area experiment, and closer to that of Krystynowicz et al. (2002). A volume of 80 mL in this sized beaker would give a ratio of approximately 0.71 cm^{-1} and would likely still provide a sufficient depth to produce high amounts of cellulose. Ratios of between 0.57 and 0.71 cm^{-1}, providing depths of 1.75 and 1.4 cm respectively would most likely give the most cost efficient amounts of media to obtain maximum bacterial cellulose. However, these ratios are also likely to depend on the media used, as HS media produces a much thinner pellicle than those produced in Yamanaka media. When 50 mL of Yamanaka media was used in the flasks, the pellicles often took up most of the space of the media, leaving very little liquid behind. This would therefore affect the optimal ratio of the surface area/volume of media relationship.

Determining a set of growth conditions must be, therefore, carefully calculated before mass producing bacterial cellulose. Increasing media volume does increase cellulose yield, but it also increases production time and cost, without significant gain.

4. Conclusions

Cellulose is an abundant polymer due to its production in plants, some bacterial and algae species. Examining the different ways to achieve the maximum amount of cellulose from the microorganism *G. xylinus* has indicated that the process of production is extremely complex. Cellulose production increases with the use of particular carbon sources in some media, but not in others, and yield is greatly affected by the selection of the media. Cellulose production increases with the surface area of static media, and with increases in media volume, but this also increases cost and production time. Many considerations need to be taken into account when determining a set of base conditions by which to produce bacterial cellulose. Cost effectiveness of the media in terms of the yield of cellulose produced is an important factor, and therefore the media composition, surface area and media volume should all be considered. Using a large surface area and high cellulose-producing media, up to 10 g of bacterial cellulose has been produced in 14 days. This cellulose will be used for further work as reinforcement material in order to achieve biodegradable composites with superior properties over the matrix alone.

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References

Atalla, R. H., & Vanderhart, D. L. (1984). Native cellulose—A composite of 2 distinct crystalline forms. *Science*, *223*(4633), 283–285.

Benziman, M., Haigler, C. H., Brown, R. M., White, A. R., & Cooper, K. M. (1980). Cellulose biogenesis—Polymerization and crystallization are coupled processes in *Acetobacter xylinum*. *Proceedings of the National Academy of Sciences of the United States of America—Biological Sciences*, *77*(11), 6678–6682.

Cannon, R. E., & Anderson, S. M. (1991). Biogenesis of bacterial cellulose. *Critical Reviews in Microbiology*, *17*(6), 435–447.

Cheng, K. C., Catchmark, J. M., & Demirci, A. (2009). Effect of different additives on bacterial cellulose production by *Acetobacter xylinum* and analysis of material property. *Cellulose*, *16*(6), 1033–1045. doi:10.1007/s10570-009-9346-5

Cook, K. E., & Colvin, J. R. (1980). Evidence for a beneficial influence of cellulose production on growth of *Acetobacter xylinum* in liquid-medium. *Current Microbiology*, *3*(4), 203–205.

Czaja, W., Romanovicz, D., & Brown, R. M. (2004). Structural investigations of microbial cellulose produced in stationary and agitated culture. *Cellulose*, *11*(3–4), 403–411.

Delmer, D. P. (1987). Cellulose biosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology*, *38*, 259–290.

El-Saied, H., El-Diwany, A. I., Basta, A. H., Atwa, N. A., & El-Ghwas, D. E. (2008). Production and characterization of economical bacterial cellulose. *BioResources*, *3*(4), 1196–1217.

Fink, H. P., Purz, H. J., Bohn, A., & Kunze, J. (1997). Investigation of the supramolecular structure of never dried bacterial cellulose. *Macromolecular Symposia*, *120*, 207–217. doi:10.1002/masy.19971200121

Hindeleh, A. M., & Johnson, D. J. (1971). Resolution of multipeak data in fibre science. *Journal of Physics D—Applied Physics*, *4*(2), 259–263.

Hutchens, S. A., Leon, R. V., O'Neill, H. M., & Evans, B. R. (2007). Statistical analysis of optimal culture conditions for *Gluconacetobacter hansenii* cellulose production. *Letters in Applied Microbiology*, *44*(2), 175–180. doi:10.1111/j.1472-765X.2006.02055.x

Jung, H. I., Jeong, J. H., Lee, O. M., Park, G. T., Kim, K. K., Park, H. C., et al. (2010). Influence of glycerol on production and structural-physical properties of cellulose from *Acetobacter* sp. V6 cultured in shake flasks. *Bioresource Technology*, *101*(10), 3602–3608. doi:10.1016/j.biortech.2009.12.111

Jung, H. I., Lee, O. M., Jeong, J. H., Jeon, Y. D., Park, K. H., Kim, H. S., et al. (2010). Production and characterization of cellulose by *Acetobacter* sp. V6 using a cost-effective molasses-corn steep liquor medium. *Applied Biochemistry and Biotechnology*, *162*(2), 486–497. doi:10.1007/s12010-009-8759-9

Keshk, S., & Sameshima, K. (2005). Evaluation of different carbon sources for bacterial cellulose production. *African Journal of Biotechnology*, *4*(6), 478–482.

Keshk, S., & Sameshima, K. (2006). The utilization of sugar cane molasses with/without the presence of lignosulfonate for the production of bacterial cellulose. *Applied Microbiology and Biotechnology*, *72*(2), 291–296. doi:10.1007/s00253-005-0265-6

Kim, S. Y., Kim, J. N., Wee, Y. J., Park, D. H., & Ryu, H. W. (2006). Production of bacterial cellulose by *Gluconacetobacter* sp. RKY5 isolated from persimmon vinegar. *Applied Biochemistry and Biotechnology*, *131*(1–3), 705–715.

Klemm, D., Schumann, D., Kramer, F., Hessler, N., Hornung, M., Schmauder, H. P., et al. (2006). Nanocelluloses as innovative polymers in research and application. *Polysaccharides II*, *205*, 49–96.

Krystynowicz, A., Czaja, W., Wiktorowska-Jezierska, A., Goncalves-Miskiewicz, M., Turkiewicz, M., & Bielecki, S. (2002). Factors affecting the yield and properties of bacterial cellulose. *Journal of Industrial Microbiology & Biotechnology*, *29*(4), 189–195. doi:10.1038/sj.jim.7000303

Masaoka, S., Ohe, T., & Sakota, N. (1993). Production of cellulose from *Acetobacter xylinum*. *Journal of Fermentation and Bioengineering*, *75*(1), 18–22.

Mikkelsen, D., Flanagan, B. M., Dykes, G. A., & Gidley, M. J. (2009). Influence of different carbon sources on bacterial cellulose production by *Gluconacetobacter xylinus* strain ATCC 53524. *Journal of Applied Microbiology*, *107*(2), 576–583. doi:10.1111/j. 1365-2672.2009.04226.x

Nguyen, V. T., Flanagan, B., Gidley, M. J., & Dykes, G. A. (2008). Characterization of cellulose production by a *Gluconacetobacter xylinus* strain from Kombucha. *Current Microbiology*, *57*(5), 449–453. doi:10.1007/s00284-008-9228-3

Oikawa, T., Morino, T., & Ameyama, M. (1995). Production of cellulose from D-arabitol by *Acetobacter xylinum* Ku-1. *Bioscience Biotechnology and Biochemistry*, *59*(8), 1564–1565.

Oikawa, T., Ohtori, T., & Ameyama, M. (1995). Production of cellulose from D-mannitol by *Acetobacter xylinum* Ku-1. *Bioscience Biotechnology and Biochemistry*, *59*(2), 331–332.

Pourramezan, G. Z., Roayaei, A. M., & Qezelbash, Q. R. (2009). Optimization of culture conditions for bacterial cellulose production by *Acetobacter* sp. 4B-2. *Biotechnology*, *8*(1), 150–154.

Quero, F., Nogi, M., Yano, H., Abdulsalami, K., Holmes, S. M., Sakakini, B. H., et al. (2010). Optimization of the mechanical performance of bacterial cellulose/poly(L-lactic) acid composites. *ACS Applied Materials & Interfaces*, *2*(1), 321–330. doi:10.1021/am900817f

Ramana, K. V., Tomar, A., & Singh, L. (2000). Effect of various carbon and nitrogen sources on cellulose synthesis by *Acetobacter xylinum*. *World Journal of Microbiology & Biotechnology*, *16*(3), 245–248.

Ross, P., Mayer, R., & Benziman, M. (1991). Cellulose biosynthesis and function in bacteria. *Microbiological Reviews*, *55*(1), 35–58.

Schramm, M., Gromet, Z., & Hestrin, S. (1957). Role of hexose phosphate in synthesis of cellulose by *Acetobacter xylinum*. *Nature*, *179*(4549), 28–29.

Schramm, M., & Hestrin, S. (1954). Factors affecting production of cellulose at the air liquid interface of a culture of *Acetobacter xylinum*. *Journal of General Microbiology*, *11*(1), 123–129.

Son, H. J., Kim, H. G., Kim, K. K., Kim, H. S., Kim, Y. G., & Lee, S. J. (2003). Increased production of bacterial cellulose by *Acetobacter* sp. V6 in synthetic media under shaking culture conditions. *Bioresource Technology*, *86*(3), 215–219.

Tarr, H. L. A., & Hibbert, H. (1931). Studies on reactions relating to carbohydrates and polysaccharides. XXXV. Polysaccharide synthesis by the action of *Acetobacter xylinus* on carbohydrates and related compounds. *Canadian Journal of Research*, *4*(1), 372–388.

Toyosaki, H., Naritomi, T., Seto, A., Matsuoka, M., Tsuchida, T., & Yoshinaga, F. (1995). Screening of bacterial cellulose-producing Acetobacter strains suitable for agitated culture. *Bioscience Biotechnology and Biochemistry*, *59*(8), 1498–1502.

Valla, S., & Kjosbakken, J. (1982). Cellulose-negative mutants of *Acetobacter xylinum*. *Journal of General Microbiology*, *128*(7), 1401–1408.

Watanabe, K., Tabuchi, M., Morinaga, Y., & Yoshinaga, F. (1998). Structural features and properties of bacterial cellulose produced in agitated culture. *Cellulose*, *5*(3), 187–200.

Yamamoto, H., Horii, F., & Hirai, A. (1996). In situ crystallization of bacterial cellulose. 2. Influences of different polymeric additives on the formation of celluloses I-alpha and I-beta at the early stage of incubation. *Cellulose*, *3*(4), 229–242.

Yamanaka, S., Watanabe, K., Kitamura, N., Iguchi, M., Mitsuhashi, S., Nishi, Y., et al. (1989). The structure and mechanical-properties of sheets prepared from bacterial cellulose. *Journal of Materials Science*, *24*(9), 3141–3145.

Zhou, L. L., Sun, D. P., Hu, L. Y., Li, Y. W., & Yang, J. Z. (2007). Effect of addition of sodium alginate on bacterial cellulose production by *Acetobacter xylinum*. *Journal of Industrial Microbiology & Biotechnology*, *34*(7), 483–489. doi:10.1007/s10295-007-0218-4