Biocomposites from Bacterial Cellulose

A thesis submitted for the degree of Doctor of Philosophy at Monash University

September 2014

Dianne Ruka, BSc (Hons), Dip. Ed.

Department of Materials Engineering

Table of Contents

Copyright Notices

Notice 1

Notice 2

Summary

Bacterial cellulose has high strength and crystallinity, and as such has been suggested as a strategically-useful material that could form the reinforcement phase in composites. In addition to any property improvement, if a biodegradable matrix is reinforced with bacterial cellulose, the entire system should be biodegradable. However, bacterial cellulose is not easily dispersed and thus has not been widely used in such composites. Investigations into the production of bacterial cellulose composites where the cellulose reinforcement component is evenly dispersed is therefore of benefit. Bacterial cellulose can be produced in high quantities by the bacterium *Gluconacetobacter xylinus* in various media and under various reaction conditions, however altering the growth conditions has been shown to change the yield and properties of the resulting cellulose.

Poly-3-hydroxybutyrate (PHB) is a bioplastic that has been hypothesised as a material that could replace traditional plastics, however it is very stiff and brittle. It is possible that its use in a composite with an effective reinforcing phase could improve these properties. Therefore this material was selected as a matrix material to be blended with bacterial cellulose.

The examination of growth conditions in this work led to a methodology by which high amounts of cellulose with high crystallinity could be obtained. In addition, methods were determined by which to achieve modified cellulose fibrils. These modifications included cellulose fibrils produced with PHB physically attached to the surface.

Solution blending and melt blending techniques were investigated as ways of producing PHB/bacterial cellulose composites. Solution blending was found to produce composites with well dispersed bacterial cellulose, however melt blending was found to degrade samples. It was found that solution blends using cellulose in a ground powder form did not achieve improved properties; however a composite with cellulose in its fibrillar form achieved improved tensile strength and modulus.

PHB/bacterial cellulose composites with cellulose in its fibrillar form were produced by dispersing the cellulose fibrils by sonication. Sonication was investigated as a method of harvesting and dispersing bacterial cellulose fibrils in various solvents, including chloroform which could directly dissolve PHB, however only small weights of individual fibrils were obtained in this way. The composite with improved mechanical properties contained 2 wt% cellulose; however it was found that these improvements were observed only if the cellulose was retained in a hydrated never-dried state.

Investigations into the biodegradability of PHB and a PHB/bacterial cellulose composite revealed that the composite degraded at a greater rate than neat PHB. This indicates that a bacterial cellulose reinforcement phase is able to assist a PHB matrix to degrade at a faster rate when submerged in compost.

It is apparent that techniques can be developed to use bacterial cellulose successfully to confer strength to composites when used as a reinforcing material, as well as increasing the rate of biodegradation of a PHB matrix. Composites of these materials should therefore be considered in the design of biodegradable materials.

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes three original papers published in peer reviewed journals, a review that has been accepted as a book chapter and two unpublished publications. The core theme of the thesis is bacterial cellulose. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Materials Engineering under the supervision of Professor George Simon and Dr. Katherine Dean from CSIRO.

[The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.]

In the case of Chapters 2 – 4 and 6 – 7, my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
2	Bacterial Cellulose and its use in Renewable Composites	Accepted for Publication (book chapter), Nanocellulose Polymer Nanocomposites: Fundamental and Applications	Planning, researching and writing, 90%
3	Altering the Growth Conditions of *Gluconacetobacter xylinus* to maximize the Yield of Bacterial Cellulose	Published, Carbohydrate Polymers 2012 89:613	Planning, researching, carrying out experiments, data analysis and writing, 90%
4	Bacterial Cellulose Growth from Media containing Ionic Liquids composed of Choline Salts	Unpublished	Planning, researching, carrying out experiments, data analysis and writing, 70%
4	*In situ* Modifications to Bacterial Cellulose by the Water Insoluble Polymer Poly-3-hydroxybutyrate	Published, Carbohydrate Polymers 2013 92:1717	Planning, researching, carrying out experiments, data analysis and writing, 85%
6	Harvesting Fibrils from Bacterial Cellulose Pellicles and Subsequent Formation of Biodegradable Poly-3-hydroxybutyrate Nanocomposites	Published, available online, Cellulose, DOI 10.1007/s10570-014-0415-z	Planning, researching, carrying out experiments, data analysis and writing, 75%
7	Biodegradability of Poly-3-hydroxybutyrate/Bacterial Cellulose Composites Measured via Evolution of Carbon Dioxide, Spectroscopic and Diffraction Methods	Unpublished	Planning, researching, carrying out experiments, data analysis and writing, 70%

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed:

Date:

Publications

Journal Articles

D. R. Ruka, G. P. Simon, K. M. Dean, Altering the growth conditions of Gluconacetobacter xylinus to maximize the yield of bacterial cellulose, Carbohydrate Polymers 89 (2012) 613-622.

D. R. Ruka, G. P. Simon, K. M. Dean, *In situ* Modifications of Bacterial Cellulose by the Water Insoluble Polymer Poly-3-hydroxybutyrate, Carbohydrate Polymers 92 (2013) 1717-1723.

D. R. Ruka, G. P. Simon, K. M. Dean. Harvesting Fibrils from Bacterial Cellulose Pellicles and Subsequent Formation of Biodegradable Poly-3-hydroxybutyrate Nanocomposites, Cellulose, DOI 10.1007/s10570-014-0415-z

Conference Proceeding

D.R. Ruka, G.P. Simon, K.M. Dean, Living Polymers: Poly-3-hydroxybutyrate/Bacterial Cellulose Composites, EUROTEC® 2013 - Proceedings of the 2nd European Technical Conference & Exhibition, Lyon, France, July 4-5, 2013. Society of Plastics Engineers, ISBN 978-0-9850112-2-2, pp. 233-237.

Book Chapter

D.R. Ruka, G.P. Simon, K.M. Dean, Bacterial Cellulose and its use in Renewable Composites, Nanocellulose/Polymer Nanocomposites: Fundamental and Applications, (2015) Edited by Vijay Kumar Thakur, Scrivener Publishing, ISBN 978118871904

Acknowledgements

I would like to thank my supervisors, Prof. George Simon and Dr. Katherine Dean, for their support throughout my PhD. They were sources of inspiration and assistance in planning the direction of the work, and assisting with editing. Thank you particularly to Katherine who helped give the publications greater impact with the additional material she suggested and worked on.

I would like to thank the CSIRO Office of the Chief Executive for the Julius Career Award, which provided the funding to carry out this work.

I would like to acknowledge several researchers at CSIRO who assisted with training me in the use of various equipment and assisting with experiments. Thank you to Dr. Parveen Sangwan, Dr. Cameron Way and Mr. Steven Petinakis for helping guide me through all the processes at CSIRO. Thanks also to Dr. Liz Goodall of the characterization lab at CSIRO, who completed all the initial XRD work and patiently explained the process and results to me, and to Dr. Aaron Seeber who assisted with later XRD work. Thanks to Mr. Mark Greaves who completed SEM on some samples and to Ms. Zoé Durrenberger, who took weekly samples of my materials out of the bioreactor so that I could repeat analysis experiments on degraded materials. In addition, thanks to Dr. Xiaoqing Zhang of CSIRO, who acted as my examiner for all the milestone requirements of my PhD, reading the reports and being on the panel.

Thanks to Monash Centre of Electron Microscopy staff Dr. Flame Burgmann, Dr. Xiya Fang and Dr. Matthew Field, who assisted with training me to use the equipment, and assisting with any microscopy problems that I had, and thanks to Dr. Wade Mosse and Dr. Jana Habsuda for training and assisting me in the use of the cryo-grinder so that I could grind my samples to powder.

Thanks to researchers who collaborated with me on papers, Prof. Douglas MacFarlane and Mr. Vijay Ranganathan from the School of Chemistry and Dr. Christopher Garvey of ANSTO.

A very special thanks to Ms. Jane Moodie of the Faculty of Engineering, who read over massive sections of my thesis in an extremely methodical manner, and patiently explained writing concepts to help make my writing better.

I need to express enormous gratitude to my family, particularly my husband Tum, who theorised what he referred to as the 'rollercoaster of study' where he could almost predict my moods. Thank you for supporting me while I worked through this. To my two wonderful children, Marshall and Carter, thank you for eventually settling into sleeping routines and for coping with a sometimes very grumpy mum, and thanks again to Tum for being such a pro-active father so that I had the chance to work on my thesis when I needed to. Thank you to my mother, Christine, for continuing to be so proud of me, and for providing practical advice throughout both my candidature and my entire life. Thanks to my friends for their support, for lending a kind ear to my hardships, particular Caroline and Nicole, for their constant support, and Kate for enduring last minute questions on the necessity of including or excluding additional commas.

Abbreviations

ATP	adenosine triphosphate
BC	bacterial cellulose
CAB	cellulose acetate butyrate
Cel-	cellulose negative bacterial mutant cells
CMC	carboxymethyl cellulose
CNW	cellulose nanowhiskers
CSL	corn steep liquor
DHP	dihydrogen phosphate
DMAc	*N,N*-dimethylacetamide
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
EVOH	ethylene vinyl alcohol
FTIR	Fourier transform-infra red
FWHM	full width at half maximum height
HPMC	hydroxypropylmethyl cellulose
HS	Hestrin-Schramm media
HS-BC	Bacterial cellulose grown in Hestrin-Schramm media
HS-PHB	Hestrin-Schramm media containing dispersed poly-3-hydroxybutyrate
ICDD	International Centre for Diffraction Data
MC	methylcellulose
MCC	microcrystalline cellulose
Mod-BC	Bacterial cellulose produced in HS-PHB media

MWCNT	multiwalled carbon nanotube
NMR	nuclear magnetic resonance
PEG	poly(ethylene glycol)
PEO	poly(ethylene oxide)
PF	paraformaldehyde
PHA	poly-hydroxyalkanoate
PHB	poly-3-hydroxybutyrate
PHBV	poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PHV	poly-3-hydroxyvalerate
P(3HB-co-4HB)	poly(3-hydroxybutyrate-co-4-hydroxybutyrate)
PLA	poly(lactic acid)
PVA	poly(vinyl alcohol)
SEM	scanning electron microscopy
TEM	transmission electron microscopy
UDP	uridine diphosphate
XRD	X-ray diffractometry
Yam-BC	Bacterial cellulose grown in Yamanaka media

Chapter 1
Introduction

1.1 Problem Statement

We live in an age where technologies are constantly changing and progressing. We are able to construct huge buildings and microscopic machines, and take advantage of a wide range of synthetic materials that have been developed over a number of years. Synthetic polymers, such as plastics, are such materials and are universally used for a wide range of purposes and products. Some plastic materials are used for packaging, often only for a single purpose and for a short period of time, after which the plastic is disposed of, typically in landfill.

We are becoming increasingly aware of the impact that the use of plastic materials has on the environment. While these types of polymers have properties that make plastics attractive as packaging materials – strength, barrier and protective properties, ease of production and low cost – they also have properties that make them undesirable for use as products in an increasingly environmentally-aware society. These traditional, synthetic plastics are produced using petroleum, making their long-term production unsustainable. In addition, plastics have properties, including high molecular weight, that make them unable to break down, causing them to persist in landfill long after disposal (Huang et al., 1990). As a result, there is a need to develop materials that are sustainable to replace these polymers. Natural materials that could be produced from renewable sources and that could break down after use, becoming part of a cyclic and sustainable process, are therefore desirable.

There are variety of natural polymers produced in the environment by organisms such as plants and bacteria. Plants can produce materials such as poly(lactic acid), starch, cellulose and various vegetable oils, whereas bacteria are known to produce materials such as glycogen, xanthan, alginate, dextran, cellulose, cyanophycin and poly-hydroxyalkanotes. In nature, the organisms that produce these materials use them for a range of purposes, such as for structure, protection, attachment and nutrients, but given the specific properties of these materials, it may be possible to adapt them for specific uses, for example as biodegradable packaging materials to replace synthetic plastics.

While natural materials have advantages of being sustainable and biodegradable, there are also disadvantages with their use. Currently, the cost of producing natural materials is high, due to the cost

of the production methods and resources used; for example, molecules such as poly-3-hydroxybutyrate (PHB) can be chemically synthesised using biological precursors, however this process is particularly expensive. It has been estimated that PHB production, which requires the precursor (R)-3-hydroxybutyryl-coA and PHB synthase, costs US$286,000 per gram of PHB, whereas PHB production in bacteria costs US$0.0025 per gram, which is still unfortunately 5 – 10 times more expensive than the cost of producing traditional polymers (Rehm, 2010). However, with continuing increases in oil prices, along with dwindling resources, it is likely that natural materials will become more competitive in terms of cost. Production costs are also likely to be improved with additional research, leading to new, more cost effective ways of producing large amounts of such materials. In addition, there are some problems with natural materials due to their properties. For example, polysaccharides are inherently polar and hydrophilic, with high moisture absorption that leads to swelling and often results in poor mechanical properties (Mensitieri et al., 2011; Yang et al., 2007). However, some of these limitations have been overcome by research into biocomposites and biofibres as, for example, modifications of the surface of biofibres have been shown to improve mechanical properties (Mohanty et al., 2000). Thus it is clear that these natural polymers have great potential as materials to replace traditional plastics in industries such as packaging.

Natural materials are similar to synthetic materials in that they can be used to develop composites. Composites are structural products made up of two or more materials whose engineering performance exceeds those of the individual components (Pommet et al., 2008). They generally consist of a matrix with fibres used as reinforcement. Composites can be made up of synthetic and/or natural biomaterials and biofibres, with those consisting of natural materials taking advantage of the biodegradable nature of such materials. However it is necessary for all components of a composite to be biodegradable if a composite is expected to completely break down in a degrading environment. In addition, to achieve good properties in a composite, it is necessary to combine the materials in such a way as to achieve well dispersed homogeneous materials. It is also necessary to have good interfacial adhesion between the materials so that they do not separate at the interface when placed under mechanical strain.

While a number of polymers are produced in plants, there are several reasons why using bacteria to produce materials is beneficial. Bacteria can easily be grown in a laboratory setting to produce small amounts of polymer for initial testing phases. They are relatively fast growing and can be used to grow multiple cultures simultaneously, providing ideal conditions for experimental investigation. Additionally, the production of bacterial cultures can be upscaled to industrial levels, using continuous cultures or large bioreactors to grow bacteria. Using bacteria rather than plants also removes the controversy behind using potential food sources for other purposes, which is of particular interest in developing countries where food sources are scarcer.

Cellulose is a material that is of much interest in material science based on its availability and properties. It has high strength and biodegradability. Cellulose is the most abundant biopolymer on earth and exists mostly in the cell wall of plants; however cellulose is also produced by bacteria. Bacterial cellulose is produced in high amounts by the bacterial species *Gluconacetobacter xylinus*. It is chemically identical to plant cellulose, but it has some advantages in that it is extremely pure and it exists naturally as nanosized fibres. This means that, unlike plant cellulose, it does not need to undergo further purification steps or treatments to obtain pure nanosized fibres.

Based on its properties, bacterial cellulose is a good candidate for use in composites as a reinforcing phase. It is highly crystalline and has a large surface area due to its nanosized fibres. This indicates that only a small amount of bacterial cellulose may need to be included in composites to confer this strength. As it is biological in nature and biodegradable, if bacterial cellulose was to be used in conjunction with a biodegradable matrix, the resulting composite would be entirely biodegradable. PHB is a bioplastic that has similar properties to polypropylene and thus has been hypothesised as a material that could eventually replace traditional plastics (Holmes, 1985; King, 1982). However, it is very stiff and brittle and is likely to be able to benefit from use in composites with an appropriate reinforcing filler material to improve its strength (Gatenholm et al., 1992). Therefore, this material was selected as a matrix to be investigated in composites with bacterial cellulose.

To develop biodegradable composites from natural materials with superior properties, it is necessary to investigate all aspects of the production of the materials. Understanding the process of biopolymer synthesis will allow us to regulate these processes and aid in the specific design of biopolymers with good material properties (Rehm, 2010). This demonstrates the importance of investigating cultivation conditions and the resulting structure and properties of biopolymers such as bacterial cellulose. The challenge, however, is to design composite materials that exhibit structural and functional stability during storage and use, but degrade in an appropriate timeframe once they have been discarded (Mohanty et al., 2000).

1.2 Aim and Scope

The aim of this work is to use bacterial cellulose as a reinforcing material in a biodegradable matrix to achieve a biodegradable composite with properties better than those of the matrix alone. In order to achieve this, there are several other aims involving the production and modification of bacterial cellulose, and evaluation of different methods used to produce composites.

The specific aims of this project thus are:

- to investigate the effects of cultivation conditions on the yield, structure and morphology of bacterial cellulose
- to identify ways to modify bacterial cellulose to achieve materials with specific properties in an attempt to “tailor-design” the cellulose for purposes such as reinforcing selected matrix materials
- to achieve and compare methods to obtain composites of PHB and bacterial cellulose, and evaluate the viability of these methods to produce useful biodegradable blends
- to establish the biodegradation characteristics of selected biomaterials and blends.

1.3 Overview of the Study

This thesis presents the results of research in the form of five journal publications (three published papers and two unpublished papers), accompanied by an introduction, a literature review comprised

mostly of an accepted book chapter, and a conclusions chapter. In addition, there is a results chapter that does not contain any publications (Chapter 5).

Together with this introduction, the literature review chapter describes the problem with unsustainable materials that end up as waste in landfill and do not degrade, and also the work that has previously been done with regards to bacterial cellulose. The first publication (*Bacterial cellulose and its use in renewable composites*) forms part of this literature review.

The subsequent publications make up the results chapters. The second publication, *Altering the growth conditions of* Gluconacetobacter xylinus *to maximize the yield of bacterial cellulose*, introduces bacterial cellulose as a product of *Gluconacetobacter xylinus* growth in laboratory culture, and researches factors that affect the production and characteristics of the cellulose. The third and fourth publications, *Bacterial cellulose growth from media containing ionic liquids composed of choline salts*, and, In situ *modifications to bacterial cellulose with the water insoluble polymer poly-3-hydroxybutyrate*, describe methods by which modifications to bacterial cellulose can be achieved by incorporating additives in the production media. The fifth publication, *Harvesting fibrils from bacterial cellulose pellicles and subsequent formation of biodegradable poly-3-hydroxybutyrate nanocomposites*, describes the production of composites using bacterial cellulose as a reinforcing phase with PHB as the matrix by way of dispersing bacterial cellulose fibrils in a solvent by sonication. The sixth publication, *Biodegradability of poly-3-hydroxybutyrate/bacterial cellulose composites under aerobic conditions measured via evolution of carbon dioxide, spectroscopic and diffraction methods*, examines the degradation of these materials in compost. Additional work describing the development and evaluation of blending conditions to produce PHB/bacterial cellulose composites is included in Chapter 5. Conclusions based on this research and recommendations future work are presented in Chapter 8.

Published papers are included in their PDF format. Accepted and unpublished manuscripts are presented in a formatted version for consistency with the main thesis.

Chapter 2
A Review of the Literature

2.1 Preface

There has been a significant amount of work done about the microbiological nature of bacterial cellulose, with many papers published relating to the growth of the bacteria and production of cellulose. By contrast, there have been few studies of the use of bacterial cellulose in composites, particularly as a reinforcement material. However an increasing number of studies published in recent years have been carried out using a range of methods to obtain a dispersion of bacterial cellulose in various matrices. These studies have focused on this material in composites often to either examine bacterial cellulose in impregnated or compressed composites, or as a dispersed reinforcement material achieved by methods such as solution or melt blending. This work is reviewed in Section 2.2.

In addition, this chapter presents background information on poly-3-hydroxybutyrate as a promising matrix material. Plant cellulose composites are also examined based on the potential to apply this work to bacterial cellulose. This work is presented in Section 2.3.

2.2 Bacterial Cellulose and its use in Renewable Composites

A review manuscript that has been accepted as a book chapter is presented here. In this review, different processes for producing bacterial cellulose are examined, as well as techniques used to produce composites, and resulting properties of these composites. The biodegradability of bacterial cellulose composites is also discussed.

This review has been formatted in order to ensure consistency with the thesis structure. Figures have been re-numbered to include the thesis chapter number. A table of contents is also included.

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conducting a critical review of the literature	90

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
George Simon	Providing supervision, proof reading and editing	N/A
Katherine Dean	Providing supervision, proof reading and editing	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	[redacted]	13/5/14
Main Supervisor's Signature	[redacted]	13/5/14

Bacterial Cellulose and its use in Renewable Composites

Dianne R. Ruka[1, 2], George P. Simon[2], Katherine M. Dean[1]

[1]CSIRO Materials Science & Engineering, Clayton, Australia

[2]Department of Materials Engineering, Monash University, Clayton, Australia

Abstract

Bacterial cellulose is a very pure form of cellulose that has high strength, is composed of nanosized fibres and is very hydrophilic. It has been suggested that this material could be used as a biodegradable filler in fully biodegradable composites. There are a wide range of methods that have been used to produce and modify bacterial cellulose, allowing the potential to achieve specific properties. There have been some reports of bacterial cellulose used in composite materials, however it is often difficult to achieve even dispersions of cellulose fibres and thus its use as a filler in composites has been limited. The discovery of ionic liquids that are capable of dissolving this typically insoluble material may allow the development of more possible processing options. Therefore further investigations into all aspects of this material are necessary.

Keywords

bacterial cellulose, nanocomposites, biodegradability, modifications

Contents

Introduction

Traditional synthetic polymers are currently used in a wide range of products and in many applications. These polymers have often superseded the use of other materials such as metals, glasses, ceramics and wood, in particular in relation to the packaging industry (Mohanty et al., 2000). The major synthetic polymer classes – polyethylene, polypropylene, polystyrene, poly(ethylene terephthalate) and poly(vinyl chloride) – are used in a vast number of applications such as films, flexible plastic bags and rigid containers (Amass et al., 1998; Mohanty et al., 2000). They have favourable properties such as being light, strong, chemically inert and inexpensive to produce. However some of their other properties also cause considerable environmental problems, with their high molecular weight, chemical stability and relatively low surface area-to-volume ratio making them resistant to degradation by microbial attack, and causing them to persist in the environment long after disposal (Huang et al., 1990). This persistence leads to problems with litter and affects animal populations, with many animals ingesting or being strangled by such waste (Jayasekara et al., 2005). It has been reported that 57.1 million tons of packaging waste was produced in 2009 (EPA, 2011) and 76 million tons in 2010 (Eurostat, 2012) in Europe and America, respectively. Not only does this cause a significant contribution to the amount of rubbish in landfills, but it also results in the generation of greenhouse gas and contaminants (Kale et al., 2007). In addition, these polymers are produced by oil-based technology, which raises a number of pertinent issues related to increasing oil prices, and dwindling resources, so the impetus to replace these polymers with renewable materials is increasing. In order to conserve resources, and avoid increased carbon emissions, materials must be developed that consume less energy and use raw materials that are derived from renewable resources. An added benefit of natural polymeric materials is that they are likely to be biodegradable and thus, if appropriately treated, would not contribute to landfill waste. The ideal situation would be to develop completely sustainable materials – using renewable sources to produce materials such as plastics that would be able to quickly break down after use, only to be reabsorbed and reused in a holistic process. This would be important for the environment in the issue of plastic bags and other debris, for

example, being swept out to sea through sewerage outflows and becoming damaging to the ocean ecosystems.

Natural polymers, or biopolymers, are polymers that are produced from renewable sources. They may be produced by biological systems such as plants or animals, or be chemically synthesised from biological materials (Flieger et al., 2003). It is also desirable to make use of natural materials which do not, for example, compete with the food chain. The use of starch from a variety of food sources is increasingly being investigated and commercially-exploited as new polymeric materials, yet a debate arises as to how this fits within the food profile of (in particular) developing countries, where much of the arable land is required to grow food stuffs, rather than crops for plastic. Therefore other sources of natural materials should be sought, and bacterially-derived polymers represent such an option.

Biopolymers are biodegradable and also often biocompatible. A biodegradable polymer can be defined as a material in which degradation results from the action of microorganisms such as bacteria, fungi and algae (Stevens, 2002). Therefore the use of biopolymers to replace synthetic polymers is attractive due to their obvious environmental advantages of being sustainable, renewable and biodegradable, being broken down into carbon dioxide and water when exposed to microbial flora. Most biodegradable polymers are thermoplastics such as poly(lactic acid) (PLA), poly-hydroxyalkanoate (PHA) and poly(vinyl alcohol) (PVA), or plant-derived polymers such as starch and cellulose (Lucas et al., 2008).

As with many polymers, synthetic or otherwise, the incorporation of a second phase to produce a composite can lead to improved properties (Zhu et al., 2006). The incorporation of a fibrous phase into a plastic can increase modulus and strength, whilst often also improving fracture toughness, the latter case being of particular importance in brittle matrices. Increasingly in synthetic polymer science and technology, additives of a nanosize scale are being used such as carbon nanotubes or nanowhiskers of ceramics or metals. Some naturally-occurring reinforcing materials are also now being investigated such as layered silicates (such as montmorillonite), or even naturally-occurring clay nanotubes such as halloysite (Paul & Robeson, 2008; Ray & Okamoto, 2003). The smaller size of

particles ensures a high surface area (and thus greater improvements for a lower concentration than using conventional fillers), whilst the high aspect ratio can allow improved stress transfer to the reinforcing phase and improved properties. Naturally-occurring polymers are no different, in that they also can benefit from improvement by addition of another phase. Poly-3-hydroxybutyrate (PHB), one such example, is by itself quite brittle, but could benefit from a second, reinforcing phase.

More desirable than incorporating synthetic nanoadditives (such as carbon nanotubes), or non-degrading naturally-occurring additives (such as nanoclays and halloysite), is the idea of using naturally-forming, degradable nanofibrous reinforcing materials. Bacterial cellulose, sometimes referred to as BC (Iguchi et al., 2000), is such a material and its incorporation into a naturally-derived, biodegradable matrix would make for a fully degradable composite.

In this review we will overview bacterial cellulose – its structure, growth conditions, surface modification possibilities (important when trying to improve interfacial adhesion to a matrix), and also its use to date in composites. We also focus on production and properties of bacterially-derived cellulose, not least because its nanofibrillar form means that it can be used as a reinforcing phase in other natural polymers, thereby creating fully degradable composites potentially with improved properties.

Cellulose Properties and Production

Introduction to Cellulose

Cellulose is a material of interest because it has the properties to make it a good reinforcing agent in composites. It is the most abundant biopolymer on earth, mostly existing in the cell wall of plants. It is composed solely of glucose molecules, linked by β-1,4 glucosidic bonds (Figure 2.1). The chains of unbranched glucose give rise to extended fibrillar structures due to the high number of free hydroxyl groups that result in extensive intra- and inter-molecular hydrogen bonding between adjacent chains (Ross et al., 1991). It is this structure that leads to its desirable features and the ability to modify the surface, giving this material the potential to be used as a reinforcing material in biodegradable composites.

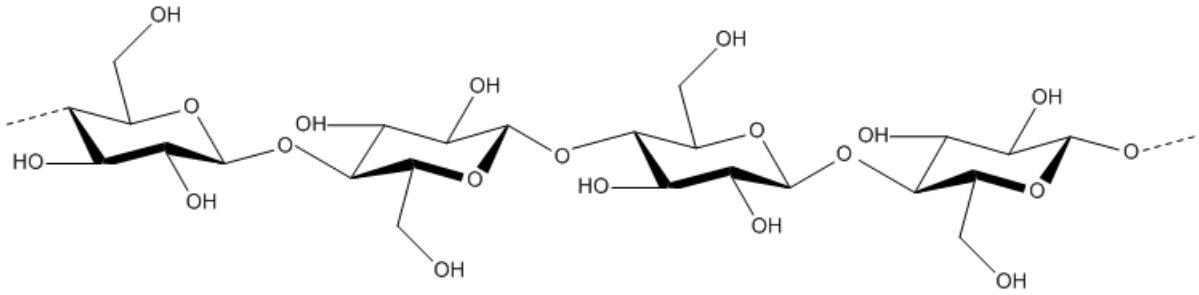


Figure 2.1: Typical structure of cellulose.

Cellulose is typically obtained from wood, but is also produced in other plants such as hemp, flax, jute, ramie and cotton. In addition to its production in plant cell walls, it is made by microorganisms such as bacteria.

There are two common crystalline forms of cellulose, cellulose I and cellulose II. Cellulose I is found in nature and is composed of parallel chains (Delmer, 1987). Cellulose II, the more stable form, is composed of antiparallel chains. There are two distinct allomorphs of cellulose I, I_α and I_β (Atalla & Vanderhart, 1984). The crystal structures of these molecules have been determined (Nishiyama et al., 2002; Nishiyama et al., 2003). The allomorph I_α has 1-chain triclinic unit cell and I_β has 2-chain monoclinic unit (Kono et al., 2002). Cellulose I_α is metastable and is readily converted to I_β. The ratio of cellulose I_α and I_β produced in nature depends on the organism producing it. For example, bacterial cellulose is composed of approximately 70% cellulose I_α, whereas other organisms are I_β rich (Atalla & Vanderhart, 1984). The mechanical properties of sheets prepared from bacterial cellulose are due to this nano-scalar network structure.

For plant cellulose to be used as a pure material, it needs first to be separated away from the hemicelluloses, lignin and pectin that it exists with naturally in the plant cell wall. In addition to this purification, nanofibrillar cellulose can be obtained by treating the cellulose in a number of ways, such as mechanical pulping, chemical pulping, homogenisation, acid hydrolysis, steam explosion and high intensity ultrasonication (Khalil et al., 2012). Each of these processes leads to different types of nanofibrillar materials. However, bacterial cellulose naturally exists as very pure nanosized fibrils and does not need additional purification. Based on these benefits, along with its high crystallinity and

stiffness, bacterial cellulose has recently become the topic of a number of areas of research in sustainable materials.

Bacterial Cellulose

Bacteria are able convert different carbon sources into a diverse range of polymers with varying chemical and material properties such as glycogen, xanthan, alginate, dextran, cellulose, cyanophycin and PHA. While many biopolymers are made in plants, bacteria offer an ideal production organism for tailor-made biopolymers (Rehm, 2010). Bacteria are fast growing organisms that can be genetically-engineered to produce specific biopolymers. However, this is a developing field and methods to specifically manipulate molecules in order to obtain specific properties require further investigation. Bacterial cellulose is such a desirable material due to its purity, properties and crystalline structure, and there has therefore been a lot of interest in developing techniques to produce bacterial cellulose for various industrial applications.

Bacterial cellulose is typically produced by a bacterial species called *Gluconacetobacter xylinus*. This species was formerly known as *Acetobacter xylinum* (Yamada et al., 1997), but will be referred to as *G. xylinus* throughout this review. This species of bacteria produces high amounts of cellulose, and thus it has been thoroughly studied and used as a model organism in the examination of cellulose, although cellulose production also occurs in other bacterial species such as *Agrobacterium tumefaciens* (Deinema & Zevenhuien, 1971), *Escherichia coli* (Zogaj et al., 2003; Zogaj et al., 2001), *Pseudomonas* species (Ude et al., 2006), *Rhizobium* species (Napoli et al., 1975) and *Salmonella* species (Römling, 2002; Zogaj et al., 2001). Bacterial cellulose is chemically the same as plant cellulose, but it differs in that it is very pure, has a higher water holding capacity, higher degree of polymerisation and is composed of a random mesh of nanosized fibres (Klemm et al., 2006). Cellulose is initially composed of a single microfibril that is extruded from the bacterial cell, and then microfibrils are packed together into larger bundles. It has a highly ordered structure, however the microfibrils are not completely crystalline, existing as a semi-crystalline structure with both crystalline and amorphous regions.

Structure

Transmission electron microscopy (TEM) of cellulose-producing bacterial cells shows the presence of pores in the outer membrane of these cells. It is believed that bacterial cellulose is produced from uridine diphosphate (UDP)-glucose (its immediate precursor) in the cytoplasmic membrane, and is extruded from the pores as microfibrils of approximately 1.5 nm in width (Ross et al., 1991). The microfibrils aggregate into ribbon-shaped fibrils approximately 40 nm in width (Hirai et al., 1998).

Bacterial cellulose forms as a thick mat, called a pellicle, at the air/surface interface when grown statically in liquid culture (Figure 2.2) (Czaja et al., 2006; Schramm & Hestrin, 1954). The pellicle is composed of randomly associated fibrils from the cells in the culture (Ross et al., 1991). It is believed that cellulose production is roughly proportional to cell growth. However, when cultures are agitated and aerated, cell growth increases and cellulose production decreases. It has also been found that shaken and agitated cultures result in macroscopic changes to the cellulose. Rather than forming as a smooth pellicle on the surface of the culture, the cellulose can accumulate as spherical pellets (Figure 2.3) (Czaja et al., 2004; Schramm & Hestrin, 1954). Microscopic differences in the cellulose also occur depending on the culture conditions, as described below. It is likely that the increased exposure to oxygen under agitated conditions causes the cellulose production to become redundant, as it is believed the purpose of the cellulose pellicle is to anchor the bacterial cells to the surface in order to obtain sufficient oxygenation.

Figure 2.2: Bacterial cellulose pellicle grown across the top of liquid culture from Czaja et al. (2006). Reprinted with permission from Elsevier.

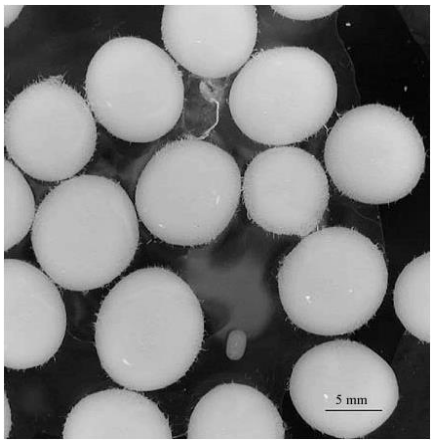


Figure 2.3: Bacterial cellulose pellets grown in agitated culture from Czaja et al. (2004). Reproduced with permission from Springer. Scale bar is 5 mm.

Properties and Methods of Characterisation

There are a number of common techniques that researchers use to characterise bacterial cellulose. Whilst this is by no means a comprehensive list, these techniques do provide a good indication of alterations that may have occurred during the cultivation of the bacteria, as cellulose polymerisation and crystallisation are closely coupled processes, and changes to the cultivation conditions can change the structure and morphology of the resulting cellulose (Benziman et al., 1980).

Scanning electron microscopy (SEM) is a common method used to observe both the surface and cross-section view of bacterial cellulose. It shows a network of random, interwoven, nanosized fibrils. TEM is less commonly used for this purpose. Figure 2.4 demonstrates the interwoven fibrils as shown by SEM and the microfibril extruding from the cell membrane by TEM (Krystynowicz et al., 2002).

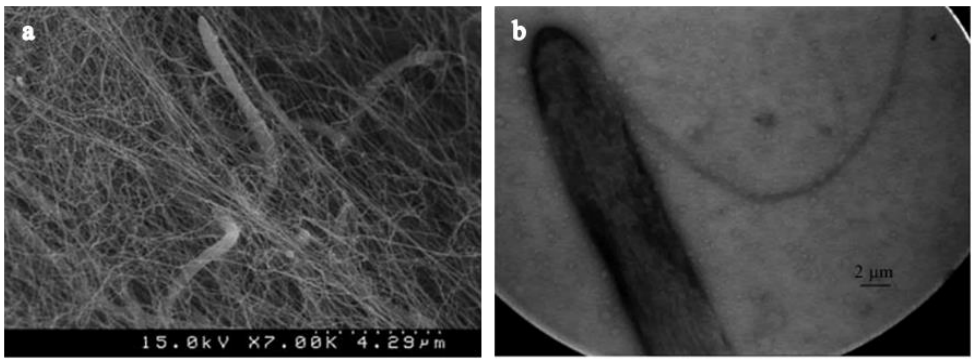


Figure 2.4: SEM (a) and TEM (b) of bacterial cellulose structure, from Krystynowicz et al. (2002). Reproduced with permission from Springer.

Nuclear magnetic resonance (NMR) and Fourier transform-infra red (FTIR) spectroscopy have been common techniques used to confirm that the material produced in bacterial culture is bacterial cellulose and to determine the I_α content of the sample. In addition, Yamamoto et al. (1996) used NMR results to determine that the cellulose I_α content be calculated by the relationship between the FTIR absorbance at peaks 750 and 710 cm^{-1}. The changes in the peaks due to additional materials with the cellulose could be due to any material present in the media, typically an additive not specifically required for cell growth. The changes to the cellulose may result from changes within the chemical bonds of the cellulose itself.

X-ray diffractometry (XRD) of bacterial cellulose samples allows for the calculation of crystallinity and crystallite sizes of the resulting cellulose, however various researchers use different peaks for these calculations (Czaja et al., 2004; Watanabe et al., 1998b; Yamamoto et al., 1996) which can result in large differences between the calculation of these values. Crystallinity has also been reported to be determined by Fourier transform Raman spectroscopy (Schenzel et al., 2005). Based on the variation of values obtained from different methods, care should be taken when comparing crystallinity and crystallite sizes between different reports in the literature.

Some researchers complete additional characterisations such as water holding capacity, and there are also reports in the literature of mechanical tests done on bacterial cellulose, resulting in calculations of Young's modulus and tensile strength (Cheng et al., 2009b; Jung et al., 2010b; Klemm et al., 2006; Nishi et al., 1990; Watanabe et al., 1998b; Yamanaka et al., 2000). These are often undertaken in order to compare the cellulose used in a composite to a matrix material alone.

Growth Conditions

There are a number of different methods that can be used to grow bacterial cellulose, however the low productivity and high cost of production of this form of cellulose has been problematic, especially if the production of this cellulose is to be upscaled for commercial applications. Several groups have examined media composition and cultivation conditions on different species and strains of *Gluconacetobacter*, often with differing results, to determine the optimal growing conditions for high

yields of bacterial cellulose. These studies, and the optimal yields of cellulose achieved as part of the testing conditions, are summarised below. Note that maximising the yield of cellulose is the predominant factor considered here, while the effects on the structure of cellulose are described in the *In situ* Modifications Section. The concentrations described in media are given in wt% or vol%, where appropriate.

Base Media

Growth of *Gluconacetobacter* has traditionally been in a complex (and expensive) medium defined by Hestrin and Schramm in 1954 (Hestrin & Schramm, 1954). This HS medium, composed of 2% glucose, 0.5% yeast extract, 0.5% peptone, 0.27% Na_2HPO_4 and 0.115% citric acid monohydrate, with a pH between 4.0 and 6.0, has been used to grow *Gluconacetobacter* under static conditions at temperatures between 25° C and 30° C. As previously stated, the pellicle produced from these conditions is believed to draw the bacterial cells to the surface, as *Gluconacetobacter* is an obligate aerobe (Cook & Colvin, 1980; Valla & Kjosbakken, 1982). As cellulose is formed at the upper-most air-layer, the older cellulose is pushed down into the media as newly formed cellulose is produced (Czaja et al., 2007). Spherical pellets can be achieved when grown under agitated conditions (Schramm & Hestrin, 1954).

Another culture medium that has been used for the cultivation of *Gluconacetobacter* was developed by Yamanaka et al. (1989). Yamanaka medium consists of 5% sucrose, 0.5% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.3% KH_2PO_4 and 0.005% $MgSO_4.7H_2O$ with a pH of 5.0. Corn steep liquor (CSL)-Fructose medium has also been used in cellulose production. CSL-Fructose medium consists of 20 ml CSL, 40 g fructose, 3.3 g $(NH_4)_2SO_4$, 14.7 mg $CaCl_2.2H_2O$, 1.0 g KH_2PO_4, 3.6 mg $FeSO_4.7H_2O$, 2.42 mg $Na_2MoO_4.2H_2O$, 250 mg $MgSO_4.7H_2O$, 1.73 mg $ZnSO_4.7H_2O$, 1.39 mg $MnSO_4.5H_2O$, 0.05 mg $CuSO_4.5H_2O$, 2 mg inositol, 0.4 mg niacin (nicotinic acid), 0.4 mg pyridoxine HCl, 0.4 mg thiamine HCl, 0.2 mg Ca pantothenate, 0.2 mg riboflavin, 0.2 mg *p*-aminobenzoic acid, 0.002 mg folic acid, 0.002 mg biotin in 1 litre of distilled water with a pH of 5.0 (Toyosaki et al., 1995).

Carbon Sources

Using glucose as the carbon source in media for growing *Gluconacetobacter* is not only expensive, but is likely not optimal for cellulose production. Glucose is oxidised to gluconic acid and the formation of gluconic acid causes a decrease in pH, which can inhibit cellulose production (Masaoka et al., 1993; Schramm et al., 1957). There are other carbon sources do not produce gluconic acid and thus do not lead to the unfavourable decrease in pH. In light of this, over the past 15 years, there has been much research activity involving investigation of the amount of cellulose produced by *Gluconacetobacter* grown in different media, often simply by substituting the carbon source in previously described media (for example, HS medium, CSL-fructose medium). 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, succinic acid, sucrose, trehalose, and xylose have been investigated (El-Saied et al., 2008; Hutchens et al., 2007; Jung et al., 2010a; Keshk & Sameshima, 2005; Kim et al., 2006; Masaoka et al., 1993; Mikkelsen et al., 2009; Nguyen et al., 2008; Oikawa et al., 1995a; Oikawa et al., 1995b; Pourramezan et al., 2009; Ramana et al., 2000). Such investigations have a long history, with a study published by Tarr and Hibbert (1931) in which they used 25 different carbon sources to investigate effects on pellicle growth. They reported that fructose, glucose and mannitol were carbon sources that resulted high amounts of cellulose to be produced, a result confirmed by many more recent studies.

Jung et al. (2010a) recently investigated different carbon sources using *Gluconacetobacter* species V6, grown under shaking conditions. By substituting the carbon source in HS media for various alternatives, it was found that glycerol (with maximum cellulose production obtained at 3%) was the best carbon source, followed by glucose in terms of cellulose production, whilst lactose also showed a high cellulose yield.

Similar findings have been reported by Keshk and Sameshima (2005) who used *G. xylinus* ATCC 10245 in HS media, and reported that the optimal cellulose yield was obtained using glycerol, followed by glucose, fructose, inositol and sucrose. Masaoka et al. (1993) reported that the carbon sources for

optimal cellulose yield for *G. xylinus* IFO13693 were glucose, fructose and glycerol. However, unlike other studies, glucose obtained a higher yield than the other carbon sources.

Mikkelsen et al. (2009) reported that *G. xylinus* ATCC 53524 produced higher cellulose levels in a modified HS medium with glycerol instead of glucose, but even higher levels with sucrose. These levels were obtained after 96 hours of growth, whereas after 48 hours, mannitol was the best carbon source, followed by glucose, with sucrose and glycerol producing very little cellulose at this time. Mannitol was also reported to be the highest cellulose producing carbon source for *G. xylinus* strain K3 (Nguyen et al., 2008) and *G. hansenii* ATCC 10821 (Hutchens et al., 2007) in HS media.

Optimal levels of cellulose were produced in an HS medium with sucrose by Pourramezan et al. (2009) using *Gluconacetobacter* species 4B-2. These authors also reported high levels of cellulose using glucose, xylose and lactose, whereas Ishihara et al. (2002) reported very little cellulose production when using xylose, using 17 different bacterial strains.

Sucrose, glucose and mannitol were reported to be the most suitable carbon sources for *G. xylinus* to achieve optimal production of cellulose using Yamanaka media (Ramana et al., 2000). Kim et al. (2006) devised an optimised medium composition using *Gluconacetobacter* species RKY5. Initially, using different carbon sources in HS media, they reported that the highest levels of cellulose were produced using glycerol, fructose and sucrose, with slightly lower levels from glucose and lactose.

Oikawa et al. (1995a; 1995b) have reported that arabitol and mannitol both produce higher levels of cellulose when used in the place of glucose. They found that cellulose production increased six-fold and three-fold by arabitol and mannitol respectively with *G. xylinus* KU-1.

In an attempt to find a cheap carbon source, some groups have used molasses instead of the other monosaccharides and disaccharides described. However the inclusion of molasses as a carbon source has been shown to produce mixed results in terms of cellulose yield. Sugar cane molasses was substituted as the carbon source in an HS medium in a study by Keshk and Sameshima (2006b) using six strains of *G. xylinus*. All strains showed an increased level of cellulose production in the sugar cane

molasses media, compared to glucose as the carbon source. Premjet et al. (2007) confirmed these findings and aimed to determine the essential ingredient of the sugar cane molasses using *G. xylinus* ATCC 10245. By adding various components to media containing different combinations of carbon sources, they found that the black colour substance component was the most effective component of sugar cane molasses in increasing the production of cellulose.

Bae and Shoda (2004) attempted to improve the production of bacterial cellulose using molasses as the carbon source, but treated the molasses to remove the heavy metals and minerals which can inhibit microbial growth. They applied a H_2SO_4–heat treatment to the molasses before adding it to their CSL medium (in the place of fructose). They reported that in CSL-treated molasses medium, *G. xylinus* BPR2001 cellulose production was less than in CSL-fructose medium (but higher than the untreated molasses medium). El-Saied et al. (2008) investigated several different types of media, including media using the cost effective H_2SO_4-heat treated molasses. They reported that when molasses was substituted for glucose in a CSL medium, a slight increase in cellulose production occurred. However, when molasses was used instead of mannitol in another medium, slightly lower levels of cellulose were produced. In accordance with the results of Bae and Shoda (2004), the H_2SO_4-heat treatment of molasses increased the cellulose produced over the untreated molasses. They also determined that the optimal concentration of molasses was 17% (El-Saied et al., 2008). Jung et al. (2010b) used two different methods to treat the molasses, by an H_2SO_4 and a $Ca_3(PO_4)_2$ treatment method. They found that the $Ca_3(PO_4)_2$ treatment method was more effective in terms of producing cellulose, and that both methods were more effective than untreated molasses. They also showed that their CSL-molasses medium resulted in higher cellulose levels than complex media with different carbon sources.

Hong and Qiu (2008) have reported using hydrolysate of konjac powder, produced from the plant *Amorphophallus rivieri Durieu*, as an alternative carbon source in a medium containing 0.5% yeast extract and 0.3% tryptone. They found that using the hydrolysates instead of glucose, mannose and a

glucose-mannose mixture as the carbon source resulted in three, six and five times higher cellulose production, respectively.

Many different carbon sources have been investigated, with many differences reported regarding which carbon source provides the highest level of cellulose. However, the sources that are routinely reported as producing high levels of bacterial cellulose are glucose, fructose, sucrose and mannitol. It may be that these differences occur due to the different strains being used, or perhaps due to experimental error. It is apparent that other strains should be examined to determine what their optimal carbon sources are. Other conditions such as nitrogen sources, additives and cultivation conditions are also likely to impact the levels of cellulose produced.

Nitrogen Sources

In a manner similar to the investigations of different carbon sources, several different nitrogen sources have been examined to determine which combination gives optimal growth of bacterial cellulose. These sources include yeast extract, peptone, ammonium sulphate, beef extract, casamino acid, casein hydrolysate, glycine, malt extract, sodium glutamate, soybean meal, soytone, and tryptone (Jung et al., 2010a; Ramana et al., 2000). In addition, Dudman (1959) reported the use of an asparagine/glutamic acid mixture as an appropriate nitrogen source.

Jung et al. (2010a) investigated a wide range of nitrogen sources and determined that increasing amounts of yeast extract (up to 2%) yielded higher bacterial cellulose in *Gluconacetobacter* species V6, whereas other organic nitrogen sources actually decreased the amount of cellulose being produced. They found that cellulose was produced at the highest level when grown with 1.6% yeast extract. In a similar study investigating different nitrogen sources, Ramana et al. (2000) reported that cellulose production was maximised by the use of peptone, ammonium sulphate and casein hydrolysate. While yeast extract has traditionally been used as a nitrogen source and several papers have shown that increasing amounts (to a point) increases bacterial cellulose synthesis, yeast extract is economically unfavourable. Therefore, some groups have been experimenting with the use of CSL as an inexpensive alternative.

CSL is a rich source of nutrients. It contains a wide range of vitamins, minerals and carbohydrates (Kona et al., 2001), perhaps not all of which are necessary for cellulose production. CSL has been found to be a potential nitrogen source for *Gluconacetobacter*. CSL-fructose medium has been increasingly used for the cultivation of *Gluconacetobacter* over the past few years as the appeal for cheaper materials develops. Toyosaki et al. (1995) grew 412 strains of *Gluconacetobacter* in both static and shaken cultures using HS and CSL-fructose media. They determined that there was a difference in the amount of cellulose produced based on the type of media used to grow the cells, stating that high cellulose production occurred only in the CSL-fructose medium in shaking cultures (however no differences in the amount of cellulose were seen between the two different media under static conditions).

Son et al. (2001) concluded that CSL may be substituted for yeast extract in media, as media containing 0.5% CSL and 0.5% yeast extract showed similar levels of cellulose production, as did 0.5% polypeptone. This was also reported by Yang et al. (1998) who reported similar cellulose concentrations with 6% CSL medium and 4% yeast extract-HS medium.

A study by Matsuoka et al. (1996) found that CSL was the most suitable nitrogen source for cellulose production in *Gluconacetobacter xylinus* subspecies *sucrofermentans* BPR2001, over yeast extract, soytone and peptone. Similar findings were reported by Nguyen et al. (2008) who found that CSL at a concentration of 4% resulted in a higher cellulose yield than peptone, yeast extract, beef extract or malt extract.

Jung et al. (2010b) investigated a CSL-molasses medium, and examined different amounts of CSL on cellulose production. They found that CSL at a concentration of 4%, with 0.1% yeast extract and 0.7% polypeptone, produced the highest levels of cellulose. They also concluded that the addition of CSL buffers the pH, avoiding the drop in pH that is observed in glucose media with the accumulation of gluconic acid, and therefore is preferable for cellulose production. Alternatively it has been reported that 8% CSL is the optimal concentration for cellulose production (El-Saied et al., 2008).

CSL-fructose medium consists of many components (Toyosaki et al., 1995), however it seems that CSL confers an increase in the amount of cellulose produced simply by being substituted for other nitrogen sources in other media. It is likely due to its complex nature and inclusion of proteins, peptides and amino acids, that CSL may strengthen the buffering capacity (Noro et al., 2004), which together with its low cost makes this nitrogen source a favourable option for cellulose production.

Additives

While there are some nutrients required to be included in the media for bacterial cell growth (such as carbon and nitrogen sources), there are additional supplements that can be included. These additives are not essential for cell growth, and therefore cellulose production, however there are a variety of additives that have been found to stimulate cellulose production. For example, the use of ethanol as a sole carbon source in media has been shown to be ineffective. However when ethanol is included with a suitable carbon source such as glucose, it has been shown, in some cases, to increase cellulose production (Dudman, 1959; Krystynowicz et al., 2002; Naritomi et al., 1998a; Park et al., 2003; Son et al., 2001). The inclusion of 1.4% ethanol increased cellulose production approximately four-fold in *Gluconacetobacter* A9 (Son et al., 2001) in an optimised medium. Ethanol added at 1.0% to a fructose based medium increased cellulose production in *G. xylinus* subspecies *sucrofermentans* BPR3001A (Naritomi et al., 1998a). The same concentration of ethanol was reported to increase cellulose production in *G. xylinus* E_{25} (Krystynowicz et al., 2002) and *G. hansenii* PJK (Park et al., 2003), where they reported that the ethanol actually decreased *G. hansenii* cell growth, but also prevented the accumulation of the cellulose non-producing mutants that can arise in agitated culture (Schramm & Hestrin, 1954). However, Dudman reported that ethanol had no stimulatory effect on cellulose production but did increase cell growth under static conditions (Dudman, 1959).

Lignosulfonate has been used at a concentration of 1% to stimulate cellulose synthesis (Keshk & Sameshima, 2006a). Keshk and Sameshima (2006a) found decreased levels of gluconic acid and concluded that this decrease was responsible for the increase in cellulose yield. However, the same researchers found no significant difference in cellulose synthesis with the addition of lignosulfonate in

a sugar cane molasses medium (Keshk & Sameshima, 2006b). Premjet et al. (1994) reported that culture media containing high molecular weight lignosulfonate showed increased cellulose yields over the low molecular weight fraction and the whole "SANPEARL CP" commercial sulphite pulping waste fraction powder.

After investigating the effect of a range of additives on the cellulose production in *G. xylinus* ATCC 700178, Cheng et al. (2009a) concluded that the addition of carboxymethyl cellulose (CMC) to CSL-fructose medium led to the highest amount of bacterial cellulose among the tested conditions. Contradictory results were published by Tantratian et al. (2005), who reported that the addition of CMC decreased cellulose production, and Chao et al. (2001) reported that CMC did not enhance cellulose production. Cheng et al. (2009a) also reported that the addition of agar produced higher levels of cellulose than the control.

Chao et al. (2001) demonstrated the impact of agar, included at 0.1%, in CSL-fructose medium. Cellulose production in *G. xylinus* subspecies *sucrofermentans* BPR2001 was increased in the presence of agar when grown in an airlift reactor. Similarly, Bae and Shoda (2005) and Bae et al. (2004) obtained optimal cellulose yields when agar was included at 0.4%. The polysaccharide xanthan was reported to yield similar increases in cellulose production when added to culture grown in an airlift reactor, however when agar and xanthan were added to static cultures, decreases in cellulose production were observed (Chao et al., 2001).

The inclusion of 0.04% sodium alginate resulted in increased cellulose production from *G. xylinus* NUST4.1 in a glucose/sucrose-CSL medium (Zhou et al., 2007). However, when Cheng et al. (2009a) included 0.2% and 0.5% sodium alginate, they showed no increase in cellulose production. It is possible at these higher concentrations, sodium alginate inhibits cellulose synthesis, which is in accordance with the findings of Zhou et al. (2007).

Inclusion of other additives such as 1.25% lactate in CSL-fructose medium was reported by Naritomi et al. (1998b) as increasing cellulose yield, as well as cell growth in *G. xylinus* subspecies *sucrofermentans* BPR3001A. Lactate at 0.15%, with 0.005% methionine, was reported to increase

cellulose production in *G. xylinus* subspecies *sucrofermentans* BPR2001 (Matsuoka et al., 1996). Pyruvate, ethanol, aldehyde and acetate were also shown to stimulate cellulose synthesis in this study, but not to the same levels as lactate. Benziman et al. (1980) investigated polymerisation kinetics in *Gluconacetobacter* using the stilbene derivative, Calcofluor White ST. In this experiment, they found that Calcofluor White ST increased the rate of glucose polymerisation into cellulose by *G. xylinus* ATCC 23769.

Toda et al. (1997) added 2% acetic acid to GPY medium. They found that *G. xylinus* DA produced high levels of cellulose in static culture with this additive, whereas other strains showed decreased levels of cellulose. When Ca^{2+} was introduced into the medium used to grow *Acetobacter aceti* subspecies *xylinus* ATCC 23770, at concentrations between 1 and 7 mmol/L, bacterial cellulose production increased in static culture (Hong & Qiu, 2008). Dudman (1959) investigated the addition of acetate, citrate and succinate to the growth media for *A. acetigenum* EA-I, and determined that these additives stimulated cellulose production, the most effective of which was succinate. However succinate decreased cellulose synthesis when used as an additive in a different medium.

In a study designed to improve the cellulose production of *G. xylinus* K3, black tea and green tea media were used. Cellulose production in these media was not as high as the control HS medium, however when 0.3% green tea was added to an HS-CSL-mannitol medium, an increase in cellulose yield was observed (Nguyen et al., 2008). Plant stimulators, caffeine and related xanthenes were previously added to media to grow *G. xylinus* BF by Fontana et al. (1991). This group determined that optimal cellulose synthesis occurred when tea infusion *Camellia sinesis* was added for growth over more than 7 days, and the addition of *Paullinia cupana* ("guarana") for shorter growth periods of less than 5 days. These stimulants are only required in small amounts and may therefore be inexpensive components with which to increase cellulose production (Sani & Dahman, 2010).

Many additives mentioned here may stimulate the synthesis of bacterial cellulose, however additives do not necessarily need to stimulate cellulose synthesis to achieve increased yields. For example, Vandamme et al. (1998) completed a study in which the maximised cellulose production was achieved

by the addition of insoluble microparticles. Diatomaceous earth, silica, sea sand, small glass beads and loam particles were added to culture media, and resulted in the cellulose yield being tripled from culture without the insoluble particles in agitated conditions. They concluded that this was due to the artificial creation of local oxygen-deprived niches around the particle surfaces, and that this favoured cellulose production over gluconic acid production.

Additives could be used as cheap ways of increasing cellulose production. Together with appropriate carbon and nitrogen sources and culture conditions, combinations of additives may maximise bacterial cellulose production, and be useful for commercial applications.

Optimised Growth Media

While many groups have used previously described media compositions and simply substituted carbon or nitrogen sources, or included additional components, others have specifically optimised the levels of each component in a medium.

Son et al. (2001; 2003), and Heo and Son (2002) have reported optimised media for strains of *Gluconacetobacter*. The inclusion of various inorganic salts, trace elements, amino acids, vitamins and co-substrates has been investigated. In 2001, Son et al. (2001) reported that a modified HS medium with 4% glucose, 1% yeast extract, 0.7% polypeptone, 0.8% $Na_2HPO_4.12H_2O$ and 1.4% ethanol was the preferred medium composition for high levels of cellulose in *Gluconacetobacter* species A9. In 2002, Heo and Son (2002) reported a medium containing 4% glucose, 0.2% $(NH_4)_2SO_4$, 0.25% KH_2PO_4, 0.3% $Na_2HPO_4.12H_2O$, 0.05% $MgSO_4.7H_2O$, 0.0002% $FeSO_4.7H_2O$, 0.00025% H_3BO_3, 0.00006% nicotinamide, 0.00025% inositol and 1.4% ethanol was better for cellulose production than the modified HS medium. In 2003, subtle changes of 1.5% glucose, 0.2% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.3% $Na_2HPO_4.12H_2O$, 0.8% $MgSO_4.7H_2O$, 0.0005% $FeSO_4.7H_2O$, 0.0003% H_3BO_3, 0.00005% nicotinamide and 0.6% ethanol were determined to make up the better medium composition for the growth of *Gluconacetobacter* species V6 (Son et al., 2003).

Other media differing from the traditional HS media structure have also been reported. Kim et al. (2006) reported an optimised medium for cellulose production in *Gluconacetobacter* species RKY5 contained 1.5% glycerol, 0.8% yeast extract, 0.3% K_2HPO_4 and 0.3% acetic acid. A study on *G. xylinus* NCIM 2526 and the maximum production of cellulose in static culture was undertaken using coconut water medium (Jagannath et al., 2008). It was reported that tender coconut water medium with 10% sucrose and 0.5% ammonium sulphate was optimal for cellulose production.

Statistical methods have also been used to optimise the components in the media for cellulose production (Bae & Shoda, 2005; Galas et al., 1999; Hutchens et al., 2007; Mohite et al., 2012). The following optimised media compositions have been reported: Optimised fructose based medium: 3.68% fructose, 5.02% yeast extract, 0.001% $(NH_4)_2NO_3$, 0.3% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (Galas et al., 1999); optimised sucrose and ethanol based medium: 5.0% sucrose, 1.36% ethanol, 1.27% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ (Galas et al., 1999); optimised CSL-fructose medium: containing 4.99% fructose, 2.85% CSL, 0.38% agar, with 28.33% dissolved oxygen (Bae & Shoda, 2005).

pH and Temperature

The effects of pH and temperature have been widely investigated on cellulose production. Optimal temperatures are typically reported to be in the range of 25 – 30° C (Pourramezan et al., 2009; Son et al., 2001), however it has also been reported that high cellulose production was achieved at 20° C (Hutchens et al., 2007). The optimal pH for cellulose production in *Gluconacetobacter* is usually reported to be between 4.0 and 6.0 (Hwang et al., 1999; Jagannath et al., 2008; Masaoka et al., 1993; Son et al., 2001; Tantratian et al., 2005; Verschuren et al., 2000), however it has been reported that a pH as high as 7.0 is preferred for cellulose synthesis (Pourramezan et al., 2009). While initial pH plays a role, possibly more important is the change in pH that occurs as a result of gluconic acid production which can inhibit cellulose synthesis (Schramm et al., 1957). Therefore, buffering components in a medium may be beneficial.

Oxygen Requirements

Gluconacetobacter is an obligate aerobe, meaning that oxygen is essential for cell growth. It is believed that the cellulose production *in vitro* is involved in exposing the bacterial cells to the required oxygen. When grown in static cultures, the cellulose anchors the cells to the surface to achieve this. Under agitated conditions, oxygen is more readily available and the use of cellulose is limited. It is found that agitated conditions lead to accelerated cellular growth, but decreased cellulose synthesis (Dudman, 1960). However, it has been shown that *G. xylinus* can grow and produce cellulose in a microaerobic environment (Williams & Cannon, 1989), indicating that the involvement of oxygen may actually be more complex than initially thought.

Kouda et al. (1997a) investigated oxygen and carbon dioxide levels on cellulose production, and found that the cellulose production rate was dependent on the oxygen transfer rate. They concluded that cellulose production was not affected by high oxygen pressures, but was decreased by high carbon dioxide pressure. Another study used agitated cultures to investigate dissolved oxygen and its effects on cellulose production, and demonstrated that increasing rotation speed increased dissolved oxygen in the media (Tantratian et al., 2005). An optimal rotation speed of 100 rpm was reported, as higher speeds increased the production of gluconic acid, which results in decreased cellulose production.

Watanabe and Yamanaka (1995) found that cellulose production was higher than at atmospheric conditions with oxygen tensions of 10% and 15%. Hwang et al. (1999) showed that the optimal dissolved oxygen concentration was 10% in fed-batch culture. Using a statistical optimisation model, Bae and Shoda (2005) reported a level of 28.33% dissolved oxygen is preferred for high cellulose yields.

Clearly, oxygen content is a variable that may require further investigation, as it would also be a key factor in a reactor designs.

Culture Conditions and Reactor Configuration

Bacterial cellulose is often grown under static conditions, that is when the container housing the culture is left undisturbed and the cellulose spreads across the surface of the liquid broth as a smooth pellicle (Figure 2.2). However, cellulose can also be produced in shaking or agitated cultures. The terms shaking and agitated are sometimes used interchangeably when used to discuss bacterial culture conditions, however some reports use them to indicate different culture conditions. The term "shaking" often refers to growth in an incubator with a rotator, whereas "agitation" can be used to describe growth in a reactor. Growth under shaking and agitated conditions, including growth in various reactors, is described below.

As previously reported, growth in a rotating culture typically has a positive impact on the growth of bacterial cells, but has been shown to decrease the production of cellulose (Dudman, 1960). Not only is this thought to be due to oxygen concentration, as previously discussed, but rotating cultivation has also been linked to the spontaneous emergence of cellulose non-producing mutants, *Cel-* (Schramm & Hestrin, 1954). *Gluconacetobacter* has been shown to contain insertion sequences in its DNA sequence that confer genetic instability (Coucheron, 1991). These *Cel-* mutants are capable of reverting to the cellulose producing state, however there is a positive correlation between the number of subcultures and the number of cells unable to revert (Cook & Colvin, 1980). While media composition can reduce the emergence of these mutants, as can the inclusion of additives such as ethanol (Krystynowicz et al., 2002), the culture conditions do influence the amount of cellulose produced.

Growth of a static culture is slow and commercially unfavourable, so an agitated culture in which high amounts of cellulose can be produced would be beneficial (Yoshinaga et al., 1997). Strains suitable for growth under agitated conditions have also been screened and various types of reactors designed with their performance investigated (Toyosaki et al., 1995). Bioreactors support biologically-active environments for the organisms growing in them and are designed to maintain optimal conditions for attachment of cells on the surface of moving rollers or discs (Krystynowicz et al., 2002). There have been various types of reactors that have been used to produce bacterial cellulose. Biofilms are grown

on the solid supports when microorganisms attach. The biofilm of cells are temporarily submerged in the media, and are exposed to the nutrients of the broth, as well as to oxygen. There are different designs of bioreactors such as the stirred tank, airlift and rotary disc bioreactors (Figure 2.5) (Sani & Dahman, 2010). Different yields of cellulose can be obtained using different reactors, as high shear caused by high stirring speed in stirred tank bioreactors can have a negative impact on cellulose synthesis (Chao et al., 2001). Several groups have aimed at maximising cellulose production in bioreactors by experimenting with variables such as rotation speed (Krystynowicz et al., 2002), oxygen transfer rate (Chao et al., 2000), media composition (Chao et al., 2001) and bioreactor materials (Cheng et al., 2009b).

Kouda et al. (1997b) investigated the impact of different agitator configurations on bacterial cellulose productivity. They identified two different impellers that were most suitable for cellulose production. These impellers mixed the culture broth well and had high oxygen transfer capacity. A high oxygen transfer rate is required for high levels of cellulose synthesis. However, this type of production requires high agitation power and a large motor, and therefore results in high energy costs (Sani & Dahman, 2010).

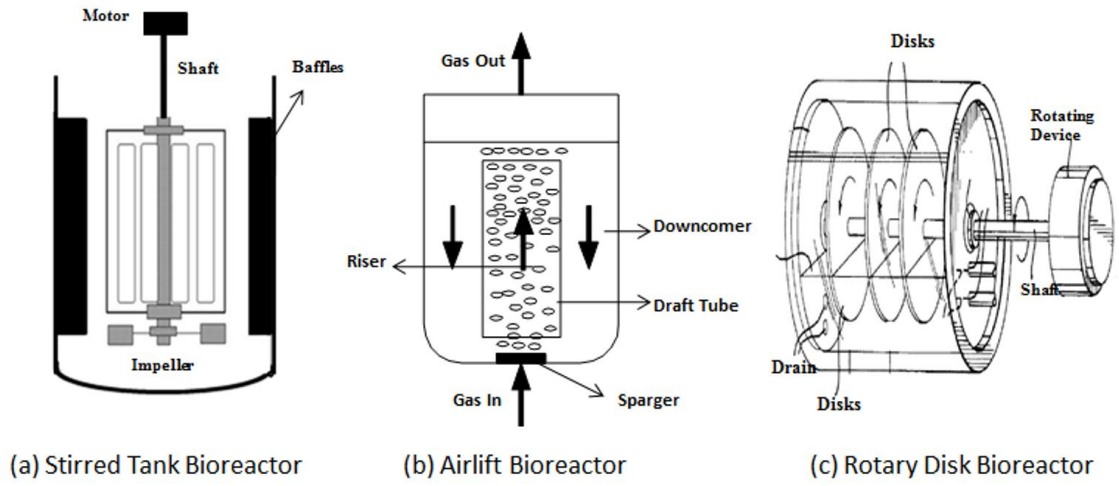


Figure 2.5: Different types of reactor designs from Sani and Dahman (2010). Reprinted with permission from Wiley.

The use of different culture vessels has also been shown to impact cellulose production. Dudman (1960) showed that flasks with smooth walls were not as effective for cellulose productivity as baffled flasks. Using an improved flask design with three baffles to avoid splashing, Toyosaki et al. (1995) presented results that supported this work. Hornung et al. (2006) described this influence as a "wall effect". Wall effects occur as the cellulose produced at the air/surface interface moves down into the substrate solution. If the walls of the vessel are sloped outwards and this sinking is prevented, a decrease in the production of cellulose occurs. Design of appropriate apparatus is therefore clearly of importance. In conical flasks, the cellulose sinks into the medium rather than sliding down the walls of the flask, thus eliminating the wall effect and leading to improved cellulose yield (Hornung et al., 2006).

Designing a cultivation system, such as a bioreactor, with a defined medium that would allow the cost-effective production of bacterial cellulose is the ultimate goal. Determining a set of conditions by which to produce this material is necessary if bacterial cellulose is to be used for other applications such as material science. Therefore further investigation into these aspects of bacterial cellulose growth is required. Changes that occur to the structure and morphology of the cellulose as a result of growth in these reactors are discussed with other *in situ* modifications that occur due to changes in media are described below.

Mutant Strains

Cellulose metabolism has been well described and occurs from hexose phosphate, via fructose or gluconate, to UDP-glucose, the immediate precursor of cellulose (Ross et al., 1991). Based on the knowledge of the production of cellulose, a number of mutant strains of cellulose-producing bacteria have been identified as having the potential to have impacted cellulose production. These mutants include strains with alterations to metabolic pathways to lead to an increase in products used for cellulose production. Several studies examining mutant strains of bacteria, either naturally-occurring or specifically created using genetic modification techniques, with increased cellulose levels have been completed. As production of gluconic acid leads to a decrease in pH and cellulose production

(with the cellulose production decreasing as a result of both the pH decrease and incorporation of the carbon source into gluconic acid instead of cellulose), an early study on *Gluconacetobacter* mutant strains focused on the isolation and cultivation of mutants with restricted gluconic acid production (DeWulf et al., 1996). Bacterial cellulose produced from a non-gluconic acid-producing mutant was found to be increased over the wild type.

An increase in cellulose production was observed in a mutant strain with resistance to sulphaguanidine (Ishikawa et al., 1995; Ishikawa et al., 1998b). This mutant was selected based on the observation that *p*-aminobenzoic acid increases cell growth and cellulose production. Resistance to sulphaguanidine, an analogue of *p*-aminobenzoic acid, is thought to enhance high-energy compounds such as ATP, which is required for cellulose production (Ishikawa et al., 1998b). Similarly, a 5-flurouridine-resistant mutant was isolated with increased cellulose production (Ishikawa et al., 1998a). This mutant was shown to have increased intracellular levels of UDP-glucose, the direct precursor of cellulose.

A strain of *G. xylinus* subspecies *sucrofermentans* named BPR2001 has been reported as being used to breed mutant strains (Watanabe et al., 1998a). BPR2001 was isolated from a natural source and was found to produce high levels of acetan, a water-soluble polysaccharide. As UDP-glucose is a precursor of acetan (and cellulose), a mutant lower in acetan production and high in cellulose production was the target. This was obtained by treatment of BPR2001 cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and the new mutant was named BPR3001A. In another study examining the effects of acetan production, wild type BPR2001 was used to make an *aceA* mutant (Ishida et al., 2002). The *aceA* gene is believed to be involved in the synthesis of acetan, and as a result of its disruption, the mutant strain, named EP1, could no longer produce acetan. However, this strain also produced significantly less cellulose than the wild type. When acetan was added to the culture medium, cellulose synthesis increased to wild type levels. The authors concluded that acetan and cellulose are not genetically related, but that acetan has a physiochemical effect on the culture conditions that stimulates cellulose production.

Increased levels of cellulose have also been obtained by introducing genes from other species into *Gluconacetobacter.* Based on the observation that plants use sucrose synthase to conserve the high energy between glucose and fructose in sucrose, and that sucrose is used for UDP-glucose synthesis and can therefore increase cellulose synthesis, a sucrose synthase gene was introduced into *G. xylinus* (Nakai et al., 1999). This gene, isolated from mung bean (*Vigna radiata*), was under the control of a *lac* promoter, and resulted in carbon directly from sucrose being incorporated into cellulose (via UDP-glucose) and also prevented UDP accumulation.

Considering *Gluconacetobacter*'s dependence on oxygen concentration, Chien et al. (2006) attempted to introduced the *Vitreoscilla* haemoglobin gene, which allows *Vitreoscilla* to grow in oxygen-poor environments. *G. xylinus* was transformed with a plasmid containing the haemoglobin gene under the control of a *bla* promoter, and demonstrated increased cellulose production in static culture under microaerophillic conditions. It is believed that lowered oxygen tensions limited the production of gluconic acid, and subsequently increased cellulose production.

Many of the mutants described here were isolated via natural means rather than by genetic manipulation techniques. They were selected specifically based on previous observation of the factors that enhance cellulose production. It may be of interest to create a transposon library and determine if any randomly created mutants lead to changes, either in structure or yield, in cellulose.

From a review of the literature, it is apparent that large changes in cellulose yield can be obtained from varying the bacterial species or strain (whether it be a mutant or a naturally occurring strain), media composition or cultivation conditions. Determining an appropriate combination of these factors to produce high amounts of cellulose at a reasonable cost is a necessary step in the development of composite materials using bacterial cellulose. Further considerations of the properties must also be taken into account and are discussed below.

Tailor-Designing Bacterial Cellulose

Modifying the Properties of Bacterial Cellulose

Bacterial cellulose exhibits properties such as nanosized fibres and high crystallinity that confers high stiffness and makes it suitable as a reinforcement material (Eichhorn et al., 2010), however it also has some significant disadvantages. While bacterial cellulose would have a natural affinity to hydrophilic matrices due to its hydrophilic nature, it would have an inherent incompatibility to hydrophobic matrices. This is a very important factor when determining the overall success of a composite material. Interaction between the two materials is important as it leads to the determination of the properties, as good mechanical properties result from good adhesion between the two materials in a composite, and weak interfacial adhesions result in poor mechanical properties (Avella et al., 2000). However it is possible to obtain a variety of modifications to bacterial cellulose due to its inherent nature of being biological and its chemical structure. These qualities provide the opportunity to alter its properties in favour of achieving specific characteristics using a variety of techniques.

This cellulose is cultivated by bacteria, allowing for samples to be produced quickly. There are also many ways to change the growth conditions, from changing the media by varying the carbon and/or nitrogen sources and including a variety of additives, to changing the actual cultivation conditions by agitating the culture or using some form of bioreactor. Changing these growth conditions can impact the structure, morphology and properties of the cellulose produced. In addition, based on the structure of this material, chemical and/or physical changes can be achieved after its growth.

Determining techniques to alter the properties of bacterial cellulose could provide methods to target and achieve specific characteristics. The ability to “tailor-design” bacterial cellulose with desirable traits would increase the potential for this material to be used as a reinforcement material for composites.

In situ Modifications

It is very easy to manipulate the growth conditions of bacterial cellulose. Due to its biological nature, changes to the growth conditions can cause changes to the cell growth and cellulose production (Ruka et al., 2013). Additional components in the media, not specifically required for cell growth or cellulose production, can stimulate (or inhibit) cellulose formation, as can the cultivation conditions. The changes that occur because of variations in the media or cultivation conditions are known as *in situ* modifications.

The inclusion of particular water-soluble compounds in the growth media may be incorporated into the cellulose as it grows, or may affect the formation of fibrils or ribbons, changing the structure of the cellulose (Klemm et al., 2006) and resulting in differences in the widths of the cellulose fibrils, the crystallinity, or the ratio of cellulose I_α and I_β produced. Some of the studies that were undertaken as part of determining the growth conditions in which bacterial cellulose production was optimised also reported on the subsequent structural changes that occurred as a result of the inclusion of selected media components, although the results are mixed. Some authors have reported that the structure of cellulose is not affected by changing the carbon or nitrogen source (Keshk & Sameshima, 2006b; Mikkelsen et al., 2009), whereas others have reported differences. El-Saied et al. (2008) reported the CSL and molasses resulted in a higher degree of crystallisation, whereas Jung et al. (2010b) reported a decrease in crystallinity in a molasses medium compared to a complex medium control. The use of glycerol as the carbon source achieved cellulose with 9% higher crystallinity over a glucose medium, however its water-holding capacity and viscosity were decreased in the glycerol medium (Jung et al., 2010a).

As mentioned previously, when *Gluconacetobacter* is grown under static conditions, the cellulose forms a pellicle at the air/surface interface. When grown under agitated conditions, growth occurs as irregular bodies within the medium (Schramm & Hestrin, 1954). It has been shown that growth under these different conditions also results in microstructural changes. Cellulose from agitated culture results in a loss of mechanical strength with decreased degree of polymerisation, lower crystallinity

index, lower cellulose I_α content, lower Young's modulus, higher water holding capacity and higher suspension viscosity in disintegrated form (Cheng et al., 2009a; Czaja et al., 2004; Krystynowicz et al., 2002; Watanabe et al., 1998b). SEM reveals that cellulose from static culture appears as a fine net, built mostly of uniaxially-oriented cellulose ribbons, whereas agitated cellulose appears as disordered, curved, denser, overlapping ribbons, with thinner microfibrils, as shown in Figure 2.6 (Czaja et al., 2004; Watanabe et al., 1998b). It has been proposed that the stress caused by agitation results in the more stable allomorph I_β (Czaja et al., 2004). However, agitated cellulose has also been shown to have a higher emulsion stabilising effect (Ougiya et al., 1997; Watanabe et al., 1998b) and the disintegrated form has higher filler retention aid function (Hioki et al., 1995; Watanabe et al., 1998b). It was concluded by Watanabe et al. (1998b) that cellulose from agitated culture exhibits more suitable properties in wet state and disintegrated form than static cellulose, for industrial applications.

When grown in an airlift reactor (see Figure 2.5), cellulose is formed as unique ellipse pellets, whereas stirtank reactors caused the cellulose to grow in a fibrous form (Chao et al., 2000). Production in an airlift reactor resulted in cellulose similar to that produced in static culture, with a higher degree of polymerisation, found to be 16,000 and 17,000 respectively, than cellulose produced in agitated culture, with 9,700 (Chao et al., 2000). Production in an airlift reactor resulted in cellulose similar to that produced in static culture, with a higher degree of polymerisation than cellulose produced in agitated culture. When grown in a plastic composite support biofilm reactor, an increase in

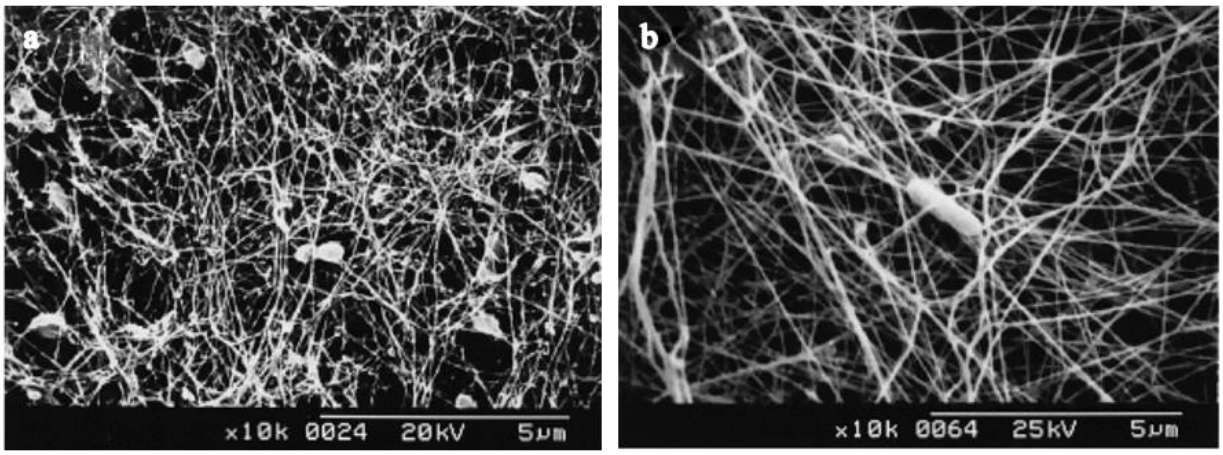


Figure 2.6: SEM images of cellulose fibrils grown under agitated (a) and static (b) conditions. Reprinted from Watanabe et al. (1998b). Reproduced with permission from Springer.

mechanical properties was observed (Cheng et al., 2009b). This cellulose had higher crystallinity, higher thermal stability and higher Young's modulus. The presence of agar in an airlift reactor caused the cellulose produced to form smaller pellets than the control. Usefully, agar also resulted in increased cellulose production (Chao et al., 2001). It was proposed that the addition of certain water-soluble polysaccharides prevented the formation of clumps of cellulose, leading to this decreased pellet size, which in turn was advantageous to transfer nutrients and oxygen into bacterial cells and led to increased cell and cellulose production.

The addition of other additives has also been shown to alter the morphology of cellulose. Antibiotics in the growth medium resulted in the elongation of cells through inhibition of cell division and, as a result, the cellulose fibres produced were wider (up to an average of 228 nm compared to a control of 117 nm wide). The opposite was true of reducing agents that caused cell shortening, and therefore thinner cellulose fibres, with an average width of 53 nm (Yamanaka et al., 2000). The inclusion of antibiotics, nalidixic acid and chloramphenicol, in culture media resulted in the production of cellulose with wider ribbons or aggregates or ribbons, and increased Young's modulus. The *Gluconacetobacter* cells were also affected (Yamanaka et al., 2000). Lignosulfonate in a culture medium not only led to higher cellulose productivity, but to higher crystallinity and I_α content in static culture (Keshk & Sameshima, 2006a). When CMC was added to the culture medium, the cellulose produced exhibited decreased crystallinity and crystal size, higher thermal stability, higher decomposition temperatures and mainly the allomorph I_β compared to the static culture control (Cheng et al., 2009a). SEM images showed that the cellulose retained its interwoven structure, but was looser in weave. There was no difference in mechanical strength shown between the CMC-altered cellulose and cellulose produced in agitated culture, but it was lower than the static control.

Sodium alginate added to the medium resulted in changes to the macromorphology of cellulose, resulting in discrete masses dispersed in the broth instead of irregular clumps (Zhou et al., 2007). This led to lower crystallinity and smaller crystallite size. Zhou et al. (2007) also demonstrated that hydrogen bonding interactions occurred between the cellulose and the sodium alginate. Tokoh et al.

(1998) showed that the presence of acetyl glucomannan in the medium changed the crystal structure of cellulose with an increased cellulose I_β fraction and caused the cellulose to form as loose bundles of microfibrils, with decreased crystallite size. Polyethylene glycol 400 and *β*-cyclodextrin in the growth medium of *G. xylinus* resulted in bacterial cellulose with increased pores. However, the addition of polyethylene glycol 4000 was shown to decrease pore size and decrease degree of polymerisation (Heßler & Klemm, 2009). Acid treated multi-walled carbon nanotubes (MWCNT) were added to the culture medium used to grow *G. xylinus* under static conditions. The cellulose produced by the bacteria under these conditions had altered crystal structure, cellulose I_α content, crystallinity index and crystallite size (Yan et al., 2008). When glucose-phosphate was added to the culture medium, either as the sole carbon source or in conjunction with glucose, phosphate-containing cellulose was produced (Basta & El-Saied, 2009). This cellulose can be used as an environmentally friendly paper additive.

In situ modifications that occur in cellulose from the inclusion of additives can directly or indirectly impact the structure of cellulose and can be used to target specific properties or characteristics. It is also possible for additives in the media to be included in the bacterial cellulose as it grows. If the additive is incorporated into the cellulose, a composite material can be produced as a result of this *in situ* modification. Ruka et al. (2013) have investigated the *in situ* modification of cellulose using poly-3-hydroxybutyrate and have shown the dispersion of PHB throughout the cellulose (Figure 2.7). This method of creating composites is discussed further below.

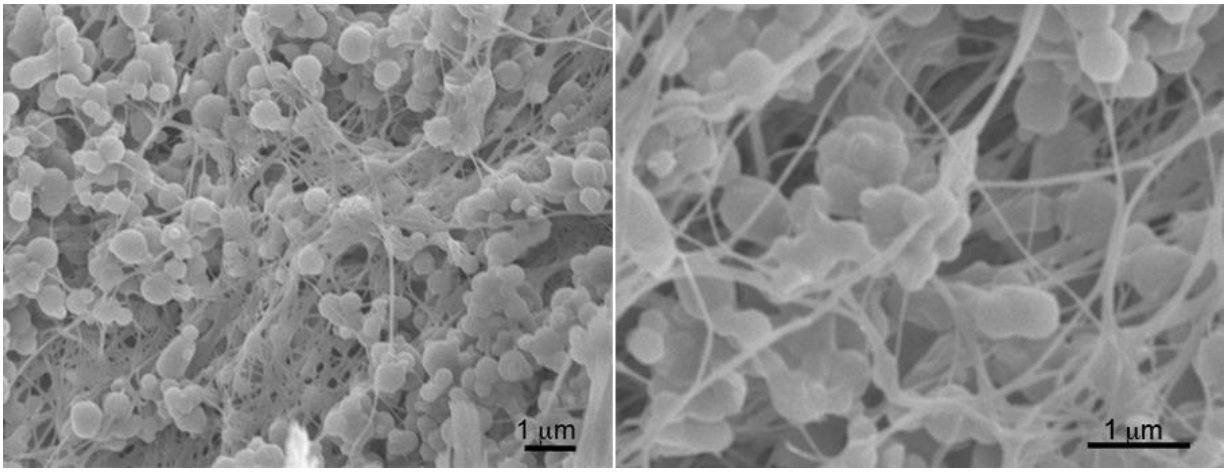


Figure 2.7: SEM images of bacterial cellulose grown with PHB in the media. Reprinted from Ruka et al. (2013). Reproduced with permission from Elsevier.

Post Modifications

Similar to *in situ* modifications, post modifications (changes to cellulose after growth) could provide the opportunity to tailor-design the material in order to achieve specific properties, which is particularly relevant if cellulose is to be included in blends and composites with other materials. These changes may be chemical or physical. For example, chemical changes may be necessary as cellulose is a hydrophilic molecule, which is a problem for cellulose fibres if they are to be used as reinforcement in plastics (Bledzki & Gassan, 1999). The glucose molecules that make up cellulose each have three free hydroxyl groups. This provides cellulose with high affinity to hydroxyl containing materials, including itself (Gardner et al., 2008). The hydrophilic nature of cellulose can weaken blends with other materials as water can become contained in the matrix (Dahman, 2009), however the large number of free hydroxyl groups does make it possible for chemical modifications to be carried out to make the cellulose more hydrophobic, and some authors have attempted to do this in order to increase the interfacial adhesion between potential matrices and fibres (Nogi et al., 2006a). There have been mixed successes in these processes, which are described below. In addition, physical changes to bacterial cellulose can result as a consequence of treating the cellulose in different ways, prior to its inclusion in blends.

Physical Modifications

Bacterial cellulose can be used in various forms in composites as a reinforcing phase in blends, but any prior treatment to inclusion in such mixtures can affect its structure and properties. It can be necessary to remove moisture prior to further treatment, and there are various ways to dry bacterial cellulose, including freeze-drying, heat or air drying, or it can be used in a never-dried state. However, it has been found that air-dried and never-dried cellulose exhibit differences in crystallite size (Fink et al., 1997), so simply selecting a method of drying, or choosing not to dry it at all could result in a change in properties.

Chemical modifications are described below, however some chemical treatments that are carried out specifically to achieve physical changes, such as dissolution, are listed here.

Dissolution

The use of cellulose is limited due to difficulties dissolving it and the limited number of appropriate solvents (Zhu et al., 2006). Cellulose is a long chain polymer composed of glucose units and is extremely hydrophilic, however it is insoluble in water and most organic solvents due to its extensive intra- and inter-molecular hydrogen bonding (Chen & Chiang, 2010). There have been some reports of cellulose solvent systems including *N,N*-dimethylacetamide/lithium chloride (DMAc/LiCl) (Shen et al., 2010), dimethyl sulfoxide-paraformaldehyde (DMSO-PF) (Masson & Manley, 1991), *N*-methylmorpholine-*N*-oxide (Biganska & Navard, 2005) and NaOH/urea aqueous solution (Zhou & Zhang, 2000). Whilst the dissolution of bacterial cellulose has been difficult in the past due its hydrogen bonds and high degree of polymerisation, the determination of ionic liquids that allow such dissolution of bacterial cellulose to occur may offer further possibilities to modify and process this material.

Ionic liquids consist entirely of ions, and are made up of at least two components, an anion and a cation, which create an enormous number of potential combinations simply by varying these components (Earle & Seddon, 2000). Ionic liquids are often referred to as "green solvents" as they have the capability of dissolving many substances, including many organic molecules such as enzymes (Fujita et al., 2007), DNA (Vijayaraghavan et al., 2010a) and collagen (Vijayaraghavan et al., 2010b), and have desirable properties, for instance chemical stability, thermal stability, low vapour pressure and high ionic conductivity (Lu et al., 2009). They are media that can affect various kinds of polymerisation and have been used to synthesise a variety of molecules, including proteins. Ionic liquids can be water soluble and, as such, have previously been included in growth media for bacteria (Sekar et al., 2013; Sekar et al., 2012).

Bacterial cellulose, while molecularly identical to plant cellulose, differs in its purity, high crystallinity and high degree of polymerisation. Several solvents suitable for plant cellulose are unable to dissolve bacterial cellulose. Shen et al. (2010) successfully dissolved bacterial cellulose in a DMAc/LiCl solvent system at a maximum concentration of 3%, provided an activation procedure, consisting of soaking

the cellulose in DMAc with trace amounts of $KMnO_4$ at 45 – 50° C for 1 hour, was completed first. They were unable to dissolve bacterial cellulose in its large grained form, and were forced to reduce the sample to a fine powder for dissolution to occur. They also determined that the optimal temperature for this process was 45° C, as higher temperatures risked degradation.

While there have been many ionic liquids reported to dissolve plant cellulose, the high molecular weight bacterial cellulose has only been successfully dissolved in a handful of reports. Schlufter et al. (2006) used 1-*N*-butyl-3-methylimidazolium chloride to efficiently dissolve bacterial cellulose powder at 80° C at a concentration of 6% in order to complete chemical modifications of the cellulose.

The ionic liquids described here are reported to be extremely fast and efficient media with which to dissolve bacterial cellulose. In addition, due to the large number of potential combination of anions and cation, it is likely that increasing numbers of ionic liquids that can dissolve this high molecular weight cellulose will be found. For example, it has been suggested that ionic liquids with acetate counter ions will be extremely effective for this task. (Liebert & Heinze, 2008). The ability to dissolve and recover bacterial cellulose, as well as the environmental benefits of using renewable and recyclable solvents, creates further possibilities to chemically modify this molecule to alter its properties in order to use bacterial cellulose to create composites.

Dispersion

Bacterial cellulose may have good properties that make it a good potential reinforcing filler material in composites, however it is difficult to process due to its extensive hydrogen bonding. It may be necessary to obtain homogeneous dispersions of this cellulose in aqueous or organic solvents to mix it with matrix materials to produce these composites or blends (Saito et al., 2006). There have been a few methods used to disperse bacterial cellulose fibres. Bacterial cellulose can be shredded, homogenised, or milled and ground to a fine powder in order to help with its dispersion in composites. It has also been reported that bacterial cellulose has been dispersed simply by vigorous stirring (Kibédi-Szabó et al., 2012). However, once again, different treatments can change the properties of this material. For example, it has been shown that cellulose homogenised for a short

period of time has been shown to experience a small decrease in crystallinity (Kose et al., 2011) and grinding cellulose can cause of loss of crystallinity (O'Connor et al., 1957; Schenzel et al., 2005).

A method involving hydrolysis of bacterial cellulose by sulphuric acid has been undertaken in order to obtain nanowhiskers. These nanowhiskers can be dispersed in various solutions, however the nanowhiskers appear stiff and rod-like, and have been reported to aggregate to some degree (Grunert & Winter, 2002). Bacterial cellulose nanowhiskers have also been prepared by hydrochloric acid digestion (Guo & Catchmark, 2012).

Electrospun bacterial cellulose fibres have been produced by first achieving dissolution in 1-allyl-3-methylimidazolium chloride at 5% bacterial cellulose at 70° C. This experiment also involved the production of electrospun composite fibres with MWCNT (Chen et al., 2010), as is discussed further below.

Sonication is another method that can be used to disperse bacterial cellulose fibres (Guhados et al., 2005; Saito et al., 2006). Tischer et al. (2010) investigated the effects of sonication on cellulose pellicles by sonicating bacterial cellulose for different time intervals and determined that this treatment achieved differences in the width and height of the cellulose fibres, the roughness of the surface and differences in the amount of crystallinity. They concluded that ultrasound energy was transferred to glucan chains in the cellulose, which resulted in a conversion of the amorphous region to crystalline regions, increasing crystallinity. Crystallite sizes were also found to increase. This provides a method to obtain cellulose fibres with specific widths and lengths, as well as high crystallinity, which may be useful when designing composites.

There has also been a recent paper that reported the achievement of individual fibres of bacterial cellulose. Kose et al. (2011) used an aqueous counter collision method that caused a separation of the interwoven mesh of fibres in the pellicle into individual fibres, dispersed in water (Figure 2.8). They completed different repetitions of this treatment and found that the width and length of the fibres changed with the number of treatments, and that cellulose I_α was converted to I_β, but that the cellulose retained its high crystallinity. This method provides further indication that the treatment of the

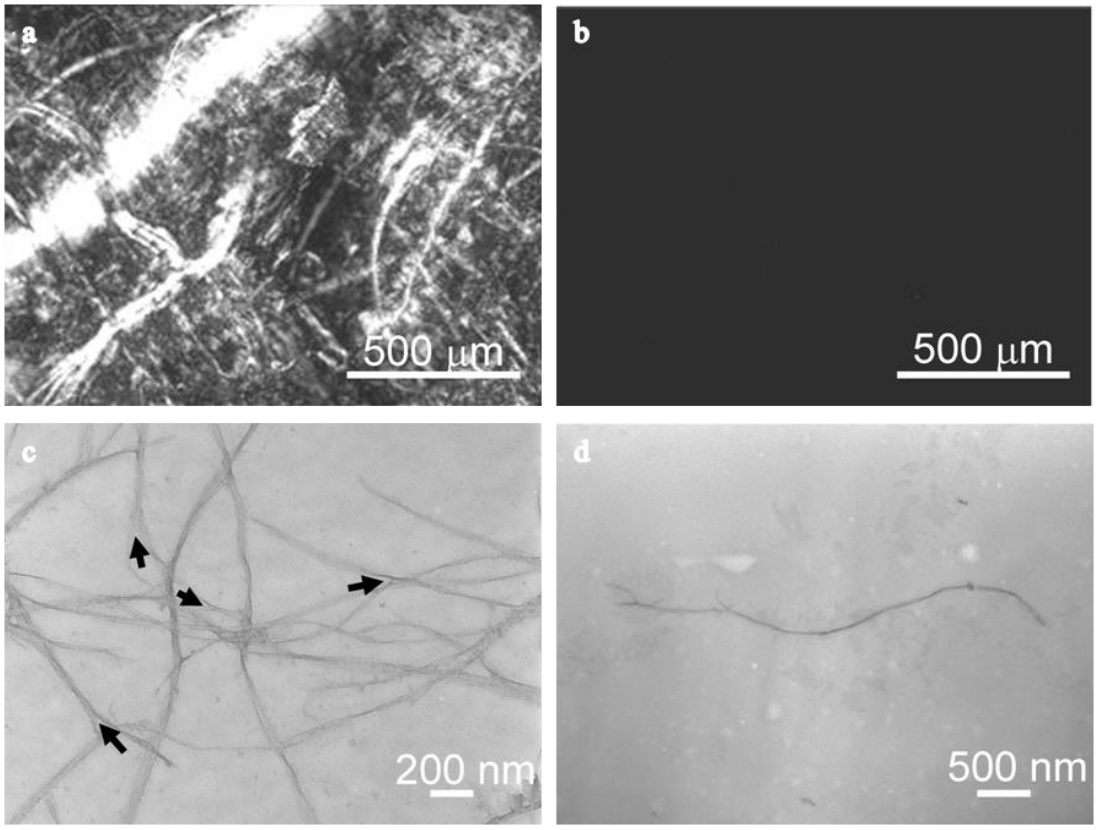


Figure 2.8: Individual fibres of bacterial cellulose obtained from aqueous counter collision method Kose et al. (2011). Reproduced with permission from ACS Publishing.

cellulose prior to its inclusion in a composite can result in different properties, with a technique that can target not only specific properties of bacterial cellulose, but can achieve individual fibres for further use.

Modifications by Impregnation

As bacterial cellulose is made up of a web of interwoven fibrils, it is possible to obtain changes to this material by physically attaching particles to the surface of the fibrils via an impregnation method. Essentially, a cellulose pellicle or sheet is soaked in a solution containing another material. Nanoparticles included in the solvent can become embedded between the cellulose fibrils, or attach to the surface of the cellulose fibres as the solvent evaporates, essentially creating surface-modified cellulose. A variety of materials have been used to modify bacterial cellulose by this impregnation method including silica (Ashori et al., 2012) and cadmium sulphide (Li et al., 2009). This can result in changes to the mechanical properties of the cellulose.

In addition, bacterial cellulose has been modified by several materials in order to improve its properties for use in biomedical applications. Montmorillonite (Ul-Islam et al., 2012a), hydroxyapatite (Wan et al., 2006) and silver nanoparticles (Hu et al., 2009; Liu et al., 2012; Maneerung et al., 2008) have all been used in this way. Montomorillonite impregnated-bacterial cellulose showed improved water release rate, as well as mechanical and thermal properties (Ul-Islam et al., 2012a). Impregnation of bacterial cellulose by soaking pellicles in hydroxyapatite solution resulted in an even covering of the cellulose by the hydroxyapatite. It was also found that impregnation for 14 days, rather than 7 days, led to increased hydroxyapatite covering, and causing much thicker fibrils as a result (Wan et al., 2006). Modifying bacterial cellulose with silver nanoparticles conferred antimicrobial activity (Hu et al., 2009; Liu et al., 2012; Maneerung et al., 2008). Similarly, bacterial cellulose pellicles have been soaked in solution containing aniline with a variety of other materials to allow polyaniline to be polymerised directly onto the cellulose fibres to achieve conducting bacterial cellulose composites (Lee et al., 2012a; Lee et al., 2012b; Marins et al., 2011; Müller et al., 2012; Shi et al., 2012).

Impregnation can be used as a method of directly modifying the surface of the bacterial cellulose, but can also be used as a method of producing bacterial cellulose composites with other materials.

Chemical Modifications

Bacterial cellulose is typically exposed to an alkaline treatment in NaOH after its removal from the growth media to remove any bacterial cell debris and to sterilise the pellicle. McKenna et al. (2009) set out to examine if this chemical treatment was able to alter the cellulose. Despite the identification of some minor damage to the cellulose fibres when visualised by SEM, they found that the low concentration of NaOH used for this process did not affect the mechanical properties of the cellulose. Nishi et al. (1990), however, found that the treatment of bacterial cellulose by a higher concentration of NaOH could actually improve its mechanical properties, likely to be due to the NaOH removing the cell debris and allowing hydrogen bonds to form within the cellulose due to increased close contact between the fibres. Conversely, highly concentrated NaOH was found to cause degradation and

decrease mechanical properties. Similar results were seen with an oxidising treatment using an NaClO solution, with even higher mechanical properties observed when both the oxidising and alkaline treatments were sequentially applied.

There are other reports of chemical modification of bacterial cellulose in the literature, using a variety of methods. Many authors do not attempt to dissolve bacterial cellulose, but rather use it in its native form and expose the film to a solvent exchange process. This method, commonly completed as part of an acetylation reaction (Geyer et al., 1994; Hu et al., 2011; Ifuku et al., 2007; Nogi et al., 2006a; Tomé et al., 2011), involves the hydrophilic hydroxyl groups being replaced with less hydrophilic acetyl groups. It consists of a progressive soaking of the bacterial cellulose pellicle in a series of solvents, such as acetone, followed by swelling in acetic acid with toluene and perchloric acid, and then exposure to acetic anhydride (Ifuku et al., 2007). However there has been a recent report of a solvent-free process of acetylating bacterial cellulose. This work involved bacterial cellulose being acetylated by acetic anhydride in the presence of iodine as a catalyst (Hu et al., 2011). Hydrophobic surfaces have resulted from these works.

Grunert and Winter (2002) treated bacterial cellulose with sulphuric acid hydrolysis in order to obtain nanocrystals. These nanocrystals were then trimethylsilylated by hexamethyldisilazane in formamide in order to make the cellulose more hydrophobic for its use in composites. They determined that the degree of substitution of the silylated crystals was an average of 0.49. The composites made from these chemically modified nanocrystals are discussed further below. Additionally, Martínez-Sanz et al. (2011a) examined the effects of the time allowed for sulphuric acid digestion to occur to obtain similar nanocrystals, which they termed bacterial cellulose nanowhiskers. They determined that increasing the hydrolysis time decreased the nanowhiskers length and increased the crystallinity. However, they also determined that thermal stability decreased following long exposure to the sulphuric acid, which made the nanowhiskers unsuitable for melt compounding (Martínez-Sanz et al., 2011a). Various bacterial cellulose nanowhisker melt blends were produced from this group and are described in more detail below.

Chemical modifications of bacterial cellulose have also been reported involving benzoylation (Wang et al., 2008), carboxymethylation (Geyer et al., 1994), phosphorylation (Oshima et al., 2008; Oshima et al., 2011) and succinylation (Yin et al., 2011). Wang et al. (2008) ground bacterial cellulose into powder before soaking it in nitrobenzene and adding different concentrations of benzoyl chloride with pyridine to achieve benzoylated bacterial cellulose with various degrees of substitution. Ground bacterial cellulose was also used for carboxymethylation, where it underwent a solvent exchange process with water-isopropanol, before NaOH and monochloroacetic acid were added with stirring (Geyer et al., 1994). Reports of phosphorylated bacterial cellulose involve the ground cellulose being soaked in *N,N*-dimethylformamide (DMF) with urea, and having phosphoric acid added (Oshima et al., 2008; Oshima et al., 2011). Similarly, Lee et al. (2009) completed a solvent exchange process with ground bacterial cellulose using methanol and pyridine, with *p*-toluenesulfonyl chloride added before functionalising the cellulose with acetic, hexanoic and dodecanoic acids in order to make the cellulose more hydrophobic.

Comparison of a solvent exchange method and a dissolution process was published by Yin et al. (2011) with succinylation. Bacterial cellulose was modified by soaking in pyridine and then adding succinic anhydrate, in the presence of 4-dimethylaminopyridine. It was also modified by dissolving bacterial cellulose in DMAc with LiCl, before triethylamine and succinic anhydride were added. Yin et al. (2011) reported that the dissolution process resulted in homogeneous modification of the bacterial cellulose, whereas the pyridine method resulted in a heterogeneous modification, with the reactions mainly occurring on the surface of the cellulose membrane. This homogeneous modification has also been seen in other systems where the bacterial cellulose was first dissolved.

There have been a number of papers recently describing the dissolution of bacterial cellulose for chemical modification. Geyer et al. (1994) created a viscous dissolution of bacterial cellulose using DMAc and LiCl before adding hexamethyldisilazane in order to silylate the cellulose. This was similar to the work of de Marco Lima et al. (2010) who acetylated bacterial cellulose at various degrees of substitution, up to 87%, by dissolving the cellulose in DMAc with LiCl and adding acetic anhydride.

With the discovery of the potential of ionic liquids to dissolve bacterial cellulose, another report involving the dissolution of bacterial cellulose in order to complete chemical modifications has been published. Schlufter et al. (2006) used the ionic liquid 1-*N*-butyl-3-methylimidazolium chloride to efficiently dissolve bacterial cellulose before acetylation and carbanilation by the addition of acetic anhydride and phenyl isocyanate, respectively, achieving extremely high degrees of substitution. The ability to completely dissolve highly polymerised bacterial cellulose presents the option to further (and homogeneously) achieve chemical modifications to this molecule, making it a more favourable biopolymer for use as reinforcement in polymer matrices. However, while dissolution does expose more surface and thus allow for higher degrees of substitution in these chemical reactions, the crystalline structure of bacterial cellulose is lost in the process (de Marco Lima et al., 2010), thereby altering the cellulose and potentially affecting the highly desirable properties that make this molecule so favourable for use as reinforcement. It will, therefore, be necessary to determine if dissolution for chemical modification conveys a greater benefit, or if surface modifications provide sufficient alteration to bacterial cellulose to improve its properties for further use in material science.

It is worth mentioning that bacterial cellulose has been modified for reasons other than its use in composites. For example, bacterial cellulose has been modified by nitrogen-containing plasma in order to improve its cell affinity, and thus increase its potential for use in biomedical applications (Pertile et al., 2010). This opens the door, not only for other uses for bacterial cellulose but also additional ways it can be modified.

Bacterial Cellulose Composites

Introduction

Composites can be entirely synthetic, a combination of synthetic and natural, or completely natural. As with most traditional synthetic polymer matrices, biopolymers could benefit from being used in conjunction with fibres to improve the mechanical properties of the matrix (Zhu et al., 2006). Desirable biocomposites could therefore benefit from being created using a biodegradable polymer as the matrix material, and biofibres as a reinforcing element (Mohanty et al., 2000). While it is possible

to combine synthetic and renewable technologies, such as composites with biodegradable cellulose fibres used as reinforcement in polymers such as polyester, epoxy, amino and phenolic resins, these would not be fully biodegradable because of the synthetic matrices (Mohanty et al., 2000; Nakagaito et al., 2005; Zadorecki et al., 1986). The use of biopolymers currently has severe limitations with inferior properties and high production costs, but should be completely biodegradable when used as both matrix and filler. In addition to traditional fibre micro-composites, nanocomposites are composites that have been reinforced with nanosized particles (Paul & Robeson, 2008). Bacterial cellulose is a good candidate for such reinforcement with its naturally produced nanosized fibrils.

Biocomposites can be developed by various methods, and the methods by which the matrix and reinforcement material are combined can strongly influence the properties of the resulting composite. For example, extrusion and injection moulding are simple methods by which composites can be produced, however processing parameters such as mixing time, speed and temperature all have been found to alter tensile strength (Saheb & Jog, 1999). As the focus of this review is bacterial cellulose, methods that have been used to create composites that involve bacterial cellulose have been described in more detail below.

Renewable Matrix Polymers

There are a large number of polymers that could potentially be used as a matrix material in combination with bacterial cellulose as a filler material. Bacterial cellulose is predicted to have a naturally high affinity with hydrophilic materials, as it too is hydrophilic. Therefore, potential matrix materials include materials such as PLA. PLA is a biodegradable thermoplastic polyester produced from renewable sources. It has previously been used in combination with cellulose nanofibres to improve the mechanical properties of the PLA (Iwatake et al., 2008). Materials such as PVA and starch from a variety of sources are also biodegradable materials that could act as matrices for reinforcement.

In addition, there are hydrophobic biodegradable materials that could potentially be improved in terms of their mechanical properties by being combined with a second phase in a composite. Of the

hydrophobic materials, the bioplastic PHB has been proposed as having the potential to replace traditional plastics (Rehm, 2010), as it has similar properties to polypropylene (Holmes, 1985; King, 1982). PHB is probably the most well-known of the PHAs, a family of homo- or hetero-polyesters produced by bacterial species that accumulate them intracellularly and use them for energy. They all consist of a single chain with a 3-carbon backbone, but differ with side chains at the 3 position. It is this side chain that determines the specific PHA. For example, PHB has a methyl group at the 3 position (Lenz & Marchessault, 2005). It has been hypothesised that an appropriate filler material could improve the properties of materials such as PHB (Gatenholm et al., 1992).

The ability to tailor design bacterial cellulose with specific properties provides the possibility that there may be a range of biodegradable matrices that could use this cellulose as the reinforcement material. Determining appropriate *in situ* or post modifications of bacterial cellulose, as well as the method of creating the composites and potential matrices, are complex processes that require further investigation. Attention should focus on biodegradable matrices and methods that can produce composites on a large scale to develop useful materials.

Bacterial Cellulose Composites

Bacterial cellulose has been used as a material in combination with many others to develop composites. It has been used with materials such as unsaturated polyester (Gao et al., 2011), the conducting polymer polyaniline (Lee et al., 2012a; Lee et al., 2012b; Marins et al., 2011; Müller et al., 2012; Shi et al., 2012; Wang et al., 2012), as well as various acrylic and phenolic resins (Nakagaito et al., 2005; Nogi et al., 2005; Nogi et al., 2006b; Trovatti et al., 2010). It has also been used with several biodegradable materials such as cellulose acetate butyrate (CAB) (Gindl & Keckes, 2004; Grunert & Winter, 2002), PLA (Kim et al., 2009; Lee et al., 2009; Quero et al., 2010; Tomé et al., 2011), PHB (Barud et al., 2011; Cai & Yang, 2011; Cai et al., 2011), PVA (Gea et al., 2010; Millon et al., 2009), and thermoplastic starch (Wan et al., 2009; Woehl et al., 2010) to produce completely biodegradable composites. Though renewable and biodegradable composites are the focus of this review, techniques and resulting composites from non-renewable sources are also mentioned.

Impregnating Bacterial Cellulose

Several researchers have used an impregnation method to develop composites with bacterial cellulose, similar to the modification method described previously, except that the material used forms a sheet rather than individual particles upon drying. The soaking of the cellulose may occur from dry or never-dried films, or films that have undergone a solvent-exchange. They have been performed under a variety of temperature and pressure conditions using materials such as CAB (Gindl & Keckes, 2004), MWCNT (Yoon et al., 2006), poly(ethylene glycol) (PEG) (Cai & Kim, 2010), PLA (Kim et al., 2009), PHB (Barud et al., 2011; Cai & Yang, 2011; Cai et al., 2011), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (Martínez-Sanz et al., 2014), PVA (Gea et al., 2010) and starch.

When included in an impregnation solution, PEG evenly penetrated the bacterial cellulose network and covered the surface of the cellulose by soaking the cellulose (Cai & Kim, 2010). PLA was used with this technique and PLA/bacterial cellulose composites were found to have increased mechanical properties over PLA alone (Kim et al., 2009). Impregnation with PVA resulted in bacterial cellulose composites consisting of 3.7% PVA, however these composites experienced a loss of tensile strength, stiffness and modulus (Gea et al., 2010). A variety of concentrations were achieved using impregnation, resulting in composites with 7.8, 15.1 and 22.0% bacterial cellulose with thermoplastic starch (Wan et al., 2009).

Recently, a number of papers have been published that describe the preliminary results of the creation of PHB/bacterial cellulose composites (Barud et al., 2011; Cai & Yang, 2011; Cai et al., 2011). PHB was first dissolved in chloroform and then the bacterial cellulose was soaked in the PHB-chloroform solution. It is believed that this process resulted in the PHB being integrated into the pores of the cellulose as the solvent evaporated. The resulting composites were shown to have improved mechanical properties, including tensile strength, over PHB alone. Similar results have been obtained using a CAB/bacterial cellulose composite with an impregnation method (Gindl & Keckes, 2004).

This method is a simple way of producing bacterial cellulose composites and provides a technique of developing composites with a range of cellulose concentrations. Composites developed from this

technique have typically been shown to have good mechanical properties. However, it is difficult to control the ratios of the materials in the resulting composite and often results in the composite consisting of large amounts of cellulose, rather than cellulose existing as the particulate reinforcing phase. There are, however, some reports of techniques that do attempt to break up the three-dimensional network of cellulose fibres to use it as a filler material (Cai et al., 2012; Chen et al., 2010; Feng et al., 2012; Grunert & Winter, 2002; Kibédi-Szabó et al., 2012; Martínez-Sanz et al., 2011a, 2012a, 2013a, 2013b; Martínez-Sanz et al., 2011b, 2012b; Martínez-Sanz et al., 2014; Millon et al., 2009; Olsson et al., 2010; Park et al., 2007; Phisalaphong et al., 2008; Stevanic et al., 2012; Stoica-Guzun et al., 2011; Tomé et al., 2011; Trovatti et al., 2012; Woehl et al., 2010; Yang et al., 2012).

Solution Blending and Casting

Solution blending is a method of combining materials with ease, however it is not a method often used for composites involving bacterial cellulose, as bacterial cellulose is difficult to disperse or dissolve. Despite this, there have recently been an increasing number of reports that are using dispersed bacterial cellulose in solution with dissolved host polymers. Such reports are listed below.

Cai et al. (2012) prepared a porous scaffold using bacterial cellulose and poly-3-hydroxybutyrate-co-4-hydroxybutyrate (P(3HB-co-4HB)) with a trifluoroacetic acid as a co-solvent, and by freeze-drying the solution to remove the co-solvent. They determined that the scaffold presented a three-dimensional network with improved mechanical properties over P(3HB-co-4HB) alone.

Solution blending has also been used to develop bacterial cellulose/alginate membranes (Phisalaphong et al., 2008). These membranes were created by dissolving bacterial cellulose in NaOH/urea solution and dissolving sodium alginate in distilled water, and subsequently mixing the two solutions at various concentrations to obtain composites ranging from 20 – 80% cellulose. Neat cellulose and alginate samples were also prepared. It was determined that the tensile properties of the membranes were improved by the addition of bacterial cellulose, over the neat alginate.

There has been a report of solution blending to combine CAB and bacterial cellulose nanocrystals (Grunert & Winter, 2002). As discussed above, bacterial cellulose was treated with sulphuric acid to obtain nanocrystals, and was subsequently trimethylsilylated. These chemically modified nanocrystals were then dispersed in acetone, and the acetone was used to dissolve CAB and was cast to form films with up to 10% cellulose. The melting temperatures of the composites showed a change, increasing with increasing concentrations of silylated cellulose, however this change was not seen with native nanocrystals. The modulus of the composites showed increasing values with increasing cellulose contents with the native crystals over most of the temperature range. The researchers concluded that the unmodified cellulose crystals had better reinforcement characteristics than the chemically modified cellulose, however the improved properties may have been due to increased native cellulose content in the composites over the silylated cellulose, as some of the weight of the silylated cellulose was due to the silyl groups.

Bacterial cellulose fibres have been dispersed in several ways to develop PVA composites. Bacterial cellulose has been homogenised (Millon et al., 2009; Yang et al., 2012), milled to powder (Jipa et al., 2012) and dispersed by vigorous stirring (Kibédi-Szabó et al., 2012; Stoica-Guzun et al., 2011) prior to mixing with PVA solution. Homogenisation of bacterial cellulose has also been used to obtain composites with arabinoxylan (Stevanic et al., 2012) and pullulan (Trovatti et al., 2012), as well as thermoplastic starch (Woehl et al., 2010). These composites were cast and dried at room temperature, 30° C and 60° C, respectively. Bacterial cellulose mixed with water and NaOH before sonication, freezing, thawing, stirring and centrifuging was added to aqueous graphene oxide to develop graphene/bacterial cellulose composites (Feng et al., 2012). These composites also used various combinations of sonication, homogenisation and stirring to achieve fibre dispersion.

PHBV has been used as a matrix material (with differing valerate contents) to develop solution blends using bacterial cellulose nanowhiskers as the reinforcing phase (Martínez-Sanz et al., 2014). The bacterial cellulose nanowhiskers were subjected to a solvent exchange process in chloroform, before being homogenised for 2 minutes, and then blended with PHBV, cast onto petri dishes and dried at

60° C under vacuum. Good dispersion of the nanowhiskers was seen at 1%, however some aggregation was observed at 3% (Martínez-Sanz et al., 2014). Despite the good dispersion, mechanical properties did not display any statistical significance from the neat materials in tensile strength and modulus.

Many of the composites described here showed good mechanical properties, however some aggregation of cellulose fibres was observed. Developing a method to maintain the desirable traits of bacterial cellulose while, at the same time, obtaining even dispersion and distribution of cellulose in solution would allow a simple method to combine materials with controlled concentrations of cellulose.

Electrospinning and Melt Blending

While many researchers use bacterial cellulose in its native form to create polymers, and some treat the bacterial cellulose by homogenisation or hydrolysation prior to casting, there is very little in the literature about the dissolution of bacterial cellulose as part of a method to create composites. One method that has used the dissolution of bacterial cellulose is electrospinning. Chen et al. (2010) used the ionic liquid 1-allyl-3-methylimidazolium chloride to dissolve freeze-dried bacterial cellulose pieces at 70° C while stirring. DMSO was added to the solution to adjust the viscosity at room temperature. Following this, MWCNT were added at a concentration of 0.02% and stirred before electrospinning was undertaken. These electrospun fibres were reported to create a composite with a smooth, continuous surface, with well dispersed MWCNT in the bacterial cellulose. Despite the extremely small concentration of included nanotubes, the composites also had increased tensile strength, thermostability and electrical conductivity, with the dissolution of the bacterial cellulose resulting in the conversion of the bacterial cellulose from the native cellulose I to cellulose II.

In addition to the dissolution of the bacterial cellulose, other electrospun composites have been developed using bacterial cellulose hydrolysed by sulphuric acid to obtain nanowhiskers. Park et al. (2007) combined dispersed bacterial cellulose nanowhiskers with poly(ethylene oxide) (PEO) dissolved in water before electrospinning occurred. The cellulose was included at concentrations of

0.2 and 0.4%, and it was found that increasing the cellulose content increased the diameter of the electrospun fibre. The cellulose nanowhiskers were well incorporated into the fibres, however some fibre surfaces were smooth whereas others appeared rough and uneven, indicating some aggregation of cellulose occurred. Regardless, the inclusion of the nanowhiskers enhanced the mechanical properties over PEO electrospun fibres alone.

Electrospun fibres of up to 20% bacterial cellulose nanowhiskers were developed with poly(methyl methacrylates) (Olsson et al., 2010). Similar to the PEO/nanowhiskers electrospun fibres, some cellulose agglomeration was observed, however the fibre diameter was shown to decrease with increasing cellulose content.

Electrospinning has also been used to develop composites from bacterial cellulose nanowhiskers using different treatments. The nanowhiskers were either centrifuged and refrigerated, or freeze-dried and ground to a powder before being mixed at varying concentrations with ethylene vinyl alcohol (EVOH) and having the solution was electrospun into fibres (Martínez-Sanz et al., 2011b). This paper concluded that the centrifuged nanowhiskers were incorporated into the composite better than the freeze-dried cellulose, and also that the diameter of the electrospun fibres decreased with increasing cellulose content.

Using electrospun bacterial cellulose nanowhiskers/EVOH fibres, melt compounding composites were produced (Martínez-Sanz et al., 2012b). Using a variety of methods, melt blending was carried out for 3 minutes at 190° C with mixing at 100 rpm with electrospun fibres and EVOH pellets. The composites were compression moulded and hot pressed. When the electrospun fibres were used, composites with good fibre dispersion and high stability were produced. When freeze-dried bacterial cellulose nanowhiskers were directly melt blended with EVOH, the composites showed the appearance of black spots which indicated that the cellulose had degraded at the high processing temperature. This method indicates that it is possible for bacterial cellulose to be evenly distributed by melt blending, but that using untreated cellulose nanowhiskers may result in high degradation and the fibres may need to be treated prior to melting. Variations on this method have also been used to produce

bacterial cellulose nanowhiskers/EVOH melt blends with good dispersion and improvements to the elastic modulus and tensile strength in the composites (Martínez-Sanz et al., 2013a, 2013b). In addition, PLA/bacterial cellulose nanowhiskers melt blends have been developed using electrospinning methods (Martínez-Sanz et al., 2012a). These PLA blends were produced by using bacterial cellulose nanowhiskers/PLA fibres by electrospinning, by using nanowhiskers from an EVOH copolymer precipitation solution, and by melt blending PLA with freeze-dried cellulose nanowhiskers as a control. This study determined that the electrospinning technique was particularly successful, resulting in good dispersion of nanowhiskers up to 3%, with increases in the elastic modulus and tensile strength from these conditions (Martínez-Sanz et al., 2012a).

Melt blending was used to develop PLA/bacterial cellulose composites, where the cellulose was disintegrated and subject to solvent exchange before being chemically modified by acetylation (Tomé et al., 2011). Melt blending was carried out at 190° C for 10 minutes at 100 rpm with the composites then injection moulded. Unmodified bacterial cellulose fibres were found to agglomerate. Despite the high processing temperature of 190° C used here, the acetylated cellulose composites demonstrated improved mechanical properties, indicating that melt blending may be useful in developing fully biodegradable composites.

These recent reports demonstrate that it may be possible to adapt a more traditional method, such as melt compounding, to disperse bacterial cellulose and use this material as a reinforcing phase in composites. This type of technique can be easily upscaled and may be adapted provide a method to produce bacterial cellulose composites for commercial applications.

In situ Composites

The inclusion of additives not specifically required for cell growth or cellulose production in the growth media can affect the cellulose produced. We have stated that some researchers observe a change in the structure, morphology and/or properties of the resulting cellulose (Cheng et al., 2009a; Tokoh et al., 2002), whereas others have determined that the host polymer present in the culture

medium can combine with the cellulose, creating *in situ* composites (Heßler & Klemm, 2009; Seifert et al., 2004).

CMC and methylcellulose (MC) have been included as additives in media for bacterial cellulose growth with differing results. Some authors reported that these additives resulted in changes to the cellulose, including decreased crystal size and crystallinity, with increased thermal stability and pore size (Cheng et al., 2009a), but did not investigate the presence of the additives in the cellulose product, instead focusing and reporting on the alterations to the cellulose. Others stated that when CMC and MC were included in the media along with the growing cellulose, composite materials were created, but the amount of the additive in these composites was not determined (Seifert et al., 2004). When acid treated MWCNT were added to the culture medium, cellulose was produced with altered structure, but it was also determined that the nanotubes became interwoven within the cellulose fibrils, effectively producing composites of these two materials (Yan et al., 2008). Weakened intermolecular hydrogen bonds also occurred as a result of weaker bonds between the MWCNT and the cellulose, compared to bonds in the cellulose alone. However, this paper did not report on the content of the MWCNT or cellulose in the product, or the mechanical properties of the resulting membranes. PVA was also added to the culture medium for bacterial cellulose, and the resultant cellulose exhibited different properties to neat cellulose, but no PVA was detected in the cellulose after the product was washed (Seifert et al., 2004). Gea et al. (2010) created bacterial cellulose/PVA nanocomposites by the inclusion of PVA in the culture media at different concentrations, and they estimated that PVA was included in the composite at a maximum value of 1.3%. It is apparent that whilst *in situ* bacterial cellulose composites can be created simply by including an appropriate additive in the growth media, many of the composites reported contained only small amounts of the additive, and only resulted in small changes to the cellulose properties.

There have been others, however, who have reported much higher concentrations of the host polymer in the composite. Brown and Laborie (2007) added PEO to the culture medium and developed several nanocomposites with different ratios, ranging from 15 to 59% bacterial cellulose, demonstrating

different compositions and morphologies. In addition, they determined that increased bacterial cellulose contents resulted in smaller cellulose fibres and an aggregation of these fibres, although at lower cellulose contents the fibres could be finely dispersed. These researchers did not attempt to remove the bacterial cells from the composites for fear that PEO would be lost in the washing process, and they found that the cell debris impacted the properties of the composite. As *in situ* composites are created directly from the bacterial culture, resulting composites may be contaminated with cell debris. The question of whether or not the cell debris needs to be removed is something that requires further investigation with a wider range of materials.

Grande et al. (2009) developed a method to create *in situ* bacterial cellulose composites by including starch in the culture medium at a concentration of 2%. The starch was partially gelatinised during the autoclaving of the media, and the bacterial cellulose/starch gels that were subsequently produced were then hot pressed in order to encourage further diffusion of the starch into the cellulose network. SEM images of these composites showed good coverage of the cellulose with starch; some fibrils were still visible (see Figure 2.9), however the bacterial cellulose was largely dispersed and the nanocomposites had good mechanical properties.

Due to the biological nature of bacterial cellulose, *in situ* methods involving the inclusion a polymer in the culture medium for cellulose to combine the materials provides an alternative method for creating composites. It is possible that composites could be made simply by the inclusion of the host polymer in the medium, however the development of subsequent treatments, for example the hot pressing of the starch/cellulose films described above, could improve the contact and interactions between the

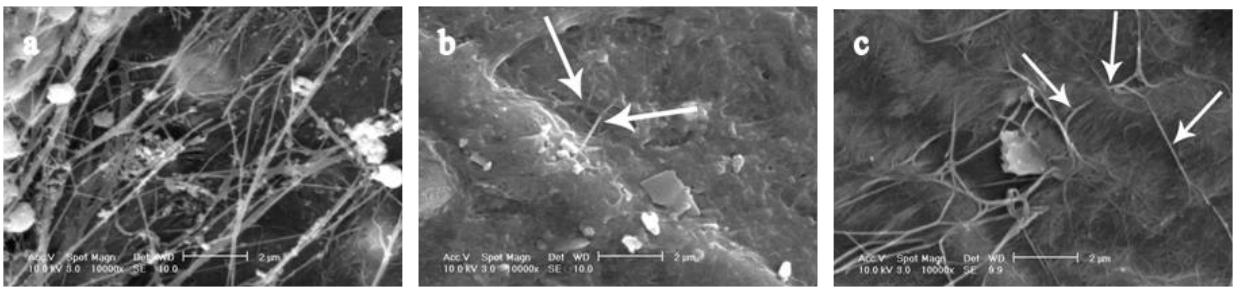


Figure 2.9: Starch/bacterial cellulose *in situ* composites, Grande et al. (2009). Some bacterial cellulose fibres can be seen amongst starch as shown by arrows. Reproduced with permission from Elsevier.

two materials. Identifying materials to be used in composites with bacterial cellulose in this way could provide improvements into the development of these types of composites.

Bacterial Cellulose Composites by Other Methods

Various impregnation methods have been used to develop composites with bacterial cellulose, with increasing numbers of solution blends and melt blends reported, however there are also reports of composites published that have used entirely different methods. Quero et al. (2010) compressed strips of bacterial cellulose between two strips of PLA films. This work found a difference between composites with bacterial cellulose that had been grown for different periods of time. Cellulose that was cultivated for 3 days showed improved interactions with the PLA over cellulose grown for 6 days due to higher total surface area, but the 6-day-old cellulose composites had better mechanical properties.

Bacterial cellulose/polyurethane based resin composites were developed by employing a similar technique. Bacterial cellulose sheets were dried between two polyterafluoroethylene membranes under pressure before impregnation in the resin and UV curing. Composites using cellulose that were dried from water provided better mechanical properties than composites with cellulose that had undergone a solvent-exchange step in ethanol, however the ethanol composite demonstrated enhanced dielectric properties (Juntaro et al., 2012).

A different method involving injection moulding was used to provide bacterial cellulose/starch composites with 1% and 5% cellulose (Martins et al., 2009). These polymers were mixed in water with glycerol to a homogeneous mixture in polyethylene bags. The mixtures had stearic acid added and were then processed by mixing at 120° C for 20 – 30 minutes at 60 rpm before they were injection moulded. This method demonstrated good fibre dispersion and good mechanical properties, with strong matrix-fibre interactions.

A method developed from temperature induced phase separation was completed to obtain PLA/bacterial cellulose composites (Lee et al., 2009). In this work, bacterial cellulose was added to

1,4-dioxane and homogenised before PLA was added and dissolved, following which the mixture was added dropwise into a liquid nitrogen bath. The precipitate was collected and freeze-dried to produce composite microspheres, which were then fed into a twin-screw extruder and were mixed at 180° C, extruded, pelletised and hot press compression moulded into films. PLA films containing bacterial cellulose showed an increase in tensile modulus, with composites containing bacterial cellulose and chemically modified bacterial cellulose shown to have improvements over PLA alone (Lee et al., 2009).

Though bacterial cellulose is difficult to process, there have been an increasing number of reports in recent years involving this material in composites. It is also possible that methods that have been used to blend plant cellulose will be able to be applied to bacterial cellulose.

Biodegradability

Biodegradation can be difficult to define and even more difficult to observe. It is often defined as an event which takes place through action of enzymes and/or chemical decomposition associated with living organisms (such as microbes) or their secretion products (Amass et al., 1998). Above, we described biodegradation in terms of the breakdown of a material as degradation resulting from the action of microorganisms. However, true biodegradation is more complex than this. In the environment there are both biotic and abiotic factors that influence the breakdown of materials. Biodegradation is made up of three stages: biodeterioration, biofragmentation and assimilation – the processes by which materials are broken down into tiny fractions, the cleavage of polymeric materials that occurs so some materials can cross the microbial cell wall, and the integration into microbial metabolism, respectively (Lucas et al., 2008).

Soil burial is a method by which researchers can observe biodegradation. The mass of a material is measured before and after soil burial for a period of time to determine if there is a loss of mass due to a material being broken down and integrated into microbial metabolism. Composting can also be used as a means of investigating biodegradation. Another method is to attempt to observe a loss of mass if a

material is placed in a culture medium with a microorganism such as a bacterium or fungus capable of degrading that specific material.

Bacterial cellulose is a typical polysaccharide, composed of carbon, hydrogen and oxygen. It is produced naturally in the soil by bacterial species such as *Gluconacetobacter*. In the environment, it is believed that bacterial cellulose is used to assist the bacterial cells in colonisation and protection (Williams & Cannon, 1989), but may also be broken down to be used as a source of nutrients for microorganisms (Costerton et al., 1987), thus being biodegraded in the environment.

Bacterial cellulose in composite materials with other polymers has been shown to break down, though there have been conflicting results in regards to the rate of this degradation due to the cellulose. Starch/bacterial cellulose composites have been shown to have lower weight loss than neat starch. Wan et al. (2009) have suggested that microbes first attack the starch in the composite, which destroys the composite integrity and eventually the cellulose is attacked, this result obtained by soil burial. In another study, Stoica-Guzun et al. (2011) reported that a higher bacterial cellulose content in PVA/bacterial cellulose composites demonstrated higher degradation over lower bacterial cellulose contents when degraded in laboratory media by a single fungal strain by visual observation (Figure 2.10). Therefore it is unclear if using bacterial cellulose in biodegradable composites will increase degradation rates, or if the polymer matrix will be attacked first. It is likely that the specific matrix used will have an impact on the overall biodegradation rate, its own biodegradability dominating, compared to bacterial cellulose. In addition, biodegradation studies have been completed using PVA/bacterial cellulose composites with and without the presence of chitosan in an activated sludge fed-batch bioreactor under aerobic and anaerobic conditions by investigating the weight loss of the composites over a period of time (Kibédi-Szabó et al., 2012). In this study, degradation was found to be higher in the composite containing chitosan, but was also higher in the anaerobic environment as opposed to the aerobic environment.

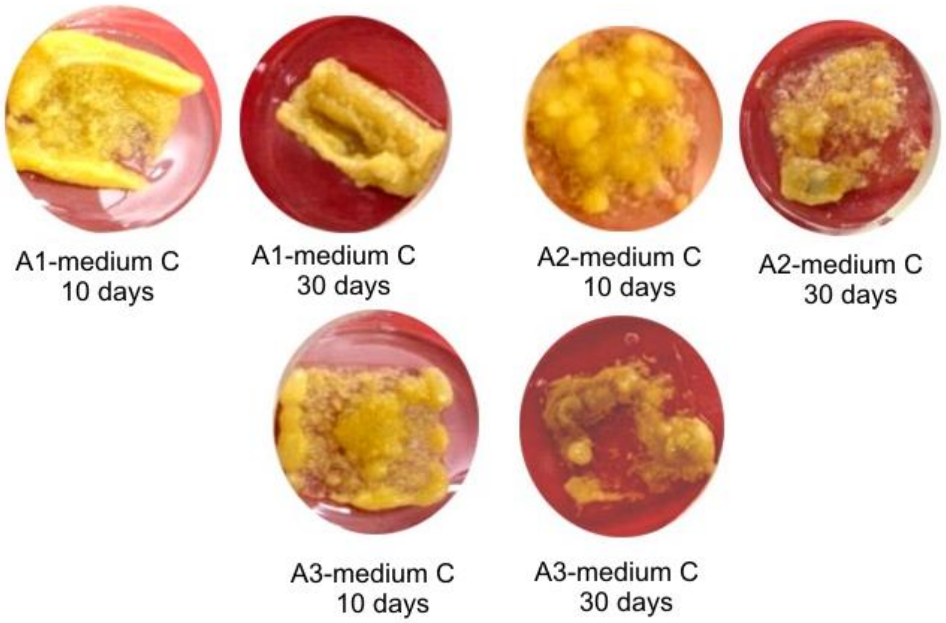


Figure 2.10: PVA/BC composites degraded over time by a single fungal strain, from Stoica-Guzun et al. (2011). Reproduced with permission from Springer.

While showing that a single species of microorganism can break down a material in a laboratory culture does show biodegradation, as does an activated sludge bioreactor, it is unrealistic of environmental conditions. When biodegradable materials reach the end of their useable life span, they will be discarded, as most current plastics are. An ideal situation would be if we were able to develop materials that could degrade in the environment, however composting may be necessary. There are also biopolymers that degrade in marine environments with their own set of conditions. With the introduction of increased biodegradable materials, there may need to be a change in waste management depending on the environments in which different materials degrade. Therefore it is necessary to test a material's biodegradability in a method that is relevant and can then be transferred to the real environment.

Conclusions

Bacterial cellulose has been fairly extensively investigated in terms of its growth and structure, with many stimulating agents determined. There have been many reports of developing bacterial cellulose

composites by impregnation methods, but the properties of the cellulose make it difficult to achieve homogeneous dispersions and therefore development of composites using this material as a reinforcing phase have been limited. Increasingly, however, techniques are being established to produce and modify bacterial cellulose. For example, it is possible to tailor-design bacterial cellulose by selecting appropriate media and cultivation conditions, through *in situ* modifications, or by post-modifications such as chemical treatments and physical modifications such as ball milling or aqueous counter collisions. There are also increasing reports of techniques used to disperse and process this material. As such, there has been an increase in the number of publications relating to bacterial cellulose composites using more traditional techniques such as solution and melt blending. Therefore, despite the difficulties in processing this material, bacterial cellulose remains a good candidate for fibre reinforcement in fully biodegradable composites in conjunction with a biodegradable matrix.

2.3 Additional Work from the Literature

The review presented in Section 2.2 critically evaluated current understandings of the composition and properties of bacterial cellulose, from extensive previous research to its recent use in composites. This section presents a review of the development and properties of PHB as a matrix material and of the characteristic approaches taken in studies of plant cellulose materials.

2.3.1 Poly-3-hydroxybutyrate as a Matrix Material

2.3.1.1 History and structure of PHB

PHB was originally discovered by Maurice Lemoigne in the 1920s, who labelled it a "lipid". However, it was not until after PHB was "re-discovered" in the 1950s, when it came to the attention of microbiologists and biochemists, that PHB was seen as a potential substitute for petroleum-derived plastics. PHB has been found to have similar properties to polypropylene. It has a high molecular weight and a melting temperature of 170 – 180° C. It also has similar tensile strength and a glass-rubber transition temperature, but PHB is stiffer and more brittle than polypropylene (Holmes, 1985; King, 1982). It becomes viscous and mouldable at temperatures above its melting point, but it degrades at temperatures above 190° C so it has a very narrow window for melt processing (Orts et al., 2008). In addition, PHB has a number of copolymers, such as PHBV and P(3HB-co-4-HB), that have been shown to have decreased melting points, strength and stiffness, but increased ductility (Jiang et al., 2008; Verhoogt et al., 1994). Copolymers are discussed further in Section 2.3.3. It has been hypothesised that an appropriate filler material could improve the properties of these materials (Gatenholm et al., 1992).

It was first suggested by Lemoigne, and later confirmed by others, that PHB is an intracellular storage molecule that can be broken down by the bacterial cell in the absence of nutrients (Braunegg et al., 1998). Because of this, it is biodegradable. PHB can be attacked by microbial enzymes, which cause it to be broken down to carbon dioxide and water (Rehm, 2010). This process can be completed by many bacteria and fungi in a range of environments. Not only is PHB biodegradable, it is also

biocompatible, allowing a wide range of potential uses, including medical implants, tissue scaffolding and delayed drug release (Wu et al., 2009).

2.3.1.2 Growth Conditions

A number of bacterial species have been identified as producing PHB as an intracellular storage molecule. These include *Actinomycetes*, *Alcaligenes*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Beijerinckia*, *Chromatium*, *Chromobacterium*, *Derxia*, *Ferrobacillus*, *Hyphomicrobium*, *Lampropaedia*, *Methylobacterium*, *Micrococcus*, *Nocardia*, *Pseudomonas*, *Rhizobium*, *Rhodopseudomonas*, *Rhodospirillum*, *Sphaerotilus*, *Spirillum*, *Streptomyces*, *Vibrio* and *Zoogloea* (Byrom, 1987). PHB accumulates as discrete, spherical granules that can make up to 80 – 90% of the cell's dry weight (Holmes, 1985; Steinbüchel & Lütke-Eversloh, 2003). It accumulates as a reserve material when there is excess carbon present, but the cell's growth is limited due to a lack of other nutrients such as nitrogen or phosphorous (Rehm, 2010). The accumulated PHB is broken down and used as a carbon source at a time when there is no extracellular carbon available (Lenz & Marchessault, 2005). When all nutrients are fully available, the cell's metabolism progresses through the tricarboxylic acid cycle where acetyl-Co-A is used for energy and the production of cell materials. However, when there is an excess of carbon, acetyl-Co-A is shunted into PHB production instead (Byrom, 1987).

The production of PHB can be further manipulated *in vitro* by the selection of the growth medium and conditions, and the microbe to produce PHB (Orts et al., 2008). Certain growth conditions, such as oxygen and phosphorous starvation, produce higher levels of PHB (Orts et al., 2008); however it has also been found that PHB-like materials can be produced under some conditions. It was determined that a bacterium, *Alcaligenes eutrophus*, could produce a PHB-like material when grown with a medium with glucose and propionic acid. The material was then identified as a random copolymer of PHB and poly-3-hydroxyvalerate (PHV) (Lenz & Marchessault, 2005). This, together with other copolymers of PHB, is described in the next section.

2.3.1.3 Copolymers

PHB has a high melting temperature and poor mechanical properties. However, it has been found to exist as a copolymer with PHV (as well as other materials), and that this copolymer has better mechanical properties. The PHB-PHV copolymer, PHBV, can exist with a range of PHV concentrations. The PHV content can be controlled by the glucose:propionic acid ratio in the medium used to grown PHBV-producing bacteria (Byrom, 1987). The melting point of this copolymer decreases as the PHV content increases (up to 40 mol%) (Orts et al., 2008). In addition, the PHBV copolymers have superior properties than those of PHB alone, with increased flexibility and toughness (Byrom, 1987). The copolymer is also highly crystalline and fully biodegradable (Lenz & Marchessault, 2005; Orts et al., 2008) lending itself to the possibility of using it as a superior material to replace petrochemical resources.

In addition to the PHBV copolymers, PHB has been found to exist as a copolymer with poly-4-hydroxybutyrate, PHV and poly-5-hydroxyvalerate, poly-4-hydroxybutyrate and PHV, hydroxypropionate, 3-hydroxyhexanoate, and poly-3-hydroxyoctanoate (Braunegg et al., 1998; Czaja et al., 2007).

Based on this information, it is apparent that PHB and copolymers could benefit from the inclusion of a reinforcing phase to produce composites. To take advantage of the biodegradable and biocompatible nature of these materials, a material such as bacterial cellulose is an appropriate choice for the investigation of PHB composites with improved mechanical properties.

2.3.2 Plant Cellulose Composites

Though there has not been a great deal of research undertaken in bacterial cellulose to investigate this material as a reinforcement material in polymer matrices, plant cellulose composites have been more extensively researched. It is likely that investigations in plant cellulose could be adapted to bacterial cellulose. A brief review of plant cellulose composites developed by solution and melt blending is presented here.

Plant cellulose has been used with a variety of matrices and various techniques, such as the more traditional blending techniques of solution blending and melt blending. In addition to the ease of completing solution and melt blending, such methods can be more easily upscaled to industrial size processes. Plant cellulose has been solution cast with matrices such as poly(styrene-co-butyl acrylate) (Siró & Plackett, 2010), gelatine (Pei et al., 2011), PLA (Sanchez-Garcia & Lagaron, 2010), and PHBV (Jiang et al., 2008; Yu et al., 2011) using various solvents. Solution blending has been traditionally used for cellulose blending, as cellulose has low thermal stability when heated, which limits its use in techniques that involve heat (Goffin et al., 2011). However, several researchers have determined that cellulose fibres can be successfully used in melt blending in conjunction with chemical modifications and/or compatibilisers.

The properties of composites formed via solution blending were compared by Jiang et al. (2008) with PHBV as the matrix material and cellulose nanowhiskers (CNW). This study determined that solution blending of the two materials in *N,N*-dimethylformamide (DMF) resulted in improved mechanical properties including tensile strength and modulus, whereas a melt processing technique (with the use of a compatibiliser) actually reduced the mechanical properties of the composite. Determining a set of parameters for melt blending can be difficult as the properties of the composites are dependent on the mixing conditions (Saheb & Jog, 1999). It is therefore crucial to study and understand the thermal properties of composites to optimise the manufacturing process (Li et al., 2008).

Microcrystalline cellulose (MCC) has been used in composites with poly(ethylene terephthalate)-poly(trimethylene terephthalate) matrices at 0 to 40% cellulose by melt blending (Kiziltas et al., 2011). It was found that the addition of MCC did not affect the glass transition temperature, melting or crystallisation temperature, but increasing the MCC content led to an increase in mechanical properties.

It is essential to achieve good fibre dispersion and interfacial adhesions in a composite in order to achieve improved mechanical properties. A variety of techniques have been investigated in order to identify a technique for improving fibre dispersion of cellulose fibres in matrices. Yang et al. (2011)

investigated the dispersion of MCC and cellulose nanofibrils as fibres in a polypropylene matrix by altering the blending conditions. Composites were created by melt blending the materials multiple times, and by single batch melt blending with increasing mixing time. Increasing the number of times the melt blending was completed and increasing the mixing time resulted in improved fibre dispersion. The mean of the mechanical properties did not change, but the variability was decreased, indicating greater reliability in the mechanical property values.

Another method that was found to improve fibre dispersion is pan milling (Zhang et al., 2011). Zhang et al. (2007) developed a novel pulverising equipment, a pan-type mill, in order to produce better composites than ball milling. They determined that pan milling cellulose could break hydrogen bonds, resulting in the presence of reactive hydroxyl groups on the surface on the cellulose. It was hypothesised that these free hydroxyl groups would react with a PVA matrix, resulting in hydrogen bonds between the matrix and fibres. It was determined that one round of pan milling did not allow for satisfactory adhesion between the matrix and fibres, but an increase in the number of milling cycles resulted in better fibre dispersion and increased mechanical properties of the composite. Composites with cellulose milled 40 times also demonstrated better biodegradability than composites with cellulose milled only once and the matrix alone, probably due to the cellulose being smaller and less crystalline because of the multiple milling cycles (Zhang et al., 2011).

Cellulose fibres have been used with and without compatibilisers with matrices such as polystyrene (Pracella et al., 2011), high density polyethylene (Tajeddin & Abdulah, 2010), ethylene-vinyl acetate (Haque & Pracella, 2010) and PHB (Rapa et al., 2010) using melt blending techniques. These studies typically report that composites created in the presence of a compatibiliser showed improved fibre dispersion and mechanical properties over a matrix and fibre alone, as composites with unmodified cellulose fires had poor adhesion. It was also determined that the matrix and fibre interactions depended on the type of copolymer compatibiliser structure and content (Haque & Pracella, 2010).

Compatibilisation was taken a step further by Goffin et al. (2011) who grafted PLA chains to the surface of CNW before melt blending the nanowhiskers to a PLA matrix. They examined

uncompatibilised fibres and found that ungrafted CNW composites showed a colour change after melt blending, appearing very dark. This suggests that thermal degradation occurred with the unmodified CNW (at 8%), whereas the surface grafted composites remained colourless. It was also found that the grafting of the CNW prevented thermal degradation by acting as a protective shell, therefore allowing processing at high temperatures (Goffin et al., 2011).

It is apparent that various forms of cellulose can be used as reinforcement in composites using traditional blending methods such as melt blending. It is possible that bacterial cellulose could be used with such a technique, however it is likely that a compatibiliser and/or surface modification would be necessary to improve fibre/matrix interactions. Developing further techniques to apply to bacterial cellulose are needed to enable the use of this form of cellulose in composites.

2.4 Conclusions

Based on this critical review of the literature, it appears likely that fully biodegradable composites can be achieved using bacterial cellulose as a reinforcing material. Methods to disperse plant cellulose, to achieve modifications to the cellulose and to produce composites could be adapted to bacterial cellulose, taking advantage of the natural purity and nanosized fibres of bacterial cellulose. Homogenous dispersion of fibres is likely to be a key challenge in the development of bacterial cellulose composites, restricting composites to low concentrations of cellulose in the blends. However, based on the surface area of bacterial cellulose fibres, low concentrations may still result in favourable properties in the composites. Therefore, this work aims to examine bacterial cellulose, including its growth and production and techniques to achieve modifications, to produce a biodegradable material for use as a reinforcing material in composites. Different ways to achieve a good dispersion of this material in composites in order to achieve good mechanical properties are also to be investigated. Finally, the biodegradability of produced composites is to be considered.

Chapter 3
Growth Conditions for Bacterial Cellulose

3.1 Preface

Bacterial cellulose is produced in high amounts by the bacterium *Gluconacetobacter xylinus*. It has been documented that the structure and morphology of the cellulose can be altered at macroscopic and nanoscopic levels based on cultivation conditions. Schramm and Hestrin (1954) developed a medium, HS, for the cultivation of *G. xylinus*, that continues to be widely used. Since then, other researchers have described a variety of media that produce higher levels of cellulose than HS medium (Son et al., 2003; Toyosaki et al., 1995; Yamanaka et al., 1989; Zhou et al., 2007). There have also been studies examining different carbon sources in some of these media. However, the high cellulose-producing media have never been directly compared in terms of cellulose yield, nor have carbon sources been examined in all of these media.

In addition to different media, there are also different methods by which bacterial cellulose can be grown, such as static or dynamic culturing conditions. This chapter explores different cultivation conditions tested in order to obtain high amounts of bacterial cellulose, alongside examination of the influence of different media, carbon sources, cultivation containers, incubation times and media volumes. The media and cultivation conditions were selected from various reports and modified in order to directly compare the different reports of cellulose yields to one another, using reports of media found to achieve high cellulose yields as well as investigating different carbon sources with these media.

3.2 Altering the Growth Conditions of *Gluconacetobacter xylinus* to Maximize the Yield of Bacterial Cellulose

The paper presented here provides a comparison of the yields and properties of bacterial cellulose obtained using a range of carbon sources with high cellulose-producing media previously reported in the literature. It examines the cost-effectiveness of using different sized containers and volumes of media to obtain this material. This information is of importance as it is necessary to know how to produce cost-effective bacterial cellulose, and whether the media or growth conditions impact the yield and structure of the cellulose produced.

This paper was published in *Carbohydrate Polymers* in 2012 and is presented here in its published form. It is reproduced here with permission from Elsevier.

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Determining which experiments to do based on gaps in the literature, carrying out the experiments, writing the initial draft of the paper and changing the paper based on advice from supervisors	90

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
George Simon	Providing supervision, proof reading and editing	N/A
Katherine Dean	Providing supervision, proof reading and editing	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	[redacted]	13/5/14
Main Supervisor's Signature	[redacted]	13/5/14

Carbohydrate Polymers 89 (2012) 613–622

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

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

Dianne R. Ruka[a,b], George P. Simon[b], Katherine M. Dean[a,*]

[a] *CSIRO Materials Science & Engineering, Gate 5 Normanby Road, Clayton 3168, Australia*
[b] *Department of Materials Engineering, Monash University, Clayton 3800, Australia*

ARTICLE INFO

Article history:
Received 29 September 2011
Received in revised form 8 March 2012
Accepted 16 March 2012
Available online 28 March 2012

Keywords:
Bacterial cellulose
Gluconacetobacter xylinus
Yield
Growth conditions
Media

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

* Corresponding author. [redacted]
URL: http://www.csiro.au (K.M. Dean).

0144-8617/$ – see front matter Crown Copyright © 2012 Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2012.03.059

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 $d_{1\,-1\,0}$ 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).

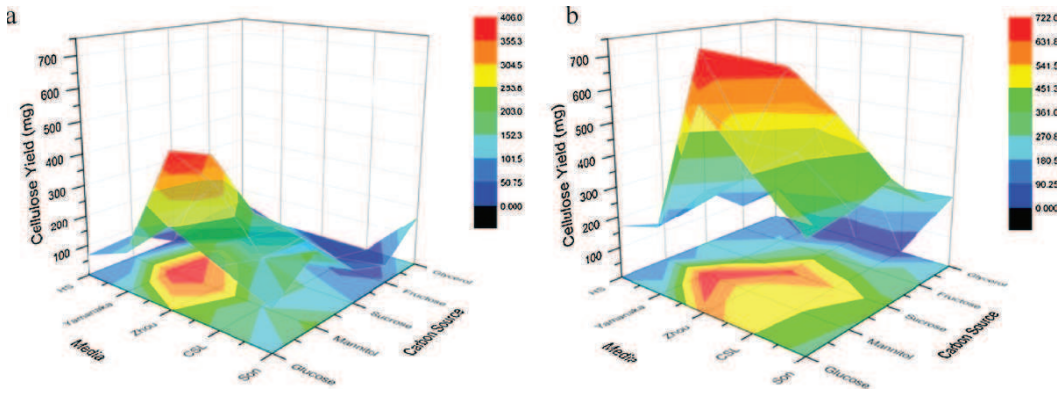


Fig. 1. Cellulose yields produced in different media with a variety of carbon sources after 4 days (a) and 7 days (b) of incubation.

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	–

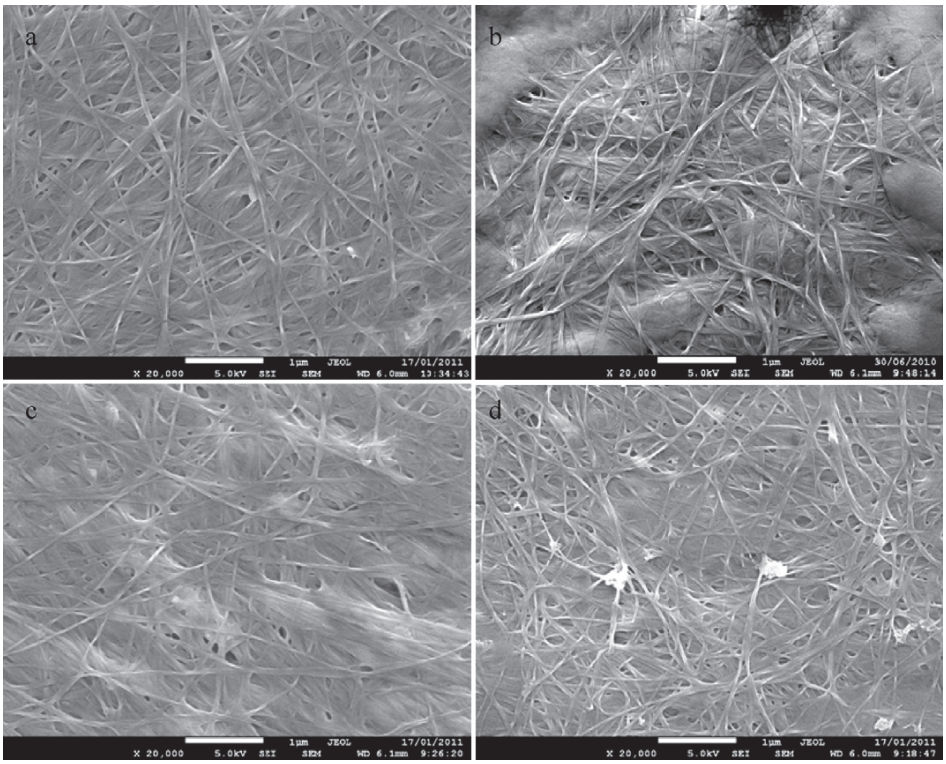


Fig. 2. SEM images of cellulose pellicles produced in various media. HS-glucose (a), Yamanaka-mannitol (b), Zhou-sucrose (c), and Zhou-mannitol (d).

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

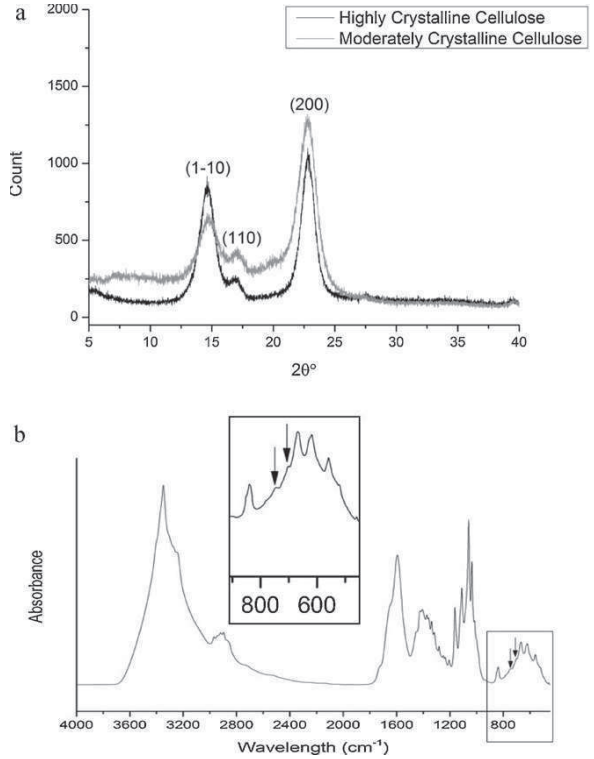


Fig. 3. Structural tests used to characterize bacterial cellulose. (a) XRD diffractograms with three peaks from cellulose produced with high and moderate crystallinity and (b) FTIR scan with I_α mass fraction determined from peaks at 750 and 710 cm^{-1}, as indicated.

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

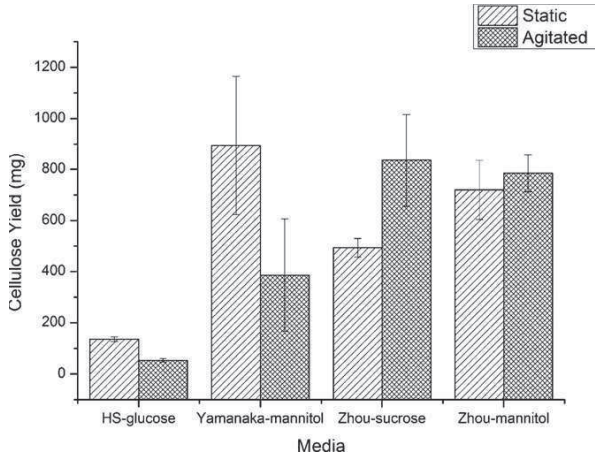


Fig. 4. Cellulose yields produced in different media under static and agitated conditions.

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),

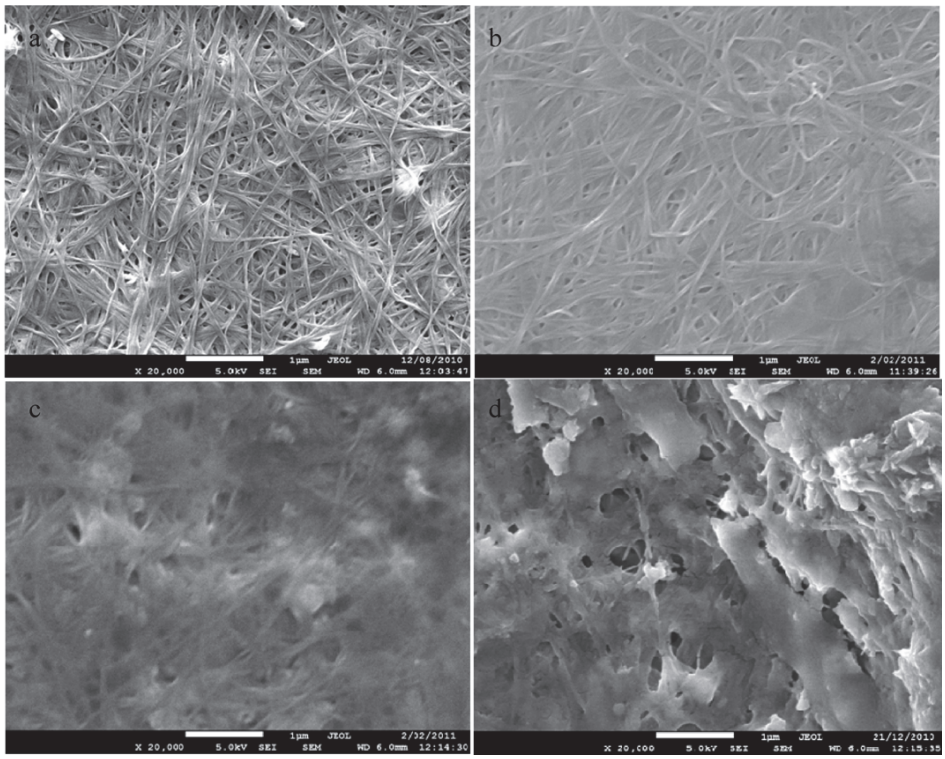


Fig. 5. SEM micrographs of cellulose produced in HS-glucose (a), Yamanaka-mannitol (b), Zhou-sucrose (c) and Zhou-mannitol (d) medium under agitated conditions.

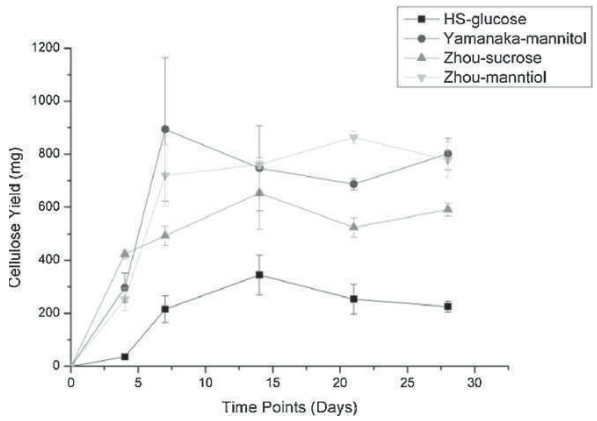


Fig. 6. Cellulose yields produced over time in high cellulose producing media.

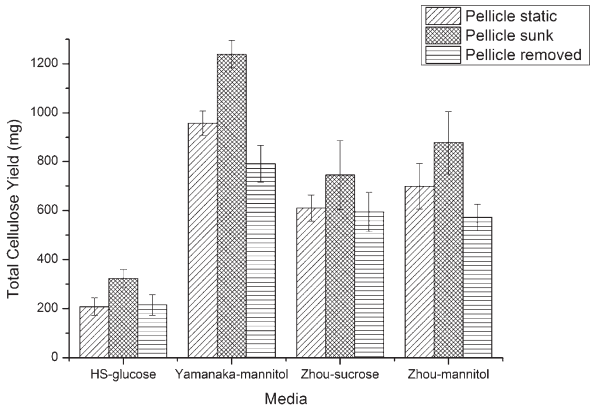


Fig. 7. Cellulose yields obtained from cultures in various media when flasks were shaken or had pellicles removed every five days.

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

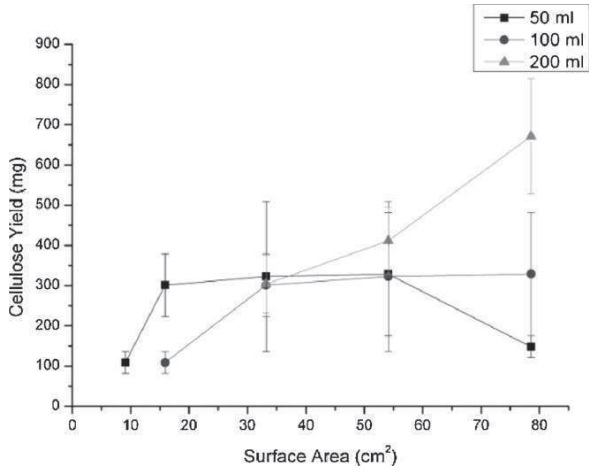


Fig. 8. Cellulose yield produced from varying volumes of media in beakers with different surface areas.

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

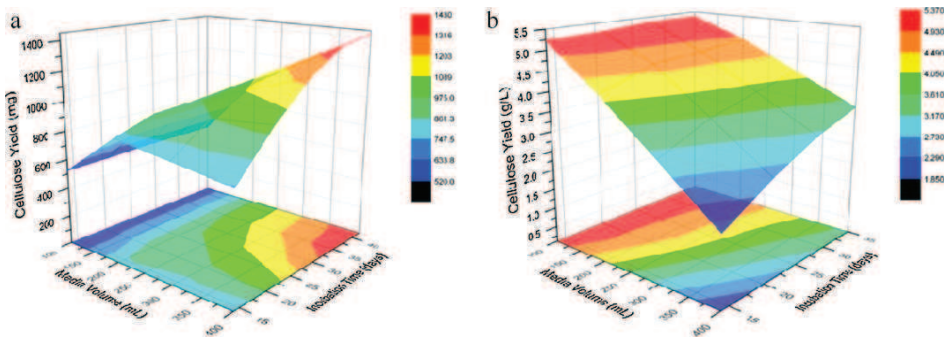


Fig. 9. Cellulose yields produced in different volumes of media with cellulose yield presented in mg (a) and g/L (b).

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.

Acknowledgments

The authors would like to thank the Monash Centre for Electron Microscopy for the use of their equipment, and Liz Goodall of CSIRO for completing the XRD work and calculations. This work was funded by a Julius Career Award from the CSIRO Office of the Chief Executive.

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

3.3 Cost Analysis of Bacterial Cellulose Production

It was found that Yamanaka-mannitol medium produced the highest level of cellulose under the selected conditions. Yamanaka-mannitol medium contains high concentrations of mannitol and yeast extract. Yeast extract has been included in several different media and has been shown to produce high levels of cellulose (Jung et al., 2010a), however this component is expensive. The individual components that make up the different media will be a factor in the price of production of bacterial cellulose. To this end, corn steep liquor, a low cost, high-nutrient source material that has been reported as producing high amounts of bacterial cellulose (El-Saied et al., 2008; Jung et al., 2010b; Nguyen et al., 2008; Son et al., 2001; Toyosaki et al., 1995; Yang et al., 1998), was included in various media, however Yamanaka-mannitol still produced higher amounts of cellulose than the CSL-containing media examined here. If the cost of the media is taken into account and related to the amount of cellulose produced, Yamanaka-mannitol can actually be considered a cost-effective medium. Figure 3.1 shows the approximate cost of the various media examined here, along with the

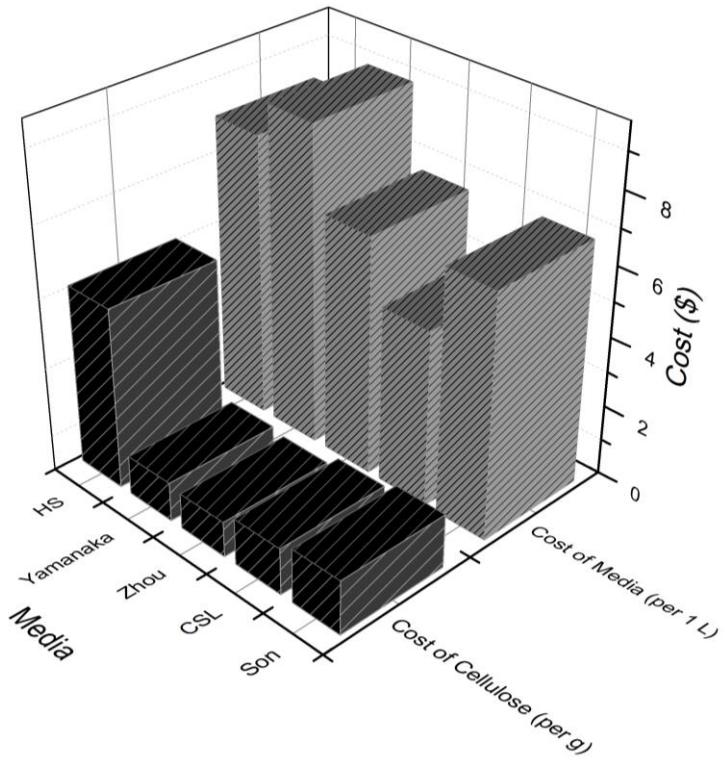


Figure 3.1: The cost of producing 1 L of each of the media examined, and the cost of cellulose produced in these media in terms of $ (AUD) per gram of cellulose.

cost of these media in terms of the amount of cellulose produced in the 50 mL flasks used in the testing conditions. The information provided here is only an estimate, as prices depend on suppliers and size of reagents purchased, and the amount of cellulose achieved depends on different growth conditions and surface areas of the reaction vessels used. However, it can be seen that Yamanaka-mannitol is one of the more expensive media to produce, but when the amount of cellulose produced is considered it actually provides the more cost effective output. In contrast, the HS medium, with its high cost and low productivity, is the least economically viable medium, producing cellulose at the highest cost (Figure 3.1). The other media vary in terms of their cost, but all produce cellulose at a similar price to that of the Yamanaka-mannitol medium. As Yamanaka-mannitol medium is cost-effective and produces high amounts of cellulose, it can be used as a successful medium for bacterial cellulose production.

3.4 Conclusions

Based on the findings presented in the paper here, it is clear that there are ways to maximise the yield of bacterial cellulose, including using vessels with greater surface area, and by using media capable of producing high amounts of cellulose. However, it should also be noted that different media produce cellulose with different crystallinity, and thus properties may vary and choice of media cannot be made on a cost basis only. Likewise, agitating particular media can cause a loss of the fibrillar structures that are obtained from static culture, demonstrating static cultures are most suitable for further experimentation. HS and Yamanaka-mannitol media were found to be appropriate media for use as standard and high-cellulose producing media, respectively, and should be considered further for cellulose production.

Chapter 4
In situ Modifications to Bacterial Cellulose

4.1 Preface

Growth conditions of *Gluconacetobacter xylinus* can impact the structure and properties of bacterial cellulose, as demonstrated in Chapter 3. The structure and properties of bacterial cellulose also can be altered by including additives not specifically required for bacterial cell growth in the media used to grow *G. xylinus*. In addition to altering the bacterial cellulose, some authors have successfully produced *in situ* nanocomposites by including a compatible host polymer into the media for incorporation into the bacterial cellulose during synthesis (Brown & Laborie, 2007; Gea et al., 2010; Grande et al., 2009). Understanding the changes that additives can make to bacterial cellulose could provide the ability to "tailor-design" cellulose with specific traits and properties. This could provide benefits if the cellulose is then to be incorporated into particular composites.

A number of additives that were previously reported in the literature as causing *in situ* modifications to bacterial cellulose were initially investigated to determine appropriate methods and protocols to examine the cellulose produced. The results from these additives and cellulose characteristics are presented in the Appendix. In addition, novel additives were investigated and are presented here. This chapter includes the investigation of additives in the form of ionic liquids and poly-3-hydroxybutyrate.

Bacterial cellulose is extremely hydrophilic and insoluble in water and most organic solvents due to the extensive hydrogen bonding in and between the glucan chains that make up cellulose. Recently, ionic liquids such as 1-*N*-butyl-3-methylimidazolium chloride (Schlufter et al., 2006) and 1-allyl-3-methylimidazolium chloride (Chen et al., 2010) were reported to dissolve even the extremely high molecular weight bacterial cellulose. Therefore some ionic liquids have the ability to impact bacterial cellulose. As additives included in the media can affect the structure and morphology of the resulting cellulose, ionic liquids may therefore also affect bacterial cellulose if included in the culture media, but may also offer the potential to include otherwise insoluble materials in the growth media for additional modifications.

Documenting the changes that occur in the structure and morphology of bacterial cellulose as a result of the inclusion of additives in the media could produce methodologies for cellulose with specific characteristics to be obtained. This would provide a means of producing particular cellulose for certain purposes, achieving “tailor-designed” bacterial cellulose with particular, desired properties.

4.2 Bacterial Cellulose Growth from Media containing Ionic Liquids composed of Choline Salts

A study was prepared and conducted to examine if nutrient-based ionic liquids could be included in cellulose-producing media as additives and still allow the production of bacterial cellulose. This paper presents five choline salts for this purposes, and the changes to the bacterial cellulose that occur as a result.

This manuscript is currently unpublished. It has been formatted to match the structure of the thesis. The figures and tables have been numbered to reflect the chapter number.

Declaration for Thesis Chapter 4

Declaration by Candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Determining how to complete the experiments based on previously completed experiments, carrying out experiments, writing the paper	70

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Vijayaraghavan Ranganathan	Synthesising the organic salts used in the experiments	N/A
Douglas MacFarlane	Synthesising the organic salts used in the experiments, writing the methods section on the synthesis of the organic salts, writing sections of the paper relating to the organic salts	N/A
George Simon	Providing supervision, proof reading and editing	N/A
Katherine Dean	Providing supervision, proof reading and editing	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	[redacted]	13/5/14
Main Supervisor's Signature	[redacted]	13/5/14

Bacterial Cellulose Growth from Media containing Ionic Liquids composed of Choline Salts

Dianne R. Ruka[1, 2], Vijay Ranganathan[3], Douglas. R. MacFarlane[3], George P. Simon[2], Katherine Dean[1]

[1]CSIRO Materials Science & Engineering, Clayton, Australia,

[2]Department of Materials Engineering, Monash University, Clayton, Australia,

[3]School of Chemistry, Monash University, Clayton, Australia

Abstract

This work demonstrates the potential for bacterial cellulose to be grown in the presence of biocompatible choline ionic liquids as growth additives. It is shown that bacterial cellulose can be more effectively grown with choline dihydrogen phosphate, tartrate and formate, compared with choline stearate and gallate as additives. In addition, bacterial cellulose production was achieved with choline formate in the absence of glucose. Ionic liquids can therefore be included in the media and allow bacterial cellulose production to occur. It may be possible to design ionic liquids to be included in growth media that subsequently dissolve materials formed, to allow *in situ* modification of bacterial cellulose to be achieved, since it is known that ionic liquids are capable of dissolving otherwise difficult to dissolve organic materials such as those used for the surface modification of fibres for the purpose of enhancing the fibre-matrix interface in the resulting composite.

Keywords

bacterial cellulose, ionic liquids, nanocomposites

Chemical compounds studied in this article

choline tartrate (PubChem CID: 6900); choline formate (PubChem CID: 115900); choline stearate (PubChem CID 90111); gallic acid (PubChem CID 370).

Introduction

Bacterial cellulose is a material that is becoming of much interest in areas such as material science and biomedical applications. It is a very pure form of cellulose, with high strength and crystallinity, along with biodegradability and biocompatibility (Czaja et al., 2007; Klemm et al., 2006), which are key characteristics for these areas of research. Bacterial cellulose is produced in liquid media by the bacterial species *Gluconacetobacter xylinus*, with the growth conditions used influencing the structure and properties (Czaja et al., 2004; Krystynowicz et al., 2002; Ruka et al., 2012; Watanabe et al., 1998b). In addition to benefits such as improved cellulose yields, modification of the bacterial cellulose can occur depending on the composition of the media, with those components in the mix not specifically required for bacterial cellulose growth potentially leading to modifications of the cellulose (Benziman et al., 1980; Uhlin et al., 1995). These *in situ* modifications can often result in material within the media becoming incorporated on the surface of the bacterial cellulose fibrils as it is produced, an example could be compatibilising materials to enhance subsequent fibre matrix interactions when these materials are incorporated in a matrix.

Ionic liquids consist entirely of ions, and are made up of at least two components, an anion and a cation, which can be varied, thereby creating an enormous number of potential combinations, and they are usually liquid at ambient or relatively low temperatures (Earle & Seddon, 2000). There are increasing numbers of uses for ionic liquids and they are often referred to as “green solvents” as they have the capability of dissolving many substances, including many organic molecules including polymers, and have good properties such as chemical stability, thermal stability, low vapor pressure and high ionic activity (Lu et al., 2009). Ionic liquids have also been used as catalysts in media that can

influence various kinds of polymerisation, and also in biotransformations (Dreyer & Kragl, 2008; Gangu et al., 2009; Shan et al., 2008).

In addition to these uses, ionic liquids have previously been included in the growth media for some bacterial species (Matsumoto et al., 2004; Sekar et al., 2013; Sekar et al., 2012). Deive et al. (2011) described that the metabolic pathways of fungal species can be altered using biocompatible ionic liquids in aqueous media, and more recently Sudharshan et al. (2012) employed choline based biocompatible ionic liquids as cosubstrates with *Staphylococcus lentus* in the biodegradation of an azo dye in aqueous solution. In addition, the lactic acid-producing bacterium *Lactobacillus rhamnosus* has been grown in media containing imidazolium-based ionic liquids (Matsumoto et al., 2004). Based on the observations that bacterial growth and lactate production were able to occur in the presence of the ionic liquids, it was concluded that may be possible to use these ionic liquids to develop an *in situ* extractive fermentation process for the lactate. It has also been shown in the literature that bacterial cellulose production (from cotton-based waste textiles) is enhanced by an imidazolium based ionic liquid (Hong et al., 2012), and a comparison of methods for detoxification of spruce hydrolysate for bacterial cellulose production has also been reported (Guo et al., 2013). Furthermore, *Staphylococcus lentus* has been grown in mineral salt media with choline salts substituted for glucose as the carbon source (Sekar et al., 2013). Choline salts were selected for *S. lentus* as choline is a water-soluble, essential nutrient present in vitamin B complex and its salts usually have nutrient properties. In this instance, the choline salts were found to be capable of acting as a sole carbon source, and were also found to increase the bacterial cell growth rate (Sekar et al., 2013).

It should be noted that, in this work, the choline ionic liquids are present as solutions in water; the liquid nature of the original choline salt increasing its solubility. In addition as water is removed throughout the drying process, the solubilising properties of ionic liquids have the potential to enhance the distribution of compatibilising materials across the surface of the fibres.

Based on the observations of other bacterial growth in choline salt-containing solutions, it may be possible for bacterial cellulose to be produced in these mixtures. We hypothesised in this study that

low levels of biocompatible choline salts included as additives would allow bacterial cellulose production, and may also enable modification of the final structure and surface. In addition to their use as modifying additives, the choline salts were also investigated as potential carbon sources in their own right, in place of the glucose.

Bacterial cellulose should not be soluble in these ionic liquids due the presence of water and the low concentration of ionic liquids. It is possible that cellulose dissolution may occur with other ionic liquids, but not the choline salts examined here, which were examined for BC growth rather than dissolution, and were selected for their nutrient value.

Material and Methods

Synthesis of Choline Salts

The synthesis of choline salts for this work followed the methods described previously (Vijayaraghavan et al., 2010b; Winther-Jensen et al., 2009). Specifically, choline dihydrogen phosphate (DHP), choline tartrate, and choline formate were synthesised by neutralising the corresponding acids with choline hydroxide. For instance, choline tartrate is made by a slow addition of aqueous tartaric acid (5.9 g, 39.5 mmol) to 20% aqueous solution of choline hydroxide (23.9 g, 197.5 mmol) in an ice bath and stirring the reaction mixture for about 2 hours at room temperature and then distilling water from the reaction mixture at reduced pressure to give a crystalline solid (9.8 g, 98% yield).

Electrospray mass spectroscopy analysis (cone ± 35 V) showed the following: Choline formate, m/z (relative intensity, %), ES^+, 103.7 ($Me_3N\ CH_2CH_2OH$, 100); ES^-, 44.8 (formate, 100); choline dihydrogen phosphate m/z (relative intensity, %), ES^+, 103.7 ($Me_3N\ CH_2CH_2OH$, 100); ES^-, 96.7 (dihydrogen phosphate, 100).

Choline gallate and choline stearate have not been described previously. These syntheses are described in more detail below:

Choline gallate was made by a slow addition of 1 mole of aqueous solution of gallic acid (36.6 mmol, 6.2 g) to 1 mole of 20% aqueous choline hydroxide solution (183.1 mmol, 22.2 g) in an ice bath and

stirring the reaction mixture for about 2 hours at room temperature (Figure 4.1). The reaction mixture was then roto-evaporated at reduced pressures to obtain crude choline gallate. To this product, activated charcoal (approx. 2 g) was added, stirred with water and filtered. The filtrate was again evaporated to obtain a pure pale yellow crystalline solid 9.7 g (97% yield) and had a melting point of 153° C. The crystal structure of the compound has also obtained and will be reported elsewhere.

Electrospray mass spectroscopy analysis of this material (cone ± 35 V) was: Choline gallate, *m/z* (relative intensity, %): ES^+, 103.9 ($Me_3N^+CH_2CH_2OH$, 100); ES^-, 169.2 ($(HO)_3C_6H_2COO^-$, 100).

Choline stearate was made by the slow addition of 1 mole of methanoic solution of stearic acid (51.6 mmol, 14.7 g) to 1 mole of 45% methanoic choline hydroxide solution (114.7 mmol, 13.6 g) in an ice bath, and stirring the reaction mixture for about 2 hours at room temperature. The reaction mixture was roto-evaporated at reduced pressures to obtain crude choline stearate. To this crude compound, activated charcoal (approx. 2 g) was added, stirred with water and filtered. The filtrate was again evaporated to obtain a pure pale yellow crystalline solid 19.3 g (96% yield) and had a melting point of 60° C.

Electrospray mass spectroscopy analysis (cone ± 35 V) was: Choline stearate, *m/z* (relative intensity, %): ES^+, 103.9 ($Me_3N^+CH_2CH_2OH$, 100); ES^-, 283.2 ($CH_3(CH_2)_{16}COO^-$, 100).

The melting points and the purity of all the choline salts used in this study are given in Table 4.1.

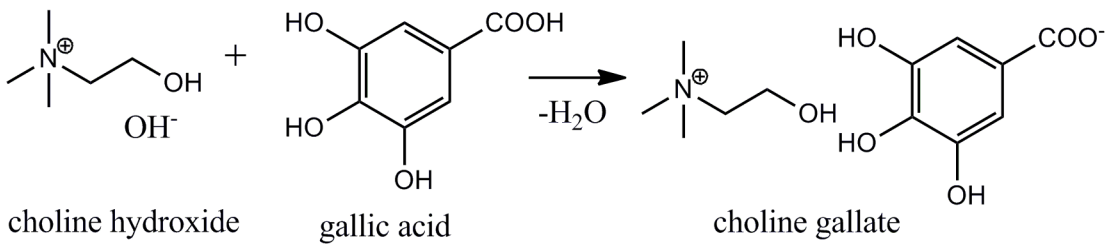


Figure 4.1: Chemical reaction to obtain choline gallate.

Table 4.1: The formula and amount of choline salts included in HS media to produce bacterial cellulose.

Choline salt	Formula	Melting Point (ºC)	Purity (based on Mass Spectrometry) m/z (ES+), RI	m/z (ES-), RI	Amount (g/L)
Choline DHP	$C_5H_{14}NO^+$ $H_2PO_4^-$	185	103.7, 100	96.7, 100	2
Choline Tartrate	$C_5H_{14}NO^+$ $C_4H_5O_6^-$	151	103.7, 100	149.3, 100	2
Choline Stearate	$C_5H_{14}NO^+$ $C_{18}H_{35}O_2^-$	153	103.7, 100	283.2, 100	0.2
Choline gallate	$C_5H_{14}NO^+$ $C_7H_5O_5^-$	60	103.7, 100	169.2, 100	0.2
*Choline formate	$C_5H_{14}NO^+$ HCO_2^-	_	103.7, 100	45.3, 100	2

*liquid at room temperature

Bacterial Strain

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

Media

HS media (Schramm & Hestrin, 1954) with various concentrations of choline salts were used to grow *G. xylinus.* The details of the media are given in Table 4.2. The media were also produced in which glucose was fully omitted (using the choline salts as the carbon source). The pH was adjusted to 5.0 with HCl or NaOH and autoclaved at 121° C for 20 minutes. The choline salts used were choline DHP, choline tartrate, choline stearate, choline gallate and choline formate.

Table 4.2: The composition of HS medium.

Chemical	Amount (g/L)
Glucose	20
Yeast extract	5
Peptone	5
Disodium hydrogen phosphate	2.7
Citric acid monohydrate	1.15

Growth Conditions

Seed cultures were prepared by selecting a single colony from a working plate of HS agar (Schramm & Hestrin, 1954) and inoculating 10 mL of HS broth. These cultures were incubated for 7 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 used for inoculations. Cultures were grown in 200 mL conical flasks containing 50 mL of media and were inoculated at a concentration of 1% with the cell suspension. Cultures were incubated for 7 days at 28° C under static conditions, and all cultures were grown in triplicate.

Treatment of Cellulose and Yield Determination

Following incubation periods, the cultures were shaken vigorously to remove the attached bacterial cells. Pellicles were then taken from the cultures and rinsed to remove any residual media. Pellicles were washed with 0.1 M NaOH at 80° C for 1 hour, and then washed repeatedly in tap water for several days until a neutral pH was obtained, and finally washed in distilled water for 1 day. Following washing, the pellicles were dried at room temperature for several days. Pellicles were weighed once dry.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed using a field-emission Nova NanoSEM 450. Air-dried film samples were mounted onto stubs, coated with a platinum coating, and examined at 2 kV. The widths of the fibrils were determined from images taken at a magnification of 100,000 and were measured using the xT microscope Control v4.7.7 software. At least ten fibrils were measured and the average determined.

X-Ray Diffractometry

X-ray diffraction (XRD) was used to monitor the $d_{1\text{-}10}$ spacing corresponding to the interlayer spacing of the crystalline structure of the bacterial celluloses. The XRD measurements were performed on the cellulose sheet samples using a Bruker D8 Diffractometer operating at 40 kV, 40 mA, CuKα radiation monochromatised 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 two major peaks was used for this calculation, as the third peak could not provide reliable FWHM values due to its low intensity. 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.

Fourier Transform – Infra Red

Fourier transform – infra red (FTIR) spectroscopy was completed on air-dried cellulose films using a Perkin-Elmer Spectrum 100 Spectrometer. Scans were taken between 4000 and 450 cm^{-1} with 16 convolutions. The baselines for each sample spectrum were normalised using the Spectrum software. I_α content was calculated using the peak heights at 750 and 710 cm^{-1} by the method determined by Yamamoto et al. (1996).

Results and Discussion

Cellulose Yield

A range of concentrations of each of the choline salts were investigated in order to determine a concentration that would allow bacterial cellulose production to occur. From this, the sample of the highest concentration of each choline salt that allowed cellulose production to occur was further examined (Table 4.1). Using these concentrations, choline salts were included in media as additives (with glucose also included). The amount of cellulose produced under each of these conditions was determined.

The presence of choline salts as additives in the media led to some changes to the bacterial cellulose yield (Figure 4.2a). Choline tartrate led to an increased bacterial cellulose yield with an increase of approximately 63% in pellicle weight. Choline DHP and formate had no impact on the yield, both

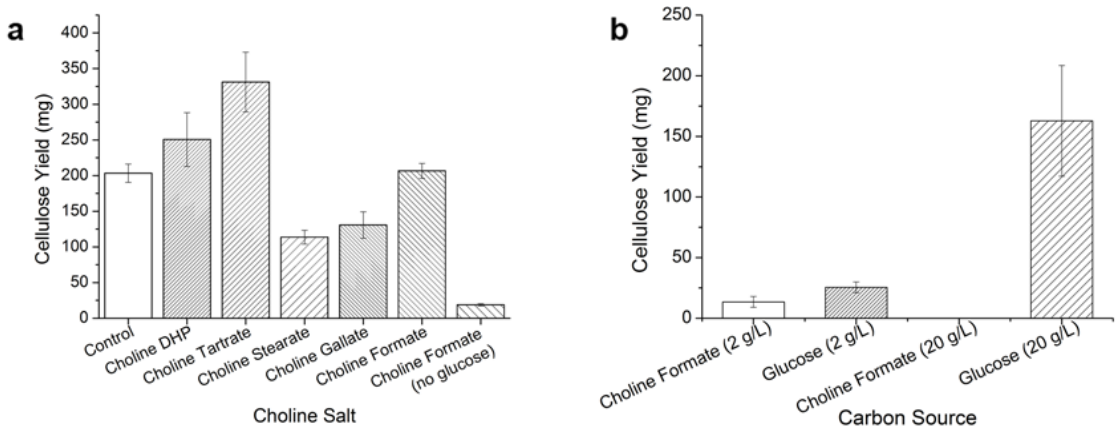


Figure 4.2: Cellulose yields obtained from media containing choline ionic salts (a) and high and low concentrations of glucose and choline formate (b). Means and standard deviations for each are given.

achieving yields within the range expected in the absence of media additives. The presence of choline stearate and gallate in the media led to a lower amount of cellulose being produced, with pellicle weights of approximately 114 and 131 mg, respectively, whereas 204 mg of cellulose was produced in the absence of choline salts.

Despite the low concentrations of choline stearate and gallate included in the media, these salts reduced the bacterial cellulose yields, demonstrating that choline stearate and gallate may have inhibitory effects on the growth of bacterial cellulose. The apparent toxicity demonstrated by choline gallate and stearate at higher concentrations can probably be explained by their structure and resulting characteristics. Gallates are known anti-oxidants, which suggests that choline gallate would also have such properties. Stearates are commonly used as detergents and perhaps may possess properties similar to those of gallates, requiring lower concentrations to allow bacterial growth and cellulose production to occur.

Of the salts examined, only choline formate allowed bacterial cellulose production to occur in the absence of glucose, though the amount of bacterial cellulose was lower than when glucose was included as the carbon source (Figure 4.2a). Under normal conditions, glucose is included in the media at 20 g/L, whereas choline formate was only included at 2 g/L. In order to examine if the difference in bacterial cellulose yield was due to the difference in the amounts of these carbon sources, media was

made up with 2 and 20 g/L of each. When both glucose and choline formate were included at 2 g/L, similar (though low) bacterial cellulose production occurred (Figure 4.2b), both conditions achieving less than 30 mg of cellulose. However, no bacterial cellulose growth was observed at 20 g/L choline formate, whereas bacterial cellulose yields were high with 20 g/L glucose, with pellicle weights ranging from approximately 140 – 215 mg. Though the ionic liquids examined here may not be appropriate as sole carbon sources for bacterial cellulose production, there may be other ionic liquids that could be developed that could act as alternate carbon source to produce bacterial cellulose.

Bacterial Cellulose Morphology and Crystal Structure

The structure and morphology of bacterial cellulose conditions were examined by SEM, XRD, and FTIR. In general, the bacterial cellulose produced in the presence of choline salts retained the same properties observed when no choline material was used, however some small differences were observed.

SEM of the bacterial cellulose grown with each choline salt demonstrated that the cellulose mainly consisted of nanosized fibrils in the normal size range of 40±5 nm (Figure 4.3), however some differences in morphology and fibril width were noted. When bacterial cellulose was produced with choline gallate, the cellulose pellicle consisted of fibrils both on and under the surface (Figure 4.3c and 4.3d), however the surface of the pellicle appeared to be covered in a layer of material with a smooth morphology. There are two possible explanations for this; firstly this is possibly the result of the ionic liquid (present at a higher concentration on the surface) modifying the morphology of the cellulose at the surface (it is known that various ionic liquids can dissolve or swell cellulose (Zhu et al., 2006)); secondly choline gallate has been shown to crystallise (unpublished data), and it is possible that the layer of material with smooth morphology is in fact crystallised choline gallate. This appearance is not an artifact of the SEM preparation method, as when the sample was re-made, a similar structure was visible. In addition, the fibrillar structure was still present on and under the surface (Figure 4.3c and 4.3d).

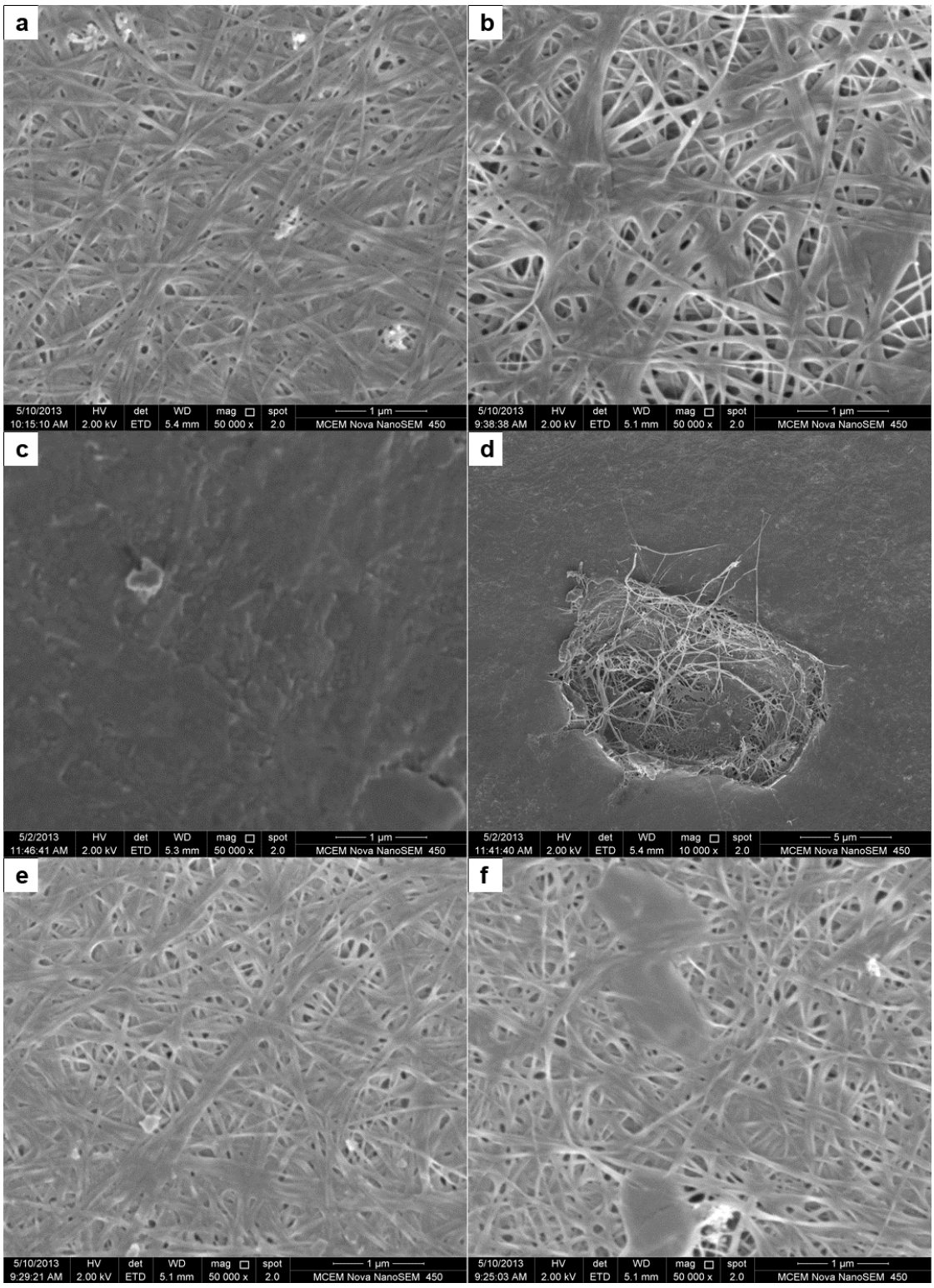


Figure 4.3: SEM morphology of bacterial cellulose grown with the absence of choline salts (a), choline stearate (b), choline gallate (c and d), choline formate in the presence and absence of glucose (e and f).

SEM also revealed that there were some very thin fibrils observed in the choline stearate and formate samples (Figure 4.3b and 4.3e). Some clumping of the bacterial cellulose was also observed when it was grown with choline formate in the absence glucose (Figure 4.3f), further suggesting that the salts interfere with bacterial cellulose production to some degree.

XRD revealed no significant differences between the bacterial cellulose samples with regards to crystallite size, with all samples ranging from 6.8 – 7.6 nm (Table 4.3). The crystallinity of the bacterial cellulose grown in the presence of all choline salts did experience a small decrease from the control (Table 4.3), with the crystallinity of the cellulose grown with choline salts ranging from 52 – 60%, and the control having 68% crystallinity. The exception to this is bacterial cellulose produced with choline formate in the absence of glucose. Under these conditions, crystallinity was calculated to be extremely high at 94%. We hypothesise that this high crystallinity is due to the low yield. We have previously noted that crystallinity is seen to decrease in media where high yields of cellulose are obtained (Ruka et al., 2012). It has been reported that cellulose is produced initially as an amorphous material and is gradually crystallised to cellulose I (Haigler et al., 1980) and it is likely that when high amounts of cellulose are rapidly produced, it cannot all be converted to the crystalline form and therefore only

Table 4.3: Structural characteristics of bacterial cellulose obtained from media containing choline ionic salts.

Choline Salt	Crystallite size (nm)	Crystallinity (%)	I_α content (%)
Control (no choline salt)	7.2	68	71
Choline DHP	7.6	55	69
Choline Tartrate	6.8	52	68
Choline Stearate	6.8	59	71
Choline Gallate	6.8	60	71
Choline Formate	7.2	56	72
Choline Formate (no glucose)	7.6	94	64

achieves moderate crystallinity. However, as choline formate only allowed small amounts of cellulose to be produced, it is possible that most crystallised, resulting in a higher level of crystallinity.

There were some differences seen in the FTIR scans between different materials (Figure 4.4). Bands characteristic of bacterial cellulose were seen in the control sample, as well as the other samples from the choline salt media. A large peak was present in the 3200 – 3400 cm^{-1} range, which is representative of the O-H bonds (Grande et al., 2009). Peaks at 1317 and 1426 cm^{-1} correspond to CH_2, with a band at 1160 cm^{-1} representative of C-O-C bonds (Kačuráková et al., 2002). Multiple peaks between 984 and 1106 cm^{-1} correspond to the C-O bonds (Maréchal & Chanzy, 2000). Small changes were seen in the peak heights in some of the samples grown in the presence of choline salts.

An increase in the FTIR peaks at approximately 1650 cm^{-1} was seen in the presence of choline salts DHP, tartrate and formate. This band likely corresponds to the amide group of the choline, and it may be that it is present in these spectra due to the higher concentration of these salts (2 g/L as compared to 0.2 g/L) included in the media, compared to choline stearate and gallate. Based on the formula of

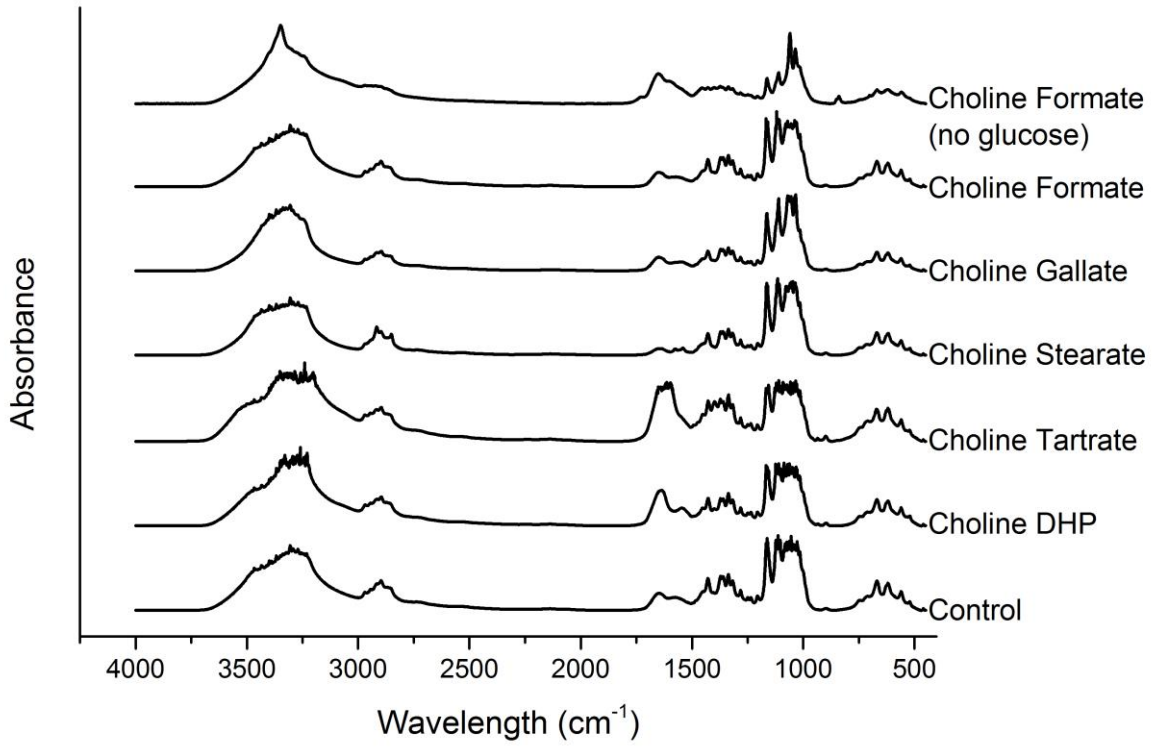


Figure 4.4: FTIR scans of bacterial cellulose in the presence of various choline salts

the choline salts (Table 4.1), it is possible that these differences are due to the presence of the choline salts becoming incorporated amongst the bacterial cellulose fibrils. This further supports the hypothesis that other chemicals could be dissolved using the ionic liquids within the bacterial cellulose growth media, such as those used for the surface modification of fibres for the purpose of enhancing the fibre-matrix interface in a resulting composite.

Calculations completed from FTIR data on the ratio of the two allomorphs of cellulose, I_α and I_β (Atalla & Vanderhart, 1984) revealed that there was very little or no impact seen on the ratio of cellulose I_α due to the inclusion of the choline salts. All media obtained I_α contents of 64 – 72% (Table 4.3), with the cellulose remaining high in the I_α allomorph, which is consistent with the ratio of the I_α cellulose produced in this organism.

Conclusions

A range of choline salts, when included in the growth media of bacterial cellulose, still allowed this nanostructured material to be produced, with only minimal changes to its structure and morphology although changes were noted in the yield of the cellulose produced. Various biocompatible choline salts allowed bacterial cellulose production when included in the media at concentrations from 0.2 – 2 g/L. This indicates that bacterial cellulose can withstand the salt conditions caused by the ionic liquid additives being included in the growth media. Based on the huge potential to create new ionic liquids with various combinations of anions and cations, it may be possible to develop ionic liquids that would not only allow for bacterial cellulose production to occur, but could also fully- or partially-dissolve otherwise insoluble bacterial cellulose and dissolve chemicals used for the surface modification of fibres, resulting in an enhanced fibre-matrix interface in the resulting composite.

4.3 *In situ* Modifications to Bacterial Cellulose with the Water Insoluble Polymer Poly-3-hydroxybutyrate

A number of water-soluble polymers have been previously used as additives in cellulose-producing media (Chao et al., 2001; Seifert et al., 2004), however insoluble materials have not been extensively reported. As PHB was selected as a matrix material, this polymer was dispersed in media for bacterial cellulose production to determine if its inclusion could result in the formation of an *in situ* composite of these two materials. Tween 80 and hydroxypropylmethyl cellulose were also included as additives for comparison, and are presented in the paper here.

This paper was published in *Carbohydrate Polymers* in 2013 and presented here in its published format. It is reproduced here with permission from Elsevier.

Declaration for Thesis Chapter 4

Declaration by candidate

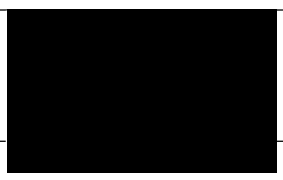
In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Determining which experiments to do based on gaps in the literature, carrying out the experiments, writing the initial draft of the paper and changing the paper based on advice from supervisors	85

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
George Simon	Providing supervision, suggesting some experiments, proof reading and editing	N/A
Katherine Dean	Providing supervision, suggesting some experiments, proof reading and editing	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature		13/5/14
Main Supervisor's Signature		13/5/14

Carbohydrate Polymers 92 (2013) 1717–1723

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

In situ modifications to bacterial cellulose with the water insoluble polymer poly-3-hydroxybutyrate

Dianne R. Ruka[a,b], George P. Simon[b], Katherine M. Dean[a,*]

[a] *CSIRO Materials Science & Engineering, Gate 5 Normanby Road, Clayton 3168, Australia*[1]
[b] *Department of Materials Engineering, Monash University, Clayton 3800, Australia*

ARTICLE INFO

Article history:
Received 17 November 2011
Received in revised form 7 September 2012
Accepted 1 November 2012
Available online 9 November 2012

Keywords:
Bacterial cellulose
In situ
Modifications
Poly-3-hydroxybutyrate
Additives
Crystallinity

ABSTRACT

Bacterial cellulose is a pure, highly crystalline form of cellulose produced from the bacteria *Gluconacetobacter xylinus* that has become of increasing interest in materials science due to its nanofibrillar structure, ideal for incorporation into other materials as a reinforcing material. The morphology and properties of bacterial cellulose can be altered by including additives not specifically required for growth of the bacteria in liquid media. The bioplastic poly-3-hydroxybutyrate (PHB), along with hydroxypropylmethyl cellulose (HPMC) and Tween 80 were selected and added to the growth media at different concentrations to examine their impact on the resulting cellulose, leading to changes in yield, crystallinity and morphology. The crystallinity index of the nanofibrils was found to vary greatly when using these different methods to calculate it from XRD data, indicating that particular care must be taken when comparing crystallinity results reported in the literature. PHB was able to be incorporated into the bacterial cellulose fibrils during production, increasing the potential for favourable interactions of the bacterial cellulose microfibrils with a neat PHB matrix with the aim of making a fully degradable nanocomposite system.

1. Introduction

Cellulose is the most abundant polymer on earth, and is becoming of increasing interest because of its fibrillar nature and potential as a reinforcing material in composites, being biodegradable, sustainable and renewable. Cellulose has long been produced from plant sources, however bacterial cellulose (BC), produced in high amounts by *Gluconacetobacter xylinus*, is particularly appealing due to its purity and highly crystalline nanostructure. There have recently been several reports on the amount of cellulose produced by *Gluconacetobacter* grown in different media, often by simply substituting the carbon and/or nitrogen components. A wide range of carbon and nitrogen sources have been investigated in this way, as has the inclusion of additional supplements.

The inclusion of additives in the growth media, that is components in the media that are not specifically required for bacterial cell growth, can affect cellulose production in different ways, as the assembly of cellulose is susceptible to chemical and physical influences by the compounds present during synthesis and aggregation (Uhlin, Atalla, & Thompson, 1995), by binding directly to the cellulose during production and interfering with the crystallization, or co-crystallizing with the cellulose. It is also possible that the additive may interfere with the bacterial cells themselves, thereby altering the cellulose production indirectly. Regardless of the method, the yield, structure, morphology and physical properties can all be affected by the presence of an additive in the media, effectively creating *in situ* modifications.

Water soluble polymers have been included in the culture media of cellulose producing bacteria with conflicting results. Some researchers note that the inclusion of such additives simply results in altered cellulose structure (Cheng, Catchmark, & Demirci, 2009; Tokoh, Takabe, Sugiyama, & Fujita, 2002b), whereas others find the creation of composites as the additive is incorporated into the growing cellulose fibrils, leading to *in situ* composites (Hessler & Klemm, 2009; Seifert, Hesse, Kabrelian, & Klemm, 2004). Water soluble polymers carboxymethyl cellulose and methylcellulose have been added to the media with claims that the inclusion of additives such as these directly affects the cellulose, causing decreased crystallinity and crystal size, as well as greater thermal stability and pore size (Cheng et al., 2009). It has also been reported that the additives become incorporated into the cellulose, creating a composite-type material (Seifert et al., 2004). Other polymers such as Tween 80 (Huang, Chen, Lin, Hsu, & Chen, 2010) and hydroxypropylmethyl cellulose (HPMC) (Huang, Chen, Lin, & Chen, 2011) have also been incorporated into the growth media of cellulose-producing bacteria, with differences observed in pore size, degree of polymerization, crystallinity, fibre widths and mechanical strength.

* Corresponding author. Tel.: [redacted]
E-mail addresses: [redacted]
[1] http://www.csiro.au.

0144-8617/$ – see front matter.
http://dx.doi.org/10.1016/j.carbpol.2012.11.007

Similarly, including additives of poly(ethylene oxide) (Brown & Laborie, 2007), poly(vinyl alcohol) (PVA) (Gea, Bilotti, Reynolds, Soykeabkeaw, & Peijs, 2010) and starch (Grande et al., 2009) in the growth media have resulted in these additives being incorporated into the bacterial cellulose resulting in *in situ* composites, however PVA levels were only achieved up to 1.3%. Composites with poly(ethylene oxide) and starch were achieved with much higher levels of the additives, indicating that it may be possible to make nanocomposites with bacterial cellulose from this method. Results from these works showed that the cellulose was well dispersed, and the nanocomposites typically had good mechanical properties.

In this work, we use poly-3-hydroxybutyrate (PHB) as the key material used for modifying the cellulosic nanofibres during the culture stage. Composites have been reported using bacterial cellulose and the water insoluble polymer PHB by an impregnation method. In these cases, the cellulose pellicle was soaked in a solvent containing dissolved PHB and, as the solvent evaporated, the PHB was incorporated into the spaces between the cellulose fibrils cellulose (Barud et al., 2011; Cai & Yang, 2011; Cai, Yang, & Kim, 2011). While water soluble polymers have been well documented as additives in the culture media for cellulose producing bacteria, the effects of water insoluble polymers in the media is unknown. However in this work, a non water soluble polymer, PHB, was directly dispersed in bacterial cellulose culture medium. HPMC and Tween 80 were selected as water soluble polymers that have previously been investigated in the media for a variety of cellulose-producing bacteria, and were examined for comparison. Alterations in the structure of bacterial cellulose may be desirable for the creation of composites in that if the fibrils become more "PHB-like", they may improve interaction if incorporated into a PHB matrix to form a reinforced, fully degradable nanocomposite.

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

The media used to cultivate *G. xylinus* was Hestrin–Schramm (HS) (Schramm & Hestrin, 1954), with different concentrations (described below) of additives added. Media were adjusted to pH 5.0 with HCl or NaOH and autoclaved at 121 °C for 20 min. The additives used were HPMC, Tween 80 and PHB. HPMC was obtained from Dow Chemical, and Tween 80 and PHB were obtained from Sigma–Aldrich.

2.3. Growth conditions

Seed cultures were prepared by selecting a single colony from a working plate of Hestrin–Schramm agar 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% of the cell suspension. Cultures were incubated for seven days at 28 °C under static conditions. All cultures were grown in triplicate. Additional pellicles were produced in HS media containing 1 wt% PHB for tensile tests.

2.4. Treatment of cellulose films

Following incubation periods, cultures were shaken vigorously to remove the attached bacterial cells. Pellicle films 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 dried at room temperature. Pellicle films were weighed once dry.

2.5. Scanning electron microscopy

Scanning electron microscopy (SEM) was performed using the field-emission SEM JEOL 7001F. Samples were coated with a gold/palladium coating, and were examined at 5 kV.

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). In addition, cellulose pellicles from HS media and HS media containing 1 wt% PHB were ground into a fine powder and mixed with potassium bromide (KBr) powder, dried under vacuum and pressed into small discs for examination by FTIR according to the protocol described above. Neat PHB powder was also examined in this way.

2.7. 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. The XRD measurements were performed on the cellulose sheet samples using a Bruker D8 Diffractometer operating at 40 kV, 40 mA, Cu Kα radiation monochromatised with a graphite sample monochromator with a diffractogram recorded between 2θ angles of 2° and 40°. Crystallite size was calculated using the software TOPAS™. The FWHM (full width at half maximum height) for the two major peaks was used for this calculation, as the third peak could not provide reliable FWHM values due to its low intensity. 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 International Centre for Diffraction Data (ICDD) PDF card 00-060-1501, amorphous cellulose. The crystalline peak positions were selected based on positions given in Czaja, Romanovicz, and Brown (2004). A pseudo Voigt Function was used to profile the peak shape and area for both the amorphous and crystalline components.

2.8. Solvent casting PHB films

A neat PHB film was prepared by dissolving 5 wt% PHB in chloroform under mechanical stirring at 80 °C for 3 h. The films were cast in glass petri dishes and the solvent was allowed to evaporate at room temperature. These films were examined for tensile properties for comparative purposes only.

2.9. Tensile properties

Tensile strength, elongation at break and modulus were determined for cellulose produced in standard HS media and media

containing 1 wt% PHB, and a solvent cast PHB film on an Instron universal testing machine (model 3366) and tested in accordance with ASTM D882 (using a type IV specimen as described in ASTM D638). The Instron was fitted with a 100 N static load cell, pneumatic grips, and the speed of extension was set to 2 mm/min. A minimum of ten specimens per each formulation were tested until fracture, from which a mean and standard deviation were calculated.

3. Results and discussion

3.1. Film weight

Each additive was added to the culture at four different concentrations (Table 1). The weight of the film from each culture was examined. The percentage increase in weight of each film from the cellulose produced in the absence of the additive is shown in Fig. 1.

Various additives have been reported to interfere with the production of cellulose by interfering with aggregation of microfibrils during production (Benziman, Haigler, Brown, White, & Cooper, 1980), which can result in decreased cellulose yield. An increase in the weight of the cellulose pellicle can indicate an increase in cellulose production, likely due to an increase in cell growth rate, or an increase in weight may be the result of the incorporation of the additive into the pellicle film. Differences observed in the structure and morphology, specifically in the fibril appearance and width, and crystallite sizes and crystallinity, are discussed below. However, even though an additive may provide beneficial characteristics for bacterial cellulose, and allow tailored design of the cellulose for specific purposes, if an additive results in significant decreases in yield of cellulose, the cost of production of the cellulose would increase, making the production undesirable for large-scale operations. Consideration should thus be given to all factors, including yield, when seeking to obtain specific characteristics in bacterial cellulose.

The inclusion of HPMC as an additive in the media decreased the weight of the film at low concentrations, but increased the weight at higher concentrations, though no difference was observed between the weight at high concentrations of HPMC and the control. It is believed that the increase observed was not due to a stimulation in the cell growth rate, but rather an indication that HPMC was incorporated into the pellicle film, thus causing the increase in weight at higher concentrations. The opposite is true, however, for Tween 80 that caused a fairly consistent decrease in the weight of the film, indicating that this additive negatively impacts cellulose production. These two additives were selected for use as comparison to PHB as they have previously been shown to have an affect on the structure of bacterial cellulose. HPMC is a water-soluble polysaccharide that can be used as an emulsifier, whereas Tween 80 is a water soluble polyethylene sorbitol ester that has a range of uses such as solubilizing proteins.

The inclusion of PHB, ranging from 0.25 to 1.0 wt% PHB in the media, resulted in a significant increase in weight of the pellicle film produced. PHB was present on the surface of the pellicle though attempts were made to remove the PHB powder from the pellicle surface during the washing steps. Based on the increase in film weight, it is likely that the product formed is a BC–PHB material. Further testing of this material by FTIR, SEM and tensile testing was completed to confirm the presence of PHB in the film.

Table 1
Concentrations added to HS media for each additive.

Additive	Concentration point (wt%)			
	1	2	3	4
Hydroxypropylmethyl cellulose	0.25	0.5	1.0	2.0
Tween 80	0.05	0.1	0.2	0.4
Poly-3-hydroxybutyrate	0.125	0.25	0.5	1.0

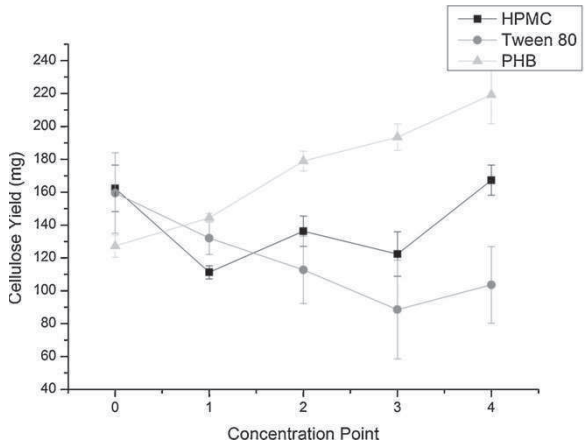


Fig. 1. Cellulose yields obtained from cultures with different concentrations of additives in the media.

3.2. Bacterial cellulose morphology and crystal structure

Bacterial cellulose is produced in the cell's cytoplasmic membrane, and is extruded as microfibrils of approximately 1.5 nm in width, and the microfibrils aggregate into a ribbon-shaped fibril approximately 40 nm in width (Ross, Mayer, & Benziman, 1991). Various additives included in the media can act as co-polymers becoming incorporated into the bacterial cellulose as it is produced, or can bind to the cellulose, affecting the morphology and structure of the cellulose (Huang et al., 2010; Klemm et al., 2006; Tokoh, Takabe, Sugiyama, & Fujita, 2002a; Yamamoto et al., 1996). By contrast, other additives, antibiotics for example, directly affect the bacterial cell and therefore the production of the cellulose as a result (Yamanaka, Ishihara, & Sugiyama, 2000).

In the work described below, one concentration of each additive was selected and the morphology of the film was examined. This involved examination by SEM for fibril morphology and width, by XRD for crystallite size and crystallinity and also by FTIR for its crystalline cellulose I_α content.

When viewed by SEM, bacterial cellulose typically presented as an interwoven mesh of fibrils of approximately 40 nm in width, although the widths of fibrils were subject to variance due to their biological nature. The inclusion of some additives resulted in some changes to the morphology of the cellulose (Fig. 2). The addition of HPMC resulted in slightly thinner fibrils, however the difference was not statistically significant, the HPMC fibrils also appeared straighter (Fig. 2b). Tween 80 as an additive resulted in slightly wider fibrils at 56 nm, over the 40 nm fibrils present without an additive (Table 2). It is possible that this additive impacted bacterial synthesis, since Tween 80 has been shown to stimulate glucan production, a glucose polysaccharide, in *Streptococcus mutans*, however no effect on bacterial cells was observed (Umesaki, Kawai, & Mutai, 1977), whereas it has been shown to decrease mechanical strength of bacterial cellulose (Huang et al., 2010).

PHB is of particular interest, its incorporation in the growth medium of bacterial cellulose not having been reported previously. PHB is water insoluble and is produced intracellularly by particular bacterial species, such as *Azotobacter*, *Bacillus* and *Pseudomonas* species (Byrom, 1987). PHB was dispersed in the media used to produce bacterial cellulose. Based on the weight of the pellicles as described above, as well as the SEM of this cellulose, it appears that PHB was integrated into the cellulose during the synthetic

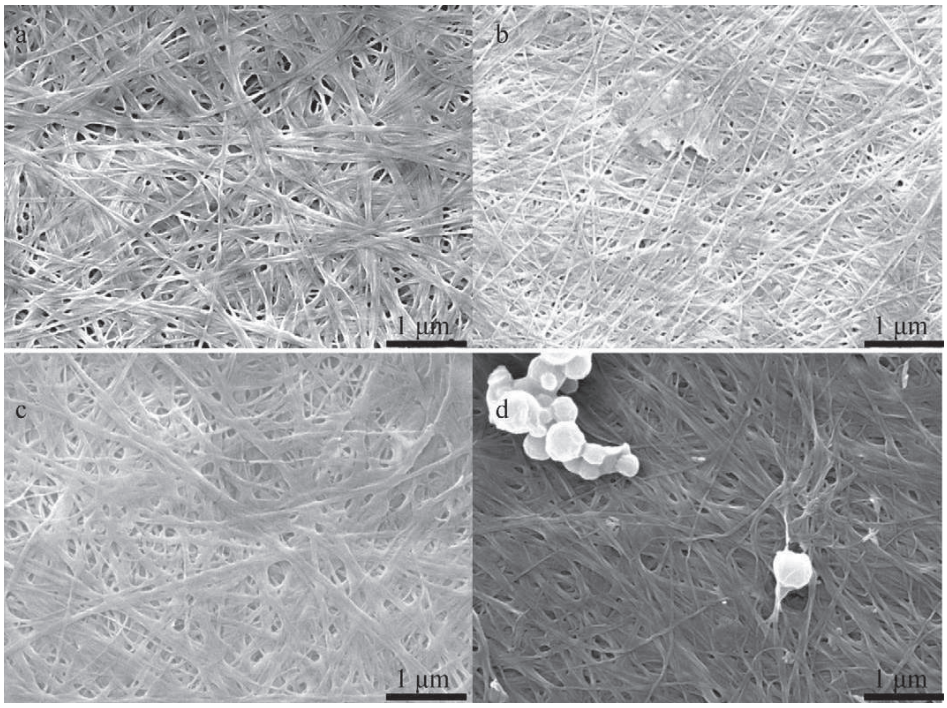


Fig. 2. SEM images of cellulose produced in media with no additives (a), HPMC (b), Tween 80 (c) and PHB (d).

process. PHB can be seen on the surface of the cellulose fibrils in Fig. 2d and was examined in further detail in Fig. 3. Fig. 3 shows the appearance of PHB powder before its inclusion in the media, and the similar appearance on the cellulose. In addition to the PHB on the surface of the pellicle (Fig 3b), PHB was also observed to be interwoven amongst the cellulose fibrils on the underside of the pellicle (Fig. 3c and d). There have been several papers published involving the inclusion of water soluble polymers in the media for cellulose producing bacteria (Chao, Mitarai, Sugano, & Shoda, 2001; Hessler & Klemm, 2009; Seifert et al., 2004; Tokoh et al., 2002a; Yamamoto et al., 1996). Some have been reported as altering the structure of the cellulose, whereas others were actually incorporated into the cellulose fibrils during synthesis. To the best of our knowledge, insoluble polymers have not been examined. Since bacterial cellulose is formed as a pellicle on the surface of the media, if an insoluble polymer is present at the air/surface interface, it is likely that it too can be incorporated into the mesh of cellulose fibrils. This was not the case with PHB as it accumulated at the bottom of the flask. It may be that some PHB remained dispersed in the medium, or that the bacterial cells were able to access the PHB from the bottom of the flask as it appeared that the PHB at the bottom of the flask become attached to the pellicle. Regardless, the PHB was incorporated in amongst the cellulose fibrils, indicating that this may represent a more general pathway for insoluble polymers be incorporated, and may provide bacterial cellulose with improved *in situ* modifications.

From Fig. 3, it is difficult to tell exactly how much PHB was incorporated into the cellulose. From the top view of the pellicle (Fig. 3b), it appears as though the PHB has largely coated the surface, as the fibrils are packed too tightly to observe any incorporated PHB, however from the bottom of the film (Fig. 3c and d), it is possible to visualize the mesh of cellulose fibrils and PHB.

As previously described, additional substrates acting as a host polymer have been added to the media used to produce bacterial cellulose in order to produce *in situ* composites with starch (Grande et al., 2009) and poly(ethylene oxide) (Brown & Laborie, 2007), the amount of matrix in those composites was not achieved with PHB, as most of the pellicle consisted of cellulose. We hypothesize that the cellulose produced in the presence of PHB will have a higher affinity to this material over cellulose produced in traditional media, and could be used as reinforcement material in a PHB matrix.

Cellulose I is the form of cellulose found in nature, it is 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).

Table 2
Structural values obtained from bacterial cellulose produced in the presence of additives.

Additive	Concentration	Fibril width (nm)	Cellulose I_α content (%)	Crystallinity (%) (calculated from one amorphous peak)	Crystallinity (%) (calculated from four amorphous peaks)	Crystallite size (nm)
No additive	–	40	68	86	79	6.9
Hydroxypropylmethyl cellulose	1%	38	70	65	60	5.8
Tween 80	0.1%	56	68	87	35	6.8
Poly-3-hydroxybutyrate	0.5%	46	69	69	52	6.8

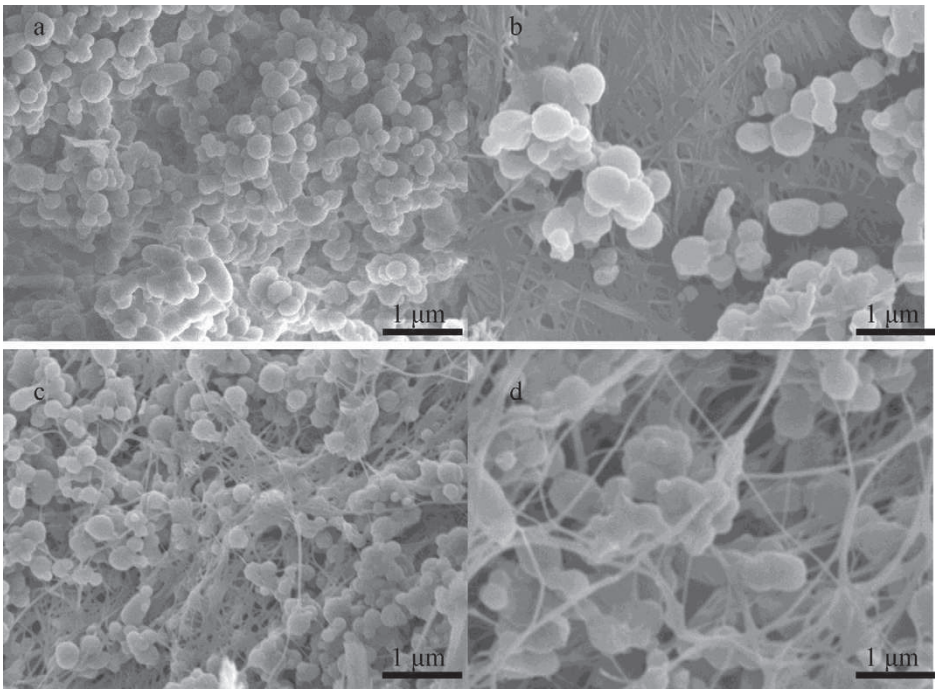


Fig. 3. SEM images of PHB powder (a) and bacterial cellulose grown with PHB in the media (b–d).

The amount of cellulose I_α can be calculated from FTIR peaks at 750 and 710 cm^{-1} (Fig. 4a). The additives included here showed no impact on I_α content, all ranging from 68 to 70% (Table 2). These results all remain very high, indicating that these additives certainly do not have a significant negative effect on the ratio of I_α/I_β content, and that the bacterial cellulose remains high in I_α content.

From the FTIR of the two pellicle films obtained from standard HS media and HS media with PHB as an additive (Fig. 4a), an additional peak was seen in the BC-PHB film at approximately 1724 cm^{-1}. This was confirmed by grinding up cellulose and cellulose grown in the presence of PHB (BC–PHB) and made into KBr discs. PHB was also examined in this way and can be seen in Fig. 4b. The peak at 1724 cm^{-1} which is seen both in the PHB and BC–PHB curves, but not in the BC curve, can be thus attributed to the C=O group which is present only in PHB, supporting the hypothesis that the pellicle produced in the presence of PHB is not simply cellulose alone, but a combination of bacterial cellulose and PHB.

If we assume that the increase in cellulose weight is due entirely to the addition of PHB then we could predict that with 1 wt% PHB in the media, we obtain a combined BC–PHB pellicle of approximately 40 wt% PHB. However it is likely that much of this PHB is superficially attached to the surface rather than being integrated amongst the fibrils. From the general appearance of the pellicle, it would not appear that it consists of such a large amount of PHB. It is also possible that PHB was used as a carbon source for the bacterial cells and led to the increased production of cellulose this way, as PHB is itself naturally an intracellular storage molecule that can be broken down by bacterial cells for cell metabolism. Based on the presence of an additional peak in the BC-PHB material at approximately 1724 cm^{-1} due to the carbonyl group in PHB, we can conclude that the pellicle film must contain some PHB.

Bacterial cellulose exists as a highly crystalline material with small crystallite sizes and XRD data was used to look at the size aspect of these crystallites from the XRD peaks (Fig. 5).

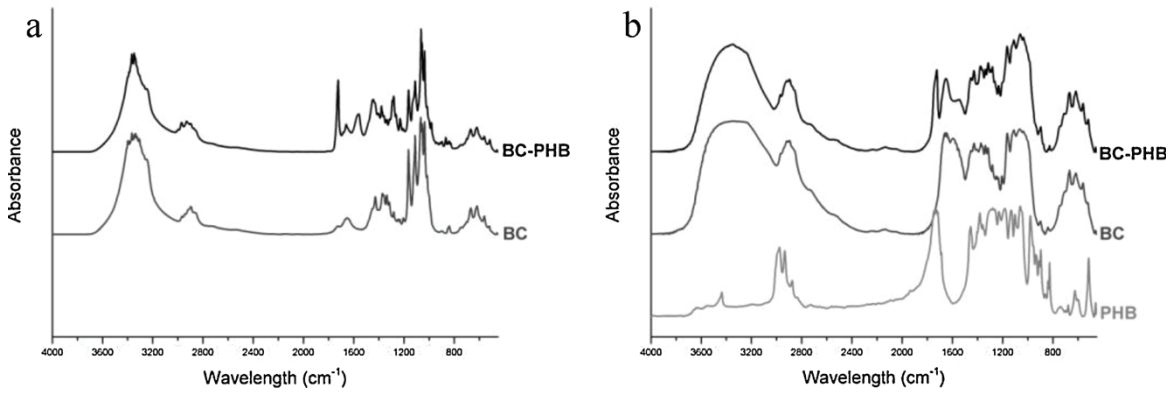


Fig. 4. FTIR obtained from BC grown in media with and without PHB as a film (a) and from KBr discs (b).

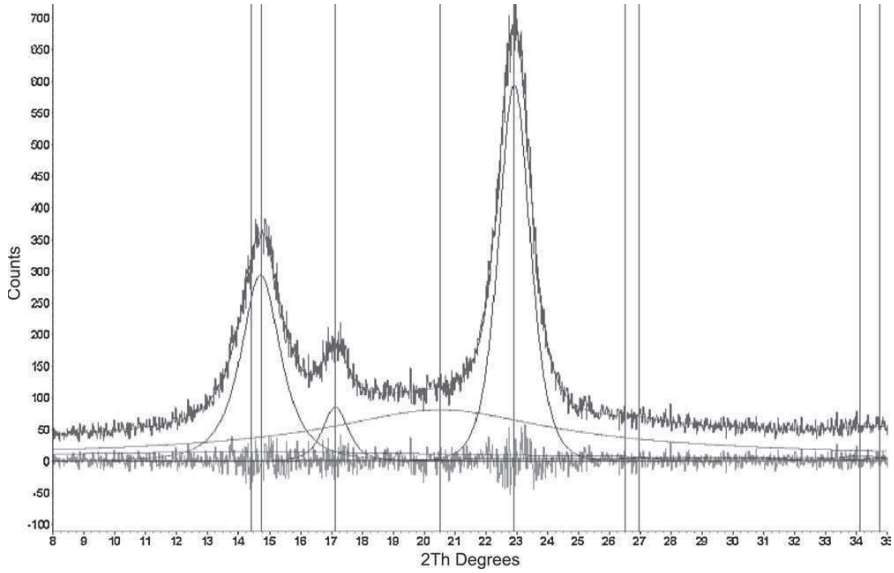


Fig. 5. XRD pattern of bacterial cellulose with peaks for the calculation of crystallite size and crystallinity.

The degree of crystallinity can be calculated using XRD data comparing a single defined amorphous peak to the crystalline peaks obtained from cellulose produced here, with the crystallinity values also typically very high. However, when these results were repeated using four peaks to define the amorphous area, the crystallinity values were very different (Table 2). Increasing the amorphous peaks from one to four caused a decrease in the calculated value for crystallinity for the cellulose produced in the absence of any additives, however this decrease was very small, indicating that bacterial cellulose is highly crystalline and that these results are generally quite robust to analysis techniques.

The inclusion of the additives caused a decrease in the crystallinity for the cellulose as demonstrated by the calculations completed using four amorphous peaks. HPMC and PHB only decreased the crystallinity to 60 and 52%, respectively, but Tween 80 caused a large decrease in the crystallinity, reducing it to 35%. This is further evidence that these additives all have an impact on the production of the cellulose. Similar to bacterial cellulose, PHB is a semi-crystalline material, however it appears that its incorporation into the cellulose pellicle interferes with the cellulose crystallization. This is not unusual as it is known that polymerization and crystallization are coupled processes in bacterial cellulose production (Benziman et al., 1980). The inclusion of additives in the media can interfere with these processes, leading to changes in the bacterial cellulose produced. Based on observations made in this work, it appears that the inclusion of many additives to the media used to produce bacterial cellulose causes undesirable changes to the cellulose.

Here, we used four amorphous peaks based on the cellulose diffraction pattern provided by the ICDD, however there are different methods that can be used to determine crystallinity (Segal, Creely, Martin, & Conrad, 1959). Based on the differences we observed between the crystallinity data calculated with one amorphous peak and four amorphous peaks, extreme caution should be taken not to over interpret the data presented in literature.

Crystallite sizes remained small, even the presence of additives, ranging from 5.8 to 7.0 nm, however these differences were not statistically significant. The addition of HPMC resulted in smaller crystallite sizes being produced of dimension 5.8 nm, further demonstrating its interference in the polymerization and crystallization process.

3.3. Tensile properties

Preliminary investigations of tensile strength in bacterial cellulose films grown in HS media with and without PHB as an additive were completed, as well as a solvent cast PHB film for comparison, in order to provide further evidence of the incorporation of PHB into the BC film. Sections were cut from the dried pellicle films and examined for the tensile properties. A decrease in tensile strength and modulus was observed from the BC to the BC–PHB film, however the elongation at yield appeared a little higher (albeit with questionable statistical significance), however the BC–PHB film exhibited better mechanical properties across all three parameters as compared to the neat PHB (Table 3).

The BC and BC–PHB films exhibited similar values for stress and strain at break (Fig. 6), however both the BC and BC–PHB films exhibited much better properties than the PHB alone.

These results are similar to mechanical properties achieved by others in the literature. Barud et al. (2011) and Cai et al. (2011) both soaked a BC pellicle in a solvent with dissolved PHB and allowed the solvent to evaporate so the PHB would be incorporated into the BC fibrils. Barud et al. (2011) reported an increase in both tensile strength and Young's modulus in a BC–PHB composite over the BC alone, but only at a low concentration of PHB. As the PHB content increased, the mechanical properties decreased. Cai et al. (2011) however reported an increase in tensile strength from the BC film to the 50:50 BC–PHB composite, but a decrease in modulus.

As there have been changes in the mechanical properties from the BC to the BC–PHB film, this further supports that the inclusion of PHB in the media results in the incorporation of this water insoluble polymer into the BC film. If the cellulose produced with this or other additives is to be considered further as a reinforcing agent, then other properties should also be considered. However based on the typical decrease in both cellulose weight and crystallinity upon

Table 3
Tensile properties of BC, PHB and BC–PHB films.

Film	Tensile strength (MPa)	Elongation at yield (%)	Modulus (MPa)
BC	105.66 ± 9.44	6.57 ± 1.73	1866 ± 451
PHB	21.30 ± 4.24	3.64 ± 0.91	852 ± 171
BC–PHB	67.41 ± 18.22	7.74 ± 1.97	1098 ± 105

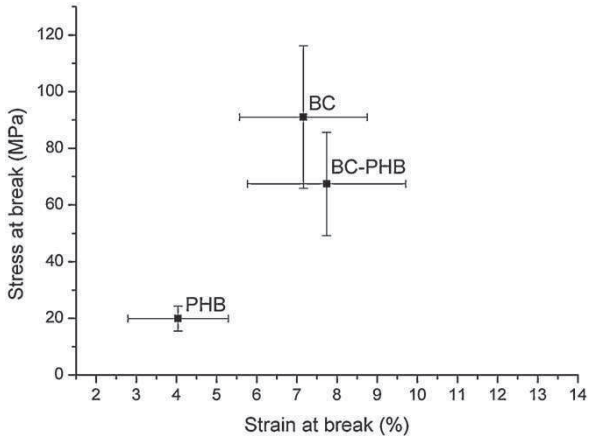


Fig. 6. Stress/strain values for BC, PHB and BC–PHB films.

inclusion of additives, and the observed decrease in tensile strength between neat cellulose and cellulose grown with PHB as an additive in the media here, a high cellulose-producing media without additional components may end up being most appropriate as an additive in nanocomposites. It is also apparent that water insoluble polymers can affect the cellulose as it is produced, creating *in situ* modifications.

4. Conclusions

Incorporation of additives not specifically required for the growth of *G. xylinus* cells or the production of bacterial cellulose can alter the yield, structure and morphology of the cellulose produced. The inclusion of PHB in the media appears to fortuitously result in a composite BC–PHB material. Such compatibilised structures may be a source of reinforcement particularly suited for incorporation in a composite, where the matrix is itself PHB.

Acknowledgments

We thank the Monash Centre for Electron Microscopy for the use of their electron microscopes, and Liz Goodall of CSIRO for the experimental XRD work and calculations. This work was funded by a Julius Career Award from the CSIRO Office of the Chief Executive.

References

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

Barud, H. S., Souza, J. L., Santos, D. B., Crespi, M. S., Ribeiro, C. A., Messaddeq, Y., et al. (2011). Bacterial cellulose/poly(3-hydroxybutyrate) composite membranes. *Carbohydrate Polymers*, *83*(3), 1279–1284.

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*, *77*(11), 6678–6682.

Brown, E. E., & Laborie, M. P. G. (2007). Bioengineering bacterial cellulose/poly(ethylene oxide) nanocomposites. *Biomacromolecules*, *8*(10), 3074–3081.

Byrom, D. (1987). Polymer synthesis by microorganisms – Technology and economics. *Trends in Biotechnology*, *5*(9), 246–250.

Chao, Y. P., Mitarai, M., Sugano, Y., & Shoda, M. (2001). Effect of addition of water-soluble polysaccharides on bacterial cellulose production in a 50-L airlift reactor. *Biotechnology Progress*, *17*(4), 781–785.

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.

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.

Gea, S., Bilotti, E., Reynolds, C. T., Soykeabkeaw, N., & Peijs, T. (2010). Bacterial cellulose-poly(vinyl alcohol) nanocomposites prepared by an in-situ process. *Materials Letters*, *64*(8), 901–904.

Grande, C. J., Torres, F. G., Gomez, C. M., Troncoso, O. P., Canet-Ferrer, J., & Martinez-Pastor, J. (2009). Development of self-assembled bacterial cellulose–starch nanocomposites. *Materials Science & Engineering C*, *29*(4), 1098–1104.

Hessler, N., & Klemm, D. (2009). Alteration of bacterial nanocellulose structure by in situ modification using polyethylene glycol and carbohydrate additives. *Cellulose*, *16*(5), 899–910.

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

Huang, H. C., Chen, L. C., Lin, S. B., & Chen, H. H. (2011). Nano-biomaterials application In situ modification of bacterial cellulose structure by adding HPMC during fermentation. *Carbohydrate Polymers*, *83*(2), 979–987.

Huang, H. C., Chen, L. C., Lin, S. B., Hsu, C. P., & Chen, H. H. (2010). In situ modification of bacterial cellulose network structure by adding interfering substances during fermentation. *Bioresource Technology*, *101*(15), 6084–6091.

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.

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

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.

Segal, L., Creely, J. J., Martin, A. E., Jr., & Conrad, C. M. (1959). An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. *Textile Research Journal*, *29*(10), 786–794.

Seifert, M., Hesse, S., Kabrelian, V., & Klemm, D. (2004). Controlling the water content of never dried and reswollen bacterial cellulose by the addition of water-soluble polymers to the culture medium. *Journal of Polymer Science Part A: Polymer Chemistry*, *42*(3), 463–470.

Tokoh, C., Takabe, K., Sugiyama, J., & Fujita, M. (2002a). Cellulose synthesized by *Acetobacter xylinum* in the presence of plant cell wall polysaccharides. *Cellulose*, *9*(1), 65–74.

Tokoh, C., Takabe, K., Sugiyama, J., & Fujita, M. (2002b). CP/MAS C-13 NMR and electron diffraction study of bacterial cellulose structure affected by cell wall polysaccharides. *Cellulose*, *9*(3–4), 351–360.

Uhlin, K. I., Atalla, R. H., & Thompson, N. S. (1995). Influence of hemicelluloses on the aggregation patterns of bacterial cellulose. *Cellulose*, *2*(2), 129–144.

Umesaki, Y., Kawai, Y., & Mutai, M. (1977). Effect of Tween-80 on flucosyl transferase production in *Streptococcus-mutans*. *Applied and Environmental Microbiology*, *34*(2), 115–119.

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., Ishihara, M., & Sugiyama, J. (2000). Structural modification of bacterial cellulose. *Cellulose*, *7*(3), 213–225.

Cai, Z., & Yang, G. (2011). Optical nanocomposites prepared by incorporating bacterial cellulose nanofibrils into poly(3-hydroxybutyrate). *Materials Letters*, *65*(2), 182–184.

Cai, Z., Yang, G., & Kim, J. (2011). Biocompatible nanocomposites prepared by impregnating bacterial cellulose nanofibrils into poly(3-hydroxybutyrate). *Current Applied Physics*, *11*(2), 247–249.

4.4 Conclusions

This chapter examined the structure and morphological changes that could occur to bacterial cellulose from the inclusion of additives in the media. Additives including a range of organic salts and PHB were added to standard HS media to investigate the impact these components had on bacterial cellulose production.

It was observed that bacterial cellulose retained its typically fibrillar structure in the presence of organic salts, however under some conditions, the fibrils were seen to be coated with a material that was likely to be the salt. Small changes were also observed in the cellulose crystallinity, crystallite size and I_α content due to the presence of the salts. Cellulose production was only able to be achieved with low concentrations of organic salts, as high concentrations appeared to confer a toxic effect, with bacterial cellulose not produced above particular levels of each organic salt. Therefore, production of bacterial cellulose in ionic liquids was not pursued further, but could be a potential source of achieving specific alterations to this material in the future.

In contrast, the inclusion of PHB in the media caused greater changes to the produced cellulose. PHB particles were seen to be physically attached to cellulose fibrils when cellulose was produced in HS-PHB media. Tensile properties of the resulting cellulose were also seen to be decreased when the cellulose was produced in HS-PHB rather than HS. Despite the decrease in tensile properties, the presence of PHB on the surface may result in an increased affinity of the cellulose to PHB. This may provide PHB-modified-bacterial cellulose an advantage for use as reinforcement in a PHB/BC composite. Therefore, the effects of this *in situ* modification on bacterial cellulose is to be examined further.

Chapter 5
Techniques for Producing PHB/BC Composites with Finely Dispersed Cellulose

5.1 Introduction

Bacterial cellulose has the potential to be used as a reinforcing phase in composites, and if a polymer such as poly-3-hydroxybutyrate is the matrix material, the composite obtained should be fully degradable due to the biodegradable nature of both components. Bacterial cellulose has been reported as being used in many different composites, often developed by way of impregnation (Ashori et al., 2012; Barud et al., 2011; Cai & Kim, 2010; Cai & Yang, 2011; Cai et al., 2011; Gea et al., 2010; Kim et al., 2011; Shah et al., 2013; Ul-Islam et al., 2012a; Wan et al., 2006; Yoon et al., 2006). In the impregnation method, the bacterial cellulose pellicle is soaked in a solvent containing another polymer, and as the solvent evaporates that polymer becomes part of the three-dimensional fibrous cellulose network. This method is advantageous because it does not require the dispersion of the cellulose fibrils, which are subject to extensive hydrogen bonding and thus problematic to disperse. However it also does not take full advantage of the nanosized fibrils that comprise bacterial cellulose, rather making use of its natural three-dimensional network structure. For bacterial cellulose to be used as a reinforcing phase, a different blending technique would need to be used. Though other blending techniques are not commonly reported as being used with bacterial cellulose, there are a number of successful plant cellulose composites that have been reported in the literature. It may be possible to adapt these techniques for use with bacterial cellulose to create PHB composites reinforced with a bacterial cellulose filler phase that have improved mechanical properties than those of the matrix alone.

Solution blending and melt blending are two techniques that can be used. Solution blending is a technique that has the potential to achieve intimate mixing, provided dispersion of the phases is good, and can readily be undertaken on a smaller (such as laboratory) scale. Melt blending is a technique that can be easily upscaled, and it can be used to develop small (if using a mini-extruder) or large blend quantities. While both techniques can be used to develop mixtures that consist of a PHB/BC composite with a high content of matrix and low content of filler material, there may be difficulties in achieving well dispersed bacterial cellulose, as cellulose is a hydrophilic material whereas PHB is

hydrophobic. Achieving a fine dispersion of cellulose is known to be a problem in the development of composites as cellulose nanofibres tend to aggregate, particularly in hydrophobic matrices, and because of their high surface area (Jipa et al., 2012; Martínez-Sanz et al., 2014; Nakagaito et al., 2009). It is therefore necessary to investigate these two blending techniques to determine a set of protocols to produce PHB/BC composites with well dispersed bacterial cellulose. Such a well dispersed reinforcing phase is necessary if improved mechanical properties are to be achieved.

Solution blending and melt blending have previously been used to produce plant cellulose/PHB and PHB-poly-3-hydroxyvalerate copolymer composites (Jiang et al., 2008; Rapa et al., 2010). Jiang et al. (2008) used PHBV with cellulose nanowhiskers and determined that cellulose improved the PHBV properties when the composite was made by solution blending, but found that melt blending resulted in poor dispersion and weak interfacial adhesion between the matrix and reinforcement material. Rapa et al. (2010) modified cellulose fibres with maleic acid and succinic anhydride before melt blending fibres with PHB, and found good mixing between the two materials and improved mechanical properties over PHB alone. These results suggest that either solution or melt blending could be adapted to PHB/BC blends.

There have been a small number of reports in the past few years that have described the production of bacterial cellulose composites using solution blending and melt blending with a range of matrix materials (Grunert & Winter, 2002; Jipa et al., 2012; Kibédi-Szabó et al., 2012; Martínez-Sanz et al., 2012a, 2013a, 2013b; Martínez-Sanz et al., 2014; Millon et al., 2009; Stoica-Guzun et al., 2011; Tomé et al., 2011; Woehl et al., 2010; Yang et al., 2012). The bacterial cellulose in these reports has been used in various forms, such as nanowhiskers or in a ground powder form. A number of methods to achieve even dispersion of cellulose in blends have also been investigated.

Melt blending is a technique that has been used to blend bacterial cellulose with poly(vinyl alcohol) (Martínez-Sanz et al., 2012a; Tomé et al., 2011) and ethylene vinyl alcohol (Martínez-Sanz et al., 2013a, 2013b; Martínez-Sanz et al., 2012b). The resultant composites showed limited cellulose dispersion, with some agglomeration of the cellulose observed. In addition, the materials showed

degradation from melt blending (Martínez-Sanz et al., 2012b; Tomé et al., 2011). However, some methods of melt blending resulted in composites with a well-dispersed cellulose phase. These well-dispersed composites were shown to possess improved mechanical properties (Martínez-Sanz et al., 2012a, 2013a, 2013b; Tomé et al., 2011). The results from these research outcomes indicate that melt blending may be a viable technique for producing composites with a well dispersed filler phase, provided that an even dispersion can be achieved.

Solution blending with bacterial cellulose with various matrix materials has been reported in the literature, including PVA (Jipa et al., 2012; Kibédi-Szabó et al., 2012; Millon et al., 2009; Stoica-Guzun et al., 2011; Yang et al., 2012), cellulose acetate butyrate (Grunert & Winter, 2002), poly-hydroxyalkanoates (Martínez-Sanz et al., 2014) and thermoplastic starch (Woehl et al., 2010). In these studies, dispersion of the cellulose was achieved using a range of methods such as homogenisation (Millon et al., 2009; Woehl et al., 2010; Yang et al., 2012), mixing acid-treated nanowhiskers (Grunert & Winter, 2002; Martínez-Sanz et al., 2014), vigorously stirring wet cellulose produced in a bioreactor (Kibédi-Szabó et al., 2012; Stoica-Guzun et al., 2011), and using cellulose in a ground form (Jipa et al., 2012). Composites achieved by homogenising bacterial cellulose in solution blends have shown good dispersion of cellulose. Homogenised bacterial cellulose solution blends have also been shown to have some improvements in mechanical properties (Millon et al., 2009; Woehl et al., 2010; Yang et al., 2012). Solution blended PHBV/BC blends (using PHBV with differing valerate contents), possessed well dispersed cellulose when cellulose was used in the form of nanowhiskers at 1 wt%, however the cellulose aggregated at 3 wt%. Despite the good dispersion, the PHBV/BC blends were found to have no significant differences in mechanical properties when cellulose nanowhiskers were incorporated, although a decrease in elongation at break was observed with one PHBV matrix with 3 wt% nanowhiskers (Martínez-Sanz et al., 2014). Reports such as these indicate that solution blending may also provide a technique to produce bacterial cellulose composites.

Several dispersion methods have been used in conjunction with solution blending to produce bacterial cellulose composites, but the mechanical properties of the composites obtained have not

been reported, with the exception of homogenised blends. Jipa et al. (2012) used bacterial cellulose in a ground powder form and blended it with PVA, with cellulose aggregation observed in these blends. Despite the limited aggregation achieved with this method in the study by Jipa et al. (2012), the use of ground bacterial cellulose provides many advantages for blending. Ground bacterial cellulose can be easily weighed for blending, ensuring accurate concentrations of the added cellulose. In addition, it may be possible that vigorously stirring the cellulose as part of the solution blend can result in a good dispersion, however based on the previous aggregation evident in PVA, additional dispersion methods may be required. One possible dispersion technique is sonication, and this technique was used as part of the solution blending process in the current research.

The possibility of "tailor-designing" bacterial cellulose with specific properties for blending was mentioned in Chapter 4, with the production of PHB-modified-BC. It was originally hypothesised that, when used in a blend, this Mod-BC may disperse well and show a greater affinity to a PHB matrix, and therefore would achieve PHB/Mod-BC composites with greater interfacial adhesions and mechanical properties than achieved from composites with traditional bacterial cellulose. Thus, in this research, the use of this Mod-BC in blends was investigated. The effects of *in situ* and post modifications, such as grinding and sonication, on bacterial cellulose prior to its inclusion in composites were also studied.

This chapter presents the investigation of different blending methods and dispersion techniques in order to determine a set of protocols to develop PHB/BC composites with finely dispersed cellulose. A comparison PHB/BC composites produced by solution and melt blending is provided, together with the use of stirring and sonicating bacterial cellulose in a ground form to achieve highly dispersed cellulose in a solution blend. The mechanical properties of PHB/BC composites with cellulose from different media, including HS-PHB media to achieve PHB-modified-BC, were also examined and the results reported here.

5.2 Materials and Methods

5.2.1 Bacterial Strain

A culture of bacterial cellulose-producing *Gluconacetobacter xylinus* ATCC 53524 was kindly provided by Mike Gidley, University of Queensland, Australia.

5.2.2 Materials

PHB was kindly provided by Metabolix. Bacterial cellulose was produced in HS media (Schramm & Hestrin, 1954), with 2 wt% glucose, 0.5 wt% yeast extract, 0.5 wt% peptone, 0.27 wt% Na_2HPO_4 and 0.115 wt% citric acid monohydrate, and Yamanaka-mannitol media (Ruka et al., 2012), with 5 wt% mannitol, 0.5 wt% yeast extract, 0.5 wt% $(NH_4)_2SO_4$, 0.3 wt% KH_2PO_4 and 0.005 wt% $MgSO_4.7H_2O$. The pH of media was adjusted to 5.0 with HCl or NaOH and autoclaved at 121° C for 20 minutes. PHB was used as an additive in HS media at 1 wt% in some cultures in order to produce surface modified-bacterial cellulose pellicles (referred to henceforth as Mod-BC) (Ruka et al., 2013). Cultures were grown in 600 mL media and were incubated for 7 days at 28° C under static conditions. Following incubation periods, cultures were shaken vigorously to remove the attached bacterial cells. Pellicles were removed and rinsed to remove any residual media. Pellicles were washed with 0.1 M NaOH at 80° C for 1 hour to remove bacterial cells, and then washed repeatedly until a neutral pH was achieved. Pellicles dried at room temperature.

5.2.3 Grinding of Bacterial Cellulose

Bacterial cellulose was ground to a fine powder using a SPEX SamplePrep Freezer/mill 6870. The cellulose was ground with a 4 minute pre-cool, and 12 cycles involving 2 minutes cooling, and 2 minutes grinding, at a frequency of 10 cps. Bacterial cellulose in various forms was examined by scanning electron microscopy and X-ray diffractometry.

5.2.4 Solution Blending for Production of PHB/BC Composites

To make solution blends, ground bacterial cellulose was added to chloroform and mixed with PHB. Dispersion was achieved by mechanical stirring alone or by sonicating the cellulose prior to PHB

dissolution. Sonication of bacterial cellulose pellicles was completed with a Branson Sonifier 250 with an 80 % duty cycle and an output of 4, with the sonicator probe kept at a consistent height for all samples. When the sonication method was used, bacterial cellulose was sonicated in chloroform at room temperature for 60 minutes. PHB was added to the cellulose-chloroform solution up to 5 wt% and was dissolved by mechanical stirring at 80° C for 3 hours. The blend was cast in glass petri dishes and stored at room temperature to allow solvent evaporation, leaving films with thicknesses of approximately 20 μm. Neat PHB and solution blends were examined by SEM and FTIR, and their swelling and tensile properties were also determined.

5.2.5 Melt Blending and Extrusion for Production of PHB/BC Composites

PHB pellets with ground bacterial cellulose at 1, 2, and 5 wt% were added to a twin screw extruder DSM Micro 15 at 180° C and were mixed at 50 rpm for 4 – 6 minutes. Melt blends were then injection moulded into tensile bars using a DSM injection-moulding machine.

5.2.6 Examination of Bacterial Cellulose, PHB and PHB/BC Composites

5.2.6.1 Scanning Electron Microscopy for Morphological Examinations

SEM was performed using a field-emission Nova NanoSEM 450. Samples were coated with platinum, and were examined at 2 kV. SEM was used to examine the surface of bacterial cellulose films, PHB and PHB/BC solution blended films, and the surface and cryo-cracked cross-section of PHB and PHB/BC melt blends.

5.2.6.2 X-ray Diffractometry and Crystallinity Calculations

XRD was used to monitor the $d_{1\text{-}10}$ spacing corresponding to the interlayer spacing of the crystalline structure of the bacterial celluloses. The XRD measurements were performed on the cellulose sheet and powder samples using a Bruker D8 Diffractometer operating at 40 kV, 40 mA, CuKα radiation monochromatised with a graphite sample monochromator with a diffractogram recorded between 2θ angles of 2° and 40°. Crystallinity was calculated using TOPAS™ based on the method of Hindeleh and Johnson (1971). The amorphous area was determined using International Centre for Diffraction Data

(ICDD) PDF card 00-060-1501, amorphous cellulose. The crystalline peak positions were selected based on positions given in Cazja et al. (2004). A fundamental parameters function was used to profile the peak shape and area for both the amorphous and crystalline components.

5.2.6.3 Tensile Properties

Tensile properties of bacterial cellulose pellicles were determined using an Instron universal testing machine (Model 3366), tested in accordance with ASTM D882 (using a type IV specimen as described in ASTM D638), fitted with a 100 N static load cell, with the rate of extension being 2 mm/min. Tensile properties for solution cast PHB and composite films were examined on an Instron universal testing machine (Model 5566) fitted with a 2.5 N static load cell, with the rate of extension being 2 mm/min. At least ten specimens per each formulation were tested until failure, where ultimate tensile strength, Young's modulus and elongation at break had mean values and standard deviations calculated.

5.2.6.4 Fourier Transform – Infra Red Spectra for Composite Characteristics

FTIR spectra were obtained using a Perkin-Elmer Spectrum 100 Spectrometer. Scans were taken between 4000 and 450 cm^{-1}, with 16 convolutions. Baselines for each sample spectrum were normalised using the Spectrum software.

5.2.6.5 Swelling Capacities for Composite Characterisation

PHB and PHB/BC solution blends were cut into 2 × 2 cm pieces and were immersed in distilled water. The weight of the films was determined before and after immersion. Swelling was determined by the following equation (Jipa et al., 2012):

$$Swelling = \left[\frac{m_{wet} - m_{dry}}{m_{dry}}\right] \times 100\%$$

5.2.6.6 Three Point Bend Tests

Flexural strength and the 1% secant modulus were determined for all PHB and PHB/BC melt blended composites on an Instron universal testing machine (model 5566) and tested in accordance with ASTM D790. The Instron was fitted with a 10 kN static load cell, a custom-built 3-point cantilever test

apparatus with a 64 mm total span, with the speed of elongation set to 1.7 mm/min (to comply with Equation 1 of ASTM D790). At least five specimens for each formulation were tested until failure or a 5% strain was achieved, from which mean values and standard deviations were calculated.

5.2.6.7 Impact Testing

Impact testing was conducted on PHB and PHB/BC melt blended composites according to ASTM 6100 using a POE Instron impact tester. A range of conditions were tested, but consistent results could not be obtained.

5.3 Results and Discussion

5.3.1 Effects of *in situ* Modifications and Post Modifications on Bacterial Cellulose

In Chapters 3 and 4, a range of different media were used to produce bacterial cellulose. These included the standard HS medium, high cellulose producing Yamanaka-mannitol medium, and HS-PHB medium which was found to produce PHB-modified-bacterial cellulose (Mod-BC) where PHB particles are physically bound to cellulose fibrils. Cellulose from these media was produced for use in blends with PHB. The structure, morphology and mechanical properties of each cellulose, referred to as HS-BC, Yam-BC and Mod-BC, respectively, was examined prior to use in composites, as it was demonstrated previously in Chapters 3 and 4 that different media can affect the structure, morphology and properties of produced cellulose, causing *in situ* modifications. The effects of post modifications on bacterial cellulose, such as grinding and sonication, were also examined in order to determine if selected post modifications had any detrimental impact on the properties of the cellulose.

The pure cellulose films, HS-BC and Yam-BC, exhibited similar morphological features when examined under SEM, however Mod-BC was found to exhibit the bound PHB on the surface of the fibrils (Figure 5.1). Similarly, the mechanical properties of the cellulose grown in the different media were similar for HS-BC and Yam-BC, with decreased ultimate tensile strength, elongation at break and Young's modulus for the Mod-BC (Table 5.1).

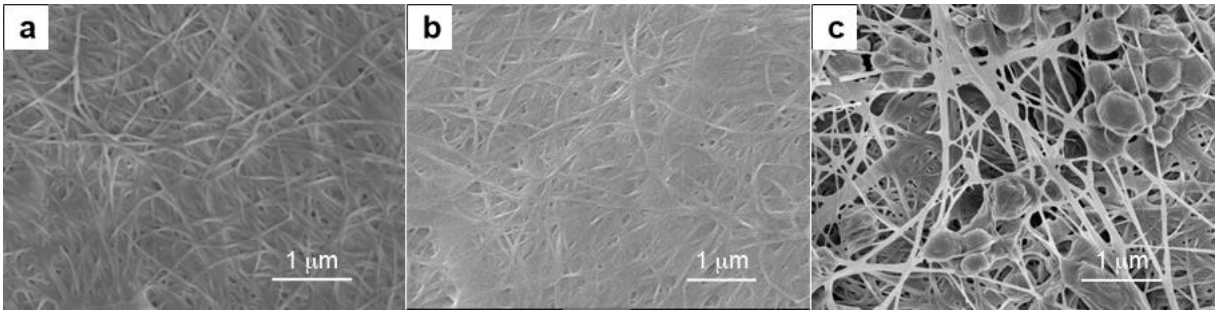


Figure 5.1: SEM images of bacterial cellulose fibrils grown in standard HS (a), Yamanaka-mannitol (b) and HS-PHB (c) media.

To achieve a homogenous dispersion of bacterial cellulose in the solution blends with specific dry weights of cellulose, bacterial cellulose was ground to a fine powder. SEM examination of the cellulose in the different states revealed that the interwoven mesh of nanosized fibrils that exist as part of the pellicle (Figures 5.2a and b) were lost when the material was subjected to grinding (Figure 5.2c and d). Similarly, Jipa et al. (2012) noted the presence of irregular particles after milling bacterial cellulose to a powder. Examination of the powder showed no fibrils of cellulose, even at very high magnifications. The nanosized fibrils potentially provide cellulose with a high aspect ratio that can be used to produce composites that are effectively reinforced with low concentrations of filler; therefore the loss of the fibrillar structure is not advantageous.

Table 5.1: Mechanical properties of bacterial cellulose produced with and without PHB in the media.

Bacterial Cellulose	Ultimate Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)
HS-BC	123.94 ± 29.40	8.19 ± 0.72	1711 ± 320
Yam-BC	143.49 ± 34.41	9.49 ± 2.53	2167 ± 336
Mod-BC	41.60 ± 16.86	3.65 ± 1.50	1260 ± 248

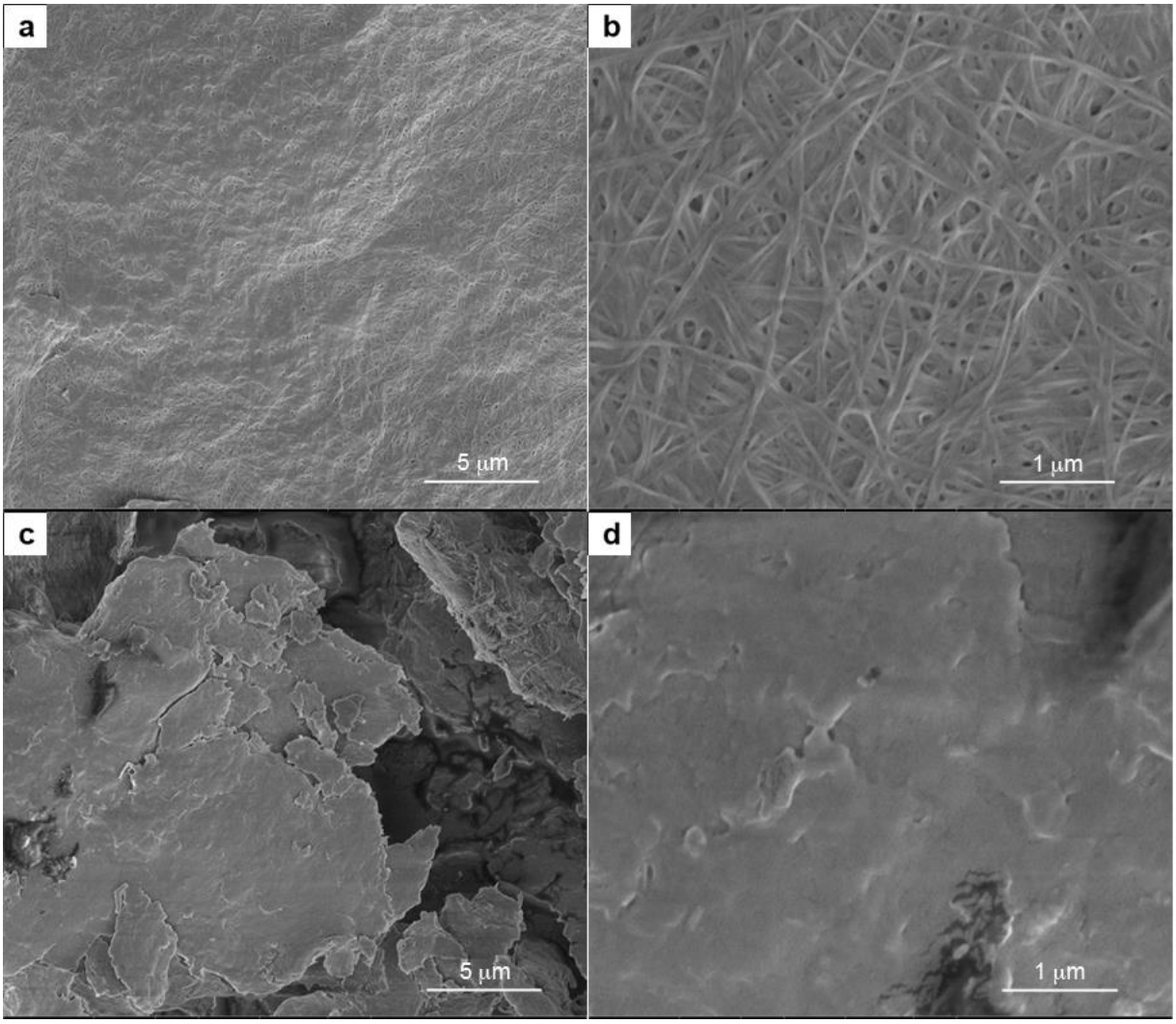


Figure 5.2: Morphology of bacterial cellulose in a pellicle (a and b) and in a ground state (c and d) from cellulose grown in HS medium.

In order to examine the characteristics of the cellulose before and after post modifications of grinding and sonication, XRD was undertaken to determine crystallinity. The examination of the bacterial cellulose after it was ground to a fine powder revealed a change in crystallinity after the grinding; however the scans revealed similarities in peaks and peak heights between the ground cellulose before and after sonication (Figure 5.3). Crystallinity calculations revealed a decrease in crystallinity of the bacterial cellulose following grinding, with an additional (albeit smaller) decrease after the sonication of the ground bacterial cellulose powder (Table 5.2).

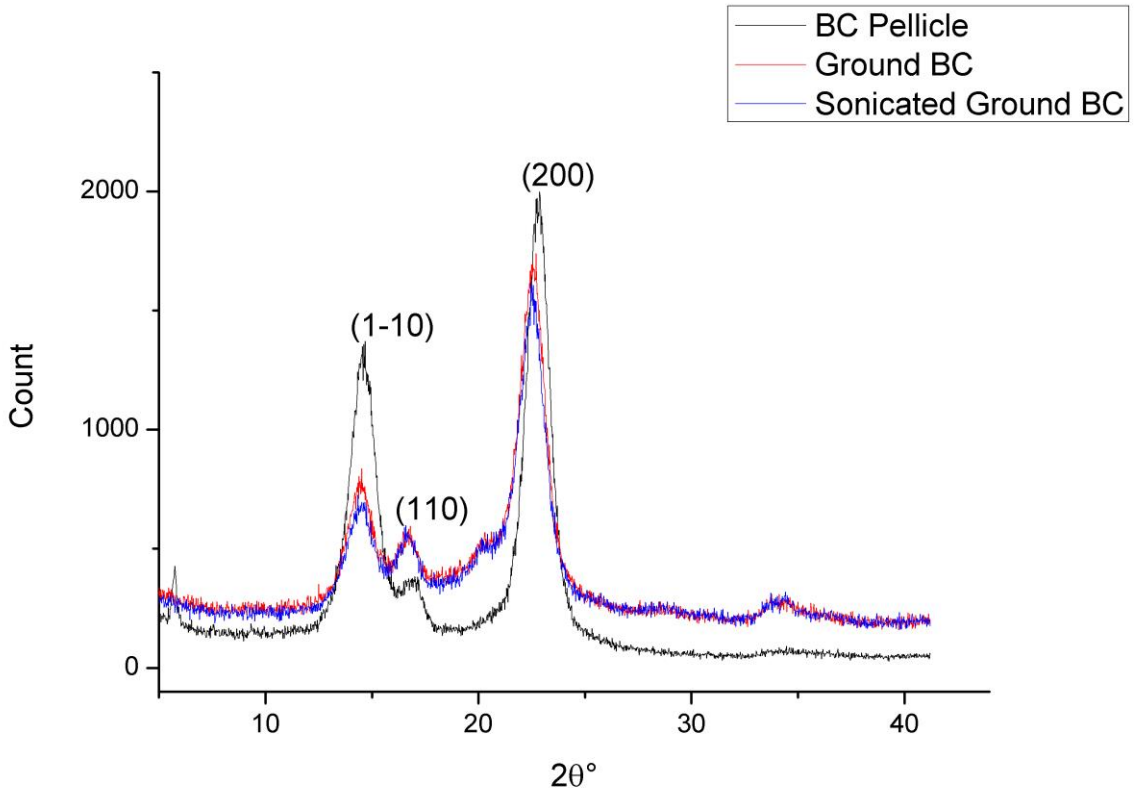


Figure 5.3: XRD of bacterial cellulose from various treatment conditions.

These results indicate that both *in situ* treatment of bacterial cellulose, by including an additive in the growth medium, and post-production treatment, such as grinding and/or sonication, lead to changes in the structure and morphology of the cellulose. However, homogenisation has previously been shown to cause a small decrease in the crystallinity of bacterial cellulose (Kose et al., 2011), but also produce composites with bacterial cellulose with good mechanical properties, suggesting that the

Table 5.2: Crystallinity of bacterial cellulose from various treatment conditions.

Bacterial Cellulose Conditions	Crystallinity (%)
BC Pellicle	80
Ground BC	71
Sonicated Ground BC	66

crystallinity decrease from sonication may too allow composites with good mechanical properties to be produced. Grinding bacterial cellulose to a fine powder may allow for the addition of specific dry weights to be added to blends to allow careful production of composites, however the grinding may also lead to changes in the cellulose that cause a loss of its desirable properties. This suggests that treatment processes must be carefully selected.

5.3.2 Solution Blending as a Technique for Producing PHB/BC Composites

5.3.2.1 Dispersion of Bacterial Cellulose in Solution Blended PHB Composites

To optimise the production of PHB/BC films, different solvents (dimethyl formamide and chloroform) and drying conditions were used. It was determined that the optimum conditions to produce PHB/BC films were achieved by using chloroform as the solvent to dissolve PHB at 5 wt%, and to use dispersion methods to incorporate ground bacterial cellulose. It was then possible to cast films of approximately 10 – 20 μm thickness.

PHB/BC composites containing 5 wt% bacterial cellulose were initially produced by dispersing the bacterial cellulose solely by mechanical stirring. In this case, the bacterial cellulose was seen to aggregate, both to the naked eye, and under SEM as shown in Figure 5.4. Similarly, Jipa et al. (2012) reported that even dispersion of bacterial cellulose was not achieved with mechanical stirring of bacterial cellulose powder in a solution containing dissolved PVA.

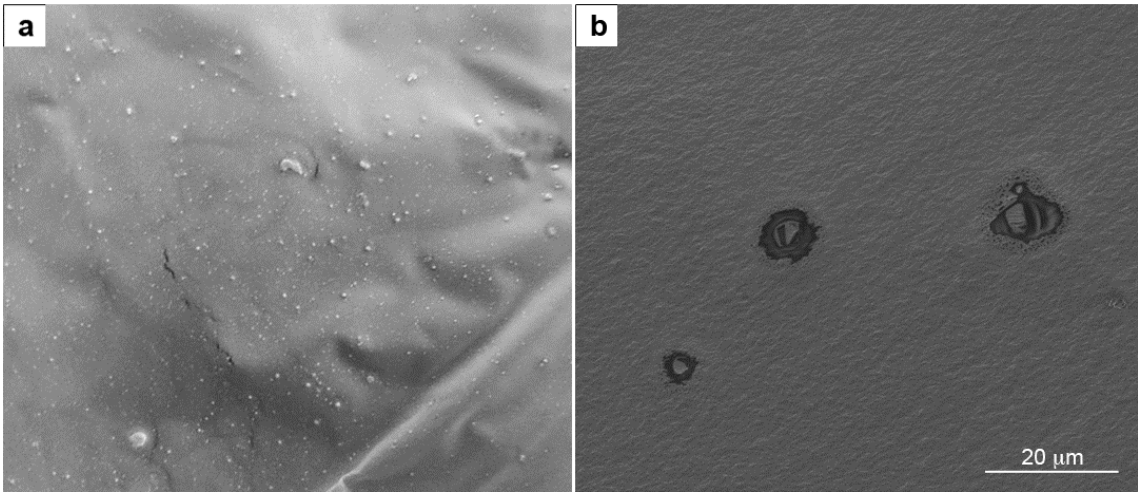


Figure 5.4: Images of aggregated bacterial cellulose in a PHB/BC solution blend.

To better disperse the bacterial cellulose powder in the solvent, a sonication step was introduced prior to the addition of PHB for dissolution. The sonication of cellulose before blending led to composites with no apparent aggregations of cellulose, with the SEM micrograph demonstrating an appearance similar to that seen with neat PHB. Using this processing protocol, PHB/BC blends were produced with cellulose contents of 1, 2 and 5 wt%.

Examination of PHB and PHB/BC composites by SEM demonstrated evenly-dispersed bacterial cellulose. Figure 5.5 demonstrates the surface and cross-section of PHB and PHB/BC with 5 wt% cellulose, with no aggregated cellulose observed. Thus the sonication step is required to disperse ground bacterial cellulose in a PHB matrix.

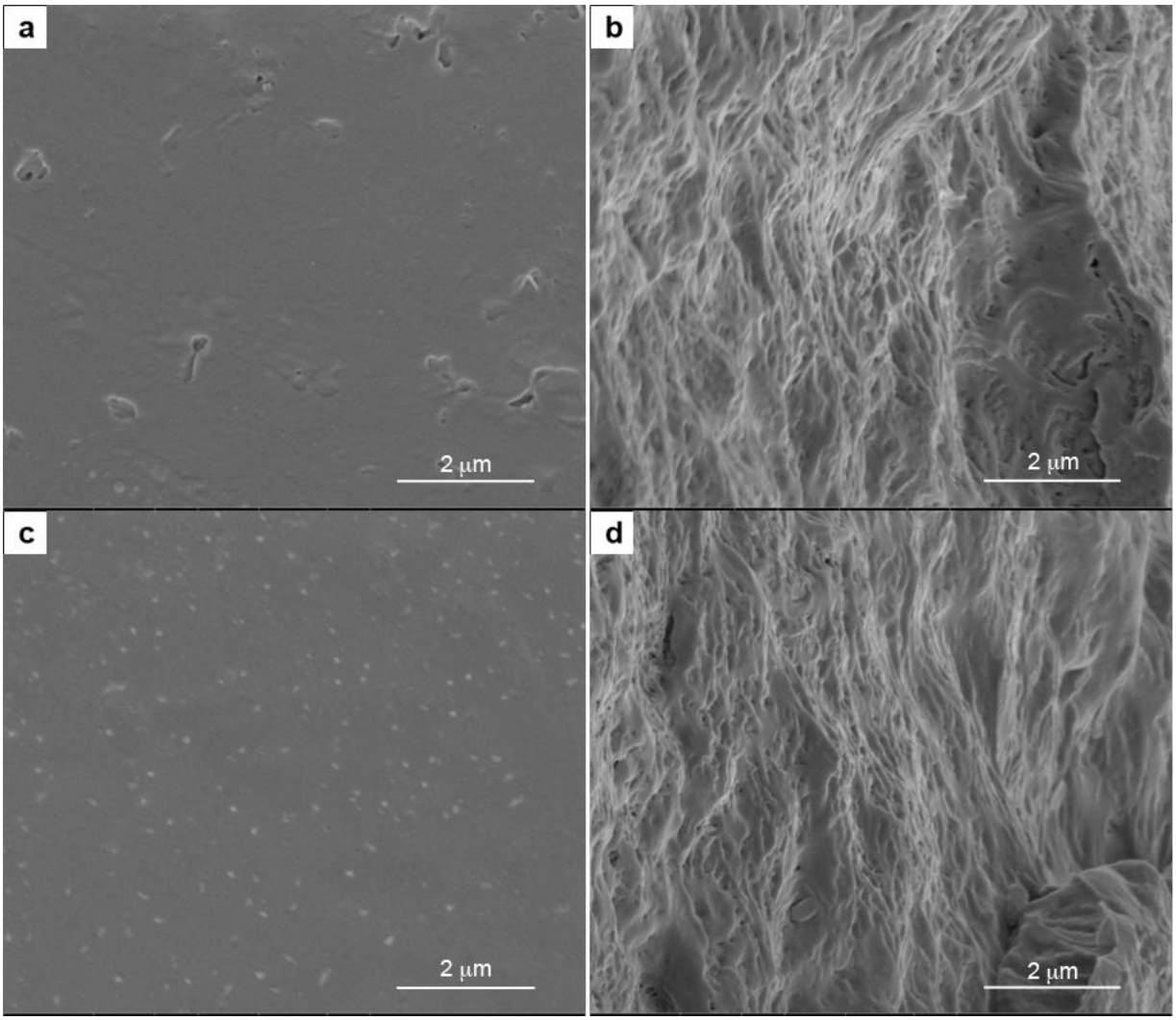


Figure 5.5: SEM of the surface (a and c) and cross-sections (b and d) of neat PHB (a and b) and PHB/5% BC solution blend (b and d) with cellulose produced from HS medium.

5.3.2.2 Mechanical Properties of PHB/BC Solution Blends

The mechanical properties of the PHB/BC composites, with up to 5 wt% cellulose, were examined to determine if the growth media for the cellulose or the cellulose content affected these mechanical properties.

The ultimate tensile strengths of neat PHB and PHB/BC blends were similar (Figure 5.6). PHB/HS-BC showed a lower tensile strength at 1 wt% BC, however the strength varied significantly. The tensile strength of PHB/HS-BC, with 5 wt% BC, was found to increase to similar levels as for the neat PHB. The ultimate tensile strength of PHB/Yam-BC blends was similar to that of the HS-BC blends. Tensile strengths of PHB/Mod-BC blends were slightly lower than that of PHB and PHB/Yam-BC at 2 and 5 wt% cellulose contents.

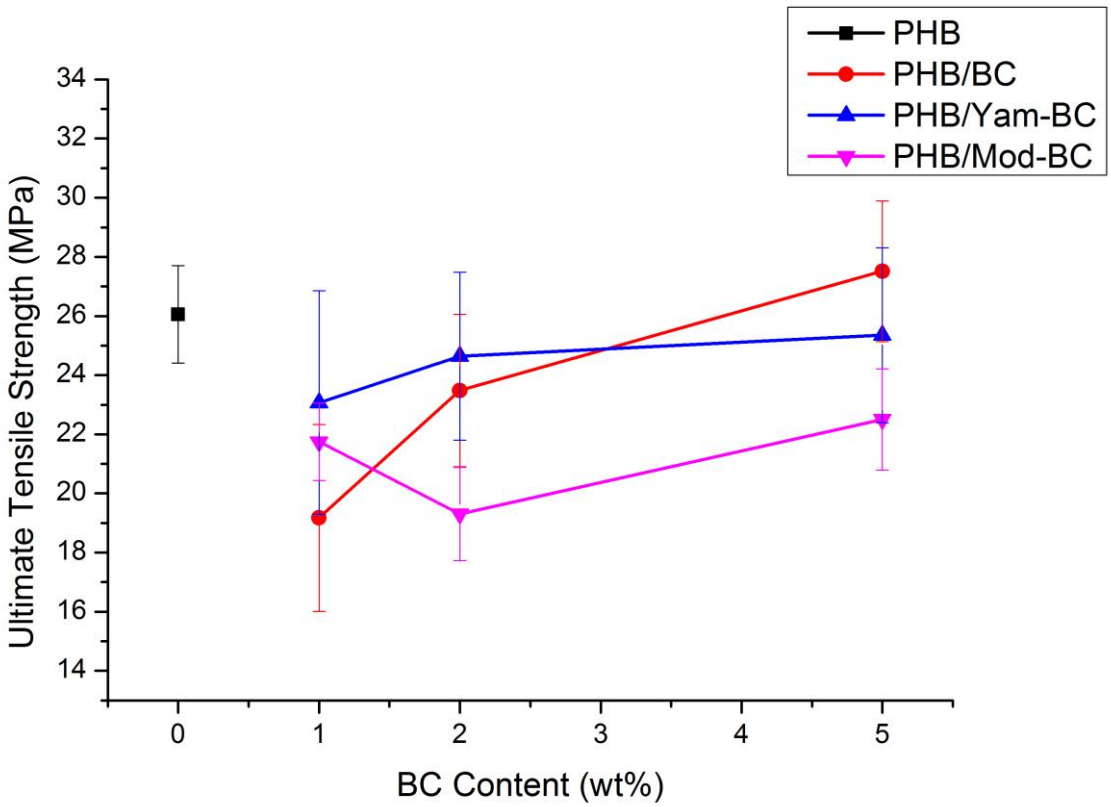


Figure 5.6: Ultimate tensile strength of PHB and PHB/BC blends.

Elongation at break of the PHB/BC composites was lower than that of PHB at all cellulose contents (Figure 5.7), however some results were not significant. The decreased values obtained indicate that the inclusion of bacterial cellulose in the PHB led to problems with interfacial adhesion, resulting in lower elongation at break values. This suggests that the Mod-BC did not improve the interfacial adhesions between the hydrophobic PHB matrix and hydrophilic cellulose.

Young's modulus values improved in some of the composites containing bacterial cellulose (Figure 5.8). The Young's modulus of the PHB/HS-BC composite at 5 wt% cellulose was greater than that of PHB alone. Young's modulus of PHB/Yam-BC showed a slight increase up to 5 wt%, however these results varied, resulting in large standard deviations. Young's modulus of PHB/Mod-BC was lower than that of PHB/HS-BC and PHB/Yam-BC, although these results were not statistically significant. The general trend of all the PHB/BC modulus values improved for the composites as the cellulose content increased from 1 wt% to 5 wt%.

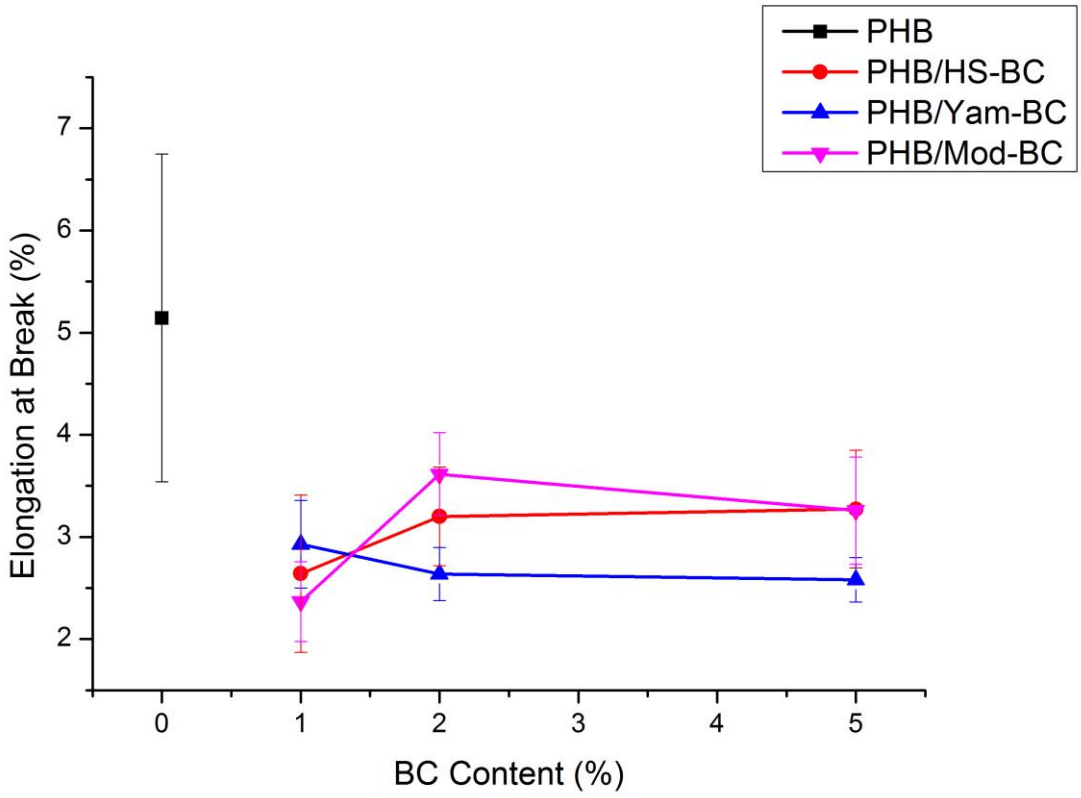


Figure 5.7: Elongation at break of PHB and PHB/BC blends.

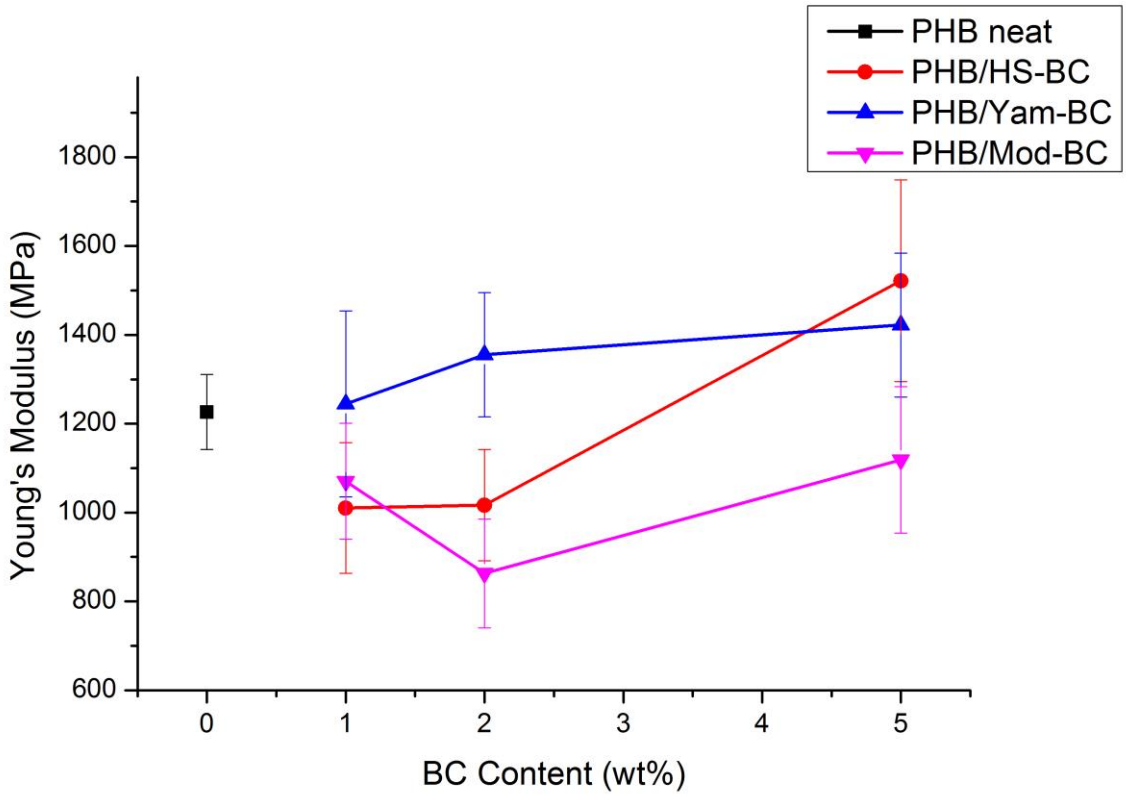


Figure 5.8: Young's modulus of PHB and PHB/BC blends.

The results from the investigation indicate that there were no significant improvements in the mechanical properties of the PHB/BC solution blends, despite a good dispersion of the cellulose phase being achieved due to sonication. It is likely that solution blending could be used to successfully blend PHB and bacterial cellulose, however the ground form of cellulose does not appear to confer improved mechanical properties. Alternate dispersion methods could be considered further to produce PHB/BC solution blends, however it is not clear if this would lead to better properties.

5.3.2.3 Comparison of Characteristics and Mechanical Properties of PHB/BC Composites with Bacterial Cellulose from Standard HS and HS-PHB Media

In Chapter 4, it was originally hypothesised that surface-modified bacterial cellulose would show a greater affinity to a PHB matrix and therefore have better mechanical properties, however it was found that the PHB/Mod-BC composites had poorer mechanical properties. There are two possible reasons for this result; firstly the lower mechanical properties in the PHB/Mod-BC blends could have

been due to the mechanical properties of the cellulose itself, as it was demonstrated that the inclusion of the PHB in the media causes a loss of tensile strength, elongation and modulus (Table 5.1). A second possibility is that the actual dry weight of the included cellulose in each of the blends is lower in the PHB/Mod-BC composites than in the PHB/HS-BC or PHB/Yam-BC composites. To determine the cause of the lower mechanical properties in the PHB/Mod-BC composites, 10 wt% blends using HS-BC and Mod-BC were produced. FTIR and swelling capabilities were determined to demonstrate how much cellulose was actually included in the PHB/Mod-BC blend compared to the PHB/HS-BC blend. Tensile properties of the two different blends were also examined.

FTIR revealed small changes in peak heights between PHB, and the PHB/HS-BC and PHB/Mod-BC solution blends (Figure 5.9a). Only small increases in peaks relating to bacterial cellulose at approximately 3200 – 3400 cm^{-1} were observed in the PHB/HS-BC and PHB/Mod-BC films due to the low concentration of bacterial cellulose compared to PHB. A large peak in this area corresponds to the O-H moiety of cellulose (Grande et al., 2009). Small peaks at 750 and 710 cm^{-1} were also present in the blends, corresponding to the cellulose I_α and I_β fractions (Yamamoto et al., 1996). The changes are present in both the PHB/HS-BC and PHB/Mod-BC composites, confirming the presence of bacterial cellulose, although these observations were not as apparent in the Mod-BC composite. These results indicate that there was a lower cellulose content in the PHB/Mod-BC composites than in the PHB/HS-BC composites. This is likely to be due to the fact that the Mod-BC powder contains the PHB that was attached to the cellulose fibrils, whereas the HS-BC sample is pure bacterial cellulose.

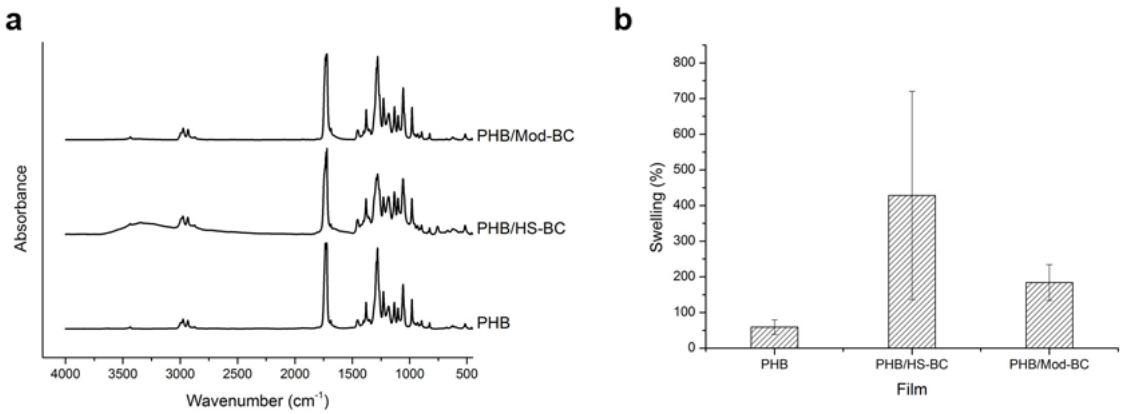


Figure 5.9: FTIR and swelling capabilities of PHB and solution blends.

The degree of swelling was found to increase for PHB/HS-BC and PHB/Mod-BC, compared to neat PHB alone, with a smaller increase in swelling found with PHB/Mod-BC (Figure 5.9b). Since PHB is a hydrophobic material, it was not expected to absorb water, whereas cellulose is extremely hydrophilic and can absorb high amounts of water (Seifert et al., 2004; Shah et al., 2013). The act of combining materials with bacterial cellulose is known to alter the swelling capacity of composites (Jipa et al., 2012; Ul-Islam et al., 2012b). Based on the hydrophobic nature of PHB and hydrophilic nature of bacterial cellulose, it is not unexpected that the inclusion of bacterial cellulose in a PHB matrix caused an increase in swelling. The lower swelling in the films containing the Mod-BC than those containing HS-BC is further evidence of a lower amount of bacterial cellulose in the Mod-BC blend, supporting the findings of the FTIR.

The 10 wt% PHB/HS-BC and PHB/Mod-BC films had similar ultimate tensile strength, elongation at break and Young's modulus (Table 5.3). This result indicates that including a higher content of Mod-BC in the 10 wt% blend accommodates for some of the Mod-BC weight being due to attached PHB, as the 10 wt% Mod-BC composite had similar mechanical properties to the PHB/HS-BC with lower cellulose contents.

The Mod-BC did not improve the mechanical properties in a PHB/BC composite by comparison to HS-BC or PHB alone. In addition, similar mechanical properties were obtained from cellulose produced in all media examined. Thus it appears that the mechanical properties of a PHB/BC blend are not impacted by the medium selected for cellulose production from the conditions tested. Because of this, and because of the ability to obtain much higher amounts of cellulose, Yamanaka-mannitol medium was selected as the production medium for future work in this research.

Table 5.3: Mechanical properties of PHB/BC blends with 10 wt% cellulose content using HS-BC and Mod-BC

Blend	Ultimate Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)
PHB/HS-BC	27.03 ± 8.78	2.21 ± 0.47	1740 ± 405
PHB/Mod-BC	24.62 ± 4.71	2.29 ± 0.65	1790 ± 179

5.3.3 Melt Blending as a Technique for Producing PHB/BC Composites

5.3.3.1 Dispersion of Bacterial Cellulose in Melt Blended PHB Composites

Melt blending is an effective method to use to create various composites as it can be easily upscaled. Using melt blending, bacterial cellulose powder was successfully blended with PHB to obtain composites with 1, 2, and 5 wt% cellulose.

Examination of the morphology of the melt blends revealed a similar morphology between the PHB and PHB/BC cross-section (Figure 5.10). There were no apparent aggregates of cellulose, suggesting that the cellulose was well dispersed. However, examination of the blends without magnification revealed some agglomeration of cellulose in the form of the occasional dark spot, but the morphology of the melt blends was generally consistent. It was also observed that, with increasing concentrations of bacterial cellulose, the colour of the composites became darker, indicating degraded samples. Figure 5.11 shows the darkening colours of the blends. The colours observed appear to be consistent throughout the blend, further supporting cellulose dispersion.

The degradation of these samples was not unexpected as it has also been observed in EVOH/BC melt blends, where the EVOH/BC melt blends resulted in composites with black spots, indicating that the cellulose had degraded at the melt temperature of 190° C (Martínez-Sanz et al., 2012b). Here, the neat PHB blend (0% BC) remained a very light colour, only darkening with increasing bacterial cellulose contents up to 5 wt%. Martínez-Sanz et al. (2012b) also demonstrated a number of methods to treat bacterial cellulose, involving acid hydrolysis and electrospinning the fibres prior to melting.. They

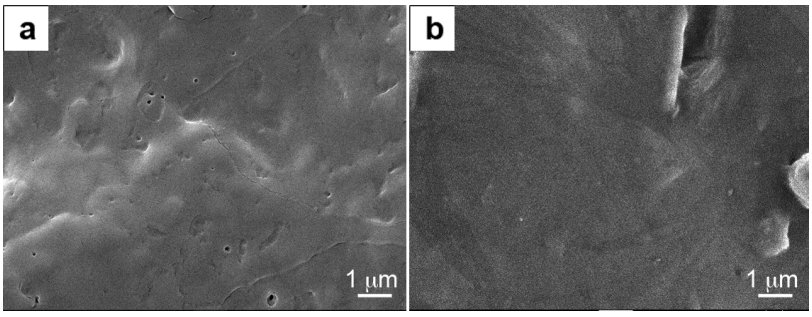


Figure 5.10: SEM microscopy of PHB and PHB/BC melt blends.

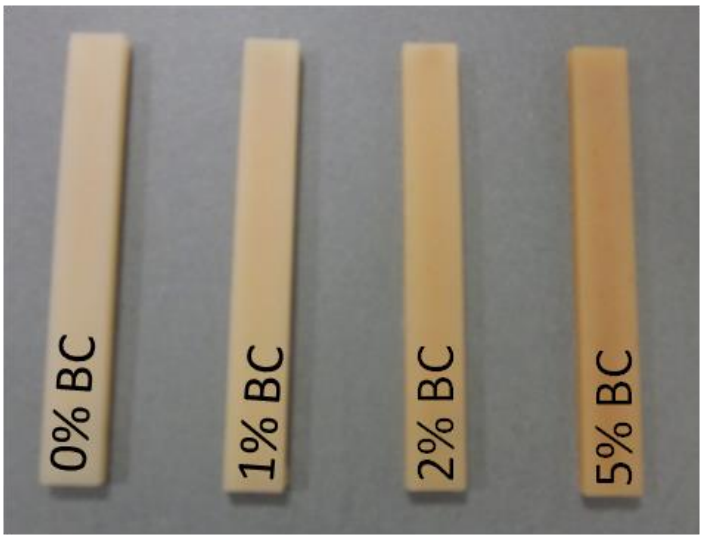


Figure 5.11: The darkening colours of PHB/BC melt blends with increasing concentration of bacterial cellulose.

concluded that it is possible for bacterial cellulose to be evenly distributed by melt blending but that using untreated cellulose nanowhiskers may result in high degradation, and that the fibres may need to be treated prior to melting. Their findings are supported by the current findings as the bacterial cellulose and PHB were not dry mixed prior to melting, but only mixed in the mini-extruder, indicating that melt blending can be used as an effective dispersion method (perhaps even more effectively if the materials are mixed prior to melting), if steps are taken to minimise bacterial cellulose degradation.

5.3.3.2 Mechanical Properties of PHB/BC Melt Blends

Three point bend tests were completed on PHB and PHB/HS-BC melt blends with 1, 2 and 5 wt% cellulose. Despite the degradation, no differences were observed in mechanical properties between the samples with and without bacterial cellulose included (data not shown). Impact testing was attempted, however there was difficulty in obtaining consistent data, so these results were discarded.

The findings from melt blending here indicate that this technique of blending could potentially be used to form PHB/BC blends. However due to the aggregation and degradation observed, it was

determined that this technique was not as appropriate for blending PHB and bacterial cellulose as solution blending.

5.4 Conclusions

An investigation of two methods for producing PHB/BC composites has been presented in this chapter. A fine dispersion of the cellulose phase and improved mechanical properties in the blended composite obtained over a PHB matrix were both desirable. Through the investigations of the achieved dispersion and mechanical properties, it was found that solution blending was a more appropriate technique for producing PHB/BC composites than melt blending.

Solution blending provided a technique for achieving PHB blended with bacterial cellulose at a range of concentrations where the cellulose was well dispersed if the cellulose was sonicated in the solvent prior to the dissolution of PHB. An investigation of composites produced with cellulose grown in different media revealed that composites with 5 wt% cellulose, with cellulose produced in HS and Yamanaka-mannitol media, exhibited improved Young's modulus values, but no differences in ultimate tensile strength and decreases in elongation. The improvement in Young's modulus was not as great for the PHB/Mod-BC composites. It was determined by FTIR and swelling capabilities that the Mod-BC composites contained lower amounts of cellulose in the blend, due to a proportion of the ground material being made up of bound PHB. A smaller cellulose content in the PHB/Mod-BC composites also explains the differences observed in mechanical properties between the blends containing HS-BC and Mod-BC.

By contrast, melt blending of PHB and bacterial cellulose powder did not result in any improvement in mechanical properties, and this technique was found to lead to degradation of the materials due to the higher processing temperatures required. It may be possible that melt blending could be used to develop bacterial cellulose composites, for example, if a higher shear stress and lower temperature were used (though care to avoid mechanical degradation must also be considered), but based on the outcomes and the investigation presented in this chapter, solution blending was chosen as the best method for achieving PHB/BC composites.

Examination of the nanostructure of ground bacterial cellulose indicated a loss of nanosized fibrils, together with decreased crystallinity. Thus, it appears that ground bacterial cellulose is not the best form of bacterial cellulose to use. This is supported by the finding that, even when sonication was incorporated in the solution blending process to achieve good dispersion in the composite, no substantial increase in mechanical properties was observed in composites containing cellulose powder.

In summary, the solution blending technique which achieves well-blended composites with reasonable mechanical properties was selected as the blending technique for further investigation. An alternative method for producing solution blends involving cellulose in its fibrillar form dispersed by sonication will be presented in the following chapter. The medium Yamanaka-mannitol was chosen to be used for further work.

Chapter 6
Composites from Sonicated Bacterial Cellulose Fibrils

6.1 Preface

Sonication is a method that is commonly used to disperse material in liquid, and has also been reported in the literature as dispersing both plant (Cheng et al., 2007; Cheng et al., 2010; Cheng et al., 2009c; Wang & Cheng, 2009) and bacterial cellulose (Guhados et al., 2005; Saito et al., 2006; Wang & Cheng, 2009). Based on the findings in Chapter 5 involving the loss of fibrillar bacterial cellulose structures by cryo-grinding, sonication was considered as an alternate method of dispersion.

Sonication is often carried out in water, however other solvents can also be used. Poly-3-hydroxybutyrate is not water-soluble; therefore, it would be of benefit to achieve a suspension of cellulose fibrils in a solvent capable of dissolving PHB to allow for subsequent blending to take place. Sonication was investigated as a process of scissioning fibrils from bacterial cellulose pellicles to achieve a suspension of dispersed fibrils that could be then blended with a matrix material.

Using different solvents for sonication can impact how much material is dispersed as the composition of the solvent has an impact on the effectiveness of the sonication process, with volatile solvents being less effective (Price et al., 1994). However, based on the advantages of achieving dispersed bacterial cellulose in chloroform (the ability to directly dissolve PHB), this solvent is of interest. Using sonication as a method of scissioning fibrils directly from cellulose pellicles to obtain a solution of dispersed fibrils for blending provides difficulty as the precise weight of dispersed cellulose will be unknown. Therefore it is also necessary to consider the weight of the fibrils that can be dispersed by such a method.

The treatment of cellulose prior to its inclusion in composites can cause changes to the material. For example, how cellulose is dried can cause changes to the properties of the composite in which it is finally blended. This has been seen in different mechanical properties obtained from composites where bacterial cellulose was treated differently prior to blending (Juntaro et al., 2012), where the cellulose was left in water or subject to a solvent exchange with ethanol before blending. Therefore this chapter also aims to examine composites produced using bacterial cellulose from never-dried (hydrated), air-dried and swelled (rewetted) states.

6.2 Harvesting Fibrils from Bacterial Cellulose Pellicles and Subsequent Formation of Biodegradable Poly-3-hydroxybutyrate Nanocomposites

A study was prepared that examined the dry weights of fibrils dispersed in solution achieved by sonicating pellicles in different solvents. Following the determination of the weights of the fibrils, dispersed fibrils in a chloroform solution were blended with PHB to produce PHB/BC composites, which were examined for mechanical properties.

This paper has been published online in Cellulose and is presented here in its published form. It is reproduced here with permission from Springer.

Declaration for Thesis Chapter 6

Declaration by candidate

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Determining which experiments to do based on gaps in the literature, carrying out the experiments, writing the initial draft of the paper and changing the paper based on advice from supervisors	75

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
George Simon	Providing supervision, suggesting some experiments, proof reading and editing	N/A
Katherine Dean	Providing supervision, suggesting some experiments, proof reading and editing	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature		23/8/14
Main Supervisor's Signature		23/8/14

Cellulose
DOI 10.1007/s10570-014-0415-z

ORIGINAL PAPER

Harvesting fibrils from bacterial cellulose pellicles and subsequent formation of biodegradable poly-3-hydroxybutyrate nanocomposites

Dianne R. Ruka · George P. Simon · Katherine Dean

Received: 29 April 2014 / Accepted: 22 August 2014

Abstract Bacterial cellulose has the potential to be used as a biodegradable, reinforcing component in composites due to its high strength and crystallinity. However it is often problematic to use in this context as it is difficult to separate its extensively bonded fibril network. This means it can be difficult for it to be incorporated as a fine dispersion into a composite and for the true benefits of the nanofibres to be realised in terms of physical property improvement in a conventional polymer format such as injection moulding. The method of sonication (using a range of experimental conditions) was utilised to harvest fibrils from the interwoven mesh of the cellulose pellicle, and then disperse them in different solvents to allow blending and subsequent casting. The novel step identified in this process was the sonication harvesting of the nanofibres undertaken on the highly hydrated as-received pellicle fresh from the reaction media (not the dried pellicle which could not be easily separated in the selected solvent). This unique step of harvesting directly from the fresh pellicle together with conventional sonication for dispersion in chloroform produced a bacterial cellulose/poly-3-hydroxybutyrate nanocomposite which showed excellent nanofibre dispersion and significant improvement in mechanical properties.

Keywords Fibres · Nanocomposites · Mechanical properties · Scanning electron microscopy

D. R. Ruka · K. Dean
CSIRO Materials Science and Engineering, Gate 5 Normanby Road, Clayton 3168, Australia
[redacted]

D. R. Ruka · G. P. Simon · K. Dean (✉)
Department of Materials Engineering, Monash University, Clayton 3800, Australia
[redacted]u

G. P. Simon
[redacted]

Introduction

Bacterial cellulose (BC) is a form of cellulose produced by the bacterial species *Gluconacetobacter xylinus*. It grows as a thick mat, called a pellicle, on the surface of liquid media (Schramm and Hestrin 1954). It is made up of an interwoven web of nanosized fibrils with extensive inter- and intra-molecular hydrogen bonds, and is very chemically pure (Ross et al. 1991).

Bacterial cellulose is rarely used as a dispersible, reinforcement material as it is difficult to process due to its insolubility and low thermal stability. However it has often been used to develop composites using an impregnation method (Ashori et al. 2012; Barud et al. 2011; Cai and Kim 2010; Cai and Yang 2011; Cai et al. 2011; Gea et al. 2010; Shah et al. 2013; Ul-Islam et al. 2012; Wan et al. 2006; Yoon et al. 2006), where the bacterial cellulose pellicle that is grown in static culture is soaked in a solution containing another material. As the solution dries, the material coats the surface of the

Published online: 28 August 2014

bacterial cellulose fibrils or becomes lodged in the spaces in the three dimensional fibrillar network. Some composites developed using this method have shown good mechanical properties (Barud et al. 2011; Ul-Islam et al. 2012). This method is advantageous as bacterial cellulose is difficult to dissolve or process by other techniques as the web produced is intrinsically interconnected (as opposed to a non-woven mesh, such as is obtained by electro spinning). For bacterial cellulose to be used as a reinforcing filler material in composites, it would be necessary to separate and scission the fibrils, to achieve a homogenous dispersion in order to develop a composite with good mechanical properties. In the past few years, there have been a few reports in the literature on blends produced by dispersing bacterial cellulose for solution casting, typically with a poly(vinyl alcohol) (PVA) matrix (Jipa et al. 2012; Kibedi-Szabo et al. 2012; Millon et al. 2009; Stoica-Guzun et al. 2011; Yang et al. 2012), though there have also reports of solution blends produced using thermoplastic starch (Woehl et al. 2010), cellulose acetate butyrate (CAB) (Grunert and Winter 2002) and poly-hydroxyalkanoates (PHA) (Martínez-Sanz et al. 2014). These reports have used a variety of different methods to disperse the bacterial cellulose in the matrices. Bacterial cellulose dispersion in the PVA matrices was achieved by milling the cellulose to a fine powder and stirring it in solution (Jipa et al. 2012), by dispersing fibrils grown in a shaking bioreactor by vigorous stirring (Kibedi-Szabo et al. 2012; Stoica-Guzun et al. 2011) and by homogenizing the cellulose (Millon et al. 2009; Yang et al. 2012). These reports focused on the biodegradation of the composites (Kibedi-Szabo et al. 2012; Stoica-Guzun et al. 2011) or the antimicrobial properties (Jipa et al. 2012), however there have been reports of improved mechanical properties in PVA/BC composites over PVA alone (Millon et al. 2009; Yang et al. 2012). Homogenizing has been used for dispersing bacterial cellulose to develop thermoplastic starch/BC composites by solution blending, and also achieved improved mechanical properties (Woehl et al. 2010). PHA composites have been produced by treating the bacterial cellulose with sulphuric acid to obtain individual nanowhiskers as the reinforcing material, however no significant improvements were obtained in the composites compared to the matrices alone (Martínez-Sanz et al. 2014). Sulfuric acid-treated bacterial cellulose was also used as reinforcement in a CAB matrix, and increased glass transition temperature and modulus with bacterial cellulose nanowhisker contents of up to 10 wt% (Grunert and Winter 2002).

When producing bacterial cellulose composites, it is necessary to consider not only how to blend the materials, but also how to treat the cellulose prior to blending, and the form that the cellulose should be used in, as different forms can have different properties. For example, bacterial cellulose can be used in freeze-dried, air-dried, heat-dried forms, or in a highly hydrated as-received state where it is retained in distilled water. It has been demonstrated that bacterial cellulose dried using different methods exhibits different fibre structures (Juntaro et al. 2012) and it has also been shown that crystallite sizes differ between dried and never-dried bacterial cellulose (Fink et al. 1997). The use of different forms of cellulose may affect the properties of resulting composites.

Sonication has been reported as a technique that can achieve fibrillation and isolation of cellulose from various plant sources (Cheng et al. 2007, 2010, 2011a, b; Wang and Cheng 2009). However, there are very few reports on using sonication as a method for separating individual bacterial cellulose fibrils (Guhados et al. 2005). Guhados et al. (2005) was able to draw out individual fibrils of bacterial cellulose using sonication, however the individual fibres still remained attached to the bulk of the original bundles. To our knowledge sonication of bacterial cellulose in solvents (without the use of pre or in situ chemical modification) to harvest individual bacterial cellulose fibres has not been reported in the literature to date.

In this work we sonicated three types of bacterial cellulose pellicles in various solvents, in order to harvest the individual fibrils. As part of the sonication process, the detached fibres were dispersed in solvents, including chloroform (capable of dissolving poly-3-hydroxybutyrate (PHB)), with the aim to develop biodegradable solution blends using PHB as the matrix material. The bacterial cellulose pellicles were used in the highly hydrated, air-dried and rehydrated forms.

Materials and methods

Bacterial strain

A culture of cellulose-producing *Gluconacetobacter xylinus* ATCC 53524 was kindly provided by Mike Gidley, University of Queensland, Australia.

Cellulose

Materials

PHB was kindly provided by Metabolix. Bacterial cellulose was produced in Yamanaka-mannitol media (Ruka et al. 2012) consisting of wt 5 % mannitol, 0.5 % yeast extract, 0.5 % $(NH_4)_2SO_4$, 0.3 % KH_2PO_4 and 0.005 % $MgSO_4{\cdot}7H_2O$, with the pH adjusted to 5.0 with HCl or NaOH and autoclaved at 121 °C for 20 min. Cultures were grown in 50 mL media and were incubated for 7 days at 28 °C under static conditions. Following incubation periods, the cultures were shaken vigorously to remove the attached bacterial cells. Pellicles were removed and rinsed to remove any residual media. Pellicles were washed with 0.1 M NaOH at 80 °C for 1 h to remove bacterial cells, and then washed repeatedly until a neutral pH was reached. Pellicle films were stored in distilled water (highly hydrated as-received) until required, or air dried at room temperature until a constant weight was achieved.

Swelling

Air-dried pellicle films were weighed to obtain the initial weight (m_{dry}). Films were then dispersed in ethanol, distilled water or chloroform for 1 h at room temperature, and were weighed again to achieve a wet weight (m_{wet}). Swelling was calculated by the equation, $Swelling = \left[\frac{m_{wet}-m_{dry}}{m_{wet}}\right] \times 100$ (Seifert et al. 2004).

Sonication

Highly hydrated as-received bacterial cellulose films were removed from distilled water and immersed in selected solvents, ethanol, distilled water or chloroform, for a minimum of 7 days prior to sonication. Air-dried pellicles were immersed in the selected solvents for 1 h prior to sonication bacterial cellulose pellicle films were sonicated in their respective solvents for 60 min at room temperature with a Branson Sonifier 250 with an 80 % duty cycle and an output of 4, with the sonicator probe kept at a consistent height for all samples. Following this, the remaining pellicle was removed and the solvent allowed to completely evaporate in order to determine the dry weight of the bacterial cellulose harvested from the pellicle.

Solution blending

Bacterial cellulose pellicles, from both the air-dried and highly hydrated as-received conditions, were sonicated in chloroform. The fibrils that were harvested from the pellicles and remained in the chloroform were subsequently used in the solution blending process. The bacterial cellulose content within the solutions was calculated (from drying sonicated solutions, five repeats were undertaken and an average taken). PHB was added to the chloroform solution containing sonicated fibrils at 2 wt% and dissolved by mechanical stirring at 80 °C for 3 h before the blend was sonicated to disperse the material. The blends were then cast in glass petri dishes and stored at room temperature to allow the solvent to evaporate. Neat PHB films were also produced for comparison.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed using a field-emission Nova NanoSEM 450. Samples were coated with a platinum coating, and were examined at 2 kV.

Fourier transform-infra red (FTIR) spectroscopy

Fourier transform-infra red (FTIR) spectroscopy was performed using a Perkin-Elmer Spectrum 100 Spectrometer with an Attenuated Total Reflectance (ATR) cell. Film samples were used for the FTIR measurements. Film samples were either PHB, PHB/BC or BC Scans ranged between 4,000 and 450 cm^{-1} wavenumbers with 64 convolutions. Baselines for each sample spectrum were normalized using the Spectrum software.

Tensile properties

Tensile properties were measured on an Instron universal testing machine (models 5566 and 3366) and tested in accordance with ASTM D882 (using a type IV specimen as described in ASTM D638), fitted with 2.5 and 100 N static load cells, respectively, with the rate of extension was set to 2 mm/min.

Results and discussion

Structure of sonicated bacterial cellulose

SEM was used to examine cellulose fibrils after various treatments. Native (air-dried) pellicles and pellicles that had been subject to sonication were mounted on SEM stubs for this purpose. In addition, solvents in which pellicles were sonicated (and thus nanofibres detached) were dropped onto silicon chips, and the solvent was allowed to evaporate (images from chloroform shown in Fig. 1). SEM of the sonicated pellicle revealed changes to the cellulose (Fig. 1). The pellicle itself experienced a change in appearance with the fibrils appearing to have a more open weave structure (Fig. 1b). Examination of the solid residue obtained by evaporating the solvent in which sonication was performed revealed that fibrils had been separated from the interwoven mesh of the pellicle and become dispersed, resulting in a suspension of fibrils that could be readily imaged in the dried state (Fig. 1c).

Tischer et al. (2010) previously completed a study in which they examined the surface of bacterial cellulose pellicles that were subjected to sonication in water for different periods of time. They found that sonication caused changes to the width and height of the bacterial cellulose fibrils, as well as increases in surface roughness, crystallite size and crystallinity. However they did not report the examination of the water after sonication, and thus it is not clear if any fibrils were harvested and dispersed. Our findings support the conclusion that sonication leads to changes to the surface morphology of the fibrils, but that in addition fibrils become separated from the pellicle itself and remain dispersed in the solution. This is therefore an effective method to cause the separation of fibrils from the bacterial cellulose network for further use.

Sonication is also a technique that is widely used to achieve an even dispersion of materials in solution and has previously been demonstrated as a method of obtaining individual cellulose fibres from sources such as wood, as well as bacteria (Cheng et al. 2007, 2010, 2011a, b; Guhados et al. 2005; Wang and Cheng 2009; Wong et al. 2009). It is thus worth considering the efficacy of this technique as a means of dispersing the bacterial cellulose fibrils in a solution, to allow subsequent blending with other polymers to take

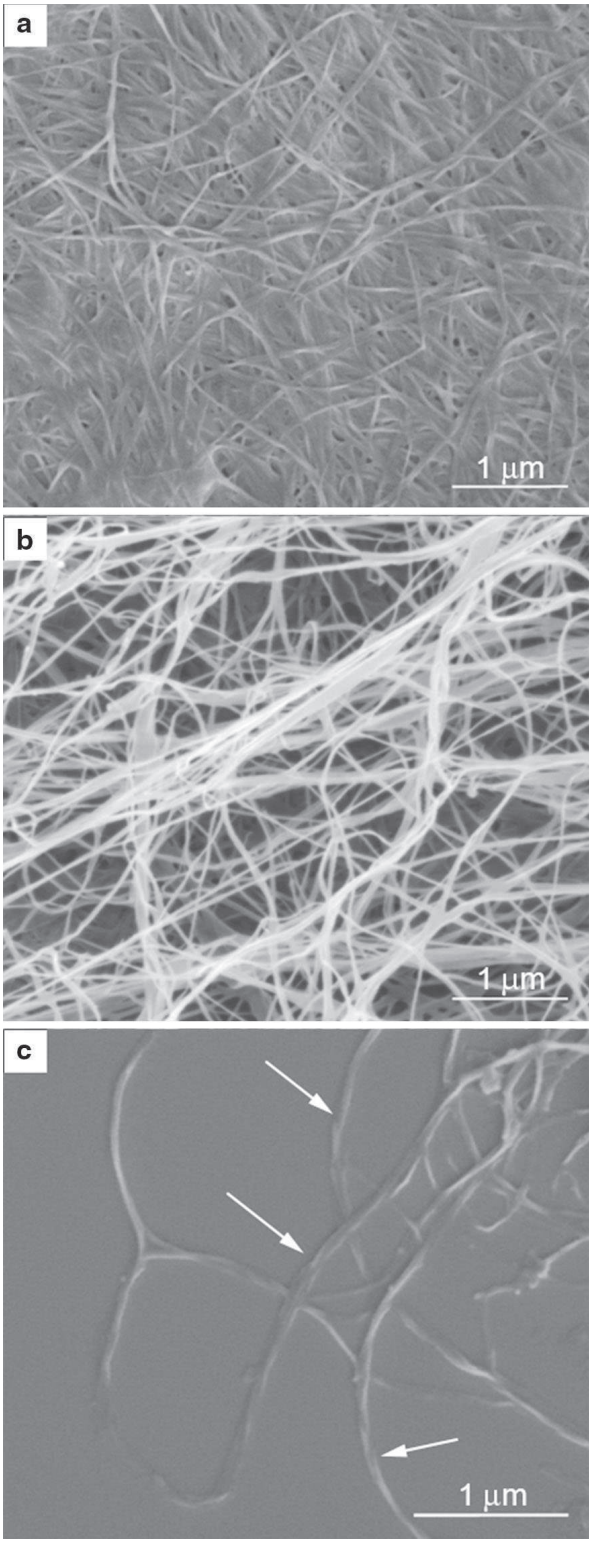


Fig. 1 SEM images of a bacterial cellulose pellicle before sonication (**a**), after sonication (**b**) and fibrils harvested from a pellicle by sonication with individual fibrils shown with *arrows* (**c**)

Table 1 The weight of pellicles achieved in their original wet never-dried, air-dried, and swollen states resulting from immersion in various solvents

Immersion solvent	Initial wet weight (g)	Dry weight (g)	Swelled weight (g)
Water	29.77 ± 2.68	0.384 ± 0.048	2.013 ± 0.262
Ethanol	28.64 ± 1.73	0.314 ± 0.016	0.376 ± 0.024
Chloroform	28.44 ± 1.31	0.358 ± 0.024	0.377 ± 0.033

place. It would also be beneficial for bacterial cellulose fibrils to be harvested from the pellicle and suspended in solvents other than water, such as chloroform. Achieving bacterial cellulose fibrils in chloroform by completing a solvent exchange on a hydrated as-received pellicle, and then sonicating that pellicle to achieve harvested and dispersed fibrils, for example, would allow the direct, subsequent dissolution of other polymers such as PHB into that same solution, and thus assist in the production of fully biodegradable PHB-cellulose nanocomposites.

Based on the observation that it was possible to directly harvest fibrils from bacterial cellulose pellicles, we then determined the amount of bacterial cellulose that could be harvested and dispersed in solution from the cellulose pellicle, using different conditions.

Determining the amount of harvested fibrils in different solvents

Sonication was carried out with pellicles from highly hydrated as-received and air-dried conditions, which were sonicated in distilled water, ethanol and chloroform to determine the dry weight of the bacterial cellulose fibrils that could be harvested. The initial wet weights (highly hydrated as-received), dry weights and the swelling capabilities of the pellicles were also considered.

Pellicles were weighed in their initial highly hydrated as-received state, and their air-dried stated. It was found that 99 % of their initial highly hydrated as-received state was due to absorbed water (Table 1). Air-dried pellicles were then resubmerged in water to determine when a constant swollen weight could be achieved.

The air-dried cellulose was found to absorb water and increase its weight, however it was not able to subsequently reach the same level of saturation when re-exposed to water after it had been dried, swelling to an average of 80 % of its dry weight upon submersion (Table 1). This finding is supported by Seifert et al. (2004) who also found that once bacterial cellulose is dried, it loses its three dimensional porous structure, its properties differ, and the original water content cannot be reached (Seifert et al. 2004). The air-dried pellicles were found to have a smaller increase in weight when the pellicles were immersed in ethanol and chloroform over the increase in weight obtained in water. The air-dried bacterial cellulose fibrils did not swell when soaked in chloroform, only achieving a ca. 5 % increase in weight, whereas swelling in ethanol and water caused approximately increases in weight of 16 and 80 %, respectively (Table 1).

Following immersion in the solvents and sonication, pellicles were removed and the solvent was allowed to evaporate to measure the dry weight of the remaining fibrils (Fig. 2). It was found that there were differences obtained in the weight of harvested fibrils between the highly hydrated as-received and air-dried pellicles. Similar amounts of harvested fibrils were achieved in all solvents from the highly hydrated as-received pellicles. This is likely due to the undisturbed three dimensional structure of the highly hydrated as-received samples. Despite being immersed in their respective sonication solvents for 7 days prior to sonication, it is likely that the cellulose remained swollen with water. Chloroform has a greater density than water (1.48 over 1.00 g/cm^3, respectively), and

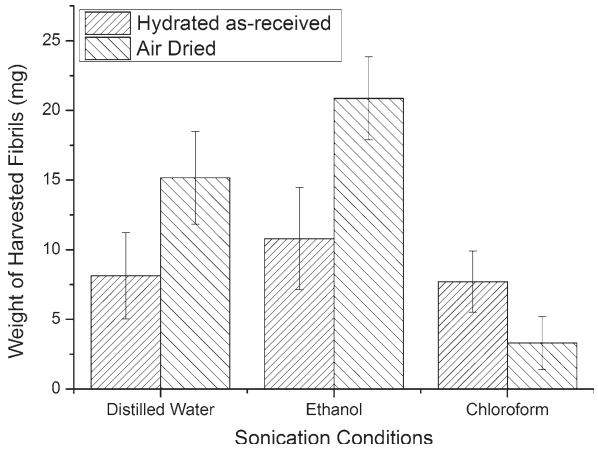


Fig. 2 Weight of dispersed fibrils harvested from hydrated as-received and air-dried pellicles in a range of solvents by sonication

thus water floats on the surface of chloroform. We have previously noted that when pellicles are grown, they remain on the surface of the media but can be easily dislodged and submerged in a solution primarily composed of water (Ruka et al. 2012), whereas the highly hydrated as-received pellicles floated on chloroform, and air-dried pellicles can be suspended. The hypothesis that the highly hydrated as-received pellicles remained swollen with water is supported by both the observation of small water bubbles on the surface on the chloroform following sonication, and also of the tendency of the buoyant, water-filled pellicle to float in the chloroform solution. Regardless, fibrils became dispersed from the bacterial cellulose pellicle network at approximately the same level when sonicated in ethanol, water and chloroform from these highly hydrated as-received pellicles.

Differences were observed in the weights of harvested fibrils achieved from air-dried pellicles when sonicated in different solvents. Sonication in ethanol and water gave higher amounts of dispersed fibrils than the never-dried pellicles, however small weights of fibrils were dispersed when air-dried pellicles were sonicated in chloroform.

Based on the differences observed between the highly hydrated as-received and air-dried pellicles in chloroform, swelling was considered as a factor capable of influencing the amount of dispersed fibrils. As the air-dried pellicle suspended in chloroform only experienced a small increase in weight, it is likely that the fibrils remained in a compacted network, rather than becoming swollen and separated from each other, whereas the hydrated as-received fibrils retain their original three-dimensional swollen structure. Therefore, it was hypothesized that a greater degree of swelling was necessary to cause fibrils to be separated from the fibril mesh. In order to investigate this, air-dried pellicles were swollen in distilled water for 1 h, and then sonicated in chloroform. However, it was found that despite the attempt to reintroduce a three dimensional network to the pellicle by swelling, with only an average weight of 3.38 ± 2.3 mg dry weight of fibrils could be harvested, similar to that achieved from the air-dried pellicles (3.3 ± 1.9 mg). It is likely that the low weights of harvested fibrils achieved in chloroform from air-dried pellicles are due to the chloroform itself, as it has been demonstrated that the volatility of a solvent can decrease the effectiveness of the sonication process (Price et al. 1994). As the weight of the fibrils achieved from sonication in chloroform from the hydrated as-received cellulose was comparable to that in water and ethanol, it may be perhaps that this is due to the inclusion of the high water content in the cellulose fibrils in this form.

Sonication is an appropriate method to separate individual bacterial cellulose fibrils from the pellicle and, although only small amounts of fibrils were harvested and dispersed by sonication, the amounts reported here are higher than any other which have been reported, for example Guhados et al. (2005) who dispersed only a 3.6 mg piece of bacterial cellulose in 1 mL of water to achieve single fibres for mechanical testing.

PHB/BC blends and properties

Based on the dry weights of the harvested fibrils achieved by sonicating highly hydrated as-received cellulose, PHB/BC blends were produced with the bacterial cellulose content of 2 wt% (±0.6).

Tensile stress–strain curves illustrate the greatest improvement in modulus and ultimate tensile strength occurred from the highly hydrated as-received bacterial cellulose nanocomposites over neat PHB (Fig. 3). More specifically the PHB nanocomposites which incorporated 2 wt% highly hydrated as-received bacterial cellulose revealed a 43 % increase in tensile strength, and a 59 % increase in modulus, increasing

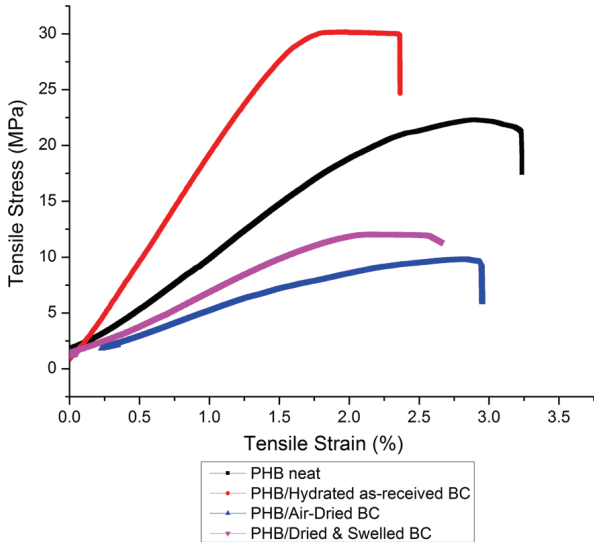


Fig. 3 Stress–strain curves obtained from neat PHB and PHB/BC blends

from 23 ± 4 to 33 ± 4 MPa, and 1,240 ± 180 to 1,970 ± 30 MPa, respectively (Fig. 4). No significant detrimental effects were observed in elongation under these conditions despite the increases in tensile strength and modulus. These increases in tensile strength and modulus were not observed, however, in composites with the air-dried and swollen fibrils, with decreases in mechanical properties with tensile strength observed with values of 9 ± 2 and 15 ± 4 MPa, and modulus values of 530 ± 40 and 850 ± 130 MPa, respectively. SEM examination of the cross-sections of fractured PHB and PHB/BC (with highly hydrated cellulose) is presented in Fig. 5. This clearly demonstrates dispersed bacterial cellulose nanofibres, with individual fibrils visible in the blend. The interactions between cellulose fibres and the PHB were also investigated using FTIR (see Fig. 6). PHB has three major characteristic peaks due to crystallinity at 1,185, 1,228 and 1,279 cm^{-1}, in particular the peak at 1,185 cm^{-1} has been used previously to calculate the crystallinity index for PHB when taken as a ratio with the peak at 1,385 cm^{-1}, which is known to be insensitive to the degree of crystallinity (Randriamahefa et al. 2003). The crystallinity index was found to increase moderately with the incorporation of 2 wt% BC for all three composites, indicating that the BC fibres could act as nucleating sites within the PHB matrix (see Table 2). However, due to the low concentration of BC in the composites no major peak shifts due to interaction between the PHB and the BC have been observed in the spectra of the composites with many of the more dominant peaks from the BC occurring in similar peak positions to PHB. Perhaps one of the more dominant series of peaks [due to the carbonyl group of the bacterial cellulose (Ruka et al. 2012)] is observed as a large should on the broad peak at 1,045 cm^{-1} between 1,000 and 1,030 cm^{-1} in all three composites.

The mechanical properties observed here support the use of sonication as a method of harvesting and dispersion to achieve PHB/BC blends when the cellulose is used in a highly hydrated as-received state. Other authors such as Martínez-Sanz et al. (2014) have shown good dispersion of cellulose fibres

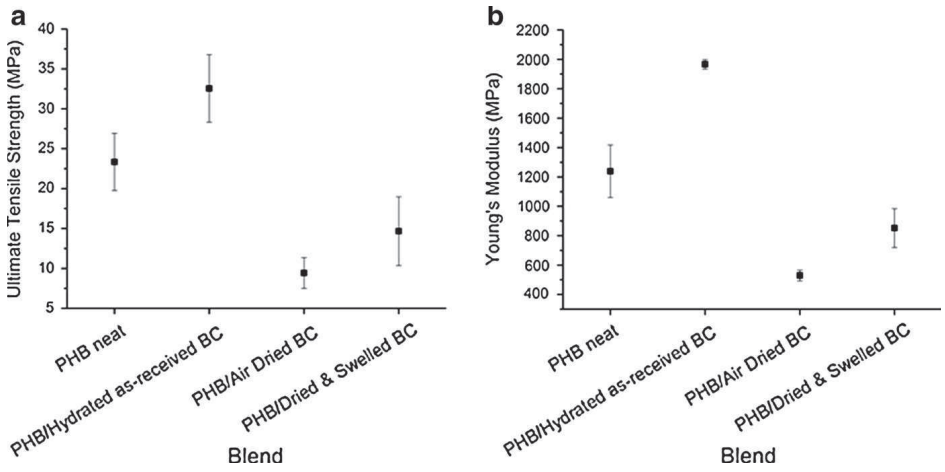


Fig. 4 Tensile properties of neat PHB and PHB/BC blends

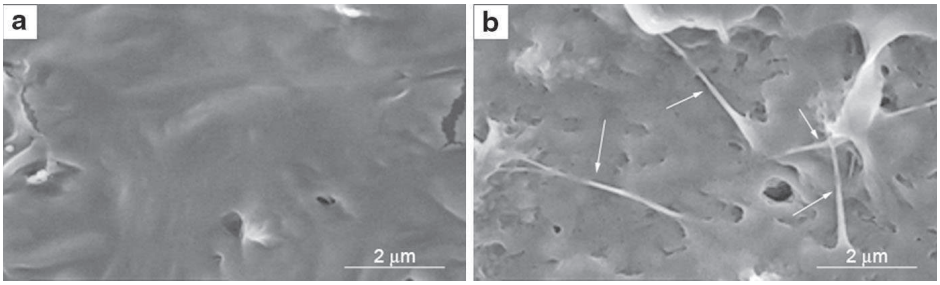


Fig. 5 SEM images of the cross-sections of fractured PHB (**a**) and PHB/BC blend (**b**)

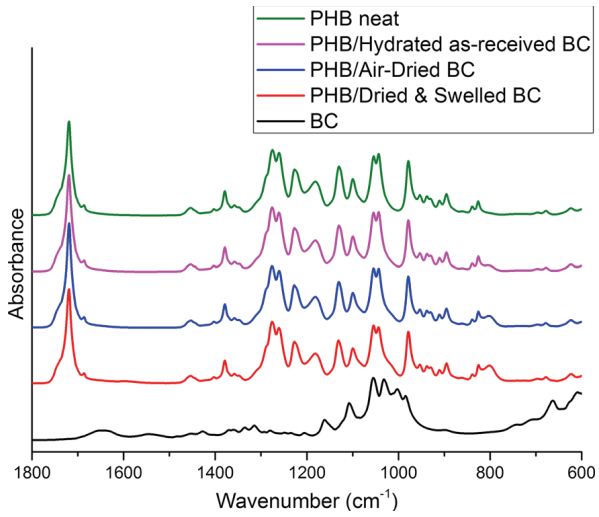


Fig. 6 FTIR spectra of PHB, BC and the resulting composites

Table 2 Crystallinity index values for PHB and PHB composites measured from FTIR data

Sample	Crystallinity Index
PHB	0.37
PHB/BC never dried	0.38
PHB/BC air dried	0.38
PHB/BC swollen	0.42

in PHA solution blends (using chloroform as a solvent). However, Martínez-Sanz et al. (2014) did not observe improvements in mechanical properties at either 1 or 3 wt% fibre loading It must also be noted that in the work by Martínez-Sanz et al. (2014) bacterial cellulose nanowhiskers were used (rather than native bacterial cellulose fibres as in our work).

The observation that bacterial cellulose fibrils are unable to re-absorb the high levels of water originally present following cultivation (Seifert et al. 2004) indicates that drying this material leads to a change in the fibrils. This is supported by the differences previously determined between the fibrils in different forms, for example, different crystallite sizes (Fink et al. 1997). Therefore, it is also likely that fibrils in the different forms exhibit different mechanical properties, and once dried the fibrils cannot reach the same levels even following swelling, as demonstrated by the superior mechanical properties of composites containing never-dried fibrils, but poor properties from air-dried fibrils. It may also be possible that the adhesions between the dried fibrils and the matrices were poor, as the inclusion of air-dried fibrils in a PHB matrix actually caused a decrease in mechanical properties.

Sonication has previously been reported as a method used for dispersing plant cellulose (not bacterial cellulose as shown in our work) in order to produce PVA/cellulose composites (Cheng et al. 2007, 2009). Cheng et al. (2009) isolated cellulose fibrils by sonication, obtaining micro and nanoscale fibrils, and then separated the fibrils by size using centrifugation. These authors produced composites using regenerated cellulose consisting of a mixture of fibrils sizes (nano and micro), as well as the small fibrils obtained by centrifugation. They found increased mechanical properties in composites with both reinforcements, but that superior properties were obtained from composites reinforced with the small fibrils, with improved tensile strength and modulus (Cheng et al. 2009). Bacterial cellulose consists of naturally nano-sized fibrils. As such, it may be that bacterial cellulose confers improved mechanical properties with only a small content due to the size of its fibrils, an advantage in using bacterial cellulose compared to micro-sized plant cellulose fibres.

These experiments indicate that it is possible to harvest and disperse bacterial cellulose fibrils in solution directly from the pellicle. To the best of our knowledge, this is the first report of achieving dispersed bacterial cellulose fibrils in solvents (other than water) for blending and casting. Developing a method to achieve increased amounts of individual never-dried fibrils in different solvents would thus be of much benefit. Sonication in different solvents for biodegradable solution blends should therefore be further investigated with other potential matrices.

Conclusion

In this work it has been shown that individual bacterial cellulose fibrils can be produced via the sonication of bacterial cellulose pellicles under defined conditions and in selected solvents. The yield of bacterial cellulose fibrils harvested from the highly hydrated as-received pellicle state was typically 2 wt% ± 0.6 in solution, the high aspect ratio of the individual fibrils led to a significant improvement in yield strength and modulus properties when they were formed into a PHB composites (compared to the neat PHB matrix). The yield strength increase by 43 %

(from 23 ± 4 to 33 ± 4 MPa) and the modulus increased by 59 % (from $1{,}240 \pm 180$ to $1{,}970 \pm 30$ MPa). This supports the hypothesis that bacterial cellulose fibrils could be an appropriate reinforcement material, using sonication as a novel method to achieve harvesting from the pellicle, as well as solution blending and casting in different solvents, leading to a good dispersion of the cellulose fibrils in the matrix, resulting in improvement in mechanical properties in the nanocomposite.

Acknowledgments We wish to thank the Monash University Centre for Electron Microscopy for the use of their equipment. This work was funded by a Julius Career award from the CSIRO Office of the Chief Executive.

References

Ashori A, Sheykhnazari S, Tabarsa T, Shakeri A, Golalipour M (2012) Bacterial cellulose/silica nanocomposites: preparation and characterization. Carbohydr Polym 90:413–418

Barud HS, Souza JL, Santos DB, Crespi MS, Ribeiro CA, Messaddeq Y, Ribeiro SJL (2011) Bacterial cellulose/poly(3-hydroxybutyrate) composite membranes. Carbohydr Polym 83:1279–1284

Cai Z, Kim J (2010) Bacterial cellulose/poly(ethylene glycol) composite: characterization and first evaluation of biocompatibility. Cellulose 17:83–91

Cai Z, Yang G (2011) Optical nanocomposites prepared by incorporating bacterial cellulose nanofibrils into poly(3-hydroxybutyrate). Mater Lett 65:182–184

Cai Z, Yang G, Kim J (2011) Biocompatible nanocomposites prepared by impregnating bacterial cellulose nanofibrils into poly(3-hydroxybutyrate). Curr Appl Phys 11:247–249

Chen WS, Yu HP, Liu YX, Chen P, Zhang MX, Hai YF (2011a) Individualization of cellulose nanofibers from wood using high-intensity ultrasonication combined with chemical pretreatments. Carbohydr Polym 83:1804–1811

Chen WS, Yu HP, Liu YX, Hai YF, Zhang MX, Chen P (2011b) Isolation and characterization of cellulose nanofibers from four plant cellulose fibers using a chemical–ultrasonic process. Cellulose 18:433–442

Cheng Q, Wang SQ, Rials TG, Lee SH (2007) Physical and mechanical properties of polyvinyl alcohol and polypropylene composite materials reinforced with fibril aggregates isolated from regenerated cellulose fibers. Cellulose 14:593–602

Cheng QZ, Wang SQ, Rials TG (2009) Poly(vinyl alcohol) nanocomposites reinforced with cellulose fibrils isolated by high intensity ultrasonication. Compos Part A Appl Sci Manuf 40:218–224

Cheng QZ, Wang SQ, Han QY (2010) Novel process for isolating fibrils from cellulose fibers by high-intensity ultrasonication. II. Fibril characterization. J Appl Polym Sci 115:2756–2762

Fink HP, Purz HJ, Bohn A, Kunze J (1997) Investigation of the supramolecular structure of never dried bacterial cellulose. Macromol Symp 120:207–217

Gea S, Bilotti E, Reynolds CT, Soykeabkeaw N, Peijs T (2010) Bacterial cellulose-poly(vinyl alcohol) nanocomposites prepared by an in situ process. Mater Lett 64:901–904

Grunert M, Winter WT (2002) Nanocomposites of cellulose acetate butyrate reinforced with cellulose nanocrystals. J Polym Environ 10:27–30

Guhados G, Wan WK, Hutter JL (2005) Measurement of the elastic modulus of single bacterial cellulose fibers using atomic force microscopy. Langmuir 21:6642–6646

Jipa IM, Dobre L, Stroescu M, Stoica-Guzun A, Jinga S, Dobre T (2012) Preparation and characterization of bacterial cellulose-poly(vinyl alcohol) films with antimicrobial properties. Mater Lett 66:125–127

Juntaro J, Ummartyotin S, Sain M, Manuspiya H (2012) Bacterial cellulose reinforced polyurethane-based resin nanocomposite: a study of how ethanol and processing pressure affect physical, mechanical and dielectric properties. Carbohydr Polym 87:2464–2469

Kibedi-Szabo CZ, Stroescu M, Stoica-Guzun A, Jinga SI, Szilveszter S, Jipa I, Dobre T (2012) Biodegradation behavior of composite films with poly (vinyl alcohol) matrix. J Polym Environ 20:422–430

Martínez-Sanz M, Villano M, Oliveira C, Albuquerque MGE, Majone M, Reis M, Lopez-Rubio A, Lagaron JM (2014) Characterization of poly hydroxyalkanoates synthesized from microbial mixed cultures and of their nano biocomposites with bacterial cellulose nano whiskers. New Biotechnol 31:364–376

Millon LE, Oates CJ, Wan WK (2009) Compression properties of polyvinyl alcohol-bacterial cellulose nanocomposite. J Biomed Mater Res Part B Appl Biomater 90B:922–929

Price GJ, West PJ, Smith PF (1994) Control of polymer structure using power ultrasound. Ultrason Sonochem 1:S51–S57

Randriamahefa S, Renard E, Guerin P, Langlois V (2003) Fourier transform infrared spectroscopy for screening and quantifying production of PHAs by *Pseudomonas* grown on sodium octanoate. Biomacromolecules 4:1092–1097

Ross P, Mayer R, Benziman M (1991) Cellulose biosynthesis and function in bacteria. Microbiol Rev 55:35–58

Ruka DR, Simon GP, Dean KM (2012) Altering the growth conditions of *Gluconacetobacter xylinus* to maximize the yield of bacterial cellulose. Carbohydr Polym 89:613–622

Schramm M, Hestrin S (1954) Factors affecting production of cellulose at the air liquid interface of a culture of *Acetobacter Xylinum*. J Gen Microbiol 11:123–129

Seifert M, Hesse S, Kabrelian V, Klemm D (2004) Controlling the water content of never dried and reswollen bacterial cellulose by the addition of water-soluble polymers to the culture medium. J Polym Sci Part A Polym Chem 42:463–470

Shah N, Ul-Islam M, Khattak WA, Park JK (2013) Overview of bacterial cellulose composites: a multipurpose advanced material. Carbohydr Polym 98:1585–1598

Stoica-Guzun A, Jecu L, Gheorghe A, Raut I, Stroescu M, Ghiurea M, Danila M, Jipa I, Fruth V (2011) Biodegradation of poly(vinyl alcohol) and bacterial cellulose composites by *Aspergillus niger*. J Polym Environ 19:69–79

Tischer PCSF, Sierakowski MR, Westfahl H, Tischer CA (2010) Nanostructural reorganization of bacterial cellulose by ultrasonic treatment. Biomacromolecules 11:1217–1224

Ul-Islam M, Khan T, Park JK (2012) Nanoreinforced bacterial cellulose-montmorillonite composites for biomedical applications. Carbohydr Polym 89:1189–1197

Wan YZ, Hong L, Jia SR, Huang Y, Zhu Y, Wang YL, Jiang HJ (2006) Synthesis and characterization of hydroxyapatite-bacterial cellulose nanocomposites. Compos Sci Technol 66:1825–1832

Wang SQ, Cheng QZ (2009) A novel process to isolate fibrils from cellulose fibers by high-intensity ultrasonication, part 1: process optimization. J Appl Polym Sci 113:1270–1275

Woehl MA, Canestraro CD, Mikowski A, Sierakowski MR, Ramos LP, Wypych F (2010) Bionanocomposites of thermoplastic starch reinforced with bacterial cellulose nanofibres: effect of enzymatic treatment on mechanical properties. Carbohydr Polym 80:866–873

Wong SS, Kasapis S, Tan YM (2009) Bacterial and plant cellulose modification using ultrasound irradiation. Carbohydr Polym 77:280–287

Yang L, Zhang HY, Yang Q, Lu DN (2012) Bacterial cellulose-poly(vinyl alcohol) nanocomposite hydrogels prepared by chemical crosslinking. J Appl Polym Sci 126:E244–E250

Yoon SH, Jin HJ, Kook MC, Pyun YR (2006) Electrically conductive bacterial cellulose by incorporation of carbon nanotubes. Biomacromolecules 7:1280–1284

6.3 Conclusions

Sonicating bacterial cellulose in solvents allowed individual fibrils to be obtained with the fibrils suspended in liquid. Completing this dispersion in chloroform allowed PHB to be directed dissolved and blended with the suspended fibrils. PHB/BC solution blends produced in this way had improved mechanical properties compared with the PHB matrix alone when bacterial cellulose was in a hydrated never-dried state. In addition, these superior mechanical properties were observed in a composite with only a small cellulose content. This indicates that solution blending can be used to disperse and blend bacterial cellulose with biodegradable materials such as PHB to produce composites with improved properties.

Chapter 7
Biodegradability of a Bacterial Cellulose Composite

7.1 Preface

Traditional plastics may have good properties but they are also resistant to microbial attack, which means they do not break down after disposal. These types of materials can be disposed of in landfill, by incineration or by recycling. Incineration can cause problems with produced pollutants, and landfill space is limited. In addition, due to their persistence in the environment, disposal of these types of materials can cause problems with litter, which can cause dangers to wildlife. Therefore it would be of benefit to produce materials that could break down after disposal to replace these types of materials.

Biodegradation is the process by which materials are broken down by microbes into carbon dioxide and water. Once broken down, these molecules can be utilised as nutrients by the degrading microbes (Lucas et al., 2008). This process is sustainable as it can be a cyclic process in which materials are produced and then broken down in order to be re-absorbed for the process to begin again. Poly-3-hydroxybutyrate and bacterial cellulose are such materials, being produced naturally in the environment by microbes for the purposes of nutrient storage, and attachment, colonisation and protection, respectively (Braunegg et al., 1998; Williams & Cannon, 1989). These materials can be broken down in soil and used for microbial cell growth.

PHB is known to be capable of breaking down in soil, and also in other environments such as seawater (Mergaert et al., 1992), but it is stable in air as it needs a microbial population to achieve degradation. However, there are conflicting reports on how long this material takes to degrade. There is even less information on the degradation rate of bacterial cellulose, which has only been reported to degrade as part of composites in a small number of publications (Kibédi-Szabó et al., 2012; Stoica-Guzun et al., 2011; Wan et al., 2009). To the author's knowledge, PHB and bacterial cellulose in a composite has not been investigated in terms of its degradation rate.

There are a number of different ways that the degradation of materials can be investigated, and a number of different environments in which this can occur. For example, materials can have degradation measured by weight loss, or by carbon dioxide production, and can be placed in environments such as soil, compost, activated sludge (Shah et al., 2008). In addition, these types of

materials can be applied to biomedical applications, so environments that mimic the body in order to determine the rate of degradation of implants could also be investigated.

Based on the focus of this work, a composting environment was selected as the biodegradation medium. Composting is a process that can be done by people in their own backyards (though backyard compost bins do not typically reach the temperatures, and therefore degradation rates, that commercial composting can), and as such, is a method that can easily be applied to real world applications. Other disposal situations may be of interest, such as in seawater, or even in landfill, however it is likely that composting will be the most efficient form of degradation for biodegradable materials in the near future.

7.2 Biodegradability of Poly-3-hydroxybutyrate/Bacterial Cellulose Composites Under Aerobic Conditions – Measured via Evolution of Carbon Dioxide, Spectroscopic and Diffraction Methods

The paper presented here sought to produce large amounts of PHB/BC solution blend to examine the rate of biodegradation for the composite and neat materials. Though PHB/BC blends with ground cellulose did not show improved mechanical properties (Chapter 5), this form of composite was produced to allow the production of large amounts of composite to be achieved to examine the biodegradability of this material.

This manuscript is currently unpublished. It has been formatted to match the structure of the thesis. The figures and tables have been numbered to reflect the chapter number.

Declaration for Thesis Chapter 7

Declaration by candidate

In the case of Chapter 7, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Determining which experiments to do based on gaps in the literature, carrying out the experiments, writing the initial draft of the paper and changing the paper based on advice from supervisors	70

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Parveen Sangwan	Assisting with biodegradation setup, assisting with biodegradation data analysis, assisting in writing biodegradation analysis for the paper	N/A
Christopher Garvey	Completing SAXS data and analysis	N/A
George Simon	Providing supervision, suggesting some experiments, proof reading and editing	N/A
Katherine Dean	Providing supervision, suggesting some experiments, proof reading and editing	N/A

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	[redacted]	23/8/14
Main Supervisor's Signature	[redacted]	23/8/14

Biodegradability of Poly-3-hydroxybutyrate/Bacterial Cellulose Composite Under Aerobic Conditions – Measured via Evolution of Carbon Dioxide, Spectroscopic and Diffraction Methods

Dianne R. Ruka[1, 2], Parveen Sangwan[1], Christopher J. Garvey[3], George P. Simon[2], Katherine M. Dean[1]

[1]CSIRO Materials Science & Engineering, Clayton, Australia,

[2]Department of Materials Engineering, Monash University, Clayton, Australia,

[3]Australian Nuclear Science and Technology Organisation, Kirrawee, Australia.

Abstract

Poly-3-hydroxybutyrate (PHB) and bacterial cellulose (BC) are both natural polymeric materials that have the potential to replace traditional, non-renewable polymers. In particular, the nanofibrillar form of bacterial cellulose makes it an effective reinforcement for PHB. Neat PHB, bacterial cellulose and a composite of PHB/BC produced with 10 wt% cellulose were composted under accelerated aerobic test conditions, with biodegradability measured by the carbon dioxide evolution method, in conjunction with spectroscopic and diffraction methods to assess crystallinity changes during the biodegradation process. It was found that the PHB/BC composite biodegraded at a greater rate and extent than that of PHB alone, reaching 80% degradation after 30 days, whereas PHB did not reach this level of degradation until close to 50 days of composting. The relative crystallinity of PHB and PHB in the PHB/BC composite was found to increase in the initial weeks of degradation, with degradation occurring primarily in the amorphous region of the material and some recrystallisation of the amorphous PHB fragments occurring contributing to a higher crystallinity index. Small angle X-ray scattering indicates that the change in PHB crystallinity is accompanied by a change in morphology of

semi-crystalline lamellae. The increased rate of biodegradability suggests that these materials could be applicable to single-use applications, and could rapidly biodegrade in compost on disposal.

Keywords

Bacterial cellulose, poly-3-hydroxybutyrate, biodegradability, solution blending

Introduction

An increasing awareness of environmental issues relating to the production and disposal of traditional polymers has brought natural materials into the spotlight. It may be possible that eventually natural polymers could replace traditional plastics in some markets. Natural materials are also often considered for biomedical applications for purposes such as implants or scaffolds that would degrade naturally in the body. Ideally, natural biodegradable materials may be developed for packaging material, as this comprises a significant proportion of the plastics market and degradability would be desirable as a waste-reduction option, as well as being advantageous if the packing becomes litter and is carried out to sea. These types of materials would be quite stable on the shelf, but could be degraded in compost or other environments after disposal. Based on this, it is important to investigate how long it would take these materials to degrade in conditions that are relevant to real world situations.

Poly-3-hydroxybutyrate (PHB) has been proposed as a material that could replace traditional plastics, as it has similar properties to polypropylene in terms of its melting temperature and tensile strength (Holmes, 1985; King, 1982). Typically the morphology PHB is organised hierarchically, with two important length-scales: alternating layers of crystalline and amorphous polymer organised in partially ordered lamellae (of the order nm), the lamellar are then organised into much larger spherulites. The size of both structures are highly dependent on the processing conditions (Owen & Bergmann, 2004; Xie et al., 2008). By comparison, bacterial cellulose possesses a high crystallinity and modulus (Eichhorn et al., 2010), and it is composed of nanosized fibrils. Given this morphology, it has

potential to be the reinforcing phase in composites, including those where the matrix is itself biodegradable. Since both these materials are produced by soil bacteria, they can be used by microbes present in the natural environments as nutrients and completely broken down into carbon dioxide and water. Therefore, blends of these materials are also expected to be completely mineralised when exposed to natural microbial flora, but this needs to be demonstrated experimentally.

There are a number of different methods used to determine the degree and rate at which materials biodegrade (Shah et al., 2008). For example, samples can be exposed to degrading enzymes or microbes in laboratory culture, or biodegraded by being submerged in soil, activated sludge or compost. There are also a number of metrics that can be obtained from such experiments to quantify and assess the rate and extent of biodegradation. The loss of weight of materials is commonly used, however this method can be problematic, as degrading materials can absorb moisture which alters their weight, or the materials could disintegrate into smaller fragments, causing difficulty in recovering the materials to determine the weight loss. Materials can also be examined for visual changes; fragmentation, with an observed loss of material confirming that degradation has taken place, although this is only a qualitative measure. The materials can also be examined for mechanical properties before and after degradation, but this too does not give a direct measure of the biodegradation or the rate of biodegradation, but rather simply provides evidence that degradation has taken place. The measurement of carbon dioxide production or oxygen consumption under aerobic conditions, however, provides a quantitative measure of degradation. The use of such respirometric data can allow the calculation of the degree and rate of biodegradation during aerobic composting process itself, which is very convenient.

There have been several reports of the biodegradation of composites that contained bacterial cellulose (Kibédi-Szabó et al., 2012; Stoica-Guzun et al., 2011; Wan et al., 2009). A starch/BC composite had degradation measured by determining the weight loss after soil burial and it was found that composites achieved 69% degradation within 3 weeks of burial (Wan et al., 2009). Bacterial cellulose contents and choice of culture medium were found to influence degradation of poly(vinyl

alcohol) (PVA)/BC composites when exposed to a single fungal strain in laboratory culture (Stoica-Guzun et al., 2011). As demonstrated by the differences in changing degradation rates with the inclusion of bacterial cellulose in composites, the rate of degradation of composites will be dependent on the matrix material used.

The degradation of PHB has been reported in the literature, with highly variable results. For example, 0.3 – 0.4 mm films of PHB were found to degrade in compost in 30 days (Savenkova et al., 2000), whereas injection-moulded dogbones were found to retain 96% of its initial weight after 151 days in compost (Mergaert et al., 1992). It has been found that the degradation rate of PHB composites is dependent of the type of material in the blends (Savenkova et al., 2000).

It must be noted that with all these previous studies weight loss has been the primary method for measuring biodegradation, but as discussed previously this method can be problematic due to moisture absorption and difficultly recovering the material to determine the weight loss.

There are a number of factors that can affect the rate of degradation. For example, nutrient supply, temperature, pH and moisture level can all change the rate of degradation, as can the microbial population and the surface area of the polymer (Byrom, 1993), which need to be kept consistent when comparing the degradation rate between samples. When a composite degrades, the rate of degradation will initially be influenced by the most biodegradable phase, which will come under microbial attack first. As a result, the composite will break down, providing more exposed surface area for the remaining biodegradable component of the composite to come under microbial attack (Shah et al., 2008).

Based on the potential for PHB and bacterial cellulose to be used to develop biodegradable composites, we produced composites of these two materials and measured the biodegradation using the precise method of evolution of carbon dioxide in conjunction with spectroscopic and diffraction methods to understand the changes in morphology occurring during the biodegradation process.

Experimental

Bacterial Strain

A culture of bacterial cellulose-producing *Gluconacetobacter xylinus* ATCC 53524 was kindly provided by Mike Gidley, University of Queensland, Australia.

Materials

PHB was purchased from Nango Tianan Biologic Material. We produced bacterial cellulose in Yamanaka-mannitol media (Ruka et al., 2012) consisting of 5 wt% mannitol, 0.5 wt% yeast extract, 0.5 wt% $(NH_4)_2SO_4$, 0.3 wt% KH_2PO_4 and 0.005 wt% $MgSO_4.7H_2O$, with the pH of media adjusted to 5.0 with HCl or NaOH and autoclaved at 121° C for 20 minutes. Cultures were grown in 600 mL media and were incubated for 7 days at 28° C under static conditions. Following incubation periods, pellicles were removed, rinsed to remove any residual media, and washed with 0.1 M NaOH at 80° C for 1 hour to remove bacterial cells. They were further repeatedly washed until a neutral pH was achieved, and the pellicles were dried at room temperature to constant weight. The pellicles were subsequently cryogenically ground to a fine powder using a SPEX SamplePrep Freezer/mill 6870.

Solution Blending and Casting

Bacterial cellulose powder was sonicated in chloroform at room temperature for 60 minutes. PHB was added to the cellulose/chloroform solution at 5 wt% and dissolved by mechanical stirring at 80° C for 3 hours. The blend was briefly sonicated, cast in glass petri dishes and stored at room temperature to allow the solvent to evaporate, producing films with thicknesses of approximately 20 μm.

Biodegradation

Films of neat PHB and PHB/BC with a bacterial cellulose content of 10 wt% were produced for biodegradation studies. Experiments were set up according to the Australian Standard AS 14855, using National Accredited Testing Authority (NATA) biodegradation facilities at CSIRO. The test specimens were cut into pieces no larger than 2 × 2 cm^2, according to the standard, and were exposed to aerobic composting conditions with a continuous supply of airflow provided to each composting vessel, temperature of 58±2° C and moisture 50 – 55% using an in-house built respirometer unit. Each

3 L bioreactor vessel contained 300 g compost and 50 g of the respective material, both on a dry weight equivalent. Microcrystalline cellulose powder (MCC) (20 microns, Sigma-Aldrich) was used as a positive reference during the biodegradation tests, and blank compost was kept in the same conditions to determine the amount of carbon dioxide given off by the compost alone. Neat bacterial cellulose powder was also examined in this way.

The theoretical amount of carbon dioxide ($THCO_2$), in grams per bioreactor, was calculated by:

$$\mathrm{THCO_2} = \mathrm{M_{TOT}} \times \mathrm{C_{TOT}} \times \frac{44}{12}$$

where, M_{TOT} is the total dry solids (in grams) in the test material at the start of the test; C_{TOT} is the proportion of total organic carbon in the total dry solids of the test material (in grams per 100 grams); 44 and 12 are the molecular mass of carbon dioxide and the atomic mass of carbon, respectively.

Percentage biodegradation (D_t) was determined by:

$$D_t = \frac{(\mathrm{CO_2})_\mathrm{T} - (\mathrm{CO_2})_\mathrm{B}}{\mathrm{THCO_2}} \times 100$$

where, $(CO_2)_T$ is the cumulative amount of carbon dioxide evolved in each bioreactor containing test material (in grams per bioreactor); $(CO_2)_B$ is the mean cumulative amount of carbon dioxide evolved in the blank vessel (in grams per bioreactor).

Following this, the cumulative amount of carbon dioxide evolved as a function of time was determined and graphed, with the test terminated after 60 days.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed under high vacuum using a field emission Philips XL30, with samples coated with iridium and viewed at an accelerating voltage of 5 kV.

Fourier Transform – Infra Red

Fourier transform – infra red (FTIR) spectroscopy was performed using a Perkin-Elmer Spectrum 100 Spectrometer with an Attenuated Total Reflectance (ATR) cell. Film samples were used for the FTIR measurements. Film samples were either the baseline PHB, PHB/BC or the fragments of the degraded samples removed from the compost (composted samples were washed is distilled water to remove compost materials and air-dried overnight prior scanning). Scans ranged between 4000 and 450 cm^{-1} wavenumbers with 64 convolutions. Baselines for each sample spectrum were normalised using the Spectrum software.

X-ray Diffractometry

A Bruker D8 Advance X-ray Diffractometer with CuKα radiation (40 kV, 40 mA) equipped with a LynxEye silicon strip detector was employed to determine the X-ray diffraction (XRD) patterns. Each sample was scanned over the 2θ range 2° to 40° with a step size of 0.02 and a count time of 0.8 seconds per step.

Analyses were performed on the collected XRD data for each sample using the Bruker XRD search match program EVA™. The samples were washed in distilled water to remove compost materials and air-dried overnight.

Small Angle X-ray Scattering

Transmission small angle X-ray scattering (SAXS) patterns were acquired on the two-dimensional detector of a Bruker Nanostar operating on a rotating anode CuKα alpha source for an acquisition time of 2 hours. Operational details of the instrument are found at the website: (http://www.ansto.gov.au/ResearchHub/Bragg/Facilities/Instruments/SAXS/BrukerSAXS/index.htm). The resulting 2-D scattering patterns were radial symmetric. The patterns were converted to the 1-*d* intensity versus *q*, the scattering vector defined by:

$$q = \frac{4\pi}{\lambda} \sin\left(\theta/2\right)$$

where λ is the wavelength of the incident radiation (1.54 Å) and is the scattering angle using the program Fit2D (Hammersley, 1998). The transformation involves radial average around beam centre, using a scattering geometry which is defined by a distance of 72.35 cm from sample to the detector and a detector consisting of 2048 × 2048 pixels 68 μm^2. Pixels around the beam stop and at the detector edges were masked out of the calculation to give an effective q-range of 0.01 to 0.45 $Å^{-1}$.

Results and Discussion

Observations of Films Degraded Over Time

Neat PHB films and bacterial cellulose powder, along with PHB/BC blends, were incubated in the compost to determine how long these materials take to biodegrade, and if the inclusion of the bacterial cellulose phase in this composite would increase or decrease degradation time. Biodegradation was determined by quantifying carbon dioxide produced during the experiment, compared to the total carbon content of materials and blank compost.

After 1 week of composting, PHB and PHB/BC films were observed to still be intact, whereas the compost in the bioreactor containing the neat bacterial cellulose powder was covered with white mycelium indicating fungal growth (Figure 7.1). This indicates that bacterial cellulose is capable of biodegrading rapidly.

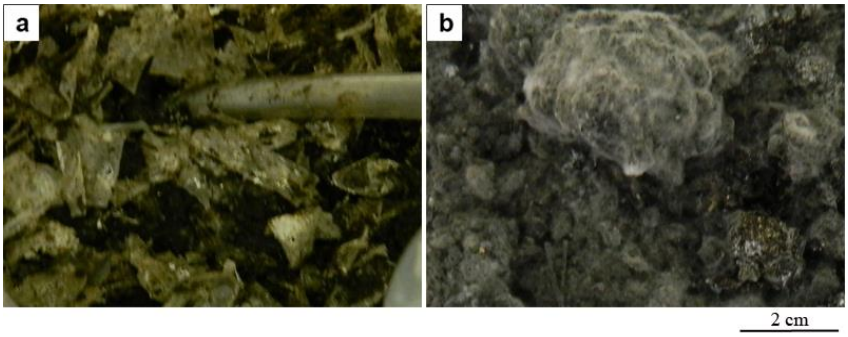


Figure 7.1: Samples in compost after 1 week: (a) neat PHB film (b) Neat BC powder covered with fungal growth.

After 2 weeks of biodegradation, it was apparent (by visual observation) that the PHB/BC films were breaking down more rapidly than the neat PHB films (Figure 7.2). At 3 weeks, much of the neat PHB films remained as large pieces; however there were only a few of the large pieces of the PHB/BC blend visible, as most of them had disintegrated into extremely small fragments. This was also apparent at 4 weeks, when there was very little visible film of PHB/BC composite left, whereas relatively large PHB film pieces persisted, along with many smaller fragments. After 6 weeks, most of the PHB films had broken down, still leaving a few large, visible pieces (Figure 7.2).

Film samples were removed each week from the compost for examination by SEM. It was observed that the films degraded over time showing distinct holes and cracks in the films (Figure 7.3). The PHB/BC showed degradation at 1 week, with many small holes and cracks, whereas this was not seen in the PHB film until the second week of degradation. Both films showed higher biodegradability after 3 weeks of composting.

At 4 weeks, the PHB/BC films had degraded to an extent that a sample could not be collected for SEM, and the PHB film continued to degrade further (data not shown). This further demonstrates the increased rate of biodegradation in the films containing the bacterial cellulose phase.

Aerobic Biodegradation

The amount of CO_2 produced from the bioreactors containing blank compost and compost with test samples was determined and values were expressed as means with standard deviations (Figure 7.4). The biodegradation of bacterial cellulose alone was unable to be repeated due to a limited amount of sample and therefore does not contain any standard deviation values. The cumulative CO_2 produced by the blank samples was relatively low and increased gradually during the test. In comparison, compost with test samples produced higher cumulative CO_2 levels during the initial 10 – 20 days, although after this time the amount of CO_2 being produced decreased and remained at low levels for the remainder of the test period (Figure 7.4). These results indicate that the test materials in the compost were actively metabolised by the microbes present, without a lag phase, producing high amounts of CO_2 from the time that they were first exposed to the compost (Dean et al., 2013).

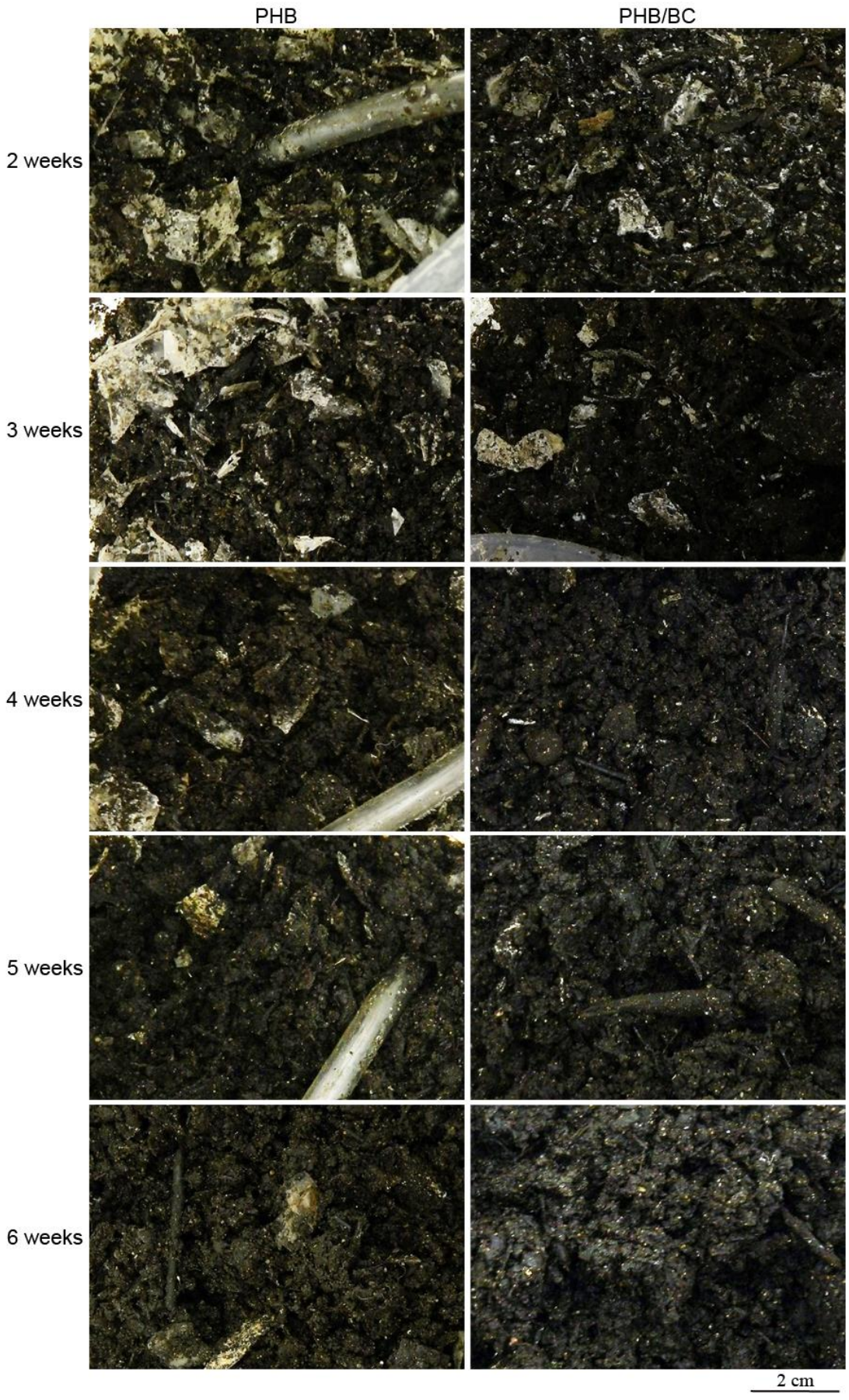


Figure 7.2: Degradation of PHB and PHB/BC films in compost over time.

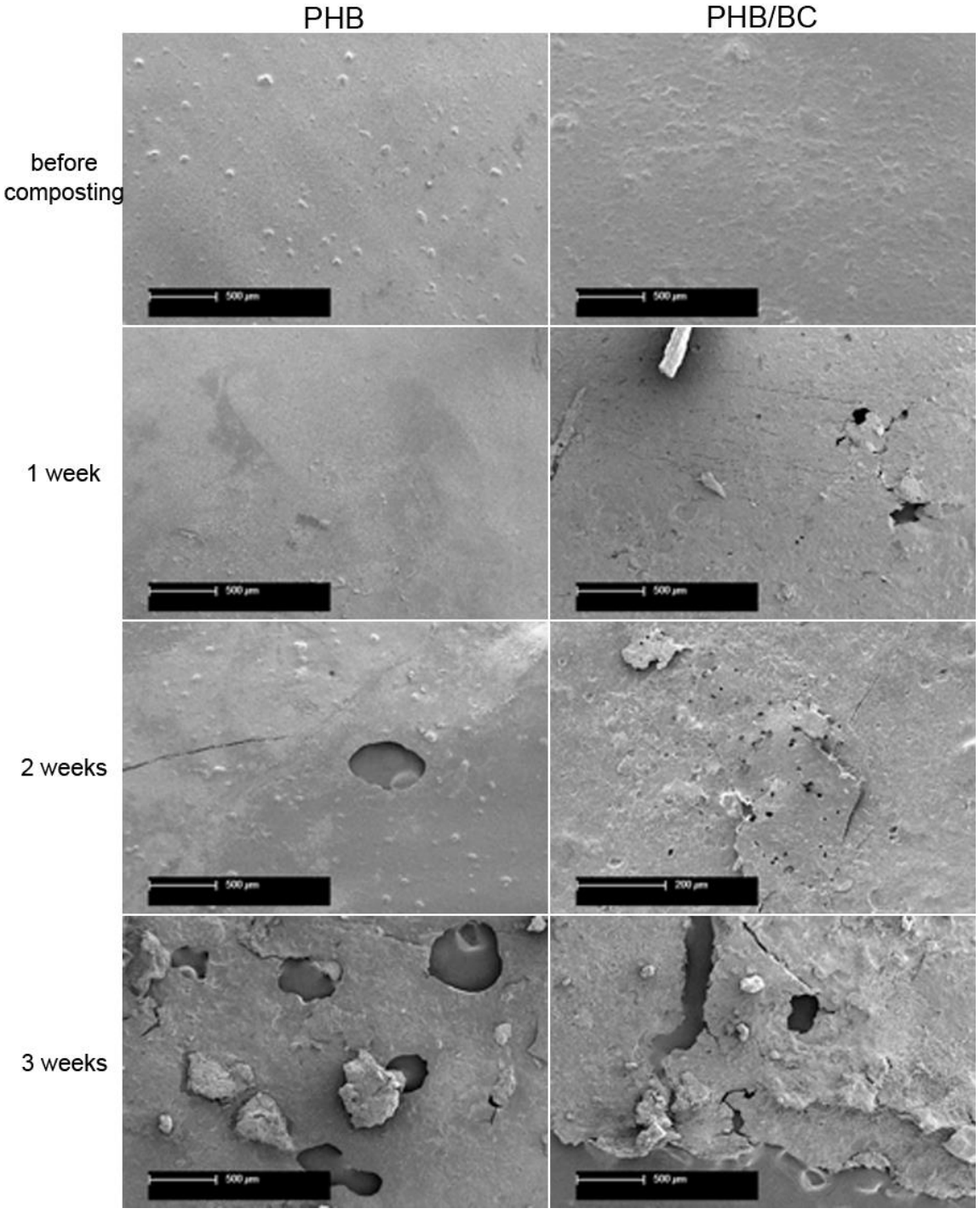


Figure 7.3: SEM of PHB and PHB/BC films during aerobic composting.

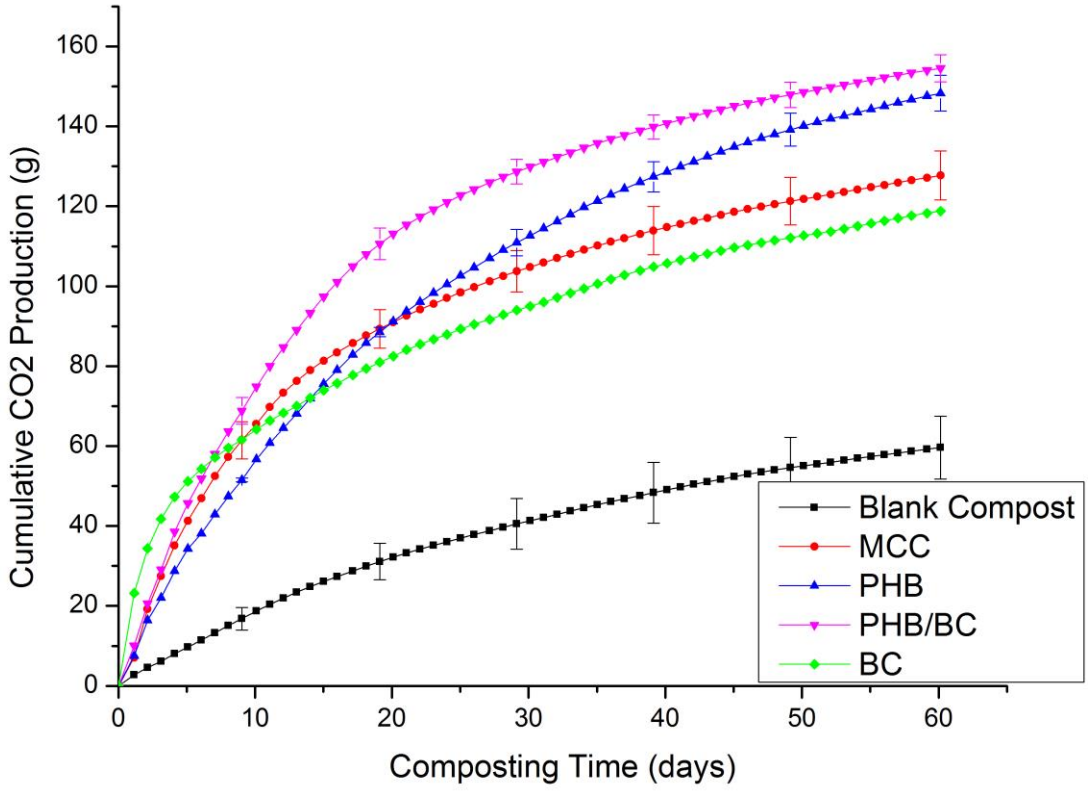


Figure 7.4: Cumulative levels of carbon dioxide evolved from bioreactors during composting.

Moreover, neat bacterial cellulose produced relatively lower cumulative CO_2, when compared to the microcrystalline cellulose, however its rate of CO_2 evolution was more rapid by comparison than MCC in the initial days of composting. The PHB/BC film produced similar amounts of CO_2 as the neat microcrystalline cellulose during initial 10 days, thereafter the PHB/BC film produced significantly higher amounts of CO_2 as compared to MCC. PHB films also produced higher cumulative CO_2 overall as compared to MCC, however the values were relatively lower than PHB/BC (Figure 7.4).

The percentage biodegradation determined from the cumulative carbon dioxide is shown in Figure 7.5, with the gradient of each curve at a given time relating to the rate at which the samples degraded. As noted above, bacterial cellulose degraded at a very fast rate, indicating that its presence in biodegradable composites may encourage overall degradation to occur quickly. This was demonstrated by the rate of degradation shown by the PHB and PHB/BC films, with the PHB/BC films experiencing a faster degradation than PHB alone, as seen by an increase in the initial gradient, supporting the visual observations made of the films in the compost (Figure 7.2). In addition to their

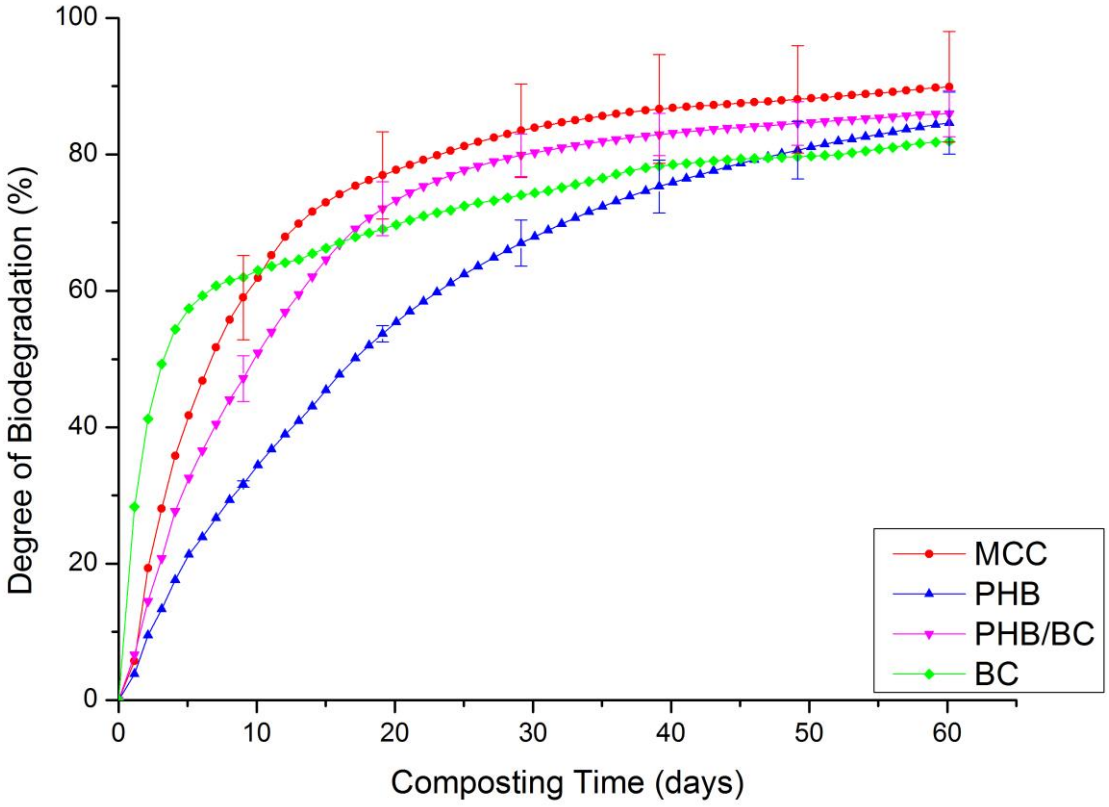


Figure 7.5: Calculated degree of biodegradation of test materials during aerobic composting.

rapid biodegradation, the inclusion of bacterial cellulose fibres within a PHB matrix could also act as nucleation sites for biodegradation of the PHB itself, in effect opening up the structure and exposing more PHB surface to the biodegradation process.

When determining the degree of biodegradation, it is likely that full degradation will never be reached in this experiment, as a certain amount of carbon will be converted into microbial biomass and other natural products, as well as into the compost itself (Shah et al., 2008). However, it is apparent from the plateau in the production of CO_2 (Figure 7.4) and the plateau in the degree of biodegradation shown here (Figure 7.5) that degradation, and subsequently CO_2 production, was essentially complete.

Due to the fast rate of degradation of neat bacterial cellulose and the potential for bacterial cellulose to act as a nucleation site for biodegradation, the PHB/BC films degraded faster than the PHB films, likely to be due to the cellulose phase being the first point of microbial attack and allowing greater

surface area for attack on PHB phase. PHB is stable in air, and is resistant to moisture, requiring exposure to specific environmental conditions and microbes for degradation (Luzier, 1992). As such, it has a potential to be used for packaging as it would remain stable for a period of application but degrade quickly in compost. Its degradation capability can be further enhanced if used in a blend with bacterial cellulose.

Structural Examination of Films Degraded Over Time

Samples were taken at weekly intervals and examined by FTIR. FTIR analysis (Table 7.1) revealed the usual peaks presented by FTIR of PHB, including peaks in the 1724 cm^{-1} region, representative on C=O bonding, and peaks from 1185 cm^{-1}, representing C– O– C bonding (Figure 7.6) (Randriamahefa et al., 2003). Peaks relating to bacterial cellulose could not be determined due similarities in peaks with PHB (Table 7.1), with most peaks relating to the matrix material due to its higher content in the blend.

The 1185 cm^{-1} peak, representative of crystalline PHB, was found to increase as degradation occurred. Peaks present at 1185, 1228 and 1279 cm^{-1} are representative of crystallinity, with the 1185 cm^{-1} peak previously used to determine the crystallinity index of PHB (Randriamahefa et al., 2003). The crystallinity index was therefore calculated as a result of the ratio of the peak at this position and the peak at 1385 cm^{-1}, which is known to be insensitive to the degree of crystallinity (Randriamahefa et al., 2003). These crystallinity indexes are presented in Table 7.2. A crystallinity index of 0.40 and 0.39 was determined for the PHB and PHB/BC, respectively, prior to degradation.

Table 7.1: Positions of some peaks given by FTIR and XRD analysis for PHB and bacterial cellulose.

	FTIR wavelength (cm^{-1})		References	XRD ($2\Theta^0$)						References
	C=O	C-O-C		020	1-10	110	021	121	200	
PHB	1724	1185	(Randriamahefa et al., 2003)	13.5	-	16.8	20.3	25.4	30.5	(Owen et al., 1992)
BC	984-1106	1160	(Kačuráková et al., 2002; Maréchal & Chanzy, 2000)	-	14.8	17.5	-	-	22.6	(Ruka et al., 2012)

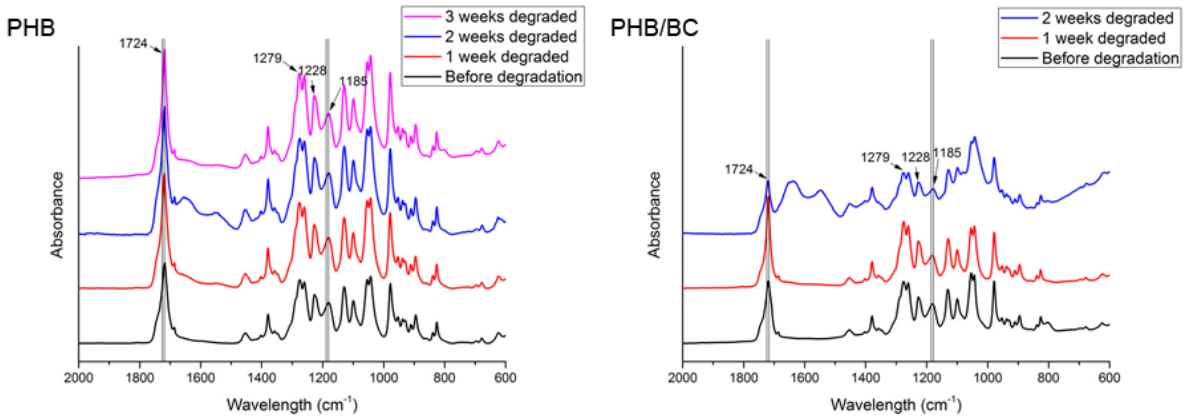


Figure 7.6: FTIR curves given by degrading PHB and PHB/BC films.

Following degradation, the crystallinity index increased up to 0.58 and 0.60 at 2 weeks biodegradation for the neat material and the composite, respectively. Beyond 2 weeks the samples continued to degrade, the PHB showing a decrease in crystallinity and no data being available for the PHB/BC as it was degraded beyond the point of sampling at this time. Some variability in sampling is inevitable using this method due to variation in compost coverage across the sample, to minimise this effect, large sample collection and multiple sampling was used. The crystallinity indexes obtained here suggest that PHB and PHB in the PHB/BC blend become more crystalline as they degrade. This indicates that the amorphous region of the material is degraded first, only achieving a loss in crystallinity after significant degradation has taken place.

Table 7.2: Crystallinity index values given from FTIR data for degraded films.

Film	Composting Time (weeks)	Crystallinity Index	Film	Composting Time (weeks)	Crystallinity Index
PHB	0	0.40	PHB/BC	0	0.39
	1	0.45		1	0.41
	2	0.58		2	0.60
	3	0.40		N/A	-

XRD patterns of semi-crystalline polymers are the linear sum of the contributions from amorphous and crystalline regions (Riello et al., 1995). Peak widths are indicative of crystallite dimensions in the direction normal to the crystallographic axis (Garvey et al., 2005). In this case, all patterns consist of relatively broad diffraction peaks superimposed on the much broader halo of the amorphous PHB (Figure 7.7). The XRD of the degrading films revealed a clear increase in crystallinity with degradation of the PHB film, demonstrated by the relative increase in the crystalline (020) peak (2θ = 13.5°) (Figure 7.7, Table 7.2). Increases in relative intensity of all major crystalline peaks were observed up to 2 weeks composting time. It also appears that some of the diffraction peaks, particularly the (020) which is clearly resolved, become sharper. Given that the peak width is inversely proportional to the width of the peak at half height (Garvey et al., 2005), this change in shape is indicative of some recrystallization process. As the time of composting increases, the contribution of the amorphous phase to the scattering pattern again increases.

For the PHB/BC film, a similar highly resolved peak was observed, at 2θ = 13.5°, corresponding to the (020) peak of PHB. Other major peaks for PHB ((110), (021), (121) and (200)) were also observed and typically increased in relative intensity in the initial period of biodegradation. In the PHB/BC film at 2θ = 23°, an additional peak was observed corresponding to the (200) peak of bacterial cellulose I. This peak also increased in intensity throughout the biodegradation process, this possibly reflective of

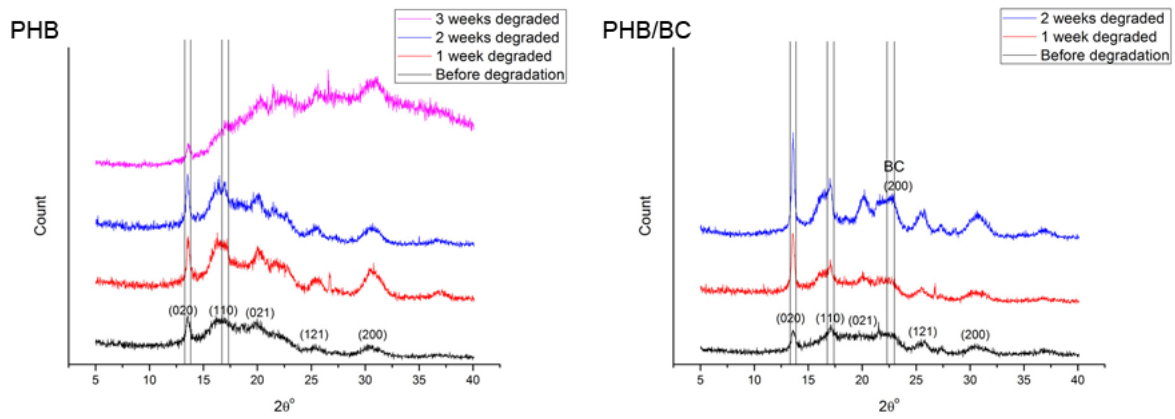


Figure 7.7: XRD peaks given by PHB and PHB/BC films with varying times and degrees of degradation.

the relative resistance of the bacterial cellulose to degradation, or increased crystallinity in the reinforcing phase. As amorphous PHB is degraded, bacterial cellulose becomes a more crystalline fraction of the remaining material. Bacterial cellulose is known to have three main crystalline peaks, the strongest being present at $2\theta = 22.6°$ (corresponding to the (200) peak) (Table 7.2) (Ruka et al., 2012). Additional peaks that are typically observed in bacterial cellulose (Table 7.2) were not clearly visible due to the dominance of the PHB phase.

Due to the nature of the samples in their degraded states, it was not possible to get an accurate quantitative measure of crystallinity from the XRD measurements, due in part to the crystalline peak width variation during biodegradation (increases of 0.13 and 0.08° 2θ for the (002) peak were observed in PHB and PHB/BC respectively) and the inability to capture the low angle data accurately (due to low sample amounts extracted from the compost). Owen et al. (1992) have discussed peak broadening in crystallisation and melting behaviour studies of PHB where they observed increased crystalline peak resolution and peak broadening and suggested that this could be due to improved ordering in the crystalline domain and a larger crystallite size. We observe there appears to be a similar effect in the early stages of biodegradation of PHB and PHB/BC. Despite the issues with XRD measurements, a qualitative increase in crystallinity could be observed (see Figure 7.7). FTIR (quantitatively) and XRD (qualitatively) clearly show an increase in crystallinity; this is similar to increases in crystallinity previously observed during the biodegradation of poly(lactic acid) (PLA)-lignocellulose composite films (Way et al., 2012), with increased crystallinity measured throughout biodegradation until the material loses most of its structural integrity. Way et al. (2012) determined that this increase and subsequent decrease in the crystallinity in PLA composites may not only be due to the preferential hydrolysis of amorphous regions, but also the ability of polymer chain alignment to occur from composting at high temperatures and loss in molecular weight caused by degradation. It was found that after 28 – 35 days, crystallinity decreased due to the breakdown of the PLA in the samples (Way et al., 2012). It is likely that after the initial increase in crystallinity observed here with PHB and PHB/BC composites, the crystallinity would decrease due to the disintegration of these films, however the films were unable to be examined further after disintegration. The increase in

crystallinity from degradation is inconsistent, however, with findings in the PHB-poly-3-hydroxyvalerate copolymer, PHBV, which was found to have the same crystallinity before degradation and after degrading in soil for 15 days, concluding that degradation occurred in both the crystalline and amorphous regions (Gonçalves et al., 2009). However, crystallinity was only determined at 0 and 15 days of degradation, so it is difficult to make a determination on which phase degraded first. It has previously been demonstrated that degradation can occur faster in soil than in compost (Dean et al., 2013), so it may be that the PHBV samples degraded faster than the PHB degraded here in compost, as it was reported that the PHBV was completed degraded after 30 days (Gonçalves et al., 2009). Here, the PHB/BC composites were found to be degraded to 80% at 30 days, whereas this level of degradation was not achieved in the PHB films until 48 days of composting.

Based on the findings here, it is likely that degradation initially occurs in the amorphous region of the PHB. The increased rate of biodegradation in the PHB/BC composite suggests that it is the bacterial cellulose phase degrading first, due to its high rate of degradation, with the PHB being quickly degraded after this time, having an increased surface area due to the cellulose degradation.

SAXS measurements were made from within each sample from pieces of film selected from within the overall sample. There was not a significant difference in the scattering curves apart from a constant scattering factor which is consistent with samples of varying thickness but similar nano-structural structural attributes (Glatter & Kratky, 1982). Figure 7.8 shows the typical SAXS curves for the PHB/BC and PHB samples. All the curves consist of a decay with one or two small peaks superimposed. At the higher values of q the scattered intensity gradually increases again as the features associated with wide angle X-ray scattering (diffraction) are encountered. For the older samples, the peaks/features become less well defined and may contain several very broad peaks. This general form of a SAXS curve is typical of a semi-crystalline polymer such as PHB with the position of the peak being characteristic of a unit lamellar cell consisting of alternating crystalline and amorphous polymer layers (Ellar et al., 1968; Strobl & Schneider, 1980). A characteristic spacing, d, may be taken from the position of the peak, q_{max}:

$$d = \frac{2\pi}{q_{max}}$$

using a simple Lorentz transformation of data, plotting $q^2.I$ versus q (inset Figure 7.8) (Cser, 2001), to locate the position of the peak more precisely on the sloping baseline.

In the case of the films at time 0, the morphology is quite simple. A single repeat spacing is observed for both samples. In the case of the PHB/BC sample, the repeat distance is approximately 52 Å, and for pure PHB it is at 49 Å. Generally in the case of pure semi-crystalline polymers, the repeat spacing is function of the processing used to form the film and the molecular weight of the polymers used (Strobl, 2007).

In this case, where the method of forming the film is the same, the presence of the cellulose appears to have an effect on the nanostructure of the PHB. As the composting time increases, the feature moves to lower values of q/larger length-scales (*cf* equation) and becomes less pronounced. These peaks are not well defined and may, in some cases, contain more than one repeat spacing. This is not surprising in view of the heterogeneous nature of degradation in compost.

Assuming that degradation does not occur within the crystalline phase since the crystalline packing excludes enzymes responsible for degradation (Jendrossek & Handrick, 2002) and a net increase in

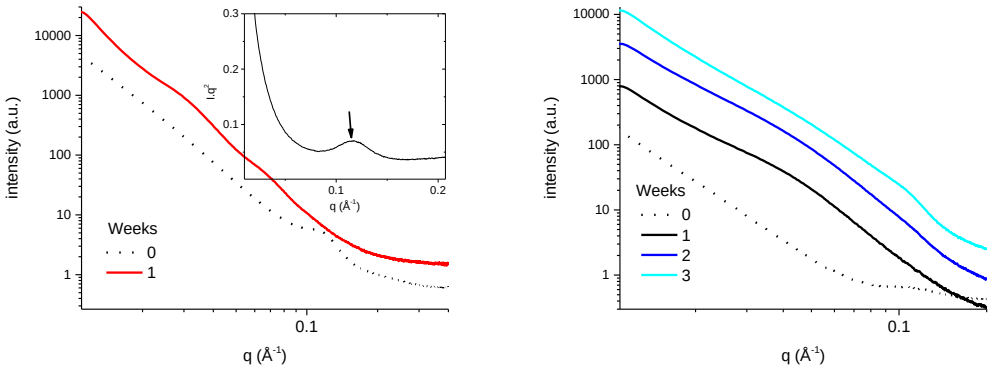


Figure 7.8: SAXS curves as a function of composting time in weeks for PHB/BC film (left) and PHB. The inset of the left hand side shows the Lorentz plot for determining *d*-spacing for the time = 0 sample. The position of the peak is shown with an arrow.

crystallinity of PHB is initially observed, changes in the structure must arise in the amorphous region. The length-scale amorphous region between crystalline lamellae is small when compared to the length-scales associated with enzymes, and it would seem likely that changes in crystallinity and subsequent formation of lamellae occurs as the result of degradation of bulk amorphous materials outside the original lamellae as the backbone of the linear polymer is cleaved statistically along chain. This produces smaller fragments, which are able to crystallise into new lamellae.

This study reports the comprehensive approach taken to measure and interpret the biodegradation of PHB and PHB/BC films in compost. CO_2 evolution was used as an accurate *in situ* measure of biodegradation, in conjunction with other techniques of SEM, FTIR, XRD and SAXS also employed to understand the morphological changes that occurred during this process. Both PHB and bacterial cellulose were found to degrade rapidly, with bacterial cellulose having an extremely high rate of degradation. The ability of this material to quickly degrade caused a faster degradation rate in the PHB/BC composite over PHB alone. In addition, the relative levels of crystallinity of the materials were found to increase during degradation. These results demonstrate the ability for these natural materials to be quickly degraded in a composting environment.

7.3 Conclusions

Bacterial cellulose was found to degrade even faster than the microcrystalline cellulose control when submerged in compost. In addition, the inclusion of bacterial cellulose in a PHB composite caused an increased rate of degradation over the PHB film alone. This indicates that these two materials could not only be used to produce biocomposites with good mechanical properties (likely using the sonication method described in Chapter 6), but could also degrade quickly in compost. Based on the ability for PHB to be degraded in other environment such as seawater, blends of these materials may, therefore, be able to be disposed of in a variety of ways, and degraded to molecules and minerals for uptake into the natural environment. The increased degradation rate seen here demonstrates a further advantage of blending bacterial cellulose with PHB, supporting the use of bacterial cellulose as a reinforcing material to produce biodegradable composites.

Chapter 8
Conclusions and Future Work

8.1 Conclusions

This work has examined the potential of bacterial cellulose for its use in composites. The entire life cycle of bacterial cellulose, from its production to decomposition, has been studied. The aims of this work were:

- to investigate the effects of cultivation conditions on the yield, structure and morphology of bacterial cellulose
- to identify ways to modify bacterial cellulose to achieve materials with specific properties in an attempt to "tailor-design" the cellulose for purposes such as reinforcing selected matrix materials
- to achieve and compare methods to obtain composites of poly-3-hydroxybutyrate and bacterial cellulose, and evaluate the viability of these methods to produce useful, biodegradable blends
- to establish the biodegradation characteristics of selected biomaterials and blends.

The conclusions of each part of the research are discussed separately in the following sections.

8.1.1 Bacterial Cellulose Cultivation

The aim of this part of the research was to investigate the effect of different cultivation conditions, such as media, physical conditions, time, vessel size and media volume, on the yield, structure and morphology of bacterial cellulose. Investigations into the use of different media considered five different media, each with a different carbon source, for the growth of cellulose. It was found that Yamanaka-mannitol medium produced the highest yield of cellulose under the selected conditions. Yamanaka-mannitol medium contains a high concentration of the carbon source, mannitol, as well as yeast extract, both of which are expensive. These components cause the price of Yamanaka-mannitol medium to be high compared to the other media. However, it was found that if the amount of cellulose produced is taken into account, Yamanaka-mannitol can actually be considered one of the most cost-effective media. Hestrin-Schramm medium has been traditionally used to produce bacterial cellulose.

However the analysis here demonstrates that it is expensive to produce, second only to Yamanaka medium, and produces only small yields of bacterial cellulose, meaning it is not a cost-effective medium. Zhou medium was found to be the most cost effective, however as Yamanaka-mannitol produced the highest yield of cellulose, Yamanaka-mannitol was selected as the growth medium for further work when large amounts of material were required (such as for the production of PHB/BC composites).

The mechanical properties of cellulose produced in Yamanaka-mannitol were compared to those of cellulose produced in HS medium. Bacterial cellulose pellicle films produced in Yamanaka-mannitol and HS media had tensile strengths of 143 and 124 MPa, elongations of 9.5 and 8.9%, and modulus values of 2.2 and 1.8 GPa, respectively. This demonstrates that the use of Yamanaka-mannitol instead of HS medium does not negatively impact the mechanical properties of the produced cellulose, and thus Yamanaka-mannitol can be used as a suitable medium to produce bacterial cellulose with good mechanical properties.

Investigations into the physical conditions of cultures used to grow bacterial cellulose, with static and agitated cultures, as well as cultures in which pellicles were removed or sunk after 5 days of growth, were undertaken. It was determined that higher yields of cellulose were achieved in static cultures compared with agitated cultures, and that sinking the pellicle after 5 days of growth for the production of a new pellicle achieved slightly higher cellulose yields than in static cultures; however this was not significantly higher in most media tested, with similar yields achieved in static conditions and cultures where the pellicles were removed to allow new pellicles to form. It was found that using agitated cultures resulted in lower yields of cellulose, with the cellulose in the form of spherical pellets that had a change in morphology, with the appearance of clumps and a loss of the fibrillar structures observed in Zhou media.

Investigations into other factors that could impact the yield of cellulose were also completed, including examination of the surface area of the growth vessel, the time allowed for growth, and the volume of media used. It was found larger surface areas of cultivation vessels allowed for larger

pellicles to be produced, and therefore achieved higher yields of cellulose. It was also found that most cellulose production occurred quickly, with cultures producing large amounts of cellulose within the first 7 – 14 days. In addition, it was found that larger volumes of media produced more cellulose than smaller volumes, and that the greater volumes of media required more time to produce higher amounts of cellulose. However, when the cost of the media was taken into account (with greater amounts of media being more expensive) it was found that it is actually more cost (and time) effective to produce cellulose in smaller volumes of media. Investigations revealed that the depth of the media is an important factor in growing static bacterial cellulose cultures, as it is necessary to have a sufficient depth of media so that the culture does not dry out and limit cellulose production. Based on all the findings presented here, it was determined that future cellulose would be produced under static conditions, using either 50 mL cultures in flasks produced for 7 days, or 600 mL cultures in beakers with a larger surface area for up to 14 days when larger amounts of cellulose needed to be produced. Yamanaka-mannitol and HS were used as the media for all further cellulose production.

8.1.2 Bacterial Cellulose Modifications

The aim of this part of the research was to identify ways to modify bacterial cellulose to achieve materials with specific properties in an attempt to “tailor-design” the cellulose. Ionic liquids have been shown to have a wide range of uses, including the ability to dissolve typically insoluble materials such as cellulose. Choline-based ionic liquids have previously been shown to act as the carbon source in other bacterial species, and thus were selected to use as additives in media to produce bacterial cellulose. The ionic liquids used included choline dihydrogen phosphate, choline tartrate, choline stearate, choline gallate and choline formate. It was found that bacterial cellulose could be successfully grown in media containing low levels of these ionic liquids, but not high levels. This indicates that the salinity of the media with high levels of ionic liquids creates a toxic environment for the bacteria. It was also found that the inclusion of these ionic liquids had little or no effect on the structure and morphology of the cellulose. It appeared that some of the ionic liquids became part of the cellulose pellicle as a type of hybrid material. Thus, since bacterial cellulose can be produced in the presence of

ionic liquids, it is possible that materials that would otherwise be insoluble could be dissolved in media containing ionic liquids, which could then be used to achieve surface modification of the cellulose. In addition, it was found that when glucose was excluded from the media, thus removing the carbon source, cellulose production was still achieved in media containing one of the ionic liquids, choline formate. Since cellulose was produced from media containing only choline formate as a carbon source, this demonstrates that there may be other materials that could be developed to act as the carbon source for cellulose production.

Investigations into the use of polymers as additives were considered, as water-soluble polymers have been included in the growth media for cellulose production and have previously been shown to successfully modify the structure and properties of the produced cellulose, and lead to the incorporation of some polymers amongst the cellulose fibrils, creating composite materials. Since PHB had been selected as the material to be used as a matrix for production of PHB/BC composites, PHB was also selected for use as an additive. Due to its insolubility, PHB was dispersed throughout the media, rather than being dissolved as has been possible with other polymers previously used. This was done in order to determine if the presence of dispersed PHB could cause modifications to the cellulose itself, and thus if a PHB/BC composite could be produced by an *in situ* technique. The inclusion of dispersed PHB in HS media (HS-PHB) led to bacterial cellulose with PHB integrated in the cellulose fibril mesh. It was found that the surface-modified cellulose had lower mechanical properties, with a reduction in tensile strength, elongation at break and Young's modulus to 42 MPa, 3.7% and 1.3 GPa, respectively. The decreases in mechanical properties are likely to be due to a decrease in interfibrillar hydrogen bonding due to the presence of the PHB particles amongst the fibrils. This demonstrates that the addition of PHB in cellulose-producing media causes modifications to the cellulose, with decreases in mechanical properties and physical binding of PHB to the cellulose fibrils. It also demonstrates that additives can become incorporated into the bacterial cellulose by simply being dispersed in the media, rather than being fully dissolved.

8.1.3 Producing Bacterial Cellulose Composites with Improved Mechanical Properties

The aim of this part of the research was to investigate whether bacterial cellulose could be used as a reinforcing material with a biodegradable matrix to achieve a biodegradable composite with properties better than those of the matrix alone. PHB was selected as the matrix material in an attempt to improve the mechanical properties of a biodegradable material that has potential to replace traditional plastics, but could benefit from improved mechanical properties. Different methods of producing these composites were considered, together with bacterial cellulose produced in different media (HS, HS-PHB and Yamanaka-mannitol). Melt blending and solution blending were selected as methods that could blend bacterial cellulose and PHB, using the cellulose as a reinforcing phase.

Melt blending was used to produce PHB/BC composites with different cellulose contents ranging from 1 – 5 wt%. The mechanical properties of the composites produced were the same as those of PHB alone. In addition, the composites demonstrated darkened colours with increasing cellulose contents. This indicates that degradation occurred when cellulose was added to the PHB melt blends, with increasing degradation occurring with increasing cellulose content. This colour change was consistent throughout the entire blend, indicating that the cellulose was well dispersed. However there were a small number of dark spots visible suggesting that some aggregation may have occurred and confirming thermal degradation of the cellulose phase. As PHB can also be subject to thermal degradation, it may be concluded that melt blending is not an effective way to make useful PHB/BC blends (particularly using bacterial cellulose in an unmodified, powder form), as the high temperatures necessary for melt blending result in degradation of the materials.

Solution blending was used to produce PHB/BC composites firstly with ground cellulose powder contents of 1, 2, 5 and 10 wt% with the cellulose grown in HS, HS-PHB and Yamanaka-mannitol media. It was found that it was necessary to sonicate the cellulose powder in the solvent prior to dissolving PHB to achieve a fine dispersion of the cellulose. Despite the dispersion achieved, the mechanical properties showed only a slight improvement in tensile strength and modulus, and decreased

elongation at break was observed in all blends. These results indicate that the interfacial adhesion between the PHB and bacterial cellulose was not good, leading to poor properties. An examination of the microscopic features of the cellulose powder revealed that the grinding process causes a loss of the cellulose fibrils. The nanosized fibrils of bacterial cellulose give it a high surface area and therefore the potential to reinforce composites at a low concentration. Since a loss of these fibrils is not desirable, grinding the cellulose to a powder may not be the optimal methodology to achieve dispersion in a composite. Despite these limitations, the solution blending method did offer the potential of producing PHB/BC composites, and was therefore studied further with sonication investigated as a dispersion method.

In order to produce solution blends with dispersed bacterial cellulose in its native fibrillar state, sonication was considered as a method of achieving individual fibrils. Cellulose pellicles were sonicated in different solvents for a period of time, after which the pellicles were removed and the solution was examined for the presence of dispersed fibrils. It was found that sonicating bacterial cellulose pellicles caused individual fibrils to become separated from the interwoven mesh of fibrils and suspended in solution. To determine how much cellulose could be dispersed in this way, pellicles were sonicated in different solvents, ethanol, water and chloroform, and the dry weights of the harvested fibrils were determined. Bacterial cellulose was also used in three different forms, specifically in the hydrated never-dried, air-dried, and air-dried and subsequently swelled (re-wetted) forms. It was found that similar weights of fibrils could be harvested from the pellicles in a never-dried state in water, ethanol and chloroform. This was possibly due to the continued presence of water amongst the cellulose fibrils, which assisted the three-dimensional structure of this material to be maintained. By contrast, greater weights of fibrils could be harvested from the pellicles when sonication occurred in water and ethanol over chloroform when cellulose was in its air-dried form. This was possibly due to drying the cellulose, during which time the fibrils are compacted and dried onto each other, resulting in the loss of the three-dimensional structure. Greater weights of fibrils were achieved with air-dried pellicles in water and ethanol compared with the same process using never-dried pellicles, however pellicles sonicated in chloroform showed greater weights of fibrils

from never-dried pellicles over air-dried pellicles. The greatest weight of fibrils was achieved from air-dried pellicles in ethanol, however these conditions still only dispersed approximately 21 mg of fibrils in the solvent. Despite the low weights of cellulose fibrils harvested and suspended in solution, this work demonstrates that it is possible to use sonication to scission fibrils directly from cellulose pellicle films to achieve suspensions of these fibrils. Solutions of fibrils harvested from cellulose pellicles in chloroform were then used for blending with PHB to produce PHB/BC composites for further examination.

Solution blending produced PHB/BC composites with cellulose contents of approximately 2 wt%, with cellulose in a fibrillar form obtained by sonication. It was found that a PHB/BC composite with cellulose from the never-dried state had better mechanical properties than PHB alone, with ultimate tensile strength and Young's modulus values of 33±4 MPa and 1970±30 MPa, compared with neat PHB values of 23±4 MPa and 1240± 80 MPa, respectively. PHB/BC composites with cellulose from air-dried and re-wetted states had lower mechanical properties than those of PHB alone. The swelled cellulose composite was found, however, to have better mechanical properties than the air-dried cellulose composite. Thus, the different mechanical properties of PHB/BC composites produced from cellulose in never-dried, air-dried and re-wetted states indicate that the drying of the cellulose causes changes to this material, and that using cellulose in the never-dried state is necessary to achieve a PHB/BC solution blended composite with improved mechanical properties. This demonstrates that solution blends can be produced with bacterial cellulose well dispersed in a fibrillar form, achieved by sonicating pellicles in a solvent and subsequently dissolving a matrix material in the solution. It is an important finding since it demonstrates that this biodegradable nanofiller, bacterial cellulose, can successfully improve the mechanical properties of a biodegradable material such as PHB.

8.1.4 Biodegradation Characteristics of Bacterial Cellulose Composite

The aim of this part of the research was to examine the biodegradability of a PHB/BC composite compared to the neat materials. PHB/BC, PHB and bacterial cellulose were all exposed to compost, with the biodegradability determined from the evolution of carbon dioxide as the materials degraded.

It was found that the PHB/BC composite degraded faster than the neat PHB film, achieving 60% degradation at 14 days, and 80% degradation at 30 days, compared to the neat material achieving the same levels of degradation at 24 and 48 days. The increased rate of biodegradation can be attributed to the inclusion of bacterial cellulose, which was shown to be a material with a high rate of biodegradation. Bacterial cellulose degraded at an extremely high rate in the initial degradation period, achieving 60% degradation after only 7 days, while the microcrystalline cellulose control reached this level of degradation after 10 days.

As PHB/BC degraded, it was found that the crystallinity increased in the initial weeks of degradation, as shown by FTIR and SAXS measurements, suggesting that degradation first occurs in the amorphous regions of the materials. These results demonstrate that PHB/BC breaks down very quickly in compost. Products developed from these materials would be stable in the air, but would biodegrade in compost, therefore avoiding the problems of litter and landfill clutter that occur with the persistence of traditional plastic material.

8.1.5 Contributions of this Research

The findings of this work indicate that bacterial cellulose can be used as an effective reinforcement material in biodegradable composites. A PHB/BC solution blend can be produced, which has improved tensile strength and Young's modulus over PHB alone. A method for producing improved PHB/BC composites was developed in which bacterial cellulose is to first have fibrils harvested and dispersed from hydrated never-dried pellicles by sonicating pellicles in chloroform. Solutions of chloroform containing suspended fibrils are then blended with PHB to dissolve the PHB, creating a blended PHB/BC material which is cast to form a film. In addition, a PHB/BC composite produced has an increased biodegradation rate over PHB. These results indicate that bacterial cellulose can be blended with an appropriate material, and can confer improved mechanical properties as well as an increased degradation rate. With the current pressing need for sustainable materials to replace traditional plastics, bacterial cellulose could provide an important reinforcement material and should be investigated further in the development of fully biodegradable composites.

8.2 Future Work

While this research has made significant steps forward in the development of biodegradable composites reinforced with bacterial cellulose, there are still areas where more work is to be done. This work established a method by which to produce PHB/BC, dispersing bacterial cellulose in its native fibrillar form. It established that improved mechanical properties can be achieved when using these fibrils in a composite from a hydrated never-dried form. However, the use of sonication only allows for small weights of fibrils to be separated from the fibril mesh and suspended in solution. It would be necessary to develop ways to achieve greater weights of dispersed fibrils in solution to produce greater amounts of fibrillar reinforcement for composites.

Investigating different factors involved in sonication, or combining sonication with an additional method to disperse bacterial cellulose in solution to increase the weight of fibrils dispersed in solution, would be of benefit. For example, never-dried bacterial cellulose pellicles could be first homogenised to a slurry, and then sonicated in order to achieve dispersed individual fibrils in solution. This process may allow for greater weights of individual fibrils to be successfully isolated and blended with PHB.

Following the establishment of a method by which high weights of individual fibrils can be dispersed in order to produce greater amounts of composite, matrices other than PHB could be investigated, together with surface-modified cellulose fibrils. For example, materials such as poly(lactic acid) or poly(vinyl alcohol), which are biodegradable materials, could be reinforced with bacterial cellulose fibrils to improve mechanical properties. It is likely that individual bacterial cellulose fibrils would be able to confer improved mechanical properties to other biodegradable matrices, particularly hydrophilic matrices due to the hydrophilic nature of the cellulose. It is also likely that chemical modifications to bacterial cellulose could improve the mechanical properties when this material is used to reinforce a hydrophobic matrix, such as PHB.

References

Amass, W., Amass, A. & Tighe, B. (1998) A review of biodegradable polymers: Uses, current developments in the synthesis and characterization of biodegradable polyesters, blends of biodegradable polymers and recent advances in biodegradation studies. *Polymer International,* 47:89-144.

Ashori, A., Sheykhnazari, S., Tabarsa, T., Shakeri, A. & Golalipour, M. (2012) Bacterial cellulose/silica nanocomposites: Preparation and characterization. *Carbohydrate Polymers,* 90:413-418.

Atalla, R.H. & Vanderhart, D.L. (1984) Native cellulose: A composite of two distinct crystalline forms. *Science,* 223:283-285.

Avella, M., Martuscelli, E. & Raimo, M. (2000) Review. Properties of blends and composites based on poly(3-hydroxy)butyrate (PHB) and poly(3-hydroxybutyrate-hydroxyvalerate) (PHBV) copolymers. *Journal of Materials Science,* 35:523-545.

Bae, S. & Shoda, M. (2004) Bacterial cellulose production by fed-batch fermentation in molasses medium. *Biotechnology Progress,* 20:1366-1371.

Bae, S. & Shoda, M. (2005) Statistical optimization of culture conditions for bacterial cellulose production using Box-Behnken design. *Biotechnology and Bioengineering,* 90:20-28.

Bae, S., Sugano, Y. & Shoda, M. (2004) Improvement of bacterial cellulose production by addition of agar in a jar fermentor. *Journal of Bioscience and Bioengineering,* 97:33-38.

Barud, H.S., Souza, J.L., Santos, D.B., Crespi, M.S., Ribeiro, C.A., Messaddeq, Y. & Ribeiro, S.J.L. (2011) Bacterial cellulose/poly(3-hydroxybutyrate) composite membranes. *Carbohydrate Polymers,* 83:1279-1284.

Basta, A.H. & El-Saied, H. (2009) Performance of improved bacterial cellulose application in the production of functional paper. *Journal of Applied Microbiology,* 107:2098-2107.

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,* 77:6678-6682.

Biganska, O. & Navard, P. (2005) Kinetics of precipitation of cellulose from cellulose-NMMO-water solutions. *Biomacromolecules,* 6:1948-1953.

Bledzki, A.K. & Gassan, J. (1999) Composites reinforced with cellulose based fibres. *Progress in Polymer Science,* 24:221-274.

Braunegg, G., Lefebvre, G. & Genser, K.F. (1998) Polyhydroxyalkanoates, biopolyesters from renewable resources: Physiological and engineering aspects. *Journal of Biotechnology,* 65:127-161.

Brown, E.E. & Laborie, M.P.G. (2007) Bioengineering bacterial cellulose/poly(ethylene oxide) nanocomposites. *Biomacromolecules,* 8:3074-3081.

Byrom, D. (1987) Polymer synthesis by microorganisms: Technology and economics. *Trends in Biotechnology,* 5:246-250.

Byrom, D. (1993) The synthesis and biodegradation of polyhydroxyalkanoates from bacteria. *International Biodeterioration & Biodegradation,* 31:199-208.

Cai, Z., Hou, C. & Yang, G. (2012) Poly(3-hydroxubutyrate-co-4-hydroxubutyrate)/bacterial cellulose composite porous scaffold: Preparation, characterization and biocompatibility evaluation. *Carbohydrate Polymers,* 87:1073-1080.

Cai, Z. & Kim, J. (2010) Bacterial cellulose/poly(ethylene glycol) composite: Characterization and first evaluation of biocompatibility. *Cellulose,* 17:83-91.

Cai, Z. & Yang, G. (2011) Optical nanocomposites prepared by incorporating bacterial cellulose nanofibrils into poly(3-hydroxybutyrate). *Materials Letters,* 65:182-184.

Cai, Z., Yang, G. & Kim, J. (2011) Biocompatible nanocomposites prepared by impregnating bacterial cellulose nanofibrils into poly(3-hydroxybutyrate). *Current Applied Physics,* 11:247-249.

Chao, Y.P., Ishida, T., Sugano, Y. & Shoda, M. (2000) Bacterial cellulose production by *Acetobacter xylinum* in a 50-L internal-loop airlift reactor. *Biotechnology and Bioengineering,* 68:345-352.

Chao, Y.P., Mitarai, M., Sugano, Y. & Shoda, M. (2001) Effect of addition of water-soluble polysaccharides on bacterial cellulose production in a 50-L airlift reactor. *Biotechnology Progress,* 17:781-785.

Chen, J.P. & Chiang, Y. (2010) Bioactive electrospun silver nanoparticles-containing polyurethane nanofibers as wound dressings. *Journal of Nanoscience and Nanotechnology,* 10:7560-7564.

Chen, P., Yun, Y.S., Bak, H., Cho, S.Y. & Jin, H.J. (2010) Multiwalled carbon nanotubes-embedded electrospun bacterial cellulose nanofibers. *Molecular Crystals and Liquid Crystals,* 519:169-178.

Cheng, K.C., Catchmark, J.M. & Demirci, A. (2009a) Effect of different additives on bacterial cellulose production by *Acetobacter xylinum* and analysis of material property. *Cellulose,* 16:1033-1045.

Cheng, K.C., Catchmark, J.M. & Demirci, A. (2009b) Enhanced production of bacterial cellulose by using a biofilm reactor and its material property analysis. *Journal of Biological Engineering,* 3:12.

Cheng, Q., Wang, S.Q., Rials, T.G. & Lee, S.H. (2007) Physical and mechanical properties of polyvinyl alcohol and polypropylene composite materials reinforced with fibril aggregates isolated from regenerated cellulose fibers. *Cellulose,* 14:593-602.

Cheng, Q.Z., Wang, S.Q. & Han, Q.Y. (2010) Novel process for isolating fibrils from cellulose fibers by high-intensity ultrasonication. II. Fibril characterization. *Journal of Applied Polymer Science,* 115:2756-2762.

Cheng, Q.Z., Wang, S.Q. & Rials, T.G. (2009c) Poly(vinyl alcohol) nanocomposites reinforced with cellulose fibrils isolated by high intensity ultrasonication. *Composites Part A-Applied Science and Manufacturing,* 40:218-224.

Chien, L.J., Chen, H.T., Yang, P.F. & Lee, C.K. (2006) Enhancement of cellulose pellicle production by constitutively expressing *Vitreoscilla* hemoglobin in *Acetobacter xylinum*. *Biotechnology Progress,* 22:1598-1603.

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,* 3:203-205.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. & Marrie, T.J. (1987) Bacterial biofilms in nature and disease. *Annual Review of Microbiology,* 41:435-464.

Coucheron, D.H. (1991) An *Acetobacter xylinum* insertion-sequence element associated with inactivation of cellulose production. *Journal of Bacteriology,* 173:5723-5731.

Cser, F. (2001) About the Lorentz correction used in the interpretation of small angle X-ray scattering data of semicrystalline polymers. *Journal of Applied Polymer Science,* 80:2300-2308.

Czaja, W., Krystynowicz, A., Bielecki, S. & Brown, R.M. (2006) Microbial cellulose - the natural power to heal wounds. *Biomaterials,* 27:145-151.

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

Czaja, W.K., Young, D.J., Kawecki, M. & Brown, R.M. (2007) The future prospects of microbial cellulose in biomedical applications. *Biomacromolecules,* 8:1-12.

Dahman, Y. (2009) Nanostructured biomaterials and biocomposites from bacterial cellulose nanofibers. *Journal of Nanoscience and Nanotechnology,* 9:5105-5122.

de Marco Lima, G., Sierakowski, M.R., Faria-Tischer, P.C.S. & Tischer, C.A. (2010) Characterisation of bacterial cellulose partly acetylated by dimethylacetamide/lithium chloride. *Materials Science and Engineering C,* 31:190-197.

Dean, K., Sangwan, P., Way, C., Zhang, X.Q., Martino, V.P., Xie, F.W., Halley, P.J., Pollet, E. & Avérous, L. (2013) Glycerol plasticised chitosan: A study of biodegradation via carbon dioxide evolution and nuclear magnetic resonance. *Polymer Degradation and Stability,* 98:1236-1246.

Deinema, M.H. & Zevenhuien, L.P.T.M. (1971) Formation of cellulose fibrils by Gram-negative bacteria and their role in bacterial flocculation. *Archiv für Mikrobiologie,* 78:42-57.

Deive, F.J., Rodríguez, A., Varela, A., Rodrígues, C., Leitão, M.C., Houbraken, J.A.M.P., Pereiro, A.B., Longo, M.A., Sanromán, M.Á., Samson, R.A., Rebelo, L.P.N. & Pereira, C.S. (2011) Impact of ionic liquids on extreme microbial biotypes from soil. *Green Chemistry,* 13:687-696.

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

DeWulf, P., Joris, K. & Vandamme, E.J. (1996) Improved cellulose formation by an *Acetobacter xylinum* mutant limited in (keto)gluconate synthesis. *Journal of Chemical Technology and Biotechnology,* 67:376-380.

Dreyer, S. & Kragl, U. (2008) Ionic liquids for aqueous two-phase extraction and stabilization of enzymes. *Biotechnology and Bioengineering,* 99:1416-1424.

Dudman, W.F. (1959) Cellulose production by *Acetobacter acetigenum* in defined medium. *Journal of General Microbiology,* 21:327-337.

Dudman, W.F. (1960) Cellulose production by *Acetobacter* strains in submerged culture. *Journal of General Microbiology,* 22:25-39.

Earle, M.J. & Seddon, K.R. (2000) Ionic liquids. Green solvents for the future. *Pure and Applied Chemistry,* 72:1391-1398.

Eichhorn, S.J., Dufresne, A., Aranguren, M., Marcovich, N.E., Capadona, J.R., Rowan, S.J., Weder, C., Thielemans, W., Roman, M., Renneckar, S., Gindl, W., Veigel, S., Keckes, J., Yano, H., Abe, K., Nogi, M., Nakagaito, A.N., Mangalam, A., Simonsen, J., Benight, A.S., Bismarck, A., Berglund, L.A. & Peijs, T. (2010) Review: Current international research into cellulose nanofibres and nanocomposites. *Journal of Materials Science,* 45:1-33.

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,* 3:1196-1217.

Ellar, D., Lundgren, D.G., Okamura, K. & Marchess.Rh. (1968) Morphology of poly-beta-hydroxybutyrate granules. *Journal of Molecular Biology,* 35:489-&.

EPA. (2011). Municipal solid waste generation, recycling, and disposal in the united states: Facts and figures for 2010 Retrieved 5th September 2012, from http://www.epa.gov/osw/nonhaz/municipal/pubs/msw_2010_rev_factsheet.pdf

Eurostat, E.C. (2012). Packaging waste, from http://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=env_waspac&lang=en

Feng, Y.Y., Zhang, X.Q., Shen, Y.T., Yoshino, K. & Feng, W. (2012) A mechanically strong, flexible and conductive film based on bacterial cellulose/graphene nanocomposite. *Carbohydrate Polymers,* 87:644-649.

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.

Flieger, M., Kantorová, M., Prell, A., Řezanka, T. & Votruba, J. (2003) Biodegradable plastics from renewable sources. *Folia Microbiologica,* 48:27-44.

Fontana, J.D., Franco, V.C., Desouza, S.J., Lyra, I.N. & Desouza, A.M. (1991) Nature of plant stimulators in the production of *Acetobacter xylinus* (tea fungus) biofilm used in skin therapy. *Applied Biochemistry and Biotechnology,* 28-9:341-351.

Fujita, K., MacFarlane, D.R., Forsyth, M., Yoshizawa-Fujita, M., Murata, K., Nakamura, N. & Ohno, H. (2007) Solubility and stability of cytochrome c in hydrated ionic liquids: Effect of oxo acid residues and kosmotropicity. *Biomacromolecules,* 8:2080-2086.

Galas, E., Krystynowicz, A., Tarabasz-Szymanska, L., Pankiewicz, T. & Rzyska, M. (1999) Optimization of the production of bacterial cellulose using multivariable linear regression analysis. *Acta Biotechnologica,* 19:251-260.

Gangu, S.A., Weatherley, L.R. & Scurto, A.M. (2009) Whole-cell biocatalysis with ionic liquids. *Current Organic Chemistry,* 13:1242-1258.

Gao, C., Wan, Y.Z., He, F., Liang, H., Luo, H.L. & Han, J. (2011) Mechanical, moisture absorption, and photodegradation behaviors of bacterial cellulose nanofiber-reinforced unsaturated polyester composites. *Advances in Polymer Technology,* 30:249-256.

Gardner, D.J., Oporto, G.S., Mills, R. & Samir, M. (2008) Adhesion and surface issues in cellulose and nanocellulose. *Journal of Adhesion Science and Technology,* 22:545-567.

Garvey, C.J., Parker, I.H. & Simon, G.P. (2005) On the interpretation of X-ray diffraction powder patterns in terms of the nanostructure of cellulose I fibres. *Macromolecular Chemistry and Physics,* 206:1568-1575.

Gatenholm, P., Kubát, J. & Mathiasson, A. (1992) Biodegradable natural composites. I. Processing and properties. *Journal of Applied Polymer Science,* 45:1667-1677.

Gea, S., Bilotti, E., Reynolds, C.T., Soykeabkeaw, N. & Peijs, T. (2010) Bacterial cellulose-poly(vinyl alcohol) nanocomposites prepared by an in-situ process. *Materials Letters,* 64:901-904.

Geyer, U., Heinze, T., Stein, A., Klemm, D., Marsch, S., Schumann, D. & Schumauder, H.-P. (1994) Formation, derivatization and applications of bacterial cellulose. *International Journal of Biological Macromolecules,* 16:343-347.

Gindl, W. & Keckes, J. (2004) Tensile properties of cellulose acetate butyrate composites reinforced with bacterial cellulose. *Composites Science and Technology,* 64:2407-2413.

Glatter, O. & Kratky, O. (1982) *Small angle X-ray scattering*: Academic Press.

Goffin, A.L., Raquez, J.M., Duquesne, E., Siqueira, G., Habibi, Y., Dufresne, A. & Dubois, P. (2011) From interfacial ring-opening polymerization to melt processing of cellulose nanowhisker-filled polylactide-based nanocomposites. *Biomacromolecules,* 12:2456-2465.

Gonçalves, S.P.C., Martins-Franchetti, S.M. & Chinaglia, D.L. (2009) Biodegradation of the films of PP, PHBV and its blend in soil. *Journal of Polymers and the Environment,* 17:280-285.

Grande, C.J., Torres, F.G., Gomez, C.M., Troncoso, O.P., Canet-Ferrer, J. & Martínez-Pastor, J. (2009) Development of self-assembled bacterial cellulose-starch nanocomposites. *Materials Science and Engineering C,* 29:1098-1104.

Grunert, M. & Winter, W.T. (2002) Nanocomposites of cellulose acetate butyrate reinforced with cellulose nanocrystals. *Journal of Polymers and the Environment,* 10:27-30.

Guhados, G., Wan, W.K. & Hutter, J.L. (2005) Measurement of the elastic modulus of single bacterial cellulose fibers using atomic force microscopy. *Langmuir,* 21:6642-6646.

Guo, J. & Catchmark, J.M. (2012) Surface area and porosity of acid hydrolyzed cellulose nanowhiskers and cellulose produced by *Gluconacetobacter xylinus*. *Carbohydrate Polymers,* 87:1026-1037.

Guo, X., Cavka, A., Jönsson, L.J. & Hong, F. (2013) Comparison of methods for detoxification of spruce hydrolysate for bacterial cellulose production. *Microbial Cell Factories,* 12:93.

Haigler, C.H., Brown, R.M. & Benziman, M. (1980) Calcofluor White ST alters the in vivo assembly of cellulose microfibrils. *Science,* 210:903-906.

Hammersley, A. (1998) FIT2D V9.129 Reference Manual V3.1. ESRF: *ESRF Internal Report*.

Haque, M.M. & Pracella, M. (2010) Reactive compatibilization of composites of ethylene-vinyl acetate copolymers with cellulose fibres. *Composites Part A-Applied Science and Manufacturing,* 41:1545-1550.

Heo, M.S. & Son, H.J. (2002) Development of an optimized, simple chemically defined medium for bacterial cellulose production by *Acetobacter* sp A9 in shaking cultures. *Biotechnology and Applied Biochemistry,* 36:41-45.

Heßler, N. & Klemm, D. (2009) Alteration of bacterial nanocellulose structure by in situ modification using polyethylene glycol and carbohydrate additives. *Cellulose,* 16:899-910.

Hestrin, S. & Schramm, M. (1954) Synthesis of cellulose by *Acetobacter xylinum*. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. *Biochemical Journal,* 58:345-352.

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

Hioki, N., Hori, Y., Watanabe, K., Morinaga, Y., Yoshinaga, F., Hibino, Y. & Ogura, T. (1995) Bacterial cellulose: As a new material for papermaking. *Japan TAPPI* 49:718-723.

Hirai, A., Tsuji, M., Yamamoto, H. & Horii, F. (1998) *In situ* crystallization of bacterial cellulose III. Influences of different polymeric additives on the formation of microfibrils as revealed by transmission electron microscopy. *Cellulose,* 5:201-213.

Holmes, P.A. (1985) Applications of PHB - a microbially produced biodegradable thermoplastic. *Physics in Technology,* 16:32-36.

Hong, F., Guo, X., Zhang, S., Han, S.F., Yang, G. & Jönsson, L.J. (2012) Bacterial cellulose production from cotton-based waste textiles: Enzymatic saccharification enhanced by ionic liquid pretreatment. *Bioresource Technology,* 104:503-508.

Hong, F. & Qiu, K.Y. (2008) An alternative carbon source from konjac powder for enhancing production of bacterial cellulose in static cultures by a model strain *Acetobacter aceti* subsp *xylinus* ATCC 23770. *Carbohydrate Polymers,* 72:545-549.

Hornung, M., Ludwig, M., Gerrard, A.M. & Schmauder, H.P. (2006) Optimizing the production of bacterial cellulose in surface culture: Evaluation of product movement influences on the bioreaction (Part 2). *Engineering in Life Sciences,* 6:546-551.

Hu, W., Chen, S., Li, X., Shi, S., Shen, W., Zhang, X. & Wang, H. (2009) In situ synthesis of silver chloride nanoparticles into bacterial cellulose membranes. *Materials Science and Engineering C,* 29:1216-1219.

Hu, W.L., Chen, S.Y., Xu, Q.S. & Wang, H.P. (2011) Solvent-free acetylation of bacterial cellulose under moderate conditions. *Carbohydrate Polymers,* 83:1575-1581.

Huang, J.-C., Shetty, A.S. & Wang, M.-S. (1990) Biodegradable plastics: A review. *Advances in Polymer Technology,* 10:23-30.

Hutchens, S.A., León, 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,* 44:175-180.

Hwang, J.W., Yang, Y.K., Hwang, J.K., Pyun, Y.R. & Kim, Y.S. (1999) Effects of pH and dissolved oxygen on cellulose production by *Acetobacter xylinum* BRC5 in agitated culture. *Journal of Bioscience and Bioengineering,* 88:183-188.

Ifuku, S., Nogi, M., Abe, K., Handa, K., Nakatsubo, F. & Yano, H. (2007) Surface modification of bacterial cellulose nanofibers for property enhancement of optically transparent composites: Dependence on acetyl-group DS. *Biomacromolecules,* 8:1973-1978.

Iguchi, M., Yamanaka, S. & Budhiono, A. (2000) Bacterial cellulose - a masterpiece of nature's arts. *Journal of Materials Science,* 35:261-270.

Ishida, T., Sugano, Y., Nakai, T. & Shoda, M. (2002) Effects of acetan on production of bacterial cellulose by *Acetobacter xylinum*. *Bioscience Biotechnology and Biochemistry,* 66:1677-1681.

Ishihara, M., Matsunaga, M., Hayashi, N. & Tišler, V. (2002) Utilization of D-xylose as carbon source for production of bacterial cellulose. *Enzyme and Microbial Technology,* 31:986-991.

Ishikawa, A., Matsuoka, M., Tsuchida, T. & Yoshinaga, F. (1995) Increase in cellulose production by sulfaguanidine-resistant mutants derived from *Acetobacter xylinum* subsp *sucrofermentans*. *Bioscience Biotechnology and Biochemistry,* 59:2259-2262.

Ishikawa, A., Tonouchi, N., Tsuchida, T. & Yoshinaga, F. (1998a) Breeding of a 5-fluorouridine-resistant mutant with increased cellulose production from *Acetobacter xylinum* subsp *nonacetoxidans*. *Bioscience Biotechnology and Biochemistry,* 62:1388-1391.

Ishikawa, A., Tsuchida, T. & Yoshinaga, F. (1998b) Relationship between sulfaguanidine resistance and increased cellulose production in *Acetobacter xylinum* BPR3001E. *Bioscience Biotechnology and Biochemistry,* 62:1234-1236.

Iwatake, A., Nogi, M. & Yano, H. (2008) Cellulose nanofiber-reinforced polylactic acid. *Composites Science and Technology,* 68:2103-2106.

Jagannath, A., Kalaiselvan, A., Manjunatha, S.S., Raju, P.S. & Bawa, A.S. (2008) The effect of pH, sucrose and ammonium sulphate concentrations on the production of bacterial cellulose (nata-de-coco) by *Acetobacter xylinum*. *World Journal of Microbiology and Biotechnology,* 24:2593-2599.

Jayasekara, R., Harding, I., Bowater, I. & Lonergan, G. (2005) Biodegradability of a selected range of polymers and polymer blends and standard methods for assessment of biodegradation. *Journal of Polymers and the Environment,* 13:231-251.

Jendrossek, D. & Handrick, R. (2002) Microbial degradation of polyhydroxyalkanoates. *Annual Review of Microbiology,* 56:403-432.

Jiang, L., Morelius, E., Zhang, J.W., Wolcott, M. & Holbery, J. (2008) Study of the poly(3-hydroxybutyrate-co-3-hydroxyvalerate)/cellulose nanowhisker composites prepared by solution casting and melt processing. *Journal of Composite Materials,* 42:2629-2645.

Jipa, I.M., Dobre, L., Stroescu, M., Stoica-Guzun, A., Jinga, S. & Dobre, T. (2012) Preparation and characterization of bacterial cellulose-poly(vinyl alcohol) films with antimicrobial properties. *Materials Letters,* 66:125-127.

Jung, H.I., Jeong, J.H., Lee, O.M., Park, G.T., Kim, K.K., Park, H.C., Lee, S.M., Kim, Y.G. & Son, H.J. (2010a) Influence of glycerol on production and structural-physical properties of cellulose from *Acetobacter* sp V6 cultured in shake flasks. *Bioresource Technology,* 101:3602-3608.

Jung, H.I., Lee, O.M., Jeong, J.H., Jeon, Y.D., Park, K.H., Kim, H.S., An, W.G. & Son, H.J. (2010b) Production and characterization of cellulose by *Acetobacter* sp V6 using a cost-effective molasses-corn steep liquor medium. *Applied Biochemistry and Biotechnology,* 162:486-497.

Juntaro, J., Ummartyotin, S., Sain, M. & Manuspiya, H. (2012) Bacterial cellulose reinforced polyurethane-based resin nanocomposite: A study of how ethanol and processing pressure affect physical, mechanical and dielectric properties. *Carbohydrate Polymers,* 87:2464-2469.

Kačuráková, M., Smith, A.C., Gidley, M.J. & Wilson, R.H. (2002) Molecular interactions in bacterial cellulose composites studied by 1D FT-IR and dynamic 2D FT-IR spectroscopy. *Carbohydrate Research,* 337:1145-1153.

Kale, G., Kijchavengkul, T., Auras, R., Rubino, M., Selke, S.E. & Singh, S.P. (2007) Compostability of bioplastic packaging materials: An overview. *Macromolecular Bioscience,* 7:255-277.

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

Keshk, S. & Sameshima, K. (2006a) Influence of lignosulfonate on crystal structure and productivity of bacterial cellulose in a static culture. *Enzyme and Microbial Technology,* 40:4-8.

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

Khalil, H., Bhat, A.H. & Yusra, A.F.I. (2012) Green composites from sustainable cellulose nanofibrils: A review. *Carbohydrate Polymers,* 87:963-979.

Kibédi-Szabó, C.Z., Stroescu, M., Stoica-Guzun, A., Jinga, S.I., Szilveszter, S., Jipa, I. & Dobre, T. (2012) Biodegradation behavior of composite films with poly (vinyl alcohol) matrix. *Journal of Polymers and the Environment,* 20:422-430.

Kim, J., Cai, Z., Lee, H.S., Choi, G.S., Lee, D.H. & Jo, C. (2011) Preparation and characterization of a bacterial cellulose/chitosan composite for potential biomedical application. *Journal of Polymer Research,* 18:739-744.

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,* 131:705-715.

Kim, Y., Jung, R., Kim, H.S. & Jin, H.J. (2009) Transparent nanocomposites prepared by incorporating microbial nanofibrils into poly(L-lactic acid). *Current Applied Physics,* 9:S69-S71.

King, P.P. (1982) Biotechnology. An industrial view. *Journal of Chemical Technology and Biotechnology,* 32:2-8.

Kiziltas, A., Gardner, D.J., Han, Y. & Yang, H.S. (2011) Thermal properties of microcrystalline cellulose-filled PET-PTT blend polymer composites. *Journal of Thermal Analysis and Calorimetry,* 103:163-170.

Klemm, D., Schumann, D., Kramer, F., Heßler, N., Hornung, M., Schmauder, H.P. & Marsch, S. (2006). Nanocelluloses as innovative polymers in research and application *Polysaccharides II* (Vol. 205, pp. 49-96).

Kona, R.P., Qureshi, N. & Pai, J.S. (2001) Production of glucose oxidase using *Aspergillus niger* and corn steep liquor. *Bioresource Technology,* 78:123-126.

Kono, H., Yunoki, S., Shikano, T., Fujiwara, M., Erata, T. & Takai, M. (2002) CP/MAS C-13 NMR study of cellulose and cellulose derivatives. 1. Complete assignment of the CP/MAS C-13 NMR spectrum of the native cellulose. *Journal of the American Chemical Society,* 124:7506-7511.

Kose, R., Mitani, I., Kasai, W. & Kondo, T. (2011) "Nanocellulose" as a single nanofiber prepared from pellicle secreted by *Gluconacetobacter xylinus* using aqueous counter collision. *Biomacromolecules,* 12:716-720.

Kouda, T., Naritomi, T., Yano, H. & Yoshinaga, F. (1997a) Effects of oxygen and carbon dioxide pressures on bacterial cellulose production by *Acetobacter* in aerated and agitated culture. *Journal of Fermentation and Bioengineering,* 84:124-127.

Kouda, T., Yano, H. & Yoshinaga, F. (1997b) Effect of agitator configuration on bacterial cellulose productivity in aerated and agitated culture. *Journal of Fermentation and Bioengineering,* 83:371-376.

Krystynowicz, A., Czaja, W., Wiktorowska-Jezierska, A., Gonçalves-Miśkiewicz, M., Turkiewicz, M. & Bielecki, S. (2002) Factors affecting the yield and properties of bacterial cellulose. *Journal of Industrial Microbiology and Biotechnology,* 29:189-195.

Lee, B.H., Kim, H.J. & Yang, H.S. (2012a) Polymerization of aniline on bacterial cellulose and characterization of bacterial cellulose/polyaniline nanocomposite films. *Current Applied Physics,* 12:75-80.

Lee, H.J., Chung, T.J., Kwon, H.J., Kim, H.J. & Tze, W.T.Y. (2012b) Fabrication and evaluation of bacterial cellulose-polyaniline composites by interfacial polymerization. *Cellulose,* 19:1251-1258.

Lee, K.Y., Blaker, J.J. & Bismarck, A. (2009) Surface functionalisation of bacterial cellulose as the route to produce green polylactide nanocomposites with improved properties. *Composites Science and Technology,* 69:2724-2733.

Lenz, R.W. & Marchessault, R.H. (2005) Bacterial polyesters: Biosynthesis, biodegradable plastics and biotechnology. *Biomacromolecules,* 6:1-8.

Li, X., Chen, S., Hu, W., Shi, S., Shen, W., Zhang, X. & Wang, H. (2009) In situ synthesis of CdS nanoparticles on bacterial cellulose nanofibers. *Carbohydrate Polymers,* 76:509-512.

Li, X., Tabil, L.G., Oguocha, I.N. & Panigrahi, S. (2008) Thermal diffusivity, thermal conductivity, and specific heat of flax fiber-HDPE biocomposites at processing temperatures. *Composites Science and Technology,* 68:1753-1758.

Liebert, T. & Heinze, T. (2008) Interaction of ionic liquids with polysaccharides. 5. Solvents and reaction media for the modification of cellulose. *BioResources,* 3:576-601.

Liu, C., Yang, D., Wang, Y.G., Shi, J.F. & Jiang, Z.Y. (2012) Fabrication of antimicrobial bacterial cellulose-Ag/AgCl nanocomposite using bacteria as versatile biofactory. *Journal of Nanoparticle Research,* 14.

Lu, J.M., Yan, F. & Texter, J. (2009) Advanced applications of ionic liquids in polymer science. *Progress in Polymer Science,* 34:431-448.

Lucas, N., Bienaime, C., Belloy, C., Queneudec, M., Silvestre, F. & Nava-Saucedo, J.E. (2008) Polymer biodegradation: Mechanisms and estimation techniques. *Chemosphere,* 73:429-442.

Luzier, W.D. (1992) Materials derived from biomass biodegradable materials. *Proceedings of the National Academy of Sciences of the United States of America,* 89:839-842.

Maneerung, T., Tokura, S. & Rujiravanit, R. (2008) Impregnation of silver nanoparticles into bacterial cellulose for antimicrobial wound dressing. *Carbohydrate Polymers,* 72:43-51.

Maréchal, Y. & Chanzy, H. (2000) The hydrogen bond network in I_β cellulose as observed by infrared spectrometry. *Journal of Molecular Structure,* 523:183-196.

Marins, J.A., Soares, B.G., Dahmouche, K., Ribeiro, S.J.L., Barud, H. & Bonemer, D. (2011) Structure and properties of conducting bacterial cellulose-polyaniline nanocomposites. *Cellulose,* 18:1285-1294.

Martínez-Sanz, M., Lopez-Rubio, A. & Lagaron, J.M. (2011a) Optimization of the nanofabrication by acid hydrolysis of bacterial cellulose nanowhiskers. *Carbohydrate Polymers,* 85:228-236.

Martínez-Sanz, M., Lopez-Rubio, A. & Lagaron, J.M. (2012a) Optimization of the dispersion of unmodified bacterial cellulose nanowhiskers into polylactide via melt compounding to significantly enhance barrier and mechanical properties. *Biomacromolecules,* 13:3887-3899.

Martínez-Sanz, M., Lopez-Rubio, A. & Lagaron, J.M. (2013a) Nanocomposites of ethylene vinyl alcohol copolymer with thermally resistant cellulose nanowhiskers by melt compounding (I): Morphology and thermal properties. *Journal of Applied Polymer Science,* 128:2666-2678.

Martínez-Sanz, M., Lopez-Rubio, A. & Lagaron, J.M. (2013b) Nanocomposites of ethylene vinyl alcohol copolymer with thermally resistant cellulose nanowhiskers by melt compounding (II): Water barrier and mechanical properties. *Journal of Applied Polymer Science,* 128:2197-2207.

Martínez-Sanz, M., Olsson, R.T., Lopez-Rubio, A. & Lagaron, J.M. (2011b) Development of electrospun EVOH fibres reinforced with bacterial cellulose nanowhiskers. Part I: Characterization and method optimization. *Cellulose,* 18:335-347.

Martínez-Sanz, M., Olsson, R.T., Lopez-Rubio, A. & Lagaron, J.M. (2012b) Development of bacterial cellulose nanowhiskers reinforced EVOH composites by electrospinning. *Journal of Applied Polymer Science,* 124:1398-1408.

Martínez-Sanz, M., Villano, M., Oliveira, C., Albuquerque, M.G.E., Majone, M., Reis, M., Lopez-Rubio, A. & Lagaron, J.M. (2014) Characterization of polyhydroxyalkanoates synthesized from microbial mixed cultures and of their nanobiocomposites with bacterial cellulose nanowhiskers. *New Biotechnology,* 31:364-376.

Martins, I.M.G., Magina, S.P., Oliveira, L., Freire, C.S.R., Silvestre, A.J.D., Neto, C.P. & Gandini, A. (2009) New biocomposites based on thermoplastic starch and bacterial cellulose. *Composites Science and Technology,* 69:2163-2168.

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

Masson, J.F. & Manley, R.S. (1991) Miscible blends of cellulose and poly(vinylpyrrolidone). *Macromolecules,* 24:6670-6679.

Matsumoto, M., Mochiduki, K., Fukunishi, K. & Kondo, K. (2004) Extraction of organic acids using imidazolium-based ionic liquids and their toxicity to *Lactobacillus rhamnosus*. *Separation and Purification Technology,* 40:97-101.

Matsuoka, M., Tsuchida, T., Matsushita, K., Adachi, O. & Yoshinaga, F. (1996) A synthetic medium for bacterial cellulose production by *Acetobacter xylinum* subsp *sucrofermentans*. *Bioscience Biotechnology and Biochemistry,* 60:575-579.

McKenna, B.A., Mikkelsen, D., Wehr, J.B., Gidley, M.J. & Menzies, N.W. (2009) Mechanical and structural properties of native and alkali-treated bacterial cellulose produced by *Gluconacetobacter xylinus* strain ATCC 53524. *Cellulose,* 16:1047-1055.

Mensitieri, G., Di Maio, E., Buonocore, G.G., Nedi, I., Oliviero, M., Sansone, L. & Iannace, S. (2011) Processing and shelf life issues of selected food packaging materials and structures from renewable resources. *Trends in Food Science & Technology,* 22:72-80.

Mergaert, J., Anderson, C., Wouters, A., Swings, J. & Kersters, K. (1992) Biodegradation of polyhydroxyalkanoates. *FEMS Microbiology Letters,* 103:317-321.

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,* 107:576-583.

Millon, L.E., Oates, C.J. & Wan, W.K. (2009) Compression properties of polyvinyl alcohol-bacterial cellulose nanocomposite. *Journal of Biomedical Materials Research Part B-Applied Biomaterials,* 90B:922-929.

Mohanty, A.K., Misra, M. & Hinrichsen, G. (2000) Biofibres, biodegradable polymers and biocomposites: An overview. *Macromolecular Materials and Engineering,* 276:1-24.

Mohite, B.V., Kamalja, K.K. & Patil, S.V. (2012) Statistical optimization of culture conditions for enhanced bacterial cellulose production by *Gluconoacetobacter hansenii* NCIM 2529. *Cellulose,* 19:1655-1666.

Müller, D., Mandelli, J.S., Marins, J.A., Soares, B.G., Porto, L.M., Rambo, C.R. & Barra, G.M.O. (2012) Electrically conducting nanocomposites: Preparation and properties of polyaniline (PAni)-coated bacterial cellulose nanofibers (BC). *Cellulose,* 19:1645-1654.

Nakagaito, A.N., Fujimura, A., Sakai, T., Hama, Y. & Yano, H. (2009) Production of microfibrillated cellulose (MFC)-reinforced polylactic acid (PLA) nanocomposites from sheets obtained by a papermaking-like process. *Composites Science and Technology,* 69:1293-1297.

Nakagaito, A.N., Iwamoto, S. & Yano, H. (2005) Bacterial cellulose: The ultimate nano-scalar cellulose morphology for the production of high-strength composites. *Applied Physics A-Materials Science & Processing,* 80:93-97.

Nakai, T., Tonouchi, N., Konishi, T., Kojima, Y., Tsuchida, T., Yoshinaga, F., Sakai, F. & Hayashi, T. (1999) Enhancement of cellulose production by expression of sucrose synthase in *Acetobacter xylinum*. *Proceedings of the National Academy of Sciences of the United States of America,* 96:14-18.

Napoli, C., Dazzo, F. & Hubbell, D. (1975) Production of cellulose microfibrils by *Rhizobium*. *Applied Microbiology,* 30:123-131.

Naritomi, T., Kouda, T., Yano, H. & Yoshinaga, F. (1998a) Effect of ethanol on bacterial cellulose production from fructose in continuous culture. *Journal of Fermentation and Bioengineering,* 85:598-603.

Naritomi, T., Kouda, T., Yano, H. & Yoshinaga, F. (1998b) Effect of lactate on bacterial cellulose production from fructose in continuous culture. *Journal of Fermentation and Bioengineering,* 85:89-95.

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,* 57:449-453.

Nishi, Y., Uryu, M., Yamanaka, S., Watanabe, K., Kitamura, N., Iguchi, M. & Mitsuhashi, S. (1990) The structure and mechanical-properties of sheets prepared from bacterial cellulose. Part 2.

Improvement of the mechanical-properties of sheets and their applicability to diaphragms of electroacoustic transducers. *Journal of Materials Science,* 25:2997-3001.

Nishiyama, Y., Langan, P. & Chanzy, H. (2002) Crystal structure and hydrogen-bonding system in cellulose 1β from synchrotron X-ray and neutron fiber diffraction. *Journal of the American Chemical Society,* 124:9074-9082.

Nishiyama, Y., Sugiyama, J., Chanzy, H. & Langan, P. (2003) Crystal structure and hydrogen bonding system in cellulose 1α from synchrotron X-ray and neutron fiber diffraction. *Journal of the American Chemical Society,* 125:14300-14306.

Nogi, M., Abe, K., Handa, K., Nakatsubo, F., Ifuku, S. & Yano, H. (2006a) Property enhancement of optically transparent bionanofiber composites by acetylation. *Applied Physics Letters,* 89.

Nogi, M., Handa, K., Nakagaito, A.N. & Yano, H. (2005) Optically transparent bionanofiber composites with low sensitivity to refractive index of the polymer matrix. *Applied Physics Letters,* 87.

Nogi, M., Ifuku, S., Abe, K., Handa, K., Nakagaito, A.N. & Yano, H. (2006b) Fiber-content dependency of the optical transparency and thermal expansion of bacterial nanofiber reinforced composites. *Applied Physics Letters,* 88.

Noro, N., Sugano, Y. & Shoda, M. (2004) Utilization of the buffering capacity of corn steep liquor in bacterial cellulose production by *Acetobacter xylinum*. *Applied Microbiology and Biotechnology,* 64:199-205.

O'Connor, R.T., Dupre, E.F. & McCall, E.R. (1957) Infrared spectrophotometric procedure for analysis of cellulose and modified cellulose. *Analytical Chemistry,* 29:998-1005.

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

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

Olsson, R.T., Kraemer, R., López-Rubio, A., Torres-Giner, S., Ocio, M.J. & Lagarón, J.M. (2010) Extraction of microfibrils from bacterial cellulose networks for electrospinning of anisotropic biohybrid fiber yarns. *Macromolecules,* 43:4201-4209.

Orts, W.J., Nobes, G.A.R., Kawada, J., Nguyen, S., Yu, G.E. & Raveneile, F. (2008) Poly(hydroxyalkanoates): Biorefinery polymers with a whole range of applications. The work of Robert H. Marchessault. *Canadian Journal of Chemistry-Revue Canadienne De Chimie,* 86:628-640.

Oshima, T., Kondo, K., Ohto, K., Onoue, K. & Baba, Y. (2008) Preparation of phosphorylated bacterial cellulose as an adsorbent for metal ions. *Reactive and Functional Polymers,* 68:376-383.

Oshima, T., Taguchi, S., Ohe, K. & Baba, Y. (2011) Phosphorylated bacterial cellulose for adsorption of proteins. *Carbohydrate Polymers,* 83:953-958.

Ougiya, H., Watanabe, K., Morinaga, Y. & Yoshinaga, F. (1997) Emulsion-stabilizing effect of bacterial cellulose. *Bioscience Biotechnology and Biochemistry,* 61:1541-1545.

Owen, A. & Bergmann, A. (2004) On the fractal character of polymer spherulites: An ultra-small-angle X-ray scattering study of poly[(R)-3-hydroxybutyrate]. *Polymer International,* 53:12-14.

Owen, A.J., Heinzel, J., Škrbić, Ž. & Divjaković, V. (1992) Crystallization and melting behavior of PHB and PHB/HV copolymer. *Polymer,* 33:1563-1567.

Park, J.K., Jung, J.Y. & Park, Y.H. (2003) Cellulose production by *Gluconacetobacter hansenii* in a medium containing ethanol. *Biotechnology Letters,* 25:2055-2059.

Park, W.I., Kang, M., Kim, H.S. & Jin, H.J. (2007) Electrospinning of poly(ethylene oxide) with bacterial cellulose whiskers. *Macromolecular Symposia,* 249:289-294.

Paul, D.R. & Robeson, L.M. (2008) Polymer nanotechnology: Nanocomposites. *Polymer,* 49:3187-3204.

Pei, Y., Zhang, L.N., Wang, H.Y., Zhang, X.Z. & Xu, M. (2011) Supermolecular structure and properties of cellulose/gelatin composite films. *Acta Polymerica Sinica,* 0:1098-1104.

Pertile, R.A.N., Andrade, F.K., Alves, C. & Gama, M. (2010) Surface modification of bacterial cellulose by nitrogen-containing plasma for improved interaction with cells. *Carbohydrate Polymers,* 82:692-698.

Phisalaphong, M., Suwanmajo, T. & Tammarate, P. (2008) Synthesis and characterization of bacterial cellulose/alginate blend membranes. *Journal of Applied Polymer Science,* 107:3419-3424.

Pommet, M., Juntaro, J., Heng, J.Y.Y., Mantalaris, A., Lee, A.F., Wilson, K., Kalinka, G., Shaffer, M.S.P. & Bismarck, A. (2008) Surface modification of natural fibers using bacteria: Depositing bacterial cellulose onto natural fibers to create hierarchical fiber reinforced nanocomposites. *Biomacromolecules,* 9:1643-1651.

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

Pracella, M., Pancrazi, C., Haque, M.M. & D'Alessio, A. (2011) Thermal and microstructural characterization of compatibilized polystyrene/natural fillers composites. *Journal of Thermal Analysis and Calorimetry,* 103:95-101.

Premjet, S., Ohtani, Y. & Sameshima, K. (1994) The contribution of high molecular lignosulfonate to the powerful bacterial cellulose production system with *Acetobacter xylinum* ATCC 10245. *Sen'i Gakkaishi,* 50:458-463.

Premjet, S., Premjet, D. & Ohtani, Y. (2007) The effect of ingredients of sugar cane molasses on bacterial cellulose production by *Acetobacter xylinum* ATCC 10245. *Sen'i Gakkaishi,* 63:193-199.

Price, G.J., West, P.J. & Smith, P.F. (1994) Control of polymer structure using power ultrasound. *Ultrasonics Sonochemistry,* 1:S51-S57.

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

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 and Biotechnology,* 16:245-248.

Randriamahefa, S., Renard, E., Guérin, P. & Langlois, V. (2003) Fourier transform infrared spectroscopy for screening and quantifying production of PHAs by *Pseudomonas* grown on sodium octanoate. *Biomacromolecules,* 4:1092-1097.

Rapa, M., Grosu, E., Degeratu, C., Scheau, A. & Stanescu, C. (2010) Biodegradable blends prepared from poly(3-hydroxybutyrate) and wood/cellulose fibers. *Materiale Plastice,* 47:503-508.

Ray, S.S. & Okamoto, M. (2003) Polymer/layered silicate nanocomposites: A review from preparation to processing. *Progress in Polymer Science,* 28:1539-1641.

Rehm, B.H.A. (2010) Bacterial polymers: Biosynthesis, modifications and applications. *Nature Reviews Microbiology,* 8:578-592.

Riello, P., Fagherazzi, G., Canton, P., Clemente, D. & Signoretto, M. (1995) Determining the degree of crystallinity in semicrystalline materials by means of the Rietveld analysis. *Journal of Applied Crystallography,* 28:121-126.

Römling, U. (2002) Molecular biology of cellulose production in bacteria. *Research in Microbiology,* 153:205-212.

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

Ruka, D.R., Simon, G.P. & Dean, K.M. (2012) Altering the growth conditions of *Gluconacetobacter xylinus* to maximize the yield of bacterial cellulose. *Carbohydrate Polymers,* 89:613-622.

Ruka, D.R., Simon, G.P. & Dean, K.M. (2013) *In situ* modifications to bacterial cellulose with the water insoluble polymer poly-3-hydroxybutyrate. *Carbohydrate Polymers,* 92:1717-1723.

Saheb, D.N. & Jog, J.P. (1999) Natural fiber polymer composites: A review. *Advances in Polymer Technology,* 18:351-363.

Saito, T., Nishiyama, Y., Putaux, J.L., Vignon, M. & Isogai, A. (2006) Homogeneous suspensions of individualized microfibrils from tempo-catalyzed oxidation of native cellulose. *Biomacromolecules,* 7:1687-1691.

Sanchez-Garcia, M. & Lagaron, J. (2010) On the use of plant cellulose nanowhiskers to enhance the barrier properties of polylactic acid. *Cellulose,* 17:987-1004.

Sani, A. & Dahman, Y. (2010) Improvements in the production of bacterial synthesized biocellulose nanofibres using different culture methods. *Journal of Chemical Technology and Biotechnology,* 85:151-164.

Savenkova, L., Gercberga, Z., Nikolaeva, V., Dzene, A., Bibers, I. & Kalnin, M. (2000) Mechanical properties and biodegradation characteristics of PHB-based films. *Process Biochemistry,* 35:573-579.

Schenzel, K., Fischer, S. & Brendler, E. (2005) New method for determining the degree of cellulose I crystallinity by means of FT Raman spectroscopy. *Cellulose,* 12:223-231.

Schlufter, K., Schmauder, H.P., Dorn, S. & Heinze, T. (2006) Efficient homogeneous chemical modification of bacterial cellulose in the ionic liquid 1-*N*-butyl-3-methylimidazolium chloride. *Macromolecular Rapid Communications,* 27:1670-1676.

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

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,* 11:123-129.

Seifert, M., Hesse, S., Kabrelian, V. & Klemm, D. (2004) Controlling the water content of never dried and reswollen bacterial cellulose by the addition of water-soluble polymers to the culture medium. *Journal of Polymer Science Part A-Polymer Chemistry,* 42:463-470.

Sekar, S., Mahadevan, S., Vijayaraghavan, R., Mandal, A.B. & MacFarlane, D.R. (2013) Bioenergetics for the growth of *Staphylococcus lentus* in biocompatible choline salts. *Applied Microbiology and Biotechnology,* 97:1767-1774.

Sekar, S., Surianarayanan, M., Ranganathan, V., MacFarlane, D.R. & Mandal, A.B. (2012) Choline-based ionic liquids-enhanced biodegradation of azo dyes. *Environmental Science & Technology,* 46:4902-4908.

Shah, A.A., Hasan, F., Hameed, A. & Ahmed, S. (2008) Biological degradation of plastics: A comprehensive review. *Biotechnology Advances,* 26:246-265.

Shah, N., Ul-Islam, M., Khattak, W.A. & Park, J.K. (2013) Overview of bacterial cellulose composites: A multipurpose advanced material. *Carbohydrate Polymers,* 98:1585-1598.

Shan, H.X., Li, Z.J., Li, M., Ren, G.X. & Fang, Y.J. (2008) Improved activity and stability of *Pseudomonas capaci* lipase in a novel biocompatible ionic liquid, 1-isobutyl-3-methylimidazolium hexafluorophosphate. *Journal of Chemical Technology and Biotechnology,* 83:886-891.

Shen, X.Y., Ji, Y.L., Wang, D. & Yang, Q. (2010) Solubility of a high molecular-weight bacterial cellulose in lithium chloride/*N*,*N*-dimethylacetamide solution. *Journal of Macromolecular Science Part B-Physics,* 49:1012-1018.

Shi, Z.J., Zang, S.S., Jiang, F., Huang, L., Lu, D., Ma, Y.G. & Yang, G. (2012) *In situ* nano-assembly of bacterial cellulose-polyaniline composites. *RSC Advances,* 2:1040-1046.

Siró, I. & Plackett, D. (2010) Microfibrillated cellulose and new nanocomposite materials: A review. *Cellulose,* 17:459-494.

Son, H.J., Heo, M.S., Kim, Y.G. & Lee, S.J. (2001) Optimization of fermentation conditions for the production of bacterial cellulose by a newly isolated *Acetobacter* sp A9 in shaking cultures. *Biotechnology and Applied Biochemistry,* 33:1-5.

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,* 86:215-219.

Steinbüchel, A. & Lütke-Eversloh, T. (2003) Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. *Biochemical Engineering Journal,* 16:81-96.

Stevanic, J.S., Bergström, E.M., Gatenholm, P., Berglund, L. & Salmén, L. (2012) Arabinoxylan/nanofibrillated cellulose composite films. *Journal of Materials Science,* 47:6724-6732.

Stevens, E.S. (2002) How green are green plastics? *Biocycle,* 43:42-45.

Stoica-Guzun, A., Jecu, L., Gheorghe, A., Raut, I., Stroescu, M., Ghiurea, M., Danila, M., Jipa, I. & Fruth, V. (2011) Biodegradation of poly(vinyl alcohol) and bacterial cellulose composites by *Aspergillus niger*. *Journal of Polymers and the Environment,* 19:69-79.

Strobl, G.R. (2007) *The physics of polymers: Concepts for understanding their structures and behavior*: Springer.

Strobl, G.R. & Schneider, M. (1980) Direct evaluation of the electron-density correlation-function of partially crystalline polymers. *Journal of Polymer Science: Polymer Physics Edition,* 18:1343-1359.

Tajeddin, B. & Abdulah, L.C. (2010) Thermal properties of high density polyethylene-kenaf cellulose composites. *Polymers and Polymer Composites,* 18:195-199.

Tantratian, S., Tammarate, P., Krusong, W., Bhattarakosol, P. & Phunsri, A. (2005) Effect of dissolved oxygen on cellulose production by *Acetobacter* sp. *Journal of Scientific Research of Chulalongkorn University* 30:179-186.

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,* 4:372-388.

Tischer, P.C.S.F., Sierakowski, M.R., Westfahl, H. & Tischer, C.A. (2010) Nanostructural reorganization of bacterial cellulose by ultrasonic treatment. *Biomacromolecules,* 11:1217-1224.

Toda, K., Asakura, T., Fukaya, M., Entani, E. & Kawamura, Y. (1997) Cellulose production by acetic acid-resistant *Acetobacter xylinum*. *Journal of Fermentation and Bioengineering,* 84:228-231.

Tokoh, C., Takabe, K., Fujita, M. & Saiki, H. (1998) Cellulose synthesized by *Acetobacter xylinum* in the presence of acetyl glucomannan. *Cellulose,* 5:249-261.

Tokoh, C., Takabe, K., Sugiyama, J. & Fujita, M. (2002) CP/MAS C-13 NMR and electron diffraction study of bacterial cellulose structure affected by cell wall polysaccharides. *Cellulose,* 9:351-360.

Tomé, L.C., Pinto, R.J.B., Trovatti, E., Freire, C.S.R., Silvestre, A.J.D., Neto, C.P. & Gandini, A. (2011) Transparent bionanocomposites with improved properties prepared from acetylated bacterial cellulose and poly(lactic acid) through a simple approach. *Green Chemistry,* 13:419-427.

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,* 59:1498-1502.

Trovatti, E., Fernandes, S.C.M., Rubatat, L., Freire, C.S.R., Silvestre, A.J.D. & Neto, C.P. (2012) Sustainable nanocomposite films based on bacterial cellulose and pullulan. *Cellulose,* 19:729-737.

Trovatti, E., Oliveira, L., Freire, C.S.R., Silvestre, A.J.D., Neto, C.P., Pinto, J.J.C.C. & Gandini, A. (2010) Novel bacterial cellulose-acrylic resin nanocomposites. *Composites Science and Technology,* 70:1148-1153.

Ude, S., Arnold, D.L., Moon, C.D., Timms-Wilson, T. & Spiers, A.J. (2006) Biofilm formation and cellulose expression among diverse environmental *Pseudomonas* isolates. *Environmental Microbiology,* 8:1997-2011.

Uhlin, K.I., Atalla, R.H. & Thompson, N.S. (1995) Influence of hemicelluloses on the aggregation patterns of bacterial cellulose. *Cellulose,* 2:129-144.

Ul-Islam, M., Khan, T. & Park, J.K. (2012a) Nanoreinforced bacterial cellulose-montmorillonite composites for biomedical applications. *Carbohydrate Polymers,* 89:1189-1197.

Ul-Islam, M., Khan, T. & Park, J.K. (2012b) Water holding and release properties of bacterial cellulose obtained by in situ and ex situ modification. *Carbohydrate Polymers,* 88:596-603.

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

Vandamme, E.J., De Baets, S., Vanbaelen, A., Joris, K. & De Wulf, P. (1998) Improved production of bacterial cellulose and its application potential. *Polymer Degradation and Stability,* 59:93-99.

Verhoogt, H., Ramsay, B.A. & Favis, B.D. (1994) Polymer blends containing poly(3-hydroxyalkanoate)s. *Polymer,* 35:5155-5169.

Verschuren, P.G., Cardona, T.D., Nout, M.J.R., De Gooijer, K.D. & Van den Heuvel, J.C. (2000) Location and limitation of cellulose production by *Acetobacter xylinum* established from oxygen profiles. *Journal of Bioscience and Bioengineering,* 89:414-419.

Vijayaraghavan, R., Izgorodin, A., Ganesh, V., Surianarayanan, M. & MacFarlane, D.R. (2010a) Long-term structural and chemical stability of DNA in hydrated ionic liquids. *Angewandte Chemie-International Edition,* 49:1631-1633.

Vijayaraghavan, R., Thompson, B.C., MacFarlane, D.R., Kumar, R., Surianarayanan, M., Aishwarya, S. & Sehgal, P.K. (2010b) Biocompatibility of choline salts as crosslinking agents for collagen based biomaterials. *Chemical Communications,* 46:294-296.

Wan, Y.Z., Hong, L., Jia, S.R., Huang, Y., Zhu, Y., Wang, Y.L. & Jiang, H.J. (2006) Synthesis and characterization of hydroxyapatite-bacterial cellulose nanocomposites. *Composites Science and Technology,* 66:1825-1832.

Wan, Y.Z., Luo, H.L., He, F., Liang, H., Huang, Y. & Li, X.L. (2009) Mechanical, moisture absorption, and biodegradation behaviours of bacterial cellulose fibre-reinforced starch biocomposites. *Composites Science and Technology,* 69:1212-1217.

Wang, H.H., Zhu, E.W., Yang, J.Z., Zhou, P.P., Sun, D.P. & Tang, W.H. (2012) Bacterial cellulose nanofiber-supported polyaniline nanocomposites with flake-shaped morphology as supercapacitor electrodes. *Journal of Physical Chemistry C,* 116:13013-13019.

Wang, S.Q. & Cheng, Q.Z. (2009) A novel process to isolate fibrils from cellulose fibers by high-intensity ultrasonication, Part 1: Process optimization. *Journal of Applied Polymer Science,* 113:1270-1275.

Wang, Y., Luo, Q.P., Peng, B.H. & Pei, C.H. (2008) A novel thermotropic liquid crystalline - benzoylated bacterial cellulose. *Carbohydrate Polymers,* 74:875-879.

Watanabe, K., Tabuchi, M., Ishikawa, A., Takemura, H., Tsuchida, T., Morinaga, Y. & Yoshinaga, F. (1998a) *Acetobacter xylinum* mutant with high cellulose productivity and an ordered structure. *Bioscience Biotechnology and Biochemistry,* 62:1290-1292.

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

Watanabe, K. & Yamanaka, S. (1995) Effects of oxygen-tension in the gaseous-phase on production and physical-properties of bacterial cellulose formed under static culture conditions. *Bioscience Biotechnology and Biochemistry,* 59:65-68.

Way, C., Dean, K., Wu, D.Y. & Palombo, E. (2012) Biodegradation of sequentially surface treated lignocellulose reinforced polylactic acid composites: Carbon dioxide evolution and morphology. *Polymer Degradation and Stability,* 97:430-438.

Williams, W.S. & Cannon, R.E. (1989) Alternative environmental-roles for cellulose produced by *Acetobacter xylinum*. *Applied and Environmental Microbiology,* 55:2448-2452.

Winther-Jensen, O., Vijayaraghavan, R., Sun, J.Z., Winther-Jensen, B. & MacFarlane, D.R. (2009) Self polymerising ionic liquid gel. *Chemical Communications*3041-3043.

Woehl, M.A., Canestraro, C.D., Mikowski, A., Sierakowski, M.R., Ramos, L.P. & Wypych, F. (2010) Bionanocomposites of thermoplastic starch reinforced with bacterial cellulose nanofibres: Effect of enzymatic treatment on mechanical properties. *Carbohydrate Polymers,* 80:866-873.

Wu, Q., Wang, Y. & Chen, G.Q. (2009) Medical application of microbial biopolyesters polyhydroxyalkanoates. *Artificial Cells Blood Substitutes and Biotechnology,* 37:1-12.

Xie, Y.P., Noda, I. & Akpaiu, Y.A. (2008) Influence of cooling rate on the thermal behavior and solid-state morphologies of polyhydroxyalkanoates. *Journal of Applied Polymer Science,* 109:2259-2268.

Yamada, Y., Hoshino, K. & Ishikawa, T. (1997) The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: The elevation of the subgenus *Gluconoacetobacter* to the generic level. *Bioscience Biotechnology and Biochemistry,* 61:1244-1251.

Yamamoto, H., Horii, F. & Hirai, A. (1996) *In situ* crystallization of bacterial cellulose. II. Influences of different polymeric additives on the formation of celluloses I_α and I_β at the early stage of incubation. *Cellulose,* 3:229-242.

Yamanaka, S., Ishihara, M. & Sugiyama, J. (2000) Structural modification of bacterial cellulose. *Cellulose,* 7:213-225.

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

Yan, Z.Y., Chen, S.Y., Wang, H.P., Wang, B., Wang, C.S. & Jiang, J.M. (2008) Cellulose synthesized by *Acetobacter xylinum* in the presence of multi-walled carbon nanotubes. *Carbohydrate Research,* 343:73-80.

Yang, H.S., Gardner, D.J. & Nader, J.W. (2011) Dispersion evaluation of microcrystalline cellulose/cellulose nanofibril-filled polypropylene composites using thermogravimetric analysis. *Journal of Thermal Analysis and Calorimetry,* 103:1007-1015.

Yang, K.K., Wang, X.L. & Wang, Y.Z. (2007) Progress in nanocomposite of biodegradable polymer. *Journal of Industrial and Engineering Chemistry,* 13:485-500.

Yang, L., Zhang, H.Y., Yang, Q. & Lu, D.N. (2012) Bacterial cellulose-poly(vinyl alcohol) nanocomposite hydrogels prepared by chemical crosslinking. *Journal of Applied Polymer Science,* 126:E244-E250.

Yang, Y.K., Park, S.H., Hwang, J.W., Pyun, W.R. & Kim, Y.S. (1998) Cellulose production by *Acetobacter xylinum* BRC5 under agitated condition. *Journal of Fermentation and Bioengineering,* 85:312-317.

Yin, X., Yu, C., Zhang, X., Yang, J., Lin, Q., Wang, J. & Zhu, Q. (2011) Comparison of succinylation methods for bacterial cellulose and adsorption capacities of bacterial cellulose derivatives for Cu^{2+} ion. *Polymer Bulletin,* 67:401-412.

Yoon, S.H., Jin, H.J., Kook, M.C. & Pyun, Y.R. (2006) Electrically conductive bacterial cellulose by incorporation of carbon nanotubes. *Biomacromolecules,* 7:1280-1284.

Yoshinaga, F., Tonouchi, N. & Watanabe, K. (1997) Research progress in production of bacterial cellulose by aeration and agitation culture and its application as a new industrial material. *Bioscience Biotechnology and Biochemistry,* 61:219-224.

Yu, H.Y., Qin, Z.Y. & Zhou, Z. (2011) Cellulose nanocrystals as green fillers to improve crystallization and hydrophilic property of poly(3-hydroxybutyrate-co-3-hydroxyvalerate). *Progress in Natural Science: Materials International,* 21:478-484.

Zadorecki, P., Karnerfors, H. & Lindenfors, S. (1986) Cellulose fibers as reinforcement in composites: Determination of the stiffness of cellulose fibers. *Composites Science and Technology,* 27:291-303.

Zhang, W., Liang, M. & Lu, C. (2007) Morphological and structural development of hardwood cellulose during mechanochemical pretreatment in solid state through pan-milling. *Cellulose,* 14:447-456.

Zhang, W., Yang, X.L., Li, C.Y., Liang, M., Lu, C.H. & Deng, Y.L. (2011) Mechanochemical activation of cellulose and its thermoplastic polyvinyl alcohol ecocomposites with enhanced physicochemical properties. *Carbohydrate Polymers,* 83:257-263.

Zhou, J.P. & Zhang, L.N. (2000) Solubility of cellulose in NaOH urea aqueous solution. *Polymer Journal,* 32:866-870.

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 and Biotechnology,* 34:483-489.

Zhu, S.D., Wu, Y.X., Chen, Q.M., Yu, Z.N., Wang, C.W., Jin, S.W., Ding, Y.G. & Wu, G. (2006) Dissolution of cellulose with ionic liquids and its application: A mini-review. *Green Chemistry,* 8:325-327.

Zogaj, X., Bokranz, W., Nimtz, M. & Römling, U. (2003) Production of cellulose and curli fimbriae by members of the family *Enterobacteriaceae* isolated from the human gastrointestinal tract. *Infection and Immunity,* 71:4151-4158.

Zogaj, X., Nimtz, M., Rohde, M., Bokranz, W. & Römling, U. (2001) The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. *Molecular Microbiology,* 39:1452-1463.

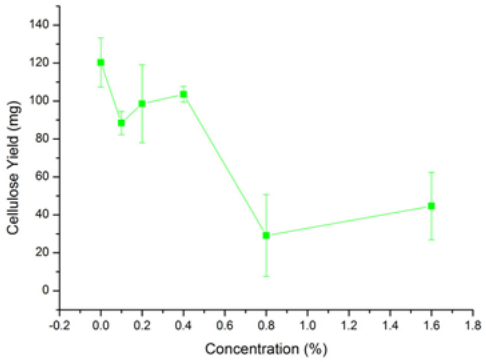
Appendix
Unpublished *in situ* Modificationsto Bacterial Cellulose

Living Polymers: *in situ* Modifications of Bacterial Cellulose

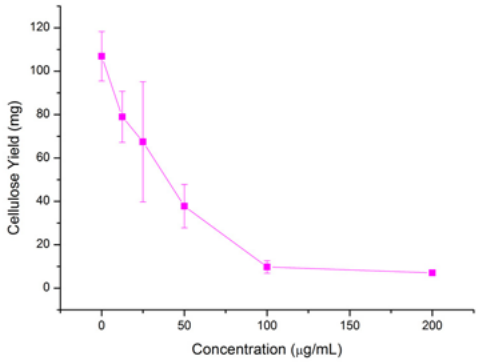
A large number of additives were originally selected from the literature to include in the base media for the growth of *Gluconacetobacter xylinus* in order to confirm the modifications that occur in the produced cellulose as reported by others. The additives were added to the media at various concentrations and the weight of the cellulose pellicles produced was recorded (Figure A.1). From this, one concentration of each additive was selected for further examination by SEM, XRD and FTIR. SEM micrographs for each additive are presented in Figure A.2.

This work was not published as a paper due to its lack of novelty, but it was completed as a poster and presented at the 32nd Australasian Polymer Symposium in 2011. The remaining findings are presented in the abstract and poster here.

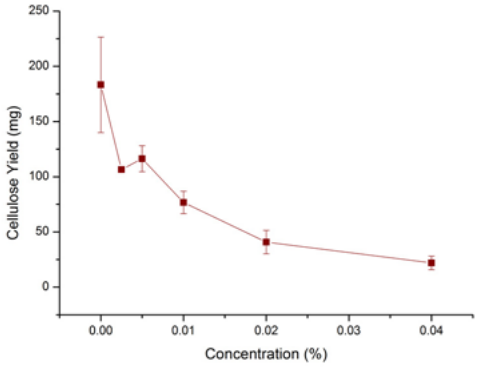
Agar



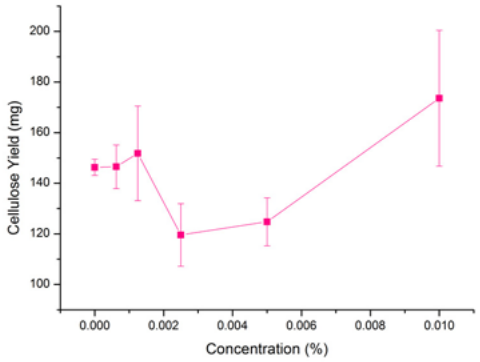
Chloramphenicol



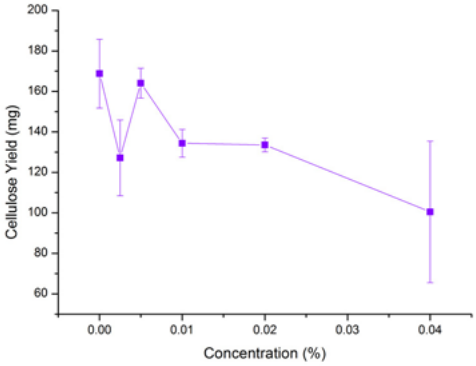
Congo red



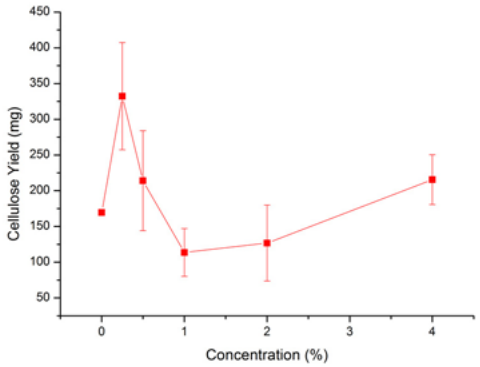
Coomassie brilliant blue



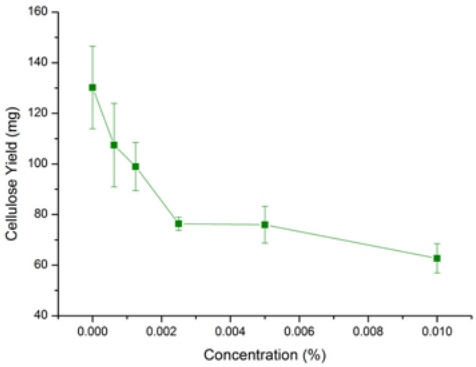
Dithiothreitol



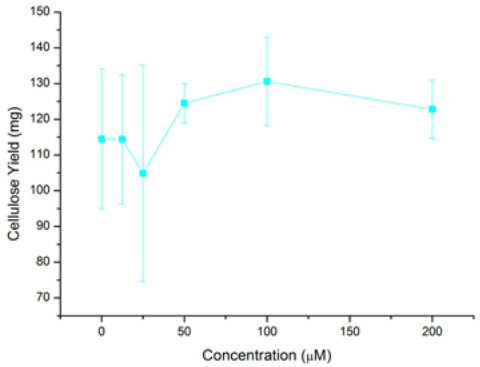
Ethanol



Fluorescent brightener 28



Iron Chloride



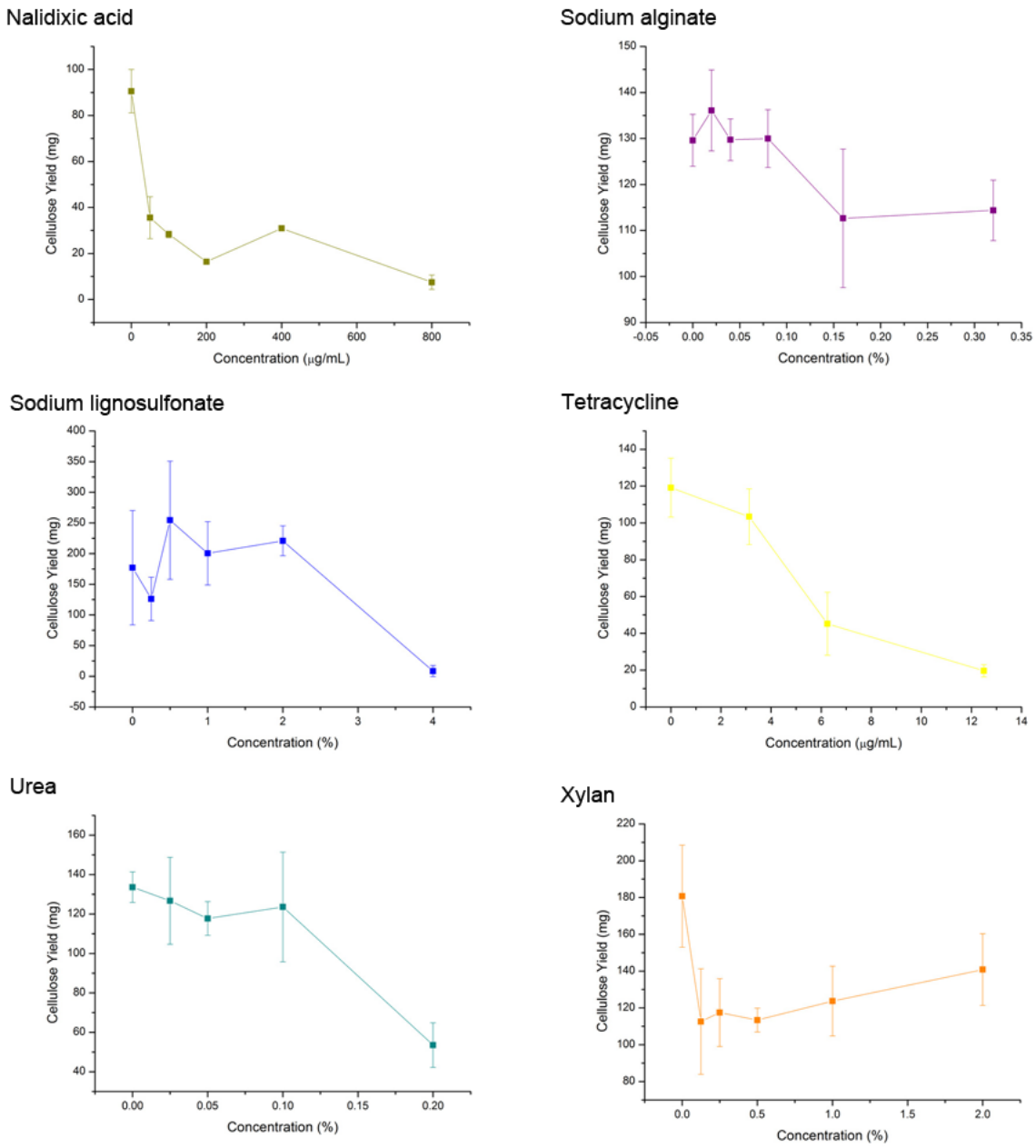
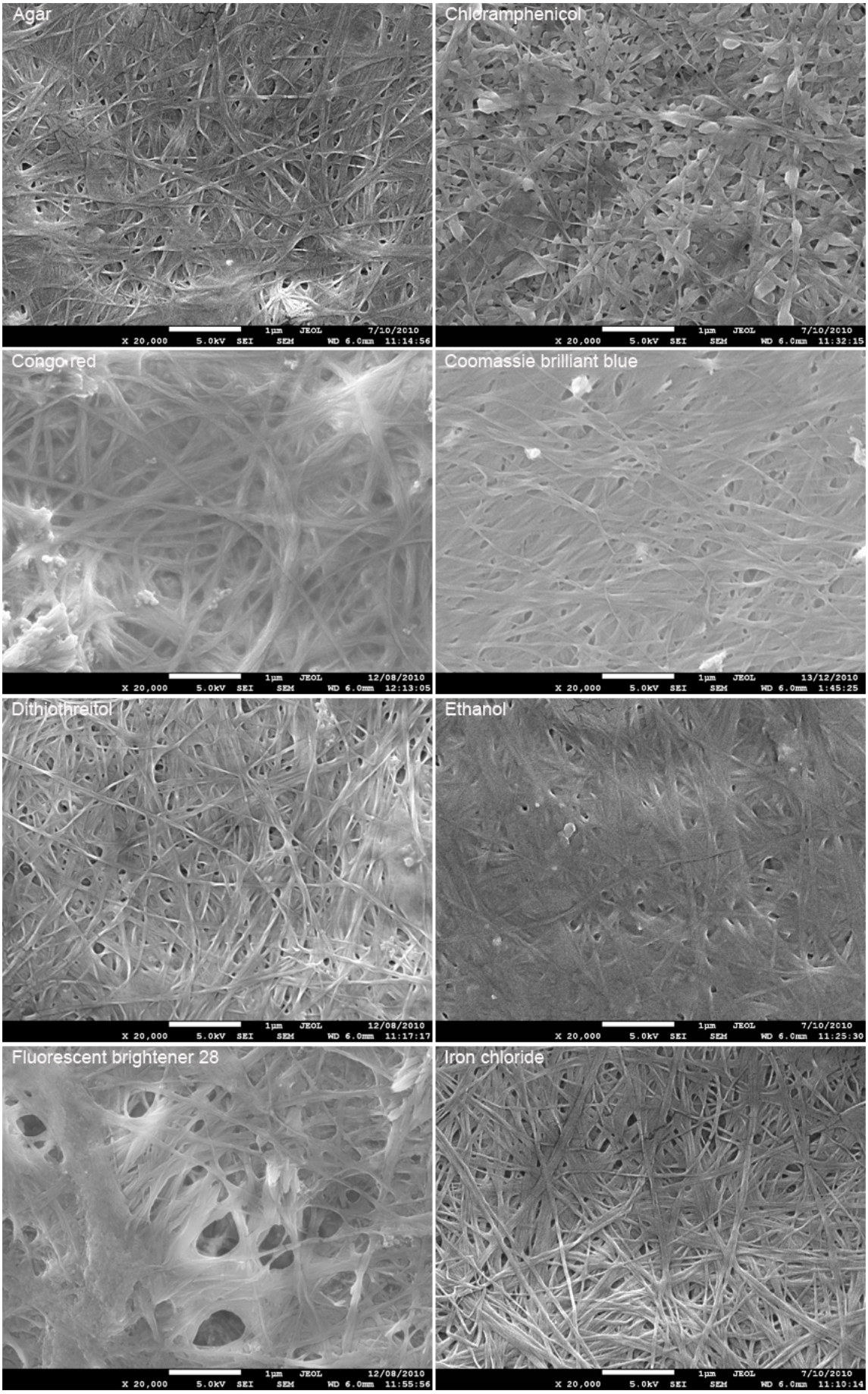


Figure A.1: The weights of bacterial cellulose pellicles grown with varying concentrations of additives in the media.


Agar
1µm JEOL 7/10/2010
X 20,000 5.0kV SEI SEM WD 6.0mm 11:14:56
Chloramphenicol
1µm JEOL 7/10/2010
X 20,000 5.0kV SEI SEM WD 6.0mm 11:32:15
Congo red
1µm JEOL 12/08/2010
X 20,000 5.0kV SEI SEM WD 6.0mm 12:13:05
Coomassie brilliant blue
1µm JEOL 13/12/2010
X 20,000 5.0kV SEI SEM WD 6.0mm 1:45:25
Dithiothreitol
1µm JEOL 12/08/2010
X 20,000 5.0kV SEI SEM WD 6.0mm 11:17:17
Ethanol
1µm JEOL 7/10/2010
X 20,000 5.0kV SEI SEM WD 6.0mm 11:25:30
Fluorescent brightener 28
1µm JEOL 12/08/2010
X 20,000 5.0kV SEI SEM WD 6.0mm 11:55:56
Iron chloride
1µm JEOL 7/10/2010
X 20,000 5.0kV SEI SEM WD 6.0mm 11:10:14

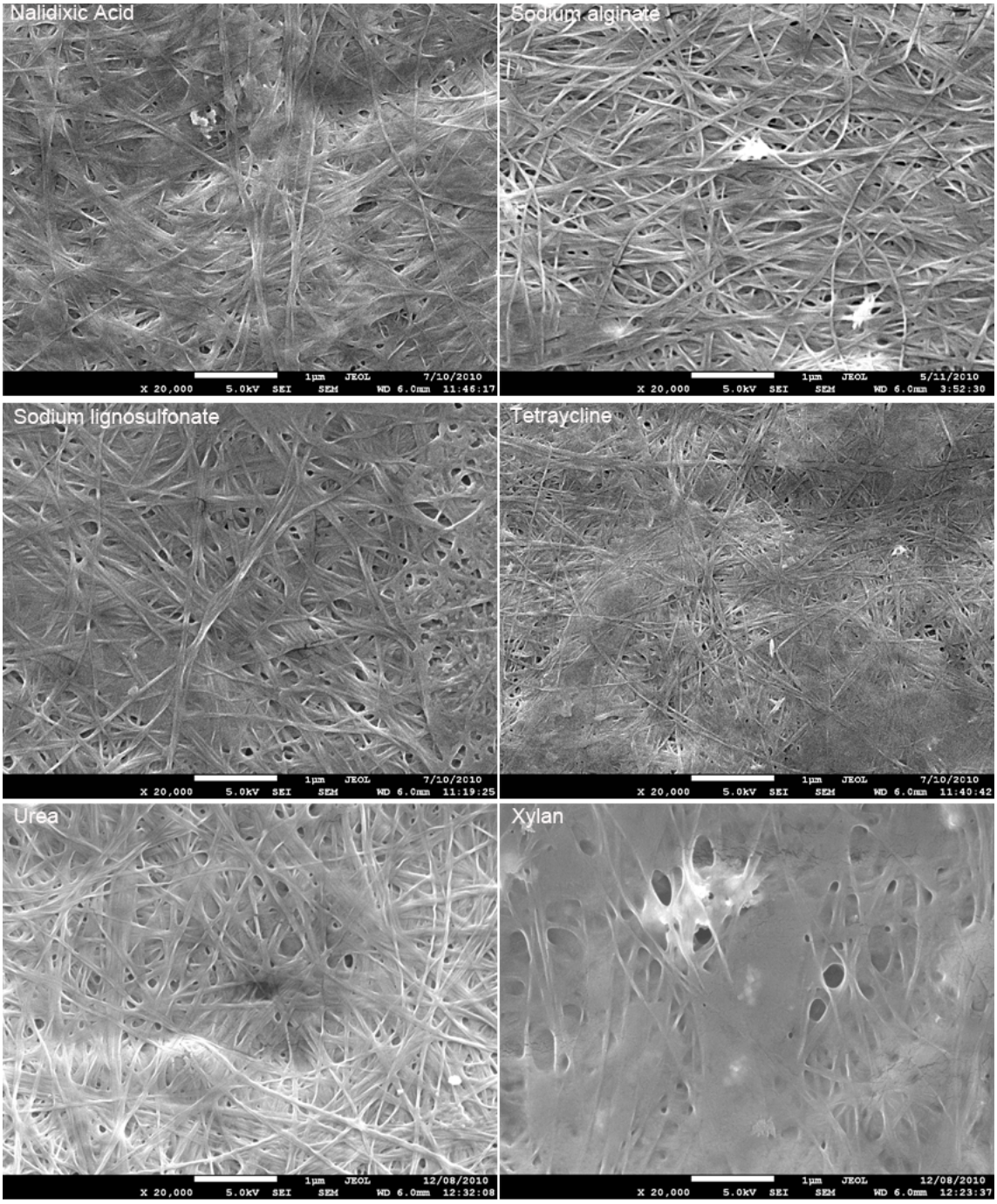


Figure A.2: SEM micrographs of bacterial cellulose fibrils produced with different additives in the media.

Living Polymers: *in situ* Modifications of Bacterial Cellulose

Dianne Ruka[1,2], *Parveen Sangwan*[1], *George Simon*[2], *Katherine Dean*[1*]

[1]CSIRO Materials Science and Engineering, Gate 5 Normanby Rd Clayton, Australia 3168
[2]Department of Materials Engineering, Monash University, Clayton, Australia 3800
[redacted]

Bacterial cellulose is a good candidate for use as a reinforcing agent in biodegradable polymers. Bacterial cellulose, produced by *Gluconacetobacter xylinus* exists as a network of randomly assembled ribbon-shaped fibrils as shown in Figure 1. Bacterial cellulose can be easily manipulated by changing the culture conditions[1, 2], and altering the composition of the growth medium can lead to changes in yield, structure and morphology of the bacterial cellulose produced[3], resulting in *in situ* modifications.

In this work we report the inclusion of additives to Hestrin-Schramm[4] media such as hydroxypropylmethyl cellulose (HPMC), Tween 80 and xylan, and show that they led to increased fibril widths (Figure 2), whereas if dithiothreitol and chloramphenicol were incorporated in the medium, thinner fibrils resulted (Figure 3). The general appearance of the cellulose was also affected by the inclusion of additives in the medium. HPMC as an additive resulted in particularly straight fibrils (Figure 2), whereas chloramphenicol caused an unusual appearance, with spherical shaped bodies on the fibrils (Figure 3). The inclusion of poly(3-hydroxybutyric acid) (PHB) in the growth medium at a range of concentrations led to thick and tough pellicles at higher concentrations of PHB. SEM micrographs (Figure 4) of this cellulose revealed a less porous surface when compared to cellulose grown in the absence of PHB. It is hypothesized that this material will have an increased compatibility to PHB for further composite work. Crystallite size and relative crystallinity were also examined.

Using cellulose produced from the determined media, PHB/bacterial cellulose composites were produced using solvent cast methods and characterized.

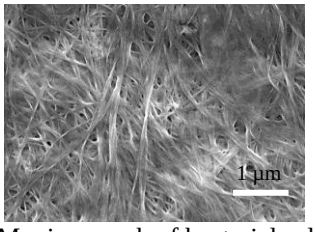


Figure 1. SEM micrograph of bacterial cellulose.

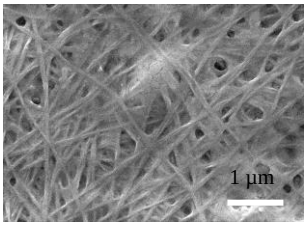


Figure 2. SEM micrograph of bacterial cellulose grown in a medium containing HPMC.

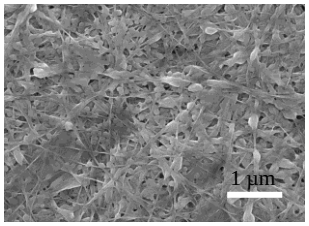


Figure 3. SEM micrograph of bacterial cellulose grown in a medium containing chloramphenicol.

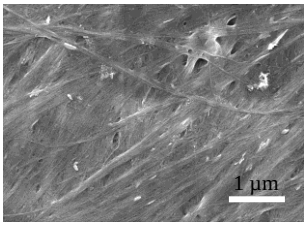


Figure 4. SEM micrograph of bacterial cellulose grown in a medium containing dissolved PHB.

[1] K. Watanabe, M. Tabuchi, Y. Morinaga, F. Yoshinaga, *Cellulose*, **1998**, *5*, 187-200
[2] W. Czaja, D. Romanovicz, R.M. Brown, *Cellulose*, **2004**, *11*, 403-411
[3] A. Hirai, M. Tsuji, H. Yamamoto, F. Horii, *Cellulose*, **1998**, *5*, 201-213
[4] M. Schramm, S. Hestrin, *J. Gen. Microbiol.*, **1954**, *11*, 123-129

Dianne Ruka
Ms
CSIRO and Monash University, Australia
[redacted]

Personal History
Since 2010 PhD at CSIRO on biocomposites from bacterial cellulose
Research interests: The interface of microbiology with materials science

Living Polymers: *in situ* Modifications of Bacterial Cellulose

Dianne Ruka[1,2], Parveen Sangwan[1], George Simon[2], Katherine Dean[1]

[1]CSIRO Materials Science and Engineering, Gate 5 Normanby Rd Clayton, Australia 3168
[2]Department of Materials Engineering, Monash University, Clayton, Australia 3800

Introduction

Bacterial cellulose is a good candidate for use as a reinforcing agent in biodegradable polymers. It is produced by *Gluconacetobacter xylinus* and exists as a network of randomly assembled ribbon-shaped fibrils as shown in Figure 1. Bacterial cellulose can be easily manipulated by changing the culture conditions[1, 2]. Altering the composition of the growth medium can lead to changes in yield, structure and morphology of the bacterial cellulose produced[3], resulting in *in situ* modifications.

Materials & Methods

Bacterial cellulose was produced from *G. xylinus* ATCC53524 in Hestrin-Schramm media[4] with different additives included at a variety of concentrations. Cellulose pellicles were harvested after seven days and were washed and boiled in 0.1M NaOH to remove bacterial cells. Air-dried films were examined by SEM, FTIR and XRD.

Results & Discussion

Morphology

- SEM of the cellulose pellicles revealed that the cellulose maintained its nanofibrillar structure regardless of the additives included in the media, however differences were observed in the width of the fibrils (see Table 1) and in the general morphology.
- The inclusion of additives to Hestrin-Schramm[4] media such as hydroxypropylmethyl cellulose (HPMC), Tween 80 and xylan led to increased fibril widths. HPMC as an additive resulted in particularly straight fibrils (Figure 2).
- The inclusion of additives dithiothreitol or chloramphenicol resulted in thinner fibrils. Chloramphenicol as an additive caused an unusual appearance, with spherical shaped bodies on the fibrils (Figure 3).
- The inclusion of poly(3-hydroxybutyric acid) (PHB) in the growth medium at a range of concentrations led to thick and tough pellicles at higher concentrations of PHB. SEM micrographs (Figure 4) of this cellulose revealed a less porous surface when compared to cellulose grown in the absence of PHB, and PHB granules on the surface. It is hypothesized that this material will have an increased compatibility to PHB for further composite work.

Figure 1: SEM micrograph of bacterial cellulose without any additives in the medium.

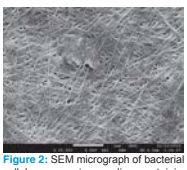


Figure 2: SEM micrograph of bacterial cellulose grown in a medium containing HPMC.

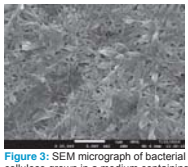


Figure 3: SEM micrograph of bacterial cellulose grown in a medium containing chloramphenicol.

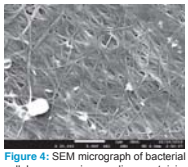


Figure 4: SEM micrograph of bacterial cellulose grown in a medium containing PHB.

Structural Characteristics

- Crystallite sizes and cellulose I_α content did not vary greatly between the different conditions.
- Crystallinity varied from 50 to 87% (with the inclusion of additives ethanol, and chloramphenicol and sodium alginate respectively) indicating that additives in the media affect the crystallinity of the resulting bacterial cellulose pellicle.
- Fibril widths varied from 26 to 65 nm with additives chloramphenicol and HPMC respectively, however fibril widths were extremely variable.

Table 1: Structural characteristics for cellulose produced in HS media containing additives.

Additive	Concentration	Crystallite size** (nm)	Crystallinity *** (%)	Cellulose I_α content (%)	Fibril width (nm)
Control	-	6.9	83	68	40
Agar	0.4%	6.5	80	68	40
Chloramphenicol	25 µg/mL	6.7	87	66	26
Congo Red	0.005%	7.1	76	70	50
Coomassie Brilliant Blue	0.00125%	*	*	67	30
Dithiothreitol	0.01%	*	*	67	28
Ethanol	0.5 %	7.0	50	78	43
Fluorescent Brightener 28	0.00125%	7.1	84	68	42
HPMC	1%	6.0	65	70	65
Iron Chloride	100 µM	7.0	84	65	38
Nalidixic Acid	50 µg/mL	6.1	78	*	35
PHB	0.5%	7.7	85	69	46
Sodium Alginate	0.04%	7.1	87	67	47
Sodium Lignosulfonate	0.5%	7.7	72	74	43
Tetracycline	3 µg/mL	6.2	86	*	35
Tween 80	0.1%	*	*	68	56
Urea	0.1%	7.3	79	86	44
Xylan	1%	*	*	66	46

*results yet to be done, **±2% error, ***±5% error

Solvent Cast Composites

The creation of solvent cast composites is currently in progress.

Conclusion

Determining a relationship between the composition of the media used to grow *G. xylinus* and the specific structure of the cellulose produced will provide the ability to tailor-design cellulose fibres with required traits for reinforcement in composites.

MONASH University

References

[1] K. Watanabe, M. Tabuchi, Y. Morinaga, F. Yoshinaga, *Cellulose*, **1998**, *5*, 187-200
[2] W. Czaja, D. Romanovicz, R.M. Brown, *Cellulose*, **2004**, *11*, 403-411
[3] A. Hirai, M. Tsuji, H. Yamamoto, F. Horii, *Cellulose*, **1998**, *5*, 201-213
[4] M. Schramm, S. Hestrin, *J. Gen. Microbiol.*, **1954**, *11*, 123-129

Acknowledgements

This work is funded by a Julius Career Award from the CSIRO Office of the Chief Executive.

Further information

contact: Katherine Dean
[redacted]
web: http://www.csiro.au/org/CMSE
www.csiro.au