Production of Bacterial Cellulose Using Low-cost Media

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Abstract

Bacterial cellulose (BC) is a polymer of glucose monomers, which has unique properties including high crystallinity and high strength. It has the potential to be used in biomedical applications such as making artificial blood vessels, wound dressings, and in the paper making industry. Unlike cellulose from plant sources, it is not contaminated with non-cellulose compounds, making it a candidate for medical use. The aim of this thesis was to optimize BC production using the Gram negative bacterium *Gluconacetobacter xylinus* DSM 46604, including identifying cheaper ingredients for the culture media. Initial trials were done on solid media and in shake flasks. Trials were then scaled and done in 3-L and 5-L conventional bioreactors. Three different processing strategies were used in the bioreactors: batch, fed-batch and continuous.

The morphology of the BC depended on the growth conditions. Thin sheets were formed in stationary cultures and pellicles were formed in agitated cultures. The scanning electron microscope micrographs showed that BC produced under static culture tends to be more densely packed than when produced in agitated shake flasks.

Exploratory trials on agar slants and in agitated shake flasks using glucose, sucrose, and lactose showed that *G. xylinus* DSM 46604 grew well on glucose and produced BC. However, there was minimal growth on the other two carbohydrates. Further trials with initial glucose concentrations between 40 and 100 g/L were done in shake flasks. Glucose concentration did not affect the BC morphology. The maximum BC concentration of 1.13 g/L was produced using 50 g/L glucose. The BC concentration using 100 g/L glucose was only 0.96 g/L.

Shake flask studies with 2 to 9 g/L yeast extract (YE) as a nitrogen source in the media showed the maximum BC concentration of 5.2 g/L was obtained using 5 g/L YE with 50 g/L of glucose. Increasing the YE to 7 or 9 g/L produced only 4.82 and 4.06 g BC/L respectively. The effect of two cheaper nitrogen sources, fish hydrolysate and fish powder prepared from waste fish, were investigated. The highest BC concentration of 0.24 g/L was obtained using 20 g/L fish hydrolysate rather than 5 g/L YE. The BC yield of 0.04 g BC /g carbon substrate used were obtained using 5 g/L YE, 20 g/L fish hydrolysate, or 15 or 20 g/L fish powder.

The effectiveness of four combinations of banana peel (as a cheaper carbon source) and glucose were investigated in shake flasks trials. The highest

BC concentration of 0.43 g/L was obtained using 10 g/L banana peel extract with 40 g/L glucose. This was similar to the BC concentration produced with 50 g/L glucose (control). Trials using the same combination of banana peel and glucose in a 3-L bioreactor produced 1 g/L BC compared with 2.2 g/L for 50 g/L glucose (control).

Shake flask fermentations using 10 to 50 g/L glycerol as the carbon source showed that the highest BC concentration of 1.43 g/L was produced with an initial glycerol of 20 g/L. Trials done in a 3-L bioreactor produced 2.87 g/L of BC, representing a yield of 0.15 g/g carbon substrate used.

The effect of aeration and agitation on BC production was studied in 3- and 5-L bioreactors. The optimal agitation was 200 rpm at constant air flow rate of 0.3 volume air per volume culture broth per minute (vvm). This produced 4.0 g/L BC and a yield of 0.06 g/g glucose. The optimal aeration rate at 150 rpm was 1.0 vvm and produced 4.4 g/L BC.

Various fermentation strategies were then investigated. The control was batch fermentation on 50 g/L glucose in a 3- or 5-L fermenter. All runs were done at 30^{O}C, 200 rpm and 1 vvm aeration. The BC yield when *G. xylinus* DSM 46604 was grown on 50 g/L glucose using a fill-and-draw fed-batch strategy was 0.05 g/g glucose or glycerol used, which was similar to the control. The BC yield increased to 0.11 g/g when using a pulse-feed fed-batch strategy but the BC yield in continuous fed-batch was only 0.03 g/g. It increased under continuous fermentation conditions and the highest yield (0.13 g/g) was achieved at a dilution rate of 0.1 h^{-1}. If dilution rate was increased further, yields began to decrease.

Trials were done by replacing 50 g/L glucose with 20 g/L glycerol. Again, BC yields were higher under continuous conditions than batch fermentation. The BC yield on 20 g/L using a fill-and-draw fed-batch strategy was 0.2 g/g BC compared with 0.15 g/g for the control. This increased to 0.39 g/g for a pulse-feed fed-batch strategy. The BC yield for continuous fed-batch at a dilution rate of 0.1 h^{-1} was 0.3 g/g. The highest BC yield under continuous conditions was 0.33 g/g when dilution rate was 0.1 h^{-1}.

These studies showed that cheaper ingredients such as fish powder or hydrolysate and banana peel extract could partially replace conventional nitrogen and carbon sources such as YE and glucose without affecting BC yield. The BC production was enhanced using fed-batch and continuous processing strategies. Higher BC yields than reported by much of the literature could be obtaining ujsing a combination of low-cost media ingredients and the best reactor conditions.

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Abbreviations

Å	Angstrom
AAB	Acetic acid bacteria
BC	Bacterial Cellulose
bcsA	cellulose synthesis operon gene, encodes cellulose synthase
bcsB	cellulose synthesis operon gene, encodes cellulose synthase
bcsC	cellulose synthesis operon gene in ‘in-vivo’ cellulose production
bcsD	cellulose synthesis operon gene in cellulose crystallization
c-di-GMP	cyclic diguanylate
$Ca_3(PO_4)_2$	tricalcium phosphate
Cel^-	non-cellulose producing cells
CCH	coffee cherry husk
CesA	cellulose synthase
CFB	continuous fed-batch
CMC	carboxymethylcellulose
CSC	cellulose synthase complex
CSL	corn steep liquor
CSL-Fru	corn steep liquor-fructose
CSTR	continuous stirred tank reactors
D-glucose	dextrorotatory glucose
DFC	direct fixed capital
DO	dissolved oxygen
DP	degree of polymerization
ESO	epoxidized soybean oil
G. xylinus	*Gluconacetobacter xylinus*
Hap	hydroxyapatite
HS	Hestrin-Schramm
I_α	crystalline phase with triclinic crystal structure
ICI	Imperial Chemical Industries
K_{La}	oxygen transfer coefficient
K_2HPO_4	dipotassium phosphate
KH_2PO_4	monopotassium phosphate
MSG	monosodium glutamate
NaAlg	sodium alginate
OTR	oxygen transfer rate

PC	purchase cost
PCS	plastic composite support
PLA	polylactic acid
PQQ-ADH	pyrroquinoline quinone-dependent alcohol dehydrogenase
PVA	polyvinyl alcohol
S1	secondary wall layer 1
S2	secondary wall layer 2
S3	secondary wall layer 3
SEM	scanning electron microscopy
TEA	triethanolamine
TMHs	transmembrane helixes
UDPG	uridine diphosphate glucose
μ_{max}	maximum specific growth rate
μ_{net}	net specific growth rate
μ_R	net specific replication rate
$Y_{P/S}$	mass of product formed per unit mass of substrate utilized
$Y_{X/S}$	mass of cells formed per unit mass of substrate utilized
YE	yeast extract

Chapter 1: Introduction

1.1 Overview

Cellulose, a glucose-based polysaccharide that makes up a large fraction of plant cell walls, is used as a raw material for pulp and paper (from trees) and clothing (as cotton). It has a very rigid structure because a cellulose bundle has approximately 36 hydrogen-bonded chains, each with 500 to 14,000 β-l, 4-linked glucose molecules (Somerville, 2006).

Plant-derived cellulose usually contains contaminants in the form of lignin, pectin and hemicellulose (Brown, 1886; Embuscado *et al.*, 1994; Delmer *et al.*, 1999; Jung *et al.*, 2005). These contaminants can be removed by various mechanical and chemical processes but this processing weakens the structure and/or decreases potential yields. Purer cellulose is produced by several bacteria. The most frequently used species is *Acetobacter xylinum*, which has been reclassified and included within the genus *Gluconacetobacter* as *G. xylinus* (Yamada *et al.*, 1997; Yamada, 2000).

Bacterial cellulose (BC), which is often produced as discrete particles, has high crystallinity, high mechanical strength and higher purity than plant-based cellulose. It does not contain lignin, hemicellulose or other contaminants present in plant-based cellulose (Bielecki *et al.*, 2005; Castro *et al.*, 2011). These excellent properties can be used in making artificial skin (Fontana *et al.*, 1990), electronic paper and composite reinforcement (Jonas and Farah, 1998). Because the BC pellicles form on the surface (Watanabe *et al.*, 1998), much of the research on producing BC has been done in static culture. However, economics of any fermentation systems are improved by increasing the scale or using aerated and agitated systems (Goelzer *et al.*, 2009).

The main disadvantage of BC is that it is a relatively expensive and therefore unlikely to be a substitute for traditional sources of cellulose. The cost of media contributes significantly to fermentation costs, especially if standard commercial media is used. Much research on producing BC utilizing low-cost components has been done to identify how to reduce BC production cost (Keshk *et al.*, 2006). Also, to meet the requirement for industrial applications, large-scale BC production needs to be developed, which involves improving fermentation conditions and identifying BC-producing strains (Vandamme *et al.*, 1998; Wu *et*

al., 2008). To obtain high productivity, it is important to optimize reactor conditions such as operating temperature and pH, dissolved oxygen (DO) and substrate concentration. Productivity is important because it ensures efficient utilization of production capacity (i.e. the bioreactor, ancillary equipment and services, and process time). If end-product concentration is low, it will be expensive to obtain product of sufficient purity (Nielsen, 2002).

The way a fermentation process is run depends on whether the desired end-product is the cells, a primary metabolite (either extra or intracellular) or a secondary metabolite. The latter often involves optimising cell growth and then identifying environmental conditions that favour secondary metabolite production yet limit further cell growth.

1.2 Significance of Research

There are many possible uses for BC, based on its special properties. Most of the BC has been produced using media made from commercially-available components, which are expensive, and consequently require many additional resources for cultivation. The main aim of this study is to investigate growth of a suitable microbe that produces BC and then investigating alternative, cheaper carbon and nitrogen sources for the media so a simple, inexpensive fermentation process for producing BC in aerated and agitated culture can be developed.

1.3 Objectives

The broad objectives of this study are:

- To identify a suitable microbe for large-scale BC production and effective ways for maintaining the stock culture
- To identify low-cost carbon and nitrogen sources that could be used in the growth media, and to develop an optimal media
- To optimize fermentation conditions and reduce BC production cost.

The literature is used to identify the best way to achieve these objectives.

1.4 Thesis Outline

The thesis is subdivided into the following chapters:

Chapter 1: Briefly introduces the background to cellulose, cellulose sources and applications. It also outlines the reasons for using BC as a bio-based polymer when manufacturing eco-friendly materials.

Chapter 2: This chapter is a critical review of the literature on BC research. Basic concepts about cellulose such as sources, crystalline structure, and the differences between plant cellulose and BC are discussed in detail. The history, properties, biochemical pathway and applications of BC are discussed, including a review of the factors affecting production of BC using *G. xylinus* bacterium.

Chapter 3: This chapter gives details on the research materials, methodology and trials done.

Chapter 4: This chapter describes how to preserve and maintain the Gram-negative *G. xylinus* strain free of contamination and how to produce BC pellicles under static and agitated cultivation. This chapter also discusses the effect of glucose and yeast extract concentrations, the effect of different nitrogen and carbon sources on BC production in shake flasks and bioreactor. Also, reactor conditions (i.e., agitation and aeration) on *G. xylinus* growth, glucose consumption, and BC production are investigated and discussed. Cost evaluation was evaluated.

Chapter 5: This chapter describes the results obtained when investigating reactor operations (i.e., fill-and-draw fed-batch, pulse feed fed-batch, continuous fed-batch (CFB) and continuous fermentation) on growth of *G. xylinus* and substrate consumption, and BC production. It also outlines the cost implications in improving BC yield.

Chapter 6: Overall conclusions and recommendations for further research are presented in this chapter.

All references used are collated in the Reference section and calibration data are given in the Appendices.

Chapter 2: Cellulose Structure, Properties, Applications and Sources

This chapter reviews the literature on cellulose structure, properties, applications and sources. It then describes bacterial cellulose (BC), a biomaterial with unique properties including high water capacity, high crystallinity, high mechanical strength and biocompatibility. These properties support a wide range of applications for human use, in medical fields, and in the food, paper and textile industries. The characteristics of *G. xylinus*, a bacterium that produces BC, are described. It is hypothesized that BC production is expensive due to the cost of the commercial culture media. This research aims to substitute components in the media such as glucose and yeast extract, to reduce culture media cost and hence decrease BC production cost.

2.1 Cellulose

The French chemist Anselme Payen (1795-1871) first used the term "cellulose" to describe the fibrous material from the cell walls of higher plants (Iguchi *et al.*, 2000) remaining after purifying various plant tissues with an acid-ammonia treatment. Elemental analysis showed that the formula of the substance was $C_6H_{10}O_5$ and that this was an isomer of starch, a polymer of glucose molecules joined by 1,6-α glycoside bonds polymer. Cellulose was found to be a linear polysaccharide made from repeating D-glucose pyranose units linked by 1, 4-β glycoside bonds (Figure 2.1). The chains are approximately 0.3 nm wide (Ioelovish, 2008).

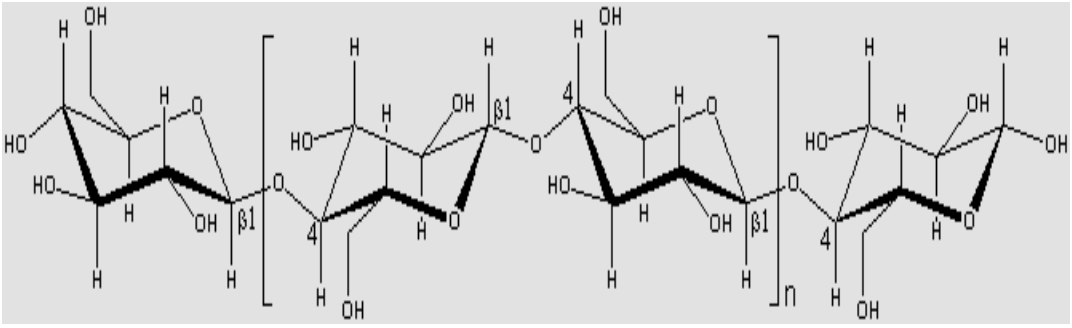

Figure 2.1: The 1,4-β glycoside chain of cellulose (Helbert *et al.*, 1996).

Cellulose is the most abundant natural biopolymer in the world and is produced by a variety of organisms ranging from vascular plants to algae and

prokaryotic organisms such as cyanobacteria and even animals (Jonas and Farah, 1998; Nobles *et al.*, 2001). It is estimated that a billion metric tonnes of cellulose is produced annually (Kwok and Wong, 2003). Forms of cellulose have been used for thousands of years and it has been extensively studied, yet there is still much to investigate about cellulose and its synthesis. This natural polymer is renewable, biodegradable, biocompatible, and has advantages such as having a low density, high modulus and high strength, being difficult to damage during processing, having few requirements for processing equipment, and is cheap raw material (Zadorecki and Michell, 1989; Joly *et al.*, 1996).

Cellulose is mainly used in packaging but is also used in many other applications such as clothing, pharmaceuticals, the automobile industry, shipping sails, and ropes. Plant cellulose has long been used in a variety of medical technologies, ranging from cotton for hemostatic wound dressings to sutures and renal dialysis membranes (Hoenich, 2006). Use of cellulose in composites has increased because it is relatively cheap compared to conventional fibre materials such as glass and aramid fibres, can be burned with energy recovery, and can compete in terms of mechanical behaviour (Eichhorn *et al.,* 2001). To extend its applications and to obtain better physical and chemical properties, cellulosic materials have been converted into various derivatives including acetate cellulose, cellulose sulphate, cellulose palmitate, and cellulose-4-nitrobenzoate (Heinze and Glasser, 1996).

Recent work showed that BC has a unique network structure of randomly assembled ribbon shaped nanofibres, increasing the scientific interest of using BC as reinforcement in polymers. It is also been incorporated into hydroxyapatite (Hap), polylactic acid (PLA), polyvinyl alcohol (PVA), and cellulose acetate butyrate (CAB) and used as a hybrid material in apple and radish pulp (Gindl and Keckes, 2004; Million and Wan, 2006; Wan *et al.*, 2006).

2.2 Sources of Cellulose

Cellulose is usually isolated from plant cell walls. The common commercial sources of cellulose include wood pulp and cotton linters (the short fibre remaining on cotton seed after the long fibres are removed). It can also be produced from other plant fibres, such as corn cobs or stalks, soybean hulls, bagasse (sugar cane stalks), oat hulls, rice hulls, wheat straw, sugar beet pulp, bamboo, and fibres such as jute, flax and ramie (Franz, 1990; Hanna, 2001).

Woody matter, seed hairs, bast, and even marine plants and peat are important sources of cellulose. Cotton fibres are about 98% cellulose (Table 2.1) and only need to be treated with hot sodium hydroxide solution to remove protein, pectic substances and wax to produce high quality cellulose. Wood, on the other hand, is 40-50% cellulose and requires extensive processing to dissolve the hemicellulose and lignin (Ockerman, 1991; Whistler and Be Miller, 1997).

Table 2.1: Chemical composition of some typical cellulose-containing materials (Klemm *et al*., 2003).

	Composition (%)			
Sources	**Cellulose**	**Hemicellulose**	**Lignin**	**Extract**
Hard wood	43-47	25-35	16-24	2-8
Soft wood	40-44	25-29	25-31	1-5
Bagasse	40	30	20	10
Coir	32-43	10-20	43-49	4
Corn cobs	45	35	15	5
Corn stalks	35	25	35	5
Cotton	95	2	1	0.4
Flax (retted)	71	21	2	6
Flax (unretted)	63	12	2	6
Hemp	70	22	6	2
Henequen	78	4-8	13	4
Istle	73	4-8	17	2
Jute	71	14	13	2
Kenaf	36	21	18	2
Ramie	76	17	1	6
Sisal	73	14	11	2
Sunn hemp	80	10	6	3
Wheat straw	30	50	15	5

Cellulose has been reported in the animal kingdom. Material, called tunicin or animal cellulose, is obtained from the outer mantles of certain marine organism belonging to the class of *Tunicata* (Ott *et al.,* 1954). Cellulose can also be produced by different microbes, including algae and fungi. Microorganisms belonging to the genera *Acetobacter, Achromobacter, Agrobacterium, Arobacter, Pseudomonas, Rhizobium* and *Sarcinar* can secrete BC, which has much higher crystallinity and chemical purity than plant cellulose (Premjet *et al.,* 2007).

Acetobacter, one of the most advanced types of non-photosynthetic bacteria can convert glucose, sugar, glycerol, or other organic substrates into pure cellulose (Brown *et al.*, 1976). Ott and coworkers (1954) reported that *Acetobacter xylinum*, now reclassified as *Gluconacetobacter xylinus* (Yamada *et al.*, 1997), is the only species capable of producing cellulose in commercially viable quantities. This cellulose-producing bacterium is used as a model organism for cellulose biosynthesis as it extrudes cellulose microfibrils from the terminal complexes into the incubation media. This means the microfibrils are affected by reagents added to the media (Tokoh *et al.*, 2002). Brown (1989) reports that "a single *G. xylinus* cell can convert up to 108 glucose molecules per hour into cellulose." Glucan chain polymers are spun into a ribbon from the linear pores on the *G. xylinus* cells. Manufacturing the cellulose ribbons involves many activities; each cell acts as a nano-spinneret and produces the sub-microscopic fibrils. A gelatinous membrane (called a pellicle) of pure cellulose is formed when the microfibrils intertwine. Cells entrapped in the cellulose membrane can be removed. The dried cellulose, which has high mechanical strength and water-holding capacity, can be used for many applications (Brown, 1989; White and Brown, 1989; Brown 1992; Brown, 1994).

2.2.1 Plant Cellulose

The main source of cellulose is plants. The degree of polymerization (DP), or number of monomer units in the cellulose polymer, is about 10,000 (Fengel and Wegener, 1983). The hydroxyl groups of cellulose form intra- and inter-molecular hydrogen bonds that hinder free rotation of the ring, and stiffen the chain. The hydrogen bonds also contribute to cellulose chains being insoluble in common solvents. Cellulose is a hydrophilic polymer; the three main hydroxyl groups per glucosidic unit are available for water adsorption (Saka, 2001).

Cellulose synthesis is central to a plant's developmental. The organized structure of the microfibril is a directly due to organization of the protein complex synthesising the cellulose. The plasma membrane-bound cellulose synthase complex (CSC) is a large protein complex consisting of multiple copies of the three different cellulose synthase CesA proteins, which are essential to performing specific functions, such as interacting with the cortical microtubules (Somerville, 2006).Taylor (2008) reports that, CSC can be visualized at the plasma membrane

in the freeze-fracture electron microscopy as hexameric structure, which gives rise to them, being known as rosettes (Figure 2.2).

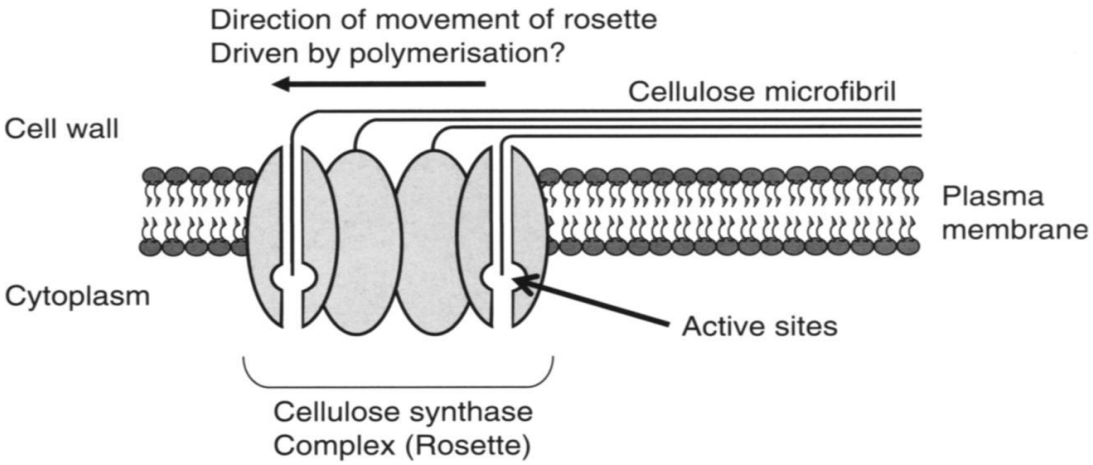

Figure 2.2: Cellulose synthase rosette (Taylor, 2008).

Rosettes are assembled in the Golgi bodies and then transported to the plasma membrane (Brown, 1986; Doblin *et al.*, 2002). Figure 2.3 showed the model of the structure, which consists of six subunits. The subunits possibly contain six CesA polypeptides, which will interact to form a rosette. Each CesA is involved in synthesis of one β-(1, 4)-glucan chain. The CesA has eight predicted TMHs (transmembrane helix), each of which can potentially form a pore in the plasma membrane to extrude the nascent chain into the wall. Once the 36 chains from the rosette are formed, they come together to form an elementary cellulose microfibril (Doblin *et al.*, 2002).

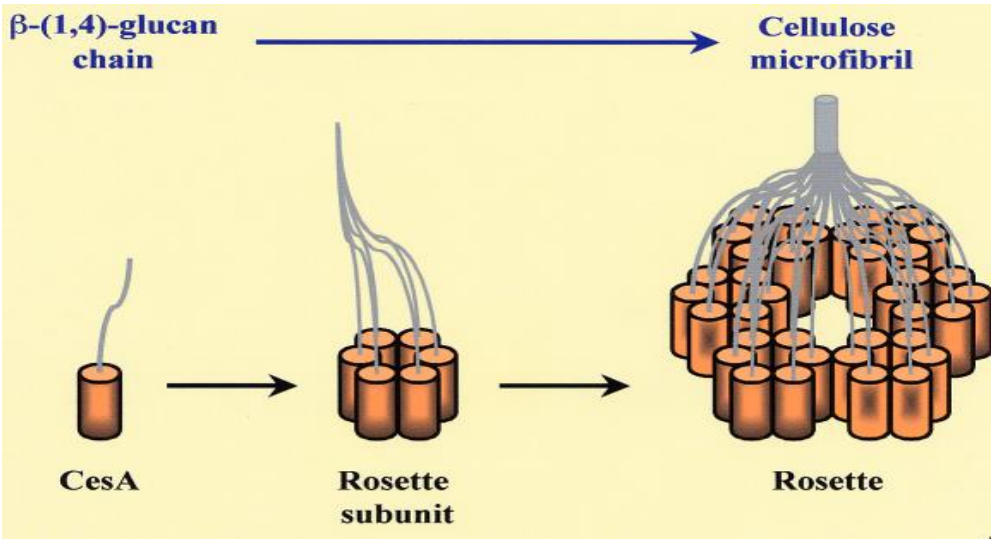

Figure 2.3: Structure of the rosette model (Brown, 1986).

The cell wall contains of the primary (P) and secondary (S) wall layers. The primary layer, which forms during surface growth of the cell wall, is a rigid skeleton of randomly arranged layers of long thin cellulose molecules united into microfibrils (Figure 2.4). After cell expansion, the secondary cell wall forms and the walls thicken. It has three sublayers, S1, S2 and S, which are categorized by the differences in microfibril orientation (Popescu *et al.*, 2011).

The glucan chains of the cellulose microfibrils within the primary and secondary cell walls differ in their degree of polymerization (DP). The cellulose in the primary and secondary walls has a DP of 1000-3000 and 15,000-25,000 respectively. The different layers within the secondary cell wall (S1, S2 and S3) are composed of microfibrils laid down in an ordered, parallel arrangement (Timell, 1969) of different thickness. The S2 layer accounts for 75-85% of the total thickness of the cell wall (Plomion *et al.*, 2001).

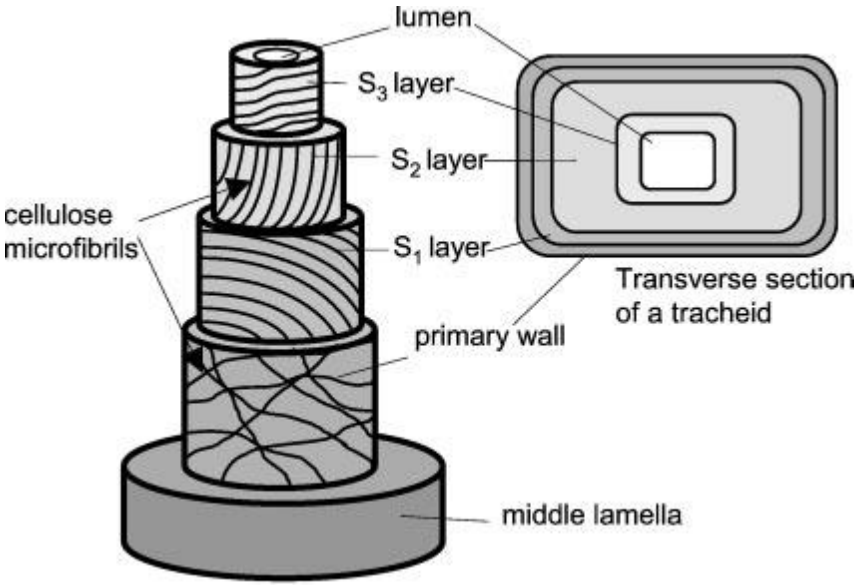

Figure 2.4: Schematic diagram of the microfibrils orientation in the primary wall and different layers of the secondary wall (Plomion *et al.*, 2001).

The wood pulping process involves first debarking and cutting wood into chips that are mechanically ground and then digested (Johnson, 1974). Both the mechanical and chemical processes can be modified to produce higher purity cellulose that is not only free of lignin and hemicellulose, but also further degraded to give reduced molecular weight products and derivatives (Kirk-Othmer, 1993; Whistler, 1997). The pulping process separates cellulose from lignin and hemicelluloses, leaving cellulose in a fibrous form that can be purified, dried, and shipped in large rolls. Various sulphite processes are used to delignify

the pulp including the bisulphite process, which uses calcium bisulphite in the presence of sulphur dioxide at a pH range of 2-6 on various wood species. Alkali processes use either caustic soda (dilute sodium hydroxide) as the pulping agent or sodium sulphate (Kraft process) as the source of alkali. The caustic soda and sodium sulphide in Kraft pulping liquor increases delignification and pulp strength. Chemicals used in the pulping process are potential pollutants. Recovering the chemicals increases pulping costs.

Newer paper mills are designed to consider energy efficiency, chemical recovery, and pollution. A newer oxygen alkali system does not use sulphur compounds but as the disadvantage of producing fibres of lower tear strength. Steam explosion is another delignification method and involves subjecting moisture saturated wood chips to high pressure and temperatures. These are milder processing conditions than the chemical methods used for manufacturing paper and moulded building materials (Kirk-Othmer, 1993).

In nature, plant is associated with other substance such as lignin and hemicelluloses. Hemicellulose, which is more closely associated with lignin than cellulose, is a common name for a group of carbohydrate heteropolymers, made primarily of xylans and glucomannan monomers. Unlike cellulose, hemicellulose is a highly-branched polymer with an amorphous structure and little inherent strength. This amorphous state is due to the many side groups, which prevent the close association between molecules required for forming crystalline regions (Klemm *et al.*, 1998). The dominant hemicelluloses in softwoods are polymers of *o*-acetyl galactomannan and arabino-(4-*o*-methylglucorono)-xylan but hardwoods contain predominantly O-acetyl-(4-*o*-methylglucorono)-xylan.

Lignin, the third major component of wood, functions as glue in the wood structure (Sjöström, 1993). It is an aromatic three-dimensional macromolecule of phenylpropane units made of p-coumaryl alcohol, coniferyl alcohol and sinaphyl alcohol (Figure 2.5). The proportions of these monomers depend on the plant species. Lignin, which is always associated with another carbohydrates, is located mainly in the middle of lamella of the cell wall but penetrates gradually, in diminishing amounts, into the primary and secondary layers of the cell walls.

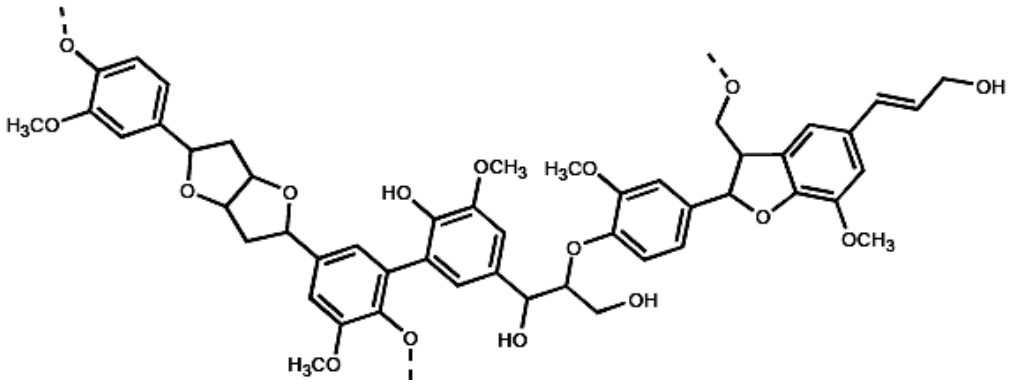

Figure 2.5: The chemical structure of lignin (Persson, 2004).

2.3 History of Bacterial Cellulose

The first scientific paper describing an extracellular gelatinous mat produced by *Acetobacter xylinum* was published by A.J. Brown in 1886 (Yamanaka *et al.,* 1989). The solid mass, referred to as a 'vinegar plant' was later identified as bacterial cellulose. *Acetobacter xylinum* become the official name under the International Code of Nomenclature of Bacteria. In 1997, *Acetobacter xylinum* was reclassified as *Gluconacetobacter xylinus* (Yamada *et al.,* 1997).

Hestrin's research group has produced several publications on BC production and its characterization. Their first publication briefly described microscopic examination of BC (Aschner and Hestrin, 1946) and the second publication described formation of a thin layer of BC at the surface of the media (Hestrin *et al.,* 1947). This group showed BC could be produced by *G. xylinus* using various substrates other than glucose. Their third publication (Schramm and Hestrin, 1954) studied BC formation and reported gas bubbles being formed within the submerged cellulose mesh before surface film formation. They concluded that the gas probably floated the cellulose and the bacteria to the surface.

Frey-Wyssling and Muhletahler (1946) studied the structure, morphology and modification of BC. X-ray analysis showed that cellulose strands are random are ribbon-like, with a cross section of 100 x 200 Å and 40 μm long. Retegi *et al.* (2012) made ESO (epoxidized soybean oil)/BC composites by an immersion process and reported that transparency and mechanical properties of the composites were influenced by curing ESO matrix. The BC nanofibres have been

functionalized by acetylation to improve both nanofibre dispersion and adhesion at the matrix interface.

Bacterial cellulose can be used in tissue engineering and has been safely implanted into living tissue. Svensson *et al.* (2005) describes a two-step chemical modification with phosphorylation and sulphation to convert BC to a scaffold material for *in vivo* tissue engineering of cartilage. Hydroxyapatite-BC composite synthesized through biometric routes can be used as artificial bone. A pre-treatment by soaking the BC in 0.1 M $CaCl_2$ at 37^oC for three days allowed the apatite to be more easily dispersed on the surface of the organic polymer. The pre-treated BC was immersed in de-ionized water and then soaked in a 1.5x simulated body fluid to trigger hydroxyapatite growth (Hong *et al.,* 2006).

2.4 Bacterial Cellulose

Bacterial cellulose is an extracellular excretion that forms aggregated fibrils, which crystallize into ribbons and assemble into a thick cellulosic mat known as a pellicle (Suwannapinunt *et al.,* 2007). The cellulose produced in a bacterial cell forms microfibrils of joined macromolecules in an ultra-fine reticulated structure. During cultivation, 12 to 70 molecules of cellulose are extruded from a cell into the media through pores located about 10 nm apart in a distinct array on the cell surface (Zaar, 1979). After biosynthesis, the cellulose molecules bind to each other via hydrogen bonds near the cell surface to produce pure cellulose (Ross *et al.,* 1991).

Hornung *et al.* (2006) report that BC obtained from aerobic fermentation is pure, has higher degree of polymerization and high water-holding capacity. Compared with cellulose from plant cells, BC is chemically pure, free of lignin and hemicelluloses (Holmes, 2004; Hong and Qiu, 2008; Karahan *et al.,* 2011). However, commercialisation of BC production has been limited because of the low yields, meaning many batches need to be processed to produce sufficient material.

The BC has a DP between 2000 and 6000 (Iguchi *et al.,* 2000), a high degree of crystallinity (above 60%), a cross-section diameter of 2 to 4 nm (Nakagaito *et al.,* 2001), and has excellent shape and strength retention. The crystalline structure of BC is mostly cellulose I and the predominant I_α in BC makes it more crystalline than plant cellulose (Bielecki *et al.,* 2002). Table 2.2 shows the comparison between plant cellulose and BC.

Table 2.2: Properties of plant cellulose and BC.

Property	Plant Cellulose	Bacterial Cellulose	Reference
Fibre width, mm	1.4-4.0 x 10^{-2}	70-80	Pecoraro *et al.,* 2008
Crystallinity, %	56-65	65-79	
DP*	13,000-14,000	2,000-6,000	
Young's modulus, GPa	Cotton: 5.5-13 Jute: 27 Flax: 28	BC sheet: 15-30 BC fibre: ~120 BC crystal: ~138	Bielecki *et al.,* 2004; Yamanaka *et al.,* 1998; Sakurada, *et al.,* 1962; Eichhorn *et al.,* 2010
Water content, %	60	98.5	Pecoraro *et al.,* 2008

* DP= degree of polymerization

Bacterial cellulose can be produced in agitated or static culture (Krystynowicz *et al.,* 2005). The BC produced by *G. xylinus* in static cultures is initially extruded from the cell surface as microfibres and entangles to form ribbons, which then intertwine to form a dense, gelatinous pellicle at the air/liquid interface. Traditional static culture, which produces pellicles on the surface of fermentation media, has been used for producing BC. In this condition, the BC pellicles grow downward as the cells entrapped in the pellicle become inactive caused by lack of oxygen (Borzani and de Souza, 1995).

Agitated culture, which has many commercial applications, is more efficient for BC production (Watanabe *et al.,* 1998). However, one problem of agitated culture is that a large proportion of the substrate glucose is converted to gluconic and keto-gluconic acids rather than cellulose. This can be decreased either by strain selection (Johnson and Neogi, 1989) or using fructose as a carbon source, which has the added advantage of lowering by-product formation (Joseph *et al.,* 2003). Under agitated culture, the microbial strain can be converted to the non-cellulose producer (Cel^-) strain, which grow more rapidly. This decreases BC production in agitated fermentation (Park *et al.,* 2004), especially when the Cel- strains outgrow the cellulose-producing strain.

Static culture is a very simple and the traditional method. It has been widely studied and used for producing several successful commercial cellulose products such as 'nata de coco', transducer diaphragms, and wound care dressing materials (Krystynowicz *et al.,* 2005). However, the number of pellicles formed on the surface of the media is lower than in other methods because the cellulose-

producing bacteria are growing relatively slowly (Son *et al.*, 2001) due to the pellicles at the air/liquid interface forming an effective barrier between atmospheric oxygen on one side and nutrient on the other. This reduces oxygen transfer through the pellicle to the cells (Dudman, 1960). Static culture is very labour intensive and requires large surface areas (Joseph *et al.*, 2003).

The pellicle produced in static culture has an ultra-fine network structure, producing ribbons 500 nm wide and 10 nm thick. These ribbons are made of smaller microfibrils of average cross-section 16 x 58 Å (Brown *et al.*, 1976; Gindle and Keckes, 2004), are 3-4 nm thick (Bielecki *et al.*, 2004). Different values have been reported for the diameter of these microfibrils, ranging from 24-86 nm (Chanliaud *et al.*, 2002), 72-175 nm (Bohn *et al.*, 2000), and 70-130 nm (Jonas and Farah, 1998; Yamanaka *et al.*, 2000). Because BC is made up of nano-sized fibres and the structure of these nanofibres determines product properties, this polymer is described as nanocellulose (Klemm *et al.*, 2003). The characteristics of BC are given in Table 2.3.

Table 2.3: Properties of BC (Bielecki *et al.*, 2005).

1.	High purity
2.	High degree of crystallinity
3.	Sheet density from 300 to 900 kg.m-3
4.	High tensile strength
5.	High absorbency
6.	High water-binding capacity
7.	High elasticity, resilience, and durability
8.	Nontoxicity
9.	Metabolic inertness
10.	Biocompatibility
11.	Susceptibility to biodegradation
12.	Good shape retention
13.	Easy tailoring of physicochemical properties

The chemical and physical stability of BC means various methods can be used to improve its mechanical and thermal properties. For example, extensive purification via alkaline and oxidative agents can increase the modulus of a BC sheet (Guhados *et al.*, 2005). Removing impurities in the cellulose matrix such as protein, nucleic acid from the bacteria and media remaining after the culture,

increases contact between individual cellulose fibrils, which allows more intra- and interfibrillar hydrogen bonds in the network (Yamanaka *et al.*, 1989). Adding a cell division inhibitor or an organic reducing agent improves Young's modulus of BC sheet by artificially changing the form of cellulose-producing bacteria to various ribbon-shaped microfibrils (Ishihara *et al.*, 2003).

Morphological properties of BC have been observed using techniques such as electron microscopy (Krystynowicz *et al.*, 2002) and atomic force microscopy (Hirai *et al.*, 2004; Guhados *et al.*, 2005). Many studies on isolating and characterizing microfibrillated cellulose, whiskers and nanocrystals have been published (Landry *et al.*, 2011). Most of the studies have investigated cellulose nanofibres as a reinforcing agent in thermoplastics. Favier *et al.* (1995) published the first work on isolating cellulose whiskers and demonstrated the reinforcing potential of cellulose whiskers in nanocomposites (Landry *et al.*, 2011).

The BC whiskers incorporated into electrospun nanofibres of poly (ethylene oxide) from aqueous dispersions, partially aggregate inside the fibres (Marta *et al.*, 2011). Costa and coworkers (2012) characterised electrospun BC and found this form has a more symmetric nanopore structure than cast films mats.

2.4.1 Biosynthesis

Cellulose synthase plays the most crucial part in cellulose biosynthesis (Brown *et al.*, 1976; Ross *et al.*, 1997). Synthesis of BC in *G. xylinus* occurs between the outer membrane and cytoplasmic membrane by a cellulose-synthesizing complex that associates with pores at the surface of the bacterium. Cyclic diguanylmonophosphate has been identified as the activator of cellulose synthase (Jonas and Farah, 1997). Brown and co-workers (1976) reported that cellulose is synthesized in close contact with the bacterial envelope. Each bacterium produces a flat ribbon containing cellulose on its own surface. These ribbons, of average diameter 10-20 to 30-40 Å, are constructed side by side in a horizontal axis. Cell motion associated with cellulose synthesis by *G. xylinus* means the cell producing the ribbon-like cellulose is propelled parallel to the microfibril (Figure 2.6) and the cell producing band-like cellulose is propelled perpendicularly to the molecular axis in the folded band (Shibazaki *et al.*, 1998).

Haigler and Benziman (1982) reported that the cellulose synthesizing sites on the cell surface have 3.5-nm diameter pores arranged in a line. Each pore covers a 10-nm particle made up of the cellulose-synthesizing enzymes involved

in the polymerization reaction and accessory proteins possibly involved in other functions. Each particle produces several glucan chains, which form a 1.5-nm sub-elementary fibril. The sub-elementary fibrils then form the microfibril. Microfibrils are connected to each other by hydrogen bonding to create escalating ribbons, flat layers and pellicle structures. Because the microfibrils are small, they are in very close contact with each other, which increases the density of inter- and intrafibrillar hydrogen bonding. The great strength and high water retention of bacterial cellulose is thought to be due to this strong hydrogen bonding (Iguchi *et al.,* 2000; Klemm *et al.,* 2001).

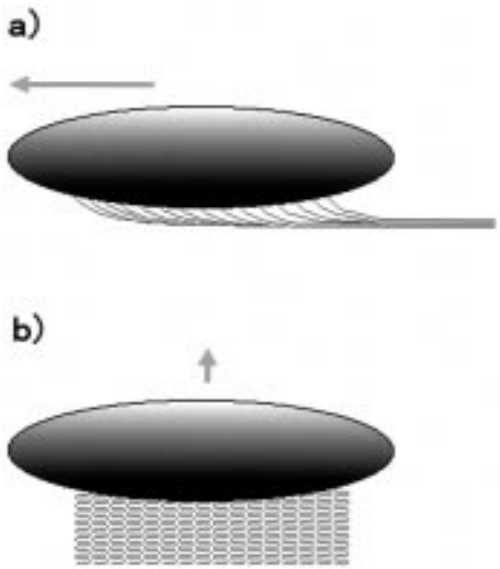

Figure 2.6: Cell motion associated with cellulose synthesis by *G. xylinus* showing (a) the cell producing ribbon-like cellulose being propelled parallel to the microfibril and (b) the cell producing band-like cellulose being propelled perpendicularly to the molecular axis in the folded band (from Shibazaki *et al.*, 1998).

The biological pathway from the substrate glucose to cellulose involves four biochemical reactions. Glucose is converted to glucose-6-phosphate by gluconase, which is then isomerised to glucose-1-phosphate by phosphor-glucomutase. Thirdly, glucose-1-phosphate is converted to UDPG (uridine diphosphate glucose) by UDPG-pyrophosphorylase. The pyrophos-phorylase in *G. xylinus* is activated by the cycling nucleotide (c-di-GMP), which is synthesized by diguanylate cyclase. Its concentration is regulated by the action of phosphor-diesterases. Finally, UDPG is polymerized into cellulose by cellulose synthase (Tal *et al.,* 1998). The carbon metabolism pathway in *G. xylinus* is shown in Figure 2.7.

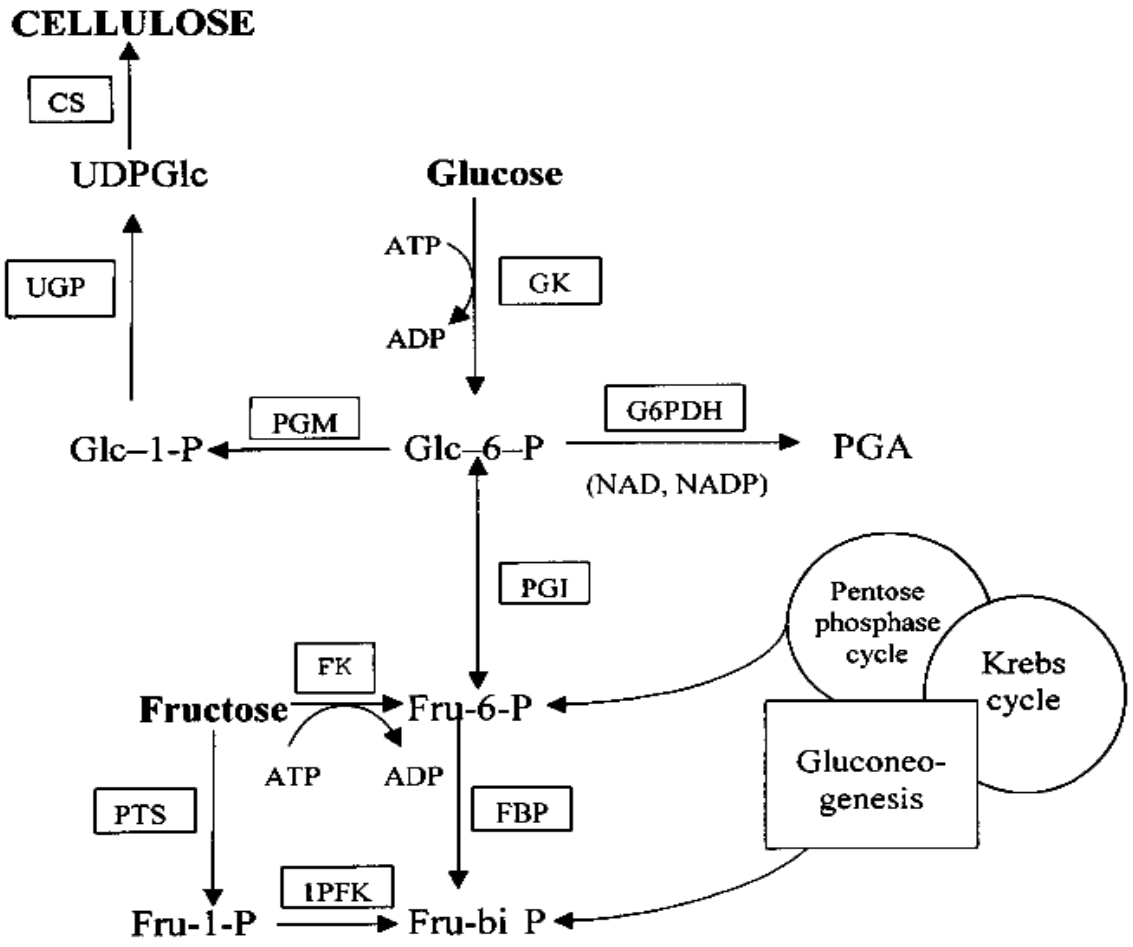

Figure 2.7: Schematic pathway of carbon metabolism in *Gluconacetobacter xylinus* (Klemm *et al.*, 2001).

Nucleotide sequence analysis indicates that the cellulose synthesis (*bcs*) operon is 9217 base pairs long and consists of four genes – *bcsA, bcsB, bcsC* and *bcsD* - which appear to be translationally coupled and transcribed as a polycistronic mRNA with an initiation site 97 bases upstream of the coding region of the first gene (*besA*) in the operon. The *bcsA* polypeptide is thought to be responsible for polymerizing UDPG into cellulose and the *bcsB* polypeptide is possibly an activator-binding subunit. The *bcsC* and *bcsD* polypeptides are situated on the outer surface of the membrane and play an important role in crystallization and/or extrusion of cellulose immediately after polymerization (Saxena *et al.,* 1994; Volman *et al.,* 1995). Results from genetic complementation tests and gene disruption analyses demonstrate that the four genes in the operon are essential for maximum BC synthesis in *G. xylinus* (Wong *et al.,* 1990).

Cellulose synthesis in bacterium cells causes a rotational movement of the cells along their longitudinal axes as they extrude and spin the cellulose fibres

(Figure 2.8). Bacterium will move forward as they secrete cellulose backward. As *G. xylinus* is aerobic, it tends to move towards the surface of the liquid media where oxygen levels are greater (Koizumi *et al.,* 2009).

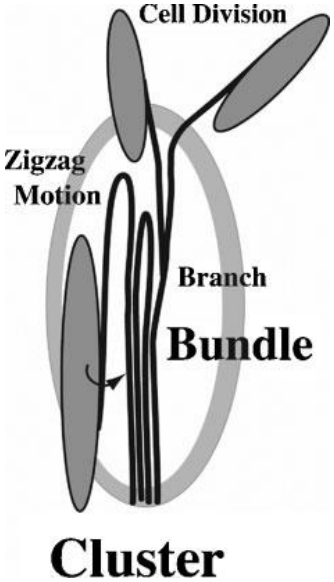

Figure 2.8: Schematic view of a cluster, in which bacteria coherently move as the cellulose bundle is formed. A bundle formed by the 'zigzag motion' and cell division makes a bundle branch (Koizumi *et al.,* 2009).

2.4.2 Applications of BC

A summary of uses for BC is given in Table 2.4. It can generally be used in any area where plant cellulose is used (Jonas and Farah, 1996). Consequently, several practical applications in biotechnology and other fields of biomedical sciences have been developed (George *et al.,* 2005).

Due to its properties such as high purity with a crystalline structure, an ability to absorb fluids and excellent histocompatibility with living tissue (Fontana *et al.,* 1990), BC has particular use as a wound dressing and artificial skin. Products include Biofill® made by a Brazilian company and XCell™ made by an American company. Mayall *et al.* (1990) used Biofill® artificial skin to treat trophic ulcerations on limbs. This material shortened the cicatrisation time, reduced contamination and decreased treatment cost. The film is applied on the lesion region that has lost epithelial tissue and acts as a new skin, eliminating pain symptoms (by isolating the nerve ending) and enhancing absorption of wound exudates (Farah, 1990). Gengiflex®, another product being developed, contains an external layer of alkali-treated BC bonded to an internal layer of crystalline BC (Novaes *et al.,* 2000).

Table 2.4: Uses for BC (Bielecki *et al.*, 2005).

Sector	Application
Cosmetics	Stabilizer of emulsions for cream, tonics, nail conditioners, polishes, and component of artificial nails
Textiles	Artificial skin and textiles; highly absorptive materials
Tourism and sport	Sport clothing, tents, camping equipment
Mining and refinery	Spilt out collecting sponge, material for toxins
Waste treatment	Absorption and recycling mineral and oils
Sewage purification	Municipal sewage purification, water ultrafiltration
Broadcasting	Sensitive diaphragms for microphone and stereo headphones
Forestry	Artificial replacement for wood, multi-layer plywood and heavy-duty containers
Paper industry	Specialty paper, archival document repair, more durable banknotes, diapers, and napkins
Machine industry	Car bodies, airplane parts, and sealing of cracks in rocket casings
Food production	Edible cellulose and nata de coco
Medicine	Temporary artificial skin for the treatment of decubitus, burns and ulcers, component of dental and arterial implants
Laboratory/research	Immobilization of proteins, chromatographic techniques, media component of *in vitro* tissue cultures

The unique physical properties of BC allow excellent moulding, with a high degree of adherence even to moving parts. This has allowed applications such as wound dressing (Figure 2.9).

Recent research (Barud *et al.*, 2011) reports on successfully preparing antimicrobial BC-silver nanoparticles composite membranes using TEA (triethanolamine) as the complexing and reducing agent and plasticizer to prevent wound infection. This new composite membrane shows strong antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli*, the bacteria usually found in contaminated wounds.

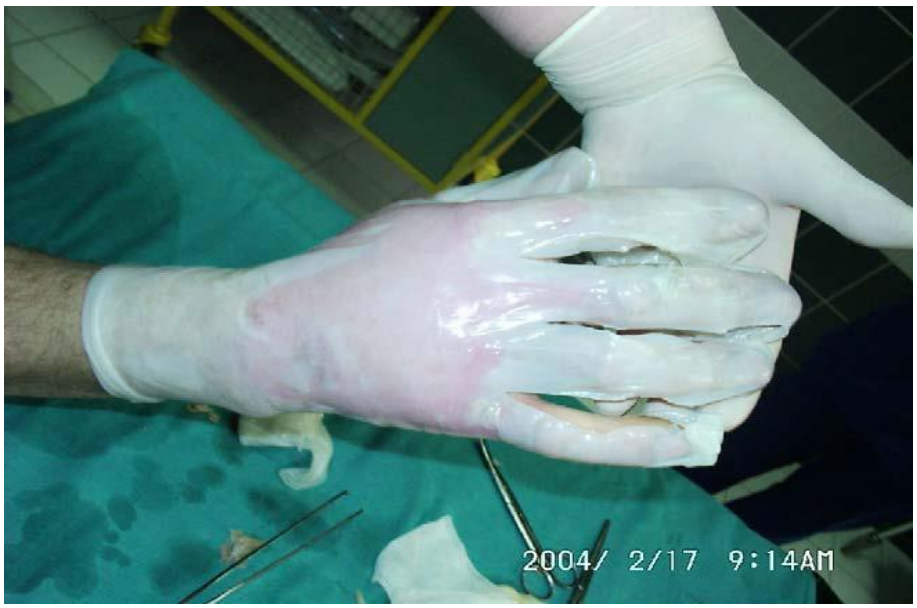

Figure 2.9: BC dressing applied on a wounded hand (image courtesy of the Center of Burn Healing, Siemianowice Slaskie, Poland; Czaja *et al.*, 2006).

The first successful commercial food application of BC is 'nata de coco' (Figure 2.10), a traditional Philippines dessert prepared by fermenting coconut milk or coconut water with sucrose and a BC-producing bacteria. Consuming the dessert is believed to protect against bowel cancer, arteriosclerosis, coronary thrombosis and prevent a sudden increase of glucose in urine. Consequently, this food is becoming increasingly common beyond Asia (Bielecki *et al.*, 2005).

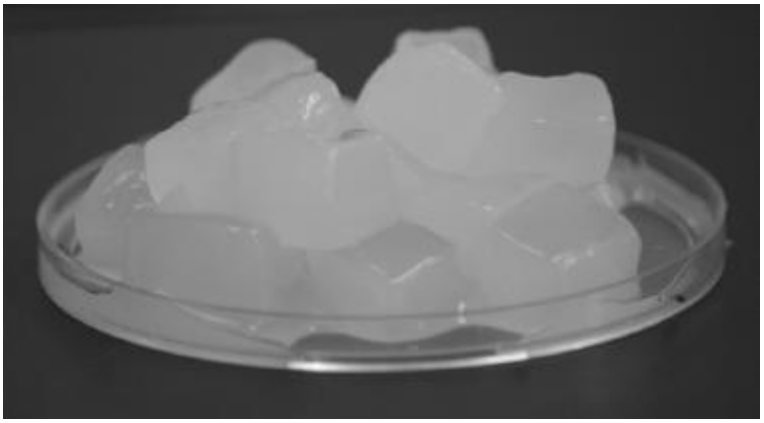

Figure 2.10: Nata de coco (Halib *et al.*, 2012).

Disintegrated BC makes an excellent component in producing paper that is stronger than normal paper. Microfibrils of the BC polymer form many hydrogen bonds when the paper is dried, thus improving chemical adhesion and tensile strength. The BC paper not only has better retention of solid additives, such as

filler and pigments but is also more elastic, air-permeable and resistant to tearing and burst forces (Iguchi *et al.,* 2000).

Researchers have also produced BC based composites with water-soluble polymers such as polyethylene glycol, carboxymethyl cellulose, carboxymethyl chitin, and other cellulose-based polymers for special applications like a filtration material. These polymers were formulated into the cellulose by in situ processes, that is, these materials were added into the media (Takai, 1994). A membrane for evaporating a binary water-ethanol mixture was also developed, and a dialysis membrane prepared from a 2-µm cleaned BC sheet which was treated with a concentrated alkali solution. The BC film had significantly higher permeation rates than a commercially-made dialysis membrane (Shibazaki *et al.,* 1993).

Treating BC with either ethanol or alginate makes it softer and gives it a texture similar to fruit and other foods, and produces edible bacterial cellulose. The mechanism of this change is immobilization of water in the gel formed, making the gel easier to bite and chew (Okiyama *et al.,* 1992).

Due to its fibrous structure, as well as the qualitative difference in protein adsorption between membranes exposed to bovine serum albumin, BC films can be used in an amperometric glucose sensor. When one side of the BC membrane is treated with Trogamid, detectable blood glucose concentrations can be increased to between 0 and 40 mM. Another feature is that a BC-covered sensor displays long-term stability pf up to over 200 h in dilute blood and around 24 h in undiluted blood. This is six to seven times more stable than probes made from plant cellulose (Ammon *et al.,* 1995).

Another successful application is acoustic diaphragms in high fidelity loudspeakers and headphones marketed by Sony Corporation (Iguchi *et al.,* 2000). This application uses sheets obtained from BC pellicles treated to have a high dynamic Young's modulus, close to 30 GPa (Nishi *et al.,* 1990; Yamanaka *et al.,* 1989). This high modulus indicated that the ultrastructure of BC had superior physical properties (Yamanaka *et al.,* 2000). Bacterial cellulose has also been used as a thickener to maintain viscosity of food, cosmetics, and similar applications (Kent *et al.,* 1991; Jonas and Farah, 1998).

2.5 Acetic Acid Bacteria

Acetic acid bacteria (AAB) have been used from ancient times for vinegar and wine production, brewing and baking, and making buttermilk, cheese and

pickles. Kutzing's demonstration of the nature of 'mother of vinegar' first showed that acetification of wine was caused by living organisms called acetic acid bacteria (AAB) that oxidize ethanol to acetic acid but and are very resistant to acetic acid (Nakano and Fukaya, 2008). Table 2.5 lists the microorganisms that produce BC.

Table 2.5: Microorganisms used in BC production.

Microorganisms	Description	References
Acetobacter lovaniensis	Gram negative, ellipsoidal to rods	Coban and Biyik, 2011
Enterobacter amnigenus	Gram negative, rods	Hungund and Gupta, 2010b
Gluconacetobacter aceti	Gram negative, ellipsoidal to rods	Panesar *et al.*, 2009
Gluconacetobacter hansenii	Gram negative, ellipsoidal to rods	Ishihara *et al.*, 2002; Park *et al.*, 2004 ; Ha *et al.*, 2008
Gluconacetobacter persimmonis	Gram negative, ellipsoidal to rods	Hungund and Gupta, 2010a
Gluconacetobacter sp.	Gram negative, ellipsoidal to rods	Son *et al.*, 2001; Heo *et al.*, 2002, Son *et al.*, 2003 ; Kim *et al.*, 2006; Seto *et al.*, 2006; Pourramezan *et al.*, 2009; Park *et al.*, 2010;
Gluconacetobacter xylinus	Gram negative, ellipsoidal to rods	Naritomi *et al.*, 1997a, 1997b; Toda *et al.*, 1997; Ramana *et al.*, 2000; Ishida *et al.*, 2002; Ishihara *et al.*, 2002; Joseph *et al.*, 2003; Bae *et al.*, 2004; Yunoki *et al.*, 2004; Chavez-Pacheco *et al.*, 2005; Shigematsu *et al.*, 2005; Keshk and Sameshima 2006a, 2006b, 2006c; Zhou *et al.*, 2007; El-Saeid *et al.*, 2008; Kongruang, 2008; Nguyen *et al.*, 2008; Goelzer *et al.*, 2009; Kurosumi *et al.*, 2009; Mikkelsen *et al.*, 2009; Dahman *et al.*, 2010; Lu *et al.*, 2011; Moosavi-Nasab *et al.*, 2011
Lactobacillus sp., L. mali, L. agilis, L. brevis, L. coryniformis, L. casei	Gram positive, rods, major part of lactic acid bacteria (LAB)	Seto *et al.*, 2006
Acetobacter from black tea	Gram negative	Goh *et al.*, 2012

The molecular mechanism of ethanol oxidation has been extensively studied in *Gluconacetobacter*. Oxidation of ethanol to acetic acid is done by a membrane-bound enzyme with transporter activity. Several molecular mechanisms involved in acetic acid resistance have been found in AAB (Nakano and Fukaya, 2008). Many are characterized as an active respiratory chain, oxidizing various alcohols, sugars, and sugar alcohols so the corresponding oxidation products accumulatin the culture media (Matsushita *et al.*, 1994).

Yakushi and Matsushita (2010) report that acetic acid production from ethanol by AAB is accomplished by two sequential membrane-bound catalytic reactions; the pyrroquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH) and membrane-bound aldehyde dehydrogenase (Figure 2.11). The PQQ-ADH, which is quinohemoprotein–cytochrome c complex bound to the periplasmic side of the cytoplasmic membrane, catalyzes the first step of acetic acid production to oxidize ethanol by transferring electrons to membranous ubiquinone (Q). The respiratory chain of AAB branches into the cytochrome bo_3 ubiquinol oxidase and a cyanide-insensitive bypass oxidase at the Q site, both of which reduce oxygen to water. Thus, PQQ-ADH functions as the primary dehydrogenase in the ethanol oxidation respiratory chain (Azuma *et al.*, 2009).

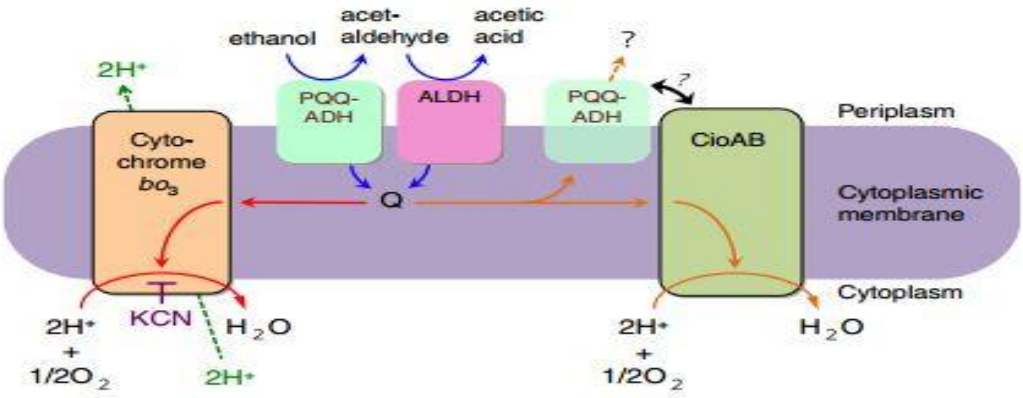

Figure 2.11: An overview of the ethanol oxidation respiratory chain of acetic acid bacteria (Yakushi and Matsushita, 2010).

2.5.1 *Gluconacetobacter xylinus*

Although several organisms can synthesize BC (Section 2.2), *G. xylinus* is reported to be the most efficient producer (Nakagaito and Yano, 2005; Brown and Laborie, 2007; Kalia *et al.*, 2011) and able to produce sufficient amounts for industrial applications (Brown, 1986; Goelzer *et al.*, 2009). *Gluconacetobacter*

xylinus was first described in 1886 (Brown, 1986; Wiegand and Klemm, 2006). This Gram negative bacterium is found in soil and can be frequently be isolated from decaying fruit. It is a strictly aerobe in the family *Acetobacteraceae* (Masaoka et *al.,* 1993; Embuscado *et al.,* 1994; Battard-Bernardo *et al.,* 2004). These efficient producers of BC (Yamada, 2000) occur singly, in pairs, or in chains, and reproduce by binary fission. They have flagella and are motile but do not form endospores. The optimum growth temperature is 25-30^{o}C and the optimum pH range is 5.4 to 6.2 (Bielecki *et al.,* 2000; Krystynowicz *et al.,* 2005). Under some growth conditions, *G. xylinus* strains form involution forms, which have swollen or elongated filaments. These can atrophy or fragment into shorter pieces, which allow normal cells to be recovered.

Gluconocetobacter xylinus produces BC on the surface of liquid and solid culture media. The gelatinous, leather-like mats formed on the surface of liquid culture media under static culture conditions contain bacterial cells entrapped in a network of cellulose fibres. Under agitated cultivation, pellicle deposition is disrupted and cellulose forms irregular granules, stellate strands and fibrous strands (Bielecki *et al.,* 2000; Krystynowicz *et al.,* 2005). On agar media, *G. xylinus* forms transparent or white, smooth or rough, flat or convex colonies, which can have undulate edges (Krystynowicz *et al.,* 2005).

The major problem for commercial production of BC using *G. xylinus* is that this organism tends to spontaneously mutate and produce non-cellulose producing (Cel^{-}) cells (Figure 2.12). This occurrence in agitated cultures was first described by Hestrin and Schramm (1954), who isolated three different types of *G. xylinus* cells, based on colony morphology and cellulose biosynthesis efficiency: Type I - wild type which are cellulose-producing (Cel^{+}) cells; Type II - cellulose-non producing forms (Cel^{-}) capable of reverting to Cel^{+}, and Type III - non reverting, non-cellulose producing forms (Cel^{-}).

The frequency of Cel^{+} to Cel^{-} conversion depends on culture conditions. Having sufficient aeration in agitated liquid culture conditions favours spontaneous appearance of Cel^{-} cells, which then become the dominate population. Thus, aeration discriminates against Cel^{+} *Gluconacetobacter xylinus* (Leisinger *et al.,* 1966; Krystynowicz *et al.,* 2005).

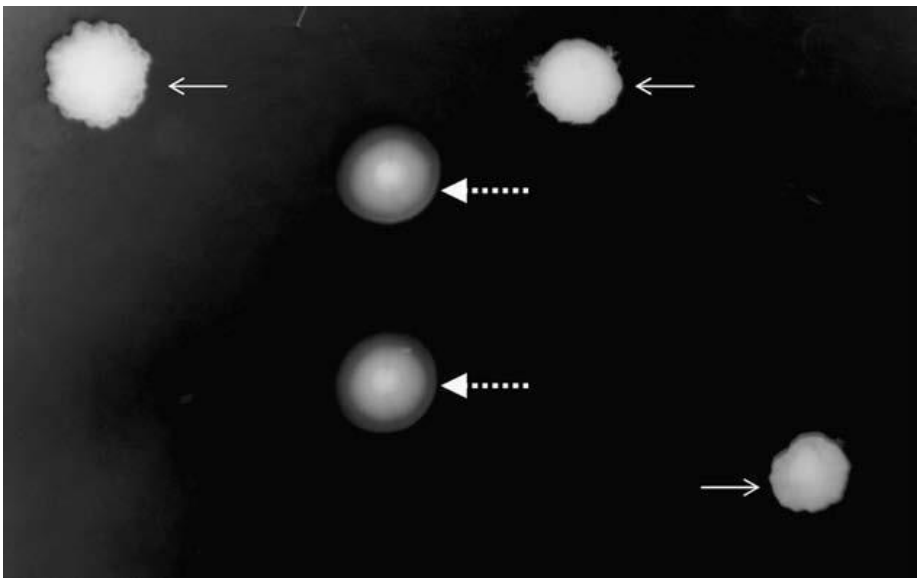

Figure 2.12: Morphology of *G. xylinus* K3 colonies formed on YPM agar. Broken arrows indicate non-mucoid colonies formed by the Cel^+ and solid arrows indicate mucoid colonies formed by mutant Cel^- (Nguyen *et al.*, 2010).

The effect of various nitrogen and carbon sources on BC biosynthesis by this organism have been widely investigated (Oikawa *et al.*, 1995; Matsuoka *et al.*, 1996; Ramana *et al.*, 2000; George *et al.*, 2005). *Gluconacetobacter xylinus* consumes sugar as its main nutrient. The researchers used HS media (Hestrin and Schramm, 1954; de Olyveira *et al.*, 2012), which contains D-glucose, peptone, yeast extract, disodium phosphate, and citric acid and distilled water. Son *et al.* (2001) studied the effect of culture media composition on the efficiency of BC synthesis by *G. xylinus* and reported that highest BC yields occurred when the media is supplemented with 0.6% ethanol.

2.6 Growth Media for Producing BC

To obtain high productivity in fermentations, it is important to identify the best media and process conditions (Desai *et al.*, 2006). Microorganisms convert media components into biomass and/or use them to synthesise metabolites. Supplying nutrients to the microbes affects product concentration, directly through metabolism and indirectly by affecting production rate of the specific metabolite (Atkinson and Mavituna, 1983). Fermentation media is important as it can affect production yield, volumetric production and process cost (Kennedy and Krousse, 1999).

Pirt (1975) reported that fermentation for biomass and/or metabolite biosynthesis involves the major elements carbon, hydrogen, oxygen and nitrogen;

the minor elements phosphate, potassium, sulphate and magnesium, and trace elements such as iron, zinc, etc; and vitamins and hormones. The effect of media components on biomass and metabolite production can be compared by yield coefficients, which is mass of cells or metabolite formed per unit mass of substrate utilized ($Y_{x/s}$ and $Y_{p/s}$ for cells and metabolite respectively). Researchers often calculate yield coefficients incorrectly (Hong, 1988), making it difficult to compare reported results.

Fermentation media can be classified into chemically defined (synthetic, defined) and undefined (natural, complex) media. Defined media consists of pure chemicals in certain known proportions, while undefined media is composed of components from natural sources, which has unknown proportions (Zhang and Greasham, 1999).

2.6.1 Effects of Carbon Source on BC Production

For economic production of BC, growth conditions that produce high BC yields need to be identified along with the how these conditions affect BC morphology and properties. Recent studies (Masaoka *et al.*, 1993; Oikawa *et al.*, 1995a; Oikawa *et al.*, 1995b; Ramana *et al.*, 2000; Keshk and Sameshima, 2005; Keshk and Sameshima, 2006; Kim *et al.*, 2006; Hutchens *et al.*, 2007; El-Saied *et al.*, 2008; Mikkelsen *et al.*, 2009; Pourramezan *et al.*, 2009; Jung *et al.*, 2010; Coban and Biyik, 2011) investigated the effect of different carbon and nitrogen components on BC production using various strains of *G. xylinus.* The media used is often HS media (Hestrin and Schramm, 1954). Carbon sources investigated include glucose, arabinose, arabitol, citric acid, ethanol, ethylene glycol, diethylene glycol, fructose, galactose, gluconolactone, glycerol, inositol, lactose, malic acid, maltose, mannitol, mannose, methanol, rhamnose, ribose, sorbose, starch, saccharide, succinic acid, sucrose, trehalose, and xylose.

Ruka *et al.* (2012) investigated the effect of different media on BC yield (Table 2.6). High BC yields were obtained on both Yamanaka and Zhou media (Figure 2.13), with Yamanaka-mannitol producing the highest. Both CSL (corn steep liquor) and Son media produced higher levels than HS media, particularly when the carbon source was mannitol. Glucose, mannitol and sucrose produced consistently high yields of BC regardless of the media used, indicating these carbon sources should be used to obtain high BC yields.

Table 2.6: Composition (w/v) of different media used in BC studies (Ruka e*t al.*, 2012).

Components	Media				
	HS	**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_20	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	-
$FeSO_4.7H_2O$	-	-	-	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	-
H_3BO_3	-	-	-	-	0.0003
p-Aminobenzoic acid	-	-	-	0.00002	-
Biotin	-	-	-	0.0000002	-
Calcium pantothenate	-	-	-	0.00002	-
Folic acid	-	-	-	0.0000002	-
Inositol	-	-	-	0.00002	-
Nicotinamide	-	-	-	0.00004	0.00005
Pyridoxine-HCl	-	-	-	0.00004	-
Riboflavin	-	-	-	0.00004	-
Thiamine-HCl	-	-	-	0.00004	-

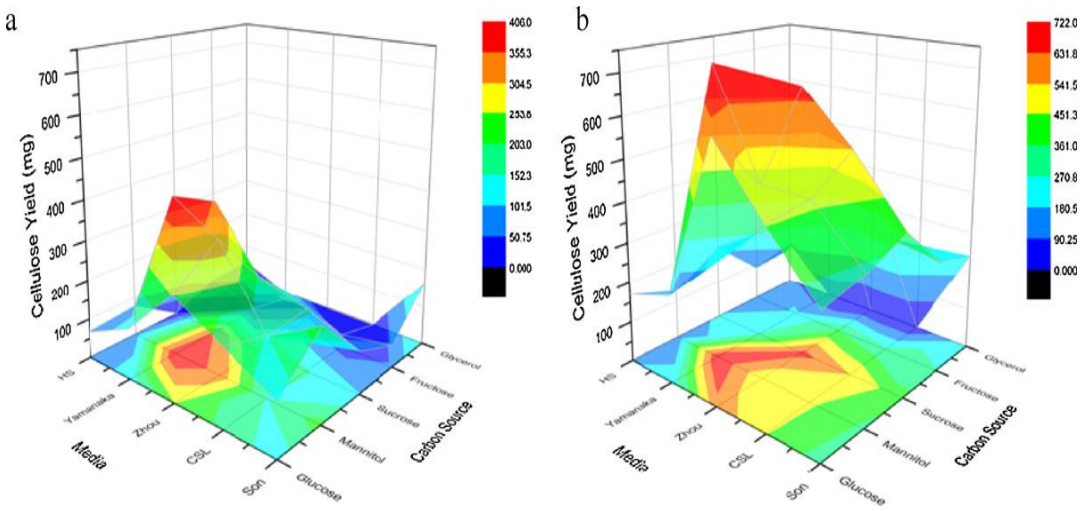

Figure 2.13: Cellulose yields produced in different media with different carbon sources after (a) four days and (b) seven days incubation (Ruka et al., 2012).

Usually, polysaccharide production is favoured by having a high ratio of the limiting nutrient (usually nitrogen), which converts 60-80% of the utilized carbon source into crude polymer (Chawla *et al.*, 2009).

Initial glucose concentration affects BC production. Gluconic acid formed as a by-product, decreases pH, which also decreases BC production. Masaoka *et al.* (1993) showed that all the glucose was consumed when initial glucose concentration was 6 g/L or 12 g/L but consumption decreased to 70% and 30% at 24 g/L and 48 g/L respectively. However, cellulose yield per unit glucose was the same.

Ishihara and coworkers (2002) grew *G. xylinus* IFO 15606 on 20 g/L xylose and obtained of 10 mg BC per 100 mL media after four weeks growth. Ramana *et al.* (2000) reported high BC yields when growing *G. xylinus* NCIM 2526 in sucrose, mannitol and glucose and Nguyen *et al*. (2008) obtained maximum BC production when *G. xylinus* isolated from kombucha (a popular fermented beverage originating in northeast China) was grown on mannitol.

One disadvantage of using glucose as a carbon source for BC production is formation of gluconic acid, which lowers pH and ultimately decreases BC production (Chawla *et al.*, 2009). Supplementing the media with lignosulphonate, which contained antioxidant and polyphenolic compounds, decreased gluconic acid production and increased BC production. They obtained BC yields from *G. xylinus* IFO 13772 of 179.8 mg in 30 mL of media containing 40 g/L sugar cane molasses with 1% lignosulphonate (Keshk and Sameshima, 2006a).

Adding 0.15% (v/v) lactate to the media containing 0.24% (w/v) (of the total nitrogen) soytone, YE or peptone gave 4.13 g/L of BC compared to the control media (without lactate) of 4.04 g/L (Matsuoka *et al.*, 1996).

Bae and Shoda (2004) found that adding sulphuric acid-treated molasses to batch fermentation of *G. xylinus* BPR2001 produced 76% more BC than using untreated molasses. The specific growth rate doubled. They also studied production of BC in fed-batch fermentation with molasses-based media and reported that 7.82 g/L of BC was produced when 200 mL of molasses media was added five times every 16 h, compared with maximum BC production when continuously feeding of 6.3 g. Investigations by Keshk and Sameshima (2006) using sugarcane molasses in a HS media indicated molasses was a better carbon source than glucose for producing BC.

Kim and coworkers (2006) reported that adding 2.0% (w/v) glucose gave a maximum BC concentration of 1.8 g/L. Supplementing with $Ca_3(PO_4)_2$ treated molasses increased the maximum concentration to 2.21 g/L BC (Jung *et al.*, 2010a). Some studies report that sugar concentration in molasses could influence metabolite production (Roukas, 1996). Jung and coworkers (2010b) used 3% of glycerol as a carbon source for BC production by *Gluconacetobacter sp.* V6 and obtained a yield of 4.98 g/L BC.

Premjet and coworkers (2007) tudied the effect of sugarcane molasses, which contains sucrose, fructose, glucose and also nitrogenous compounds, non-nitrogenous acids, nucleic acids, vitamins, other carbohydrates, minerals and black colour substances, individually or in combinations on BC production by *G. xylinus* ATCC 10245. They concluded that vitamins, amino acids, other carbohydrates, minerals and black colour substances in the molasses increased BC yield by up to 210%. In their report, yields are defined as ratio to that produced in Hestrin-Schramm media.

Although the effect of single sugars on BC production, whether using sucrose or fructose as the carbon source has widely been investigated, there are also some studies using multiple sugars. Dahman and coworkers (2010) studied the effect of single sugars or mixtures of fructose, sucrose, glucose, arabinose, galactose, mannose and xylose with compositions similar to acid hydrolysates of different agriculture residues. The highest BC production (5.65 g/L) was obtained on fructose; 96% of the fructose was used. The highest BC concentration was

obtained using 40 g/L glucose with corn fibres, followed by 40 g/L glucose with wheat straw, which produced 19.2 g/L.

Rani and Appaiah (2011) reported that supplementing coffee cherry husk (CCH) as a carbon source with corn steep liquor (CSL), urea, ethyl alcohol and acetic acid produced 5.24 g/L BC. The ratio of 1:1 (w/v) of CCH was used, along with 2-10% (v/v) CSL, 0.5% ethyl alcohol and 1.13% (v/v) acetic acid. This is the first study reporting on using components with pectin and high polyphenol content.

Some studies on BC production utilize renewable sources. For instance, Zeng *et al.* (2011) used maple syrup for BC production from *G. xylinus* BPR2001 and found that supplementing 0 g carbohydrate/L maple syrup in the media produced 1.51 g/L BC, which was comparable with 20 g/L fructose as a control, which yielded 1.60 g/L BC. They concluded that maple syrup could be used as a cheaper alternative to fructose.

Kurosami and coworkers (2009) used fruit juices for BC production and reported that supplementing the fruit juices (containing glucose, fructose and sucrose) with nitrogen sources (2.0% peptone, 0.5% yeast extract and 0.12% citric acid) enhanced BC yield. They obtained a BC yield of 7% of the sugars.

Panesar and coworkers (2009) achieved maximum BC on mannitol (1.8 g/L), followed by glucose (1.5g/L). A similar result was also reported by Jonas and Farah (1998), who reported that maximum BC was achieved by supplementing the 2% (w/v) glucose. Glucose was selected as the carbon source throughout the study due to the cost of mannitol.

2.6.2 Effect of Additives on BC Production

Chao *et al.* (2001) investigated the effect of adding water-soluble polysaccharides on BC production in a 50-L airlift reactor. Adding 0.1% (w/w) agar or 0.06% (w/w) xanthan increased BC concentration to 8.7 g/L and 7.2 g/L respectively compared with 6.3 g/L in the CSL-fructose media (without added polysaccharides. They also showed that adding 0.1%-0.2% dextran was not effective, and that 1% dextran inhibited *Acetobacter xylinum* BPR2001 growth. They concluded that not all polysaccharides improve BC productivity.

Joseph and coworkers (2003) studied the effect of PA (polyacrylamide), a polymeric flocculant that can form strong hydrogen bonds (Ben-Bassat *et al.*,

1992; Joseph *et al.*, 2003), on BC production. They reported that adding up to 3 g/L of PA enhanced BC production.

Zhou *et al.* (2007) investigated the effect of sodium alginate on BC production by *G. xylinus* NUST41. Adding 0.04% (w/v) sodium alginate stimulated BC yield to 6.0 g/L in shake flasks cultivation, which was 27% of the total sugar initially added, compared to 3.7 g/L and 24% in the control media, respectively. Similar improvements in a stirred-tank bioreactor were observed. The maximum BC yield of 1.89 g/L was obtained by adding 0.04% sodium alginate. This was 1.7 times higher than the 1.09 g/L obtained in the control media without sodium alginate.

The effect of additives such as CMC (carboxymethylcellulose), microcrystalline cellulose and sodium alginate on BC production also have been studied. Cheng and coworkers (2009) reported that adding 0.5% CMC gave the highest BC concentration of 7.2 g/L compared with 1.3 g/L for the control. Adding 0.5% microcrystalline cellulose and 0.2% agar improved BC concentration to 4.37 and 4.49 g/L respectively. They also reported that sodium alginate has an adverse effect and decreased BC production. Thus, CMC was selected as the best additive for the study. The optimal concentration of CMC was 0.8% (w/v) and it produced 8.2 g/L, which was 6.3 times greater than the control (CSL-fructose media without additives).

Ishida *et al.* (2003) did a similar study using two different strains of *G. xylinus*, BPR2001 and EP1. The EP1 strain was derived by disrupting BPR2001 strain *ace*A gene. *Gluconacetobacter xylinus* BPR2001 produced water-insoluble BC and acetan in CSL-fructose media but *G. xylinus* EP1 was incapable of producing acetan. These researchers initially found that BC production by EP1 strain (2.88 g/L) was lower than BPR2001 strain (4.6 g/L). Adding purified acetan (0.15%) to the media enhanced BC production and the BC final yield with the EP1 strain was almost same as from the BPR2001 strain. Similar results were obtained by adding 0.1%-0.4% agar to the media. They concluded that adding acetan or agar enhanced culture media viscosity, which may delay BC coagulation and cell growth, thus stimulating growth of BPR2001 and BC production by BPR2001 strain.

2.6.3 Effect of Nitrogen Source on BC Production

Yeast extract and peptone are the most commonly used nitrogen sources when producing BC and provide convenient nitrogen and growth factors for *Gluconacetobacter* strains (Chao *et al.*, 2001; Kim *et al.*, 2003). Yang *et al.* (1998) examined the effect of adding 0.5% to 6% yeast extract to a media containing 20 g/L sugar. The maximum BC concentration (6.7 g/L) was obtained by adding 4% yeast extract. Coban and Biyik (2011) found that using 20 g/L glucose and 5 g/L casein hydrolysate enhanced BC production by 12.1%, which was same production as obtained by combining 20 g/L fructose and yeast extract.

Adding 1% (w/v) yeast extract, tryptone or CSL (corn steep liquor) enhanced BC yield (Kim *et al.*, 2006). Adding yeast extract gave the highest BC yield (4.49 g/L), followed by tryptone (3 g/L) and CSL (2.16 g/L). As yeast extract produced the highest BC yield, optimizing trials were done for yeast extract concentrations of up to 8 g/L. They found that adding 8 g/L yeast extract increased BC yield and concluded it as the optimum nitrogen source concentration for BC production by *Gluconacetobacter sp.* RKY5.

Kurosami *et al*. (2009) reported that adding supplemental fruit juices, which contained glucose, fructose and sucrose with the nitrogen sources in HS media (2.0% peptone, 0.5% yeast extract and 0.12% citric acid) enhanced BC production *G. xylinus* NBRC13693 by 7%.

Pourramezan *et al*. (2009) obtained BC concentrations of 11.65 g/L with 7 g/L yeast extract and 9 g/L peptone. They concluded both nitrogen sources were essential for high BC production.

Hungund and Gupta (2010) investigated the effect of different nitrogen sources on BC production by *Gluconacetobacter persimmonis* GH-2. With an initial beef extract concentration of 0.5% (w/v), they obtained a BC concentration of 6.25 g/L. Casein hydrolysate and peptone gave 5.25 g/L and 5.16 g/L BC respectively. In trials on the effect of adding beef extract, they obtained the highest BC production (5.89 g/L) using 0.6% (w/v) beef extract.

Panesar *et al.* (2010) also investigated the effect of nitrogen sources on BC production and found sodium nitrate, peptone and methionine were effective for cell growth and BC production. Based on cost factors, sodium nitrate was selected for further trials on BC production. The maximum BC production (1.6 g/L) was obtained with 1% (w/v) sodium nitrate. Matsuoka and coworkers (1996)

reported that vitamins and amino acids are crucial for cell growth and BC production.

2.7 Factors Affecting for BC Production

As well as studies on stimulatory factors such as ethanol (Naritomi *et al.*, 1998a), organic acids (Toda *et al.*, 1997), and water-soluble polysaccharides (Ishida *et al.*, 2003; Bae *et al.*, 2004), other approaches to optimize and maximize BC production include investigating the effect of agitation and aeration in agitated culture. High oxygen supply and high volumetric agitation power are essential to enhance BC production (Kouda *et al.*, 1996; Kouda *et al.*, 1997b; Yoshinaga *et al.*, 1997). Agitation and aeration influence BC production and can trigger formation of cellulose non-producing mutants (Cel^-) in the culture media.

2.7.1 Effect of Agitation and Agitator Configuration

Many researchers have found that more BC can be produced in static than in agitated cultivation. One problem in agitated culture is formation of cellulose non-producing mutants, which give low BC concentrations and a BC with a non-uniform structure (Valla and Kjosbakken, 1982; Park *et al.*, 2004). Cellulose mutant formation is related to BC hydrolysis by beta glucosidase in the cell wall (Cannon and Anderson, 1991). As optimization strategies in static culture are limited, conditions that give efficient, cost-effective BC production in agitated and aerated conditions needs to be investigated (Ross *et al.*, 1991).

Toyosaki *et al.* (1995), Kouda *et al.* (1997b), Lee and Zhao (1999), Bae and Shoda (2004) cited by Hungund and Gupta (2010) suggested that aerated, agitated fermentation may be suitable for enhanced BC productivity on an industrial scale. Hungund and Gupta (2010) found the BC production increased rapidly in an agitated fermenter and about 30% more was produced than in static cultivation. This may be due to the high mass transfer of nutrients and oxygen and optimum control of pH and temperature. They commented that static cultivation is not suitable for industrial scale of BC production because it is laborious and long cultivation times are needed. The cultivation period of 12-14 days in static cultivation decreased to 5-7 days in agitated cultivation.

Some studies report that adding water-soluble polysaccharides such as agar, polyacrylamide acid (PA), xanthan and acetan can reduce shear stress during agitation and also reduce BC coagulation (Chao *et al.*, 2001; Ishida *et al.*, 2003;

Joseph *et al.*, 2003; Bae *et al.*, 2004). This favours nutrient transfer into the bacterial cells and further stimulates BC productivity in the culture media.

Joseph *et al.* (2003) reported that adding 0-0.3% PA to *G. xylinus* BPR2001 fermentations increased BC production from 2.7 to 6.5 g/L at 175 rpm and from 1.7 to 3.7 g/L at 375 rpm. Zhou *et al.* (2007) obtained 6.0 g/L BC by adding 0.04% NaAlg in shake flask cultivation, which was 1.6 times greater than the 3.7 g/L in media without NaAlg. The effect of agar addition was also reported by Bae *et al*. (2004). Growing *G. xylinus* BPR2001 on agar-supplemented media increased BC production to 12.8 g/L BC compared with 8 g/L on media without agar. These researchers concluded that adding reduced BC clumping and reduced shear stress.

As well as studies on using water-soluble polysaccharides to reduce shear effect, some studies have been done in airlift reactors, which have low shear force. However, these reactors may induce oxygen exhaustion, which will decrease BC production. Oxygen transfer is not efficient as BC lumps accumulate in the lower section of the liquid circulation (Chao *et al.*, 2001; Kim *et al.*, 2012). To eliminate this, Kim *et al.* (2012) supplied agar to the up and down circulation type bubble column reactor (Figure 2.14) newly developed for BC mass production.

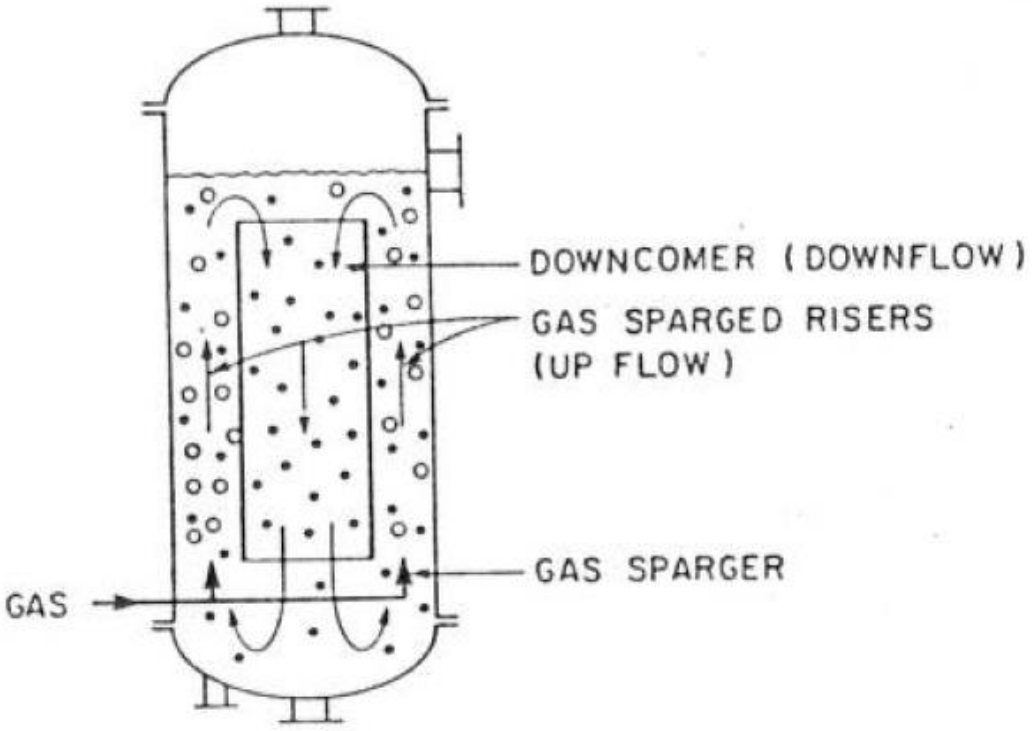

Figure 2.14: Schematic diagram of airlift reactor (Chisti and Moo-Young, 1987).

A study on the effect of agitation led researchers to elucidate the effect of viscosity due to adding water-soluble polysaccharides to the culture media. Kouda *et al*. (1997a) investigated the effect of agitator configuration on two properties: (1) the level of mixing achieved and (2) oxygen transfer capacity. Mixing the viscous culture media was described in terms of mixing time; cavern formation, flow visualization (Nienow *et al.,* 1988; Kouda *et al.,* 1997a) and circulation time measured using a tracer (Funahashi *et al.,* 1988; Kouda *et al.,* 1997a). The oxygen transfer capacity of each agitator was determined by measuring K_{La}. This value can be measured by static gassing-out, dynamic gassing-out and gas balance methods (Kouda *et al.,* 1997a).

Recent research by Kim *et al.* (2012) investigated the effect of viscosity on BC production by determining the effect of water-soluble polysaccharides (agar) on K_{La} quantitatively and insoluble BC (the main viscosity-inducing ingredient in the culture broth) on viscosity and $K_{La.}$ Supplementing the media with 0.4% agar increased viscosity and BC production. However, K_{La} value decreased when agar concentration increased from 0 to 0.4%. They concluded that adding water-soluble polysaccharides reduces shear stress in agitated culture and adding water-soluble polysaccharides such as agar had a greater effect on BC production than the K_{La}. The data indicated that controlling the two major factors viscosity and K_{La} could enhance BC production and also reduce shear stress during agitation.

Kouda *et al*. (1997a) found that the Maxblend (Figure 2.15) and gate with turbine impellers can mix culture media efficiently and give larger K_{La} values. They recommend that impellers should be configured to give a high shear region around the sparger. This is crucial for dispersing the air bubbles and thus increasing K_{La}. These factors affect agitation and oxygen transfer, which are both important when producing BC in agitated and aerated culture.

2.7.2 Effect of Aeration/Oxygen Pressure

The effect of oxygen on BC production in aerated and agitated culture also has also been studied. Kouda *et al*. (1997b) recommended the effect of oxygen pressure on BC production is determined because media containing BC is difficult to mix due to its high viscosity. A second reason is that BC production rate and BC yield are affected by oxygen transfer rate (OTR) and K_{La} (oxygen transfer coefficient).

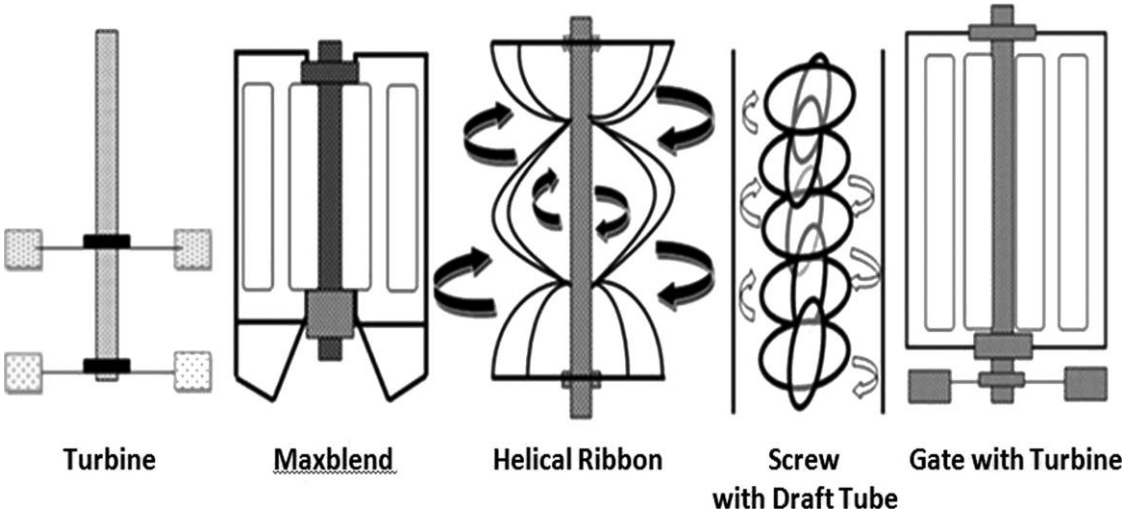

Figure 2.15: Agitators used in fermenters (Kouda *et al.*, 1997a).

The BC production rate increases when K_{La} is high, not because mass transfer around cells is enhanced but because oxygen transfer from the gaseous phase to the culture media is rapid (Kouda *et al.*, 1997b). Increasing oxygen partial pressure by using oxygen-enriched air and/or raising the operating pressure improves oxygen supply. Kouda *et al.* (1997b) also reported that BC production rate depended on oxygen transfer rate, which decreased as the culture media viscosity increased (due to BC accumulation).

2.8 Factors that Affect BC Production

2.8.1 Substrate

Carbon sources including monosaccharide, disaccharides, and polysaccharides have been used for growing *Gluconacetobacter* strains and producing BC. Most studies used glucose as the sole carbon source because it is relatively cheap. However, other carbon sources such as sucrose, fructose, maltose, xylose, molasses, ethanol, mannitol, glycerol and inositol have been investigated.

The BC yields reported range from 0.01g per g substrate to 0.80g per g substrate used. As the current research is on maximising BC yields, any literature on effect of substrate, additives and growing conditions on BC yields that obtained BC yields of less than 0.01 g/g substrate (i.e. 1% conversion) were disregarded.

Pourramezan *et al.* (2009), used sucrose and glucose as carbon sources, to obtain BC yields of 0.78 g/g ad 0.58 g/g respectively. Some researchers have found that glycerol and mannitol were good carbon sources for producing BC. For example, Kim *et al.* (2006) obtained BC yields of 0.375 g/g BC using glycerol and Moosavi-Nasab *et al.* (2011) obtained 0.9 g/g when using glucose.

Some studies report that BC production is strongly affected by adding ethanol to the media. For example, adding 1% (v/v) ethanol enhances BC production but increasing ethanol concentration above 1.5% (v/v) reduced BC production (Park *et al.*, 2003). Naritomi *et al.* (1998a) found that adding 1% (v/v) ethanol to 30 g/L CSL-fructose media increased BC production, cell concentration and fructose consumption rate. Under continuous fermentation conditions, they obtained a BC yield of 1.53 g/g. However, if ethanol was ≥1.5% (v/v), BC production decreased because cell growth was inhibited. Other studies on the effect of ethanol confirmed Naritomi and co-workers' (1998a) findings. Son *et al.* (2001) found that adding 1.4% (v/v) g/L ethanol to 40 g/L glucose increased BC production to 0.745 g/g, which was about four times than that of media without ethanol. Park *et al.* (2003) added 1% (v/v) g/L ethanol to the media for BC production by *G. hansenii* and successfully eliminated production of BC non-producing cells .The BC production increased from 1.3 g/L to 2.31 g/L. However, if ethanol was ≥ 1.5% (v/v), BC production decreased, confirming Naritomi and coworkers' (1998a) findings.

Lu *et al.* (2011) investigated the effect of different alcohols on BC production. They found that that methanol, n-butanol, ethylene glycol, n-propanol, glycerol and mannitol affected BC production and concluded that alcohols stimulate BC production and may be crucial for BC biosynthesis.

Some studies investigated the effect of renewable sources (i.e. undefined media) as a cheaper substrate for producing BC. Keshk and Sameshima (2006a) Bae and Shoda (2004) and Jung *et al.* (2010) used molasses. Keshk and Sameshima (2006a) reported that molasses with lignosulfonate enhanced BC production and decreased gluconic acid formation (a problem when using glucose as a carbon source). They assumed that the antioxidant and polyphenolic compounds in lignosulfonate inhibited gluconic acid production, thereby increasing BC production.

Bae and Shoda (2004) treated molasses with sulphuric acid and obtained 0.22 g/g BC yields on molasses media, compared to 0.20 g/g in CSL-Fruc (corn

steep liquor-fructose) media. They found that adding 0.2 L molasses to 4.5 L of media improved BC yield.

Some studies have used fruit waste as a carbon source for BC production. Studies by Moosavi-Nasab *et al*. (2011) and Kurosumi *et al.* (2009) showed promising results by obtaining 0.69 g/g BC on orange juice supplied with nitrogen sources. Moosavi-Nasab *et al.* (2011) replaced sucrose with date syrup and obtained a BC yield of 1.5 g/g on 20 g/L date syrup. Kongruang (2008) grew *Acetobacter xylinum* 998, 893 and 975 strain on coconut juice. Strain 998 gave 0.01 g/g of BC yield and 0.03 g/g of BC yield by both 893 and 975 strain. Kurosumi *et al.* (2009) used several types of fruit juices and found orange juice was suitable for BC production. Nitrogen sources such as yeast extract and peptone were added to the fruit slurries.

Nitrogen, which is a component of proteins, is essential in cell metabolism, making up 8-14 % of bacterial dry cell mass (Ramana *et al*., 2000). Yeast extract and peptone are the most widely used nitrogen sources and also act as growth stimulators in BC production. Other nitrogen sources used to improve BC production include corn steep liquor, casein hydrolysates, beef extract, malt extract, soybean meal, urea, and MSG (monosodium glutamate) (Appendix 1). Yang *et al*. (1998) found that 4% yeast extract (range 4-6%) gave the best BC yield (0.335 g/g on various carbon substrates).The highest BC yield was obtained on 6% CSL. Ramana *et al.* (2000) found that using casein hydrolysate or peptone gave better BC concentrations than other nitrogen sources such as soybean meal, ammonium sulphate, monosodium glutamate (MSG) and glycine. Other researchers (El-Saeid *et al.*, 2008; Hungund and Gupta, 201a; Hungund and Gupta, 2010b) also report that casein hydrolysates and peptones can markedly increase BC yield compared with inorganic nitrogen sources.

2.8.2 Substrate Concentration

The concentration of carbon source used for producing BC ranged from 5 g/L to 200 g/L (Appendix 1). Most studies used 20 g/L of sugar (whether glucose, sucrose, fructose, mannitol, glycerol etc) for producing BC (19 of the 38 references) and obtained BC yields of 0.01 to 0.90 g/g substrate. Eight further studies used 40 g/L sugar and produced BC yields of 0.02-0.75 g/g; four studies used 50 g/L sugar and produced BC yields of 0.016-0.233 g/g. Overall, an initial sugar concentration 20 g/L appears to be the most favourable. For example,

Pourremezan *et al.* (2009) obtained a BC yield of 0.78 g/g on 20 g/L sucrose and 0.58 g/g on 20 g/L glucose; Son *et al.* (2001) obtained 0.75 g/g on 20 g/L glucose.

The initial carbon source concentration affects BC production and increasing carbon source concentration can often decrease BC production (for example, Son *et al.,* 2001; Masaoka *et al.,* 1998). With 4.8 g glucose per flask (a glucose concentration of approximately 70 g/L), the amount of gluconic acid, a major by-product in BC production was increased (Masaoka *et al.,* 1993), which lowers pH of the culture broth and inhibits BC production (Takai, 1989).

2.8.3 Bacterial Species

Gluconacetobacter genus contains several strains that are classified as an efficient BC producers (Castro *et al.,* 2012). Researchers have isolated *Gluconacetobacter sp.* from sources such as fruit (Dellaglio *et al.,* 2005), flowers, fermented foods, (Lisdiyanti *et al.,* 2001; Alauzet *et al.,* 2011) and vinegar (Sokollek *et al.,* 1998). Of the 38 literature references on BC production obtained (Appendix 1), 36 used *Gluconacetobacter sp.* to produce BC, one study used *Enterobacter sp*, one study used a tea fungus, and one study used *Lactobacillus sp.* *Gluconacetobacter sp.* is the most favoured microbe for producing BC and gives the highest BC production (Jonas and Farah, 1998; Klemm *et al.,* 2001; Brown *et al.,* 2004; Klemm *et al.,* 2005; Aydin and Aksoy, 2009). The highest BC concentration of 9.7 g/L was obtained by growing a sulfaguanidine-resistant mutant of *G. xylinus* subsp. *sucrofermentans* on 40 g/L sucrose (Seto *et al.,* 2006).

2.8.4 pH

The literature indicates that pH, bacterial strain and media affect BC production. Most researchers use *Gluconacetobacter spp.* on media with a pH of 4.0 to 7.0 (Iguchi *et al.,* 2000).

Fermentation pH affects cell growth and BC production so the effect of pH needs to be evaluated. *Gluconacetobacter sp.* can produce BC at pH values from 4 to 7 (Iguchi *et al.,* 2000). It is reported that the optimal pH value for BC production has been reported as 4 to 6 (Fontana *et al.,* 1991) and pH 4 to 7 (Galas *et al.,* 1999). Jonas and Farah (1998) recommend pH 4 and pH 4.5 for commercial applications. Jahan *et al.* (2012) studied media with pH of 3 to 10 and found that highest BC concentration of was 0.92 g/L was obtained by growing *G sp.* F6 on 20 g/L glucose at pH 6.

Other researchers also reported higher yields of BC when fermentations were done at pH 5 or pH 6. According to Coban and Biyik (2011), Fiedler *et al.* (1989), Joris *et al.* (1990), Ishikawa *et al.* (1995) reported that pH 5 was optimum, while Hestrin and Schramm (1954) and Masaoka *et al.* (1993) reported pH 6 was optimum. Of the 38 reported studies on producing BC using bacteria (Appendix 1), ten used an initial pH of 5, ten used an initial pH of 6 and a further three further studies used pH 5.5. The remaining studies were done using initial pH values from 2.7 to 6.8. In general, BC production was not encouraged if pH was below 4 or above 7. For example, Bae *et al.* (2004) obtained 0.48 g BC/g substrate when growing *G. xylinus* on 40 g/L fructose at pH 5, Keshk and Sameshima (2006c) obtained 0.8 g/g at pH 6, and Kurosumi *et al.* (2009) obtained 0.69 g/g on fruit juices at pH 6. Son *et al*. (2001) obtained 0.75 g/g of BC on media with 40 g/L glucose and 1.4 mL/L ethanol at pH 6.5. Moosavi-Nasab *et al.* (2011) successfully produced BC by growing *G. xylinus* PTCC1734 on 50 g/L sucrose and 50 g/L date syrup at pH 6.8.

2.8.5 Temperature

Bacterial cellulose yield is temperature dependent because temperature affects both cell growth and BC production. Most research has been done at temperatures in the mid to high 20s. For instance, Jonas and Farah (1991) and Masaoka *et al.* (1993) reported the optimal temperature for BC production and cell growth to be 25 to 30^{o}C.

Of the research summarised (Appendix 1), most used 28 to 30^{o}C (for example, Hestrin and Schramm, 1954; Romano *et al.*, 1989; Geyer *et al.*, 1994; Pourramezan *et al.*, 2009). An analysis of the 38 references shows 24 studies used 30^{o}C, nine used 28^{o}C and two further studies used 29^{o}C. One study (Ramana *et al.*, 2000) used 35^{o}C.

Fermenting 20 g/L at 30^{o}C appears to be the most favoured temperature. The BC yields vary. For example, Pourramezan *et al.* (2009) obtained 0.78 g/g substrate, Son *et al.* (2001) obtained 0.75 g/g , Kurosumi *et al.* (2009) obtained 0.69 g/g, Goh *et al.* (2012) obtained 0.67 g/g, Bae *et al.* (2004) obtained 0.48 g/g, Heo *et al.* (2002) obtained 0.47 g/g , Naritomi *et al.* (1998b) obtained 0.46 g/g and Kim *et al.* (2006) obtained 0.375 g/g .

Some studies indicate 28^{o}C is the optimum temperature. For example; Keshk and Sameshima (2006c) obtained 0.8 g/g by fermenting *G. xylinus*

ATCC10245 on 18 g/L molasses with 2 g/L glucose; Joseph *et al.* (2003) obtained 0.325 g/g on 20 g/L fructose, and Ishihara *et al.* (2002) obtained 0.312 g/g on 20 g/L glucose. Even a slight increase in temperature appears to decrease yields. For example, Dahman *et al.* (2010) obtained only 0.141 g/g of BC when growing *G. xylinus* on 40 g/L fructose at 29^{o}C compared to Keshk and Sameshima (2006a) at 28^{o}C, whom obtained 0.145 g/g on 40 g/L molasses.

A recent study (Jahan *et al.*, 2012) on the effect of 25 to 40^{o}C for growing *Gluconacetobacter sp.* F6 found that the optimum temperature for producing BC was 30^{o}C and they obtained a BC concentration of 0.91 g/L. This concentration decreased if temperature changed slightly from 30^{o}C. Minimum BC production occurred at 35^{o}C and none was produced at 40^{o}C. Another study on the effect of temperatures between 4 and 37^{o}C also reported that the optimum temperature for BC production was 30^{o}C (Coban and Biyik, 2011).

2.8.6 Conclusions

An analysis of the literature showed that supplementing 20 g/L carbon source with 5 g/L yeast extract and peptone appeared favourable for producing BC. Adding other materials such as 1%-1.4% ethanol or 1-1.5 g/L water-soluble polysaccharides (agar, acetan) increased BC production. Adding treated materials such as lignosulfonate-molasses and acid-treated molasses improved BC production. Fruit juices supplemented with sufficient nitrogen sources also gave enhanced BC productivity.

Producing BC using *G. xylinus* at pH 6 and 30^{o}C improved BC production. Further optimization of the reactor conditions such as pH, temperature, aeration rate, agitator configuration and agitation speed are needed to increase BC production, especially for industrial scale.

2.9 Fermentation, Bioreactor Design and Operation

An effective bioreactor provides a controlled environment for growing microorganisms and producing biological reaction. The two important factors for optimising growth and/or yield are: identifying processing parameters to obtain the desired biological, chemical and physical effects (including microbial growth and metabolites); and controlling the bioreactor, which includes environmental conditions such as pH, temperature mixing and aeration, and product and by-product harvesting (Williams, 2002).

2.9.1 Formulating Fermentation Media

Media can be defined or complex. Defined media has specific concentrations of chemical components with known compositions. Complex (or undefined) media is made from natural components and their exact chemical compositions may not be known. Complex media is usually cheaper than defined media and may give higher yields. However, researchers often prefer to use defined media because it gives greater reproducibility and end-product purification is often easier and cheaper (Shuler and Kargi, 2002). Media composition for growing *G. xylinus* to produce BC was discussed in Section 2.6. Most researches used defined media but some researchers used complex media such as molasses and fruit juices.

Nutrients for bacterial growth can be classified as macro or micronutrients. The former are required in large concentrations (more than 10^{-4} M) and include carbon, nitrogen, oxygen, hydrogen, sulphur, phosphorus, magnesium and potassium. Micronutrients are required in small amounts (less than 10^{-4} M) and too high a concentration may be toxic. They include molybdenum, zinc, copper, manganese, calcium, sodium, vitamins, hormones and metabolic precursors. These are often co-factors in metabolic cycles.

The carbon source is the major supply of cellular energy in fermentation media. Sources such as glucose, sucrose and fructose are widely used in laboratory-scale fermentations and most of the reported work on BC production used these substrates. Carbon in the form of alcohols such as glycerol and mannitol has been also used for producing BC (Table 2.7).

Nitrogen is important in cell structure and function as well as for nitrogen catabolite repression (Farrell *et al.*, 1993; Fleet *et al.*, 1998; Júnior *et al.*, 2008). Nitrogen sources also play a role in structure and function (Júnior *et al.*, 2008).

Ammonia, ammonium salts, peptides and amino acids are widely used in fermentation. Yeast extract (which contain vitamins, minerals and amino acids) and peptone (made by hydrolysing proteins) are expensive nitrogen sources used by most organisms for synthesising nitrogenous compounds (Greasham, 2003).

Phosphorus, in salts such as KH_2PO_4 and K_2HPO_4 commonly used in fermentation media, favour cell metabolism. The concentration should be less than 1 mM for secondary metabolite formation. The macronutrient magnesium is a cofactor for some enzymes and has a role in carbohydrate metabolism (Shuler and Kargi, 2002).

Micronutrients, such as trace elements, help decrease the lag phase. A lack of a specific micronutrient may decrease specific growth rate and yield. Zinc (Zn), iron (Fe), and manganese (Mn) are the most common trace elements used in fermentation. Iron (Fe), zinc (Zn) and manganese have crucial a regulatory role. Copper (Cu) favours antibiotic and citric acid fermentations while sodium (Na) has a role in cell transport and also ion balance (Shuler and Kargi, 2002).There are many challenges when designing media, whether for laboratory scale experiments or for industrial scale production (Table 2.8).

Designing media is important for strain development. The main objective in choosing an optimum media is to ensure all required minerals are present in sufficient amounts yet not inhibiting cell growth and production (Greasham, 2003). There are two options for choosing the best media. Firstly, to identify the best composition based on results using one microbial strain and then identify the best strain using this media. Secondly, to identify the optimal strain using one general media followed by media optimisation (Kennedy and Krouse, 1999). The best media gives high productivity, and a consistent and cost-effective fermentation process (Greasham, 2003).

Microbial growth and metabolite production

Microorganisms consume nutrients from the culture media and convert them into cell mass and/or metabolites/by-products.

$$\text{Substrates} \xrightarrow{\text{cells}} \text{Products + Cell replication}$$

Microbial growth rate (μ_{net}) describes cell concentration (X) at time (t):

$$\mu_{net} = 1/x \; . \; dX/dt$$

They can also be characterized in terms of cell number (N):

$$\mu_R = 1/N \; . \; dN/dt$$

where, μ_R is the specific replication rate (h^{-1}).

Table 2.7: Summary of Carbon Sources used for Producing BC

Carbon source	Conc^{n} (g/L)	References
Glucose	10	Heo *et al.,* 2002; Park *et al.,* 2004; Keshk and Sameshima, 2006c
	14	Keshk and Sameshima, 2006c
	15	Pourramezan *et al.,* 2009; Son *et al.,* 2003; Kim *et al.,* 2006
	18	Keshk and Sameshima, 2006c
	20	Coban and Biyik, 2011; El-Saeid *et al.,* 2008; Hungund and Gupta, 2010a; Hungund and Gupta, 2010b; Ishihara *et al.,* 2002; Keshk and Sameshima, 2006a; Keshk and Sameshima, 2006c; Kim *et al.,* 2006; Lu *et al.,* 2011, Mikkelsen *et al.,* 2009; Nguyen *et al.,* 2008; Panesar *et al.,* 2009; Park et *al.,* 2010; Pourramezan *et al.,* 2009; Son *et al.,* 2001; Toda *et al.,* 1997; Yunoki *et al.,* 2004
	21	Zhou *et al.,* 2007
	25	El-Saeid *et al.,* 2008
	30	Naritomi *et al.,* 1997a; Naritomi *et al.,* 1997b
	40	Dahman *et al.,* 2010; Goelzer *et al.,* 2009; Heo *et al.,* 2002; Seto *et al.,* 2006; Son *et al.,* 2001; Shigematsu *et al.,* 2005
	50	Chavez-Pacheco *et al.,* 2005; Ha *et al.,* 2008; Ramana *et al.,* 2000
Fructose	10	Keshk and Sameshima, 2006b
	15	Kim *et al.,* 2006
	20	Coban and Biyik, 2011; Hungund and Gupta, 2006a, Hungund and Gupta, 2006b; Joseph *et al.,* 2003; Kim *et al.,* 2006; Mikkelsen *et al.,* 2009; Nguyen *et al.,* 2008; Panesar *et al.,* 2009; Son *et al.,* 2001; Toda *et al.,* 1997
	40	Bae et al., 2004; Dahman *et al.,* 2010; Ishida *et al.,* 2002; Seto *et al.,* 2006; Son *et al.,* 2001; Shigematsu *et al.,* 2005
	50	Chavez-Pacheco *et al.,* 2005; Goh *et al.,* 2012; Moosavi-Nasab *et al.,* 2011; Ramana *et al.,* 2000

Carbon source	Conc^{n} (g/L)	References
Sucrose	15	Kim *et al.,* 2006; Pourramezan *et al.,* 2009
	18	Zhou *et al.,* 2007
	20	Coban and Biyik, 2011; Hungund and Gupta, 2010a; Hungund and Gupta, 2010b; Kim *et al.,* 2006; Mikkelsen *et al.,* 2009; Nguyen *et al.,* 2008; Panesar *et al.,* 2009; Pourramezan *et al.,* 2009; Son *et al.,* 2001; Toda *et al.,* 1997
	21	Zhou *et al.,* 2007
	50	Chavez-Pacheco *et al.,* 2005; Goh *et al.,* 2012; Moosavi-Nasab *et al.,* 2011; Ramana *et al.,* 2000
	40	Seto *et al.,* 2006; Son et al., 2001
Glycerol	20	Hungund and Gupta, 2010a; Hungund and Gupta, 2010b; Kim *et al.,* 2006; Mikkelsen *et al.,* 2009; Park *et al.,* 2010; Toda *et al.,* 1997
	25	Park *et al.,* 2010
	50	Park *et al.,* 2010
	100	Chavez-Pacheco *et al.,* 2005
Mannitol	20	Hungund and Gupta, 2010a; Hungund and Gupta, 2010b; Lu *et al.,* 2011; Nguyen *et al.,* 2008; Panesar *et al.,* 2009; Son *et al.,* 2001; Toda *et al.,* 1997
	25	El-Saeid *et al.,* 2008
	40	Son *et al.,* 2001
	50	Ramana *et al.,* 2000
Fruit juices	200	Kongruang, 2008
	73-103	Kurosumi *et al.,* 2009

Table 2.8: Challenges during media design (Kennedy and Krouse, 1999)

Encountered in laboratory scale	Encountered in industrial scale
• Development time • Development cost • Limited shaker space • Precipitation reactions • Water quality • Dispersion of solid components • Effect of components on assay techniques • Effect of components on downstream processing • Foaming	• Availability of raw materials • Fluctuation in cost of media • Stability of supplier company • Handling of media components • Pest problems • Transportation cost for media components • Effect of broth viscosity on power consumption • Disposal cost of the media • Dust hazards

Microbial growth involves (1) a lag phase, (2) a log/exponential phase, (3) a deceleration phase, (4) a stationary phase and (5) a death phase (Figure 2.16). In the lag phase, cells and new enzymes are synthesized and the internal systems of cells adapt to the new culture conditions. In the log phase, cells multiply so cell mass increases exponentially. The deceleration phase occurs because nutrients have been depleted or growth is inhibited. This condition causes a reconstituting of the cell to increase the prospect of cellular survival. In the stationary phase, growth rate is equal to death rate and cell division may not occur. Cells may produce secondary metabolites and still metabolise. The number of viable cells may decrease but total cell mass may be static. In the death phase, viable cells decrease and cell lysis may occur (Shuler and Kargi, 2002).

Commercial fermentations involve growing cells from a stock culture. It is important to limit the lag period or fermentations will take a long time, tying up expensive equipment and facilities. Long lag times may also allow contaminant microbes to outgrow the desired organisms. The normal method is to use an inoculum 'train', which involves transferring actively growing cells (e.g. from the exponential growth phase) to successively larger reactors.

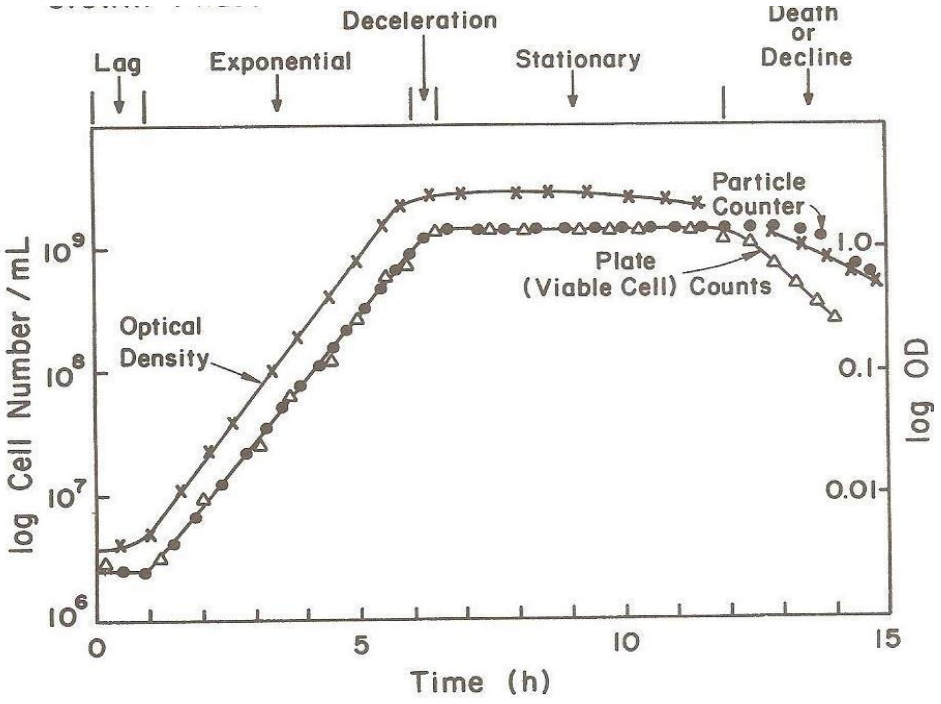

Figure 2.16: Microbial growth phases (Schuler and Kargi, 2002).

Limiting nutrient(s)

Nutrient limitation can be defined as the minimum concentration of a specific nutrient that gives less than optimum growth rate (Ferenci, 2001). The growth can be limited by a single or multiple nutrients. For example, carbon limitation is the most widely-used mode and has been used to study cell metabolism. In carbon-depleted media, growth is limited (Larsson *et al.*, 1993; Larsson *et al.*, 1997; Sauer *et al.*, 1999; Dauner *et al.*, 2001; Wick *et al.*, 2001; Hua *et al.*, 2004). Other nutrient limitations include nitrogen, phosphorus, or sulphur, with nitrogen limitation being the most commonly investigated (Larsson *et al.*, 1993; Sauer *et al.*, 1999; Dauner *et al.*, 2001; Wick *et al.*, 2001; Hua *et al.*, 2004).

To grow, most microbes require an organic compound (which acts as a carbon and/or energy source), carbon dioxide, hydrogen acceptors and inorganic ions. Some microbes may also require specific compounds as growth factors. Any of the required nutrients has the potential to be limiting (Monod, 1949).

"Hunger" can be described as a condition where the specific growth rate, μ, is neither zero nor the maximum rate to have the saturating concentration of nutrient, μ_{max} (Figure 2.17). Thus, "hungry" bacteria or microbes have a finite, nutrient limited growth rate characterized as $\mu_{max} > \mu \neq 0$

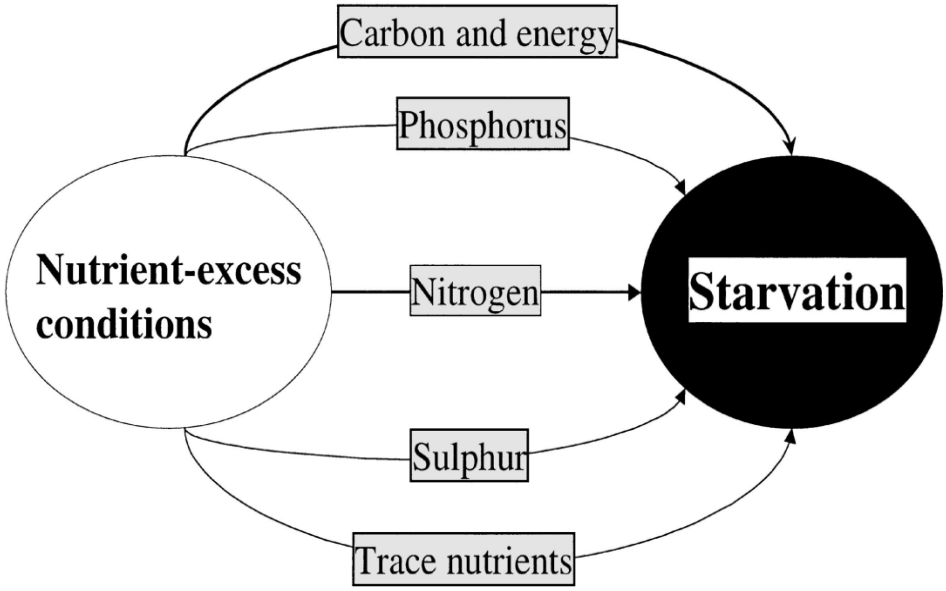

Figure 2.17: Schematic of bacterial response between nutrient excess and nutrient-starved states (Ferenci, 2001).

Inoculum preparation

Inoculum or seed culture preparation is an essential stage for obtaining productive, viable cells for fermentation (Gershater *et al.*, 2009). The process begins with transfer of variable cells from agar slants or stock culture to a shake flask (Meyrath and Suchanek, 1972; Webb and Kamat, 1993). The following factors need to be considered in preparing inoculum (Gershater *et al.*, 2009):

i. Culture storage conditions
ii. Preparing stock culture for culture maintenance
iii. Culture assessment
iv. Inoculum criteria

Stock culture is the capital in a fermentation process. In qualitative terms, it represents product formation capacity in a defined environment. In quantitative terms, the stock culture is defined by controlling the holding conditions so the cultivar is suitable for production. Cultures are usually grown on agar slants or plates and involve sub-culturing or transferring microbe colonies from one solid media to a new fresh media to revive the cells (Gershater *et al.*, 2009). Stock cultures are often generated and stored at -80^{o}C but need to be replenished when there is qualitative change in the culture component (Figure 2.18). Gershater *et al*. (2009) recommends that the replenishing process is initiated when 60% to 70% of the existing stock has been used but this can be delayed until 70% to 80% of the stock is used.

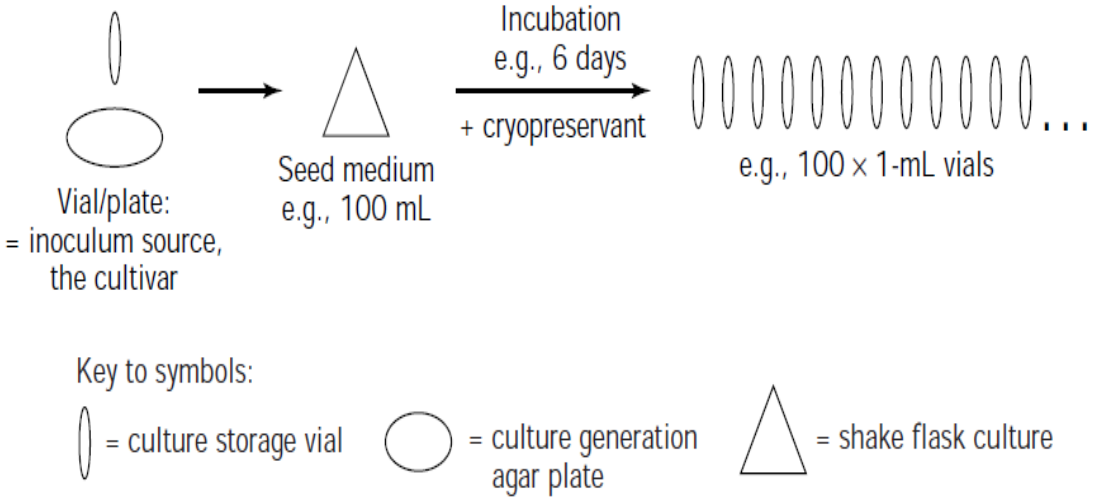

Figure 2.18: Stock culture generation models (Gershater *et al.*, 2009).

High quality inoculum is needed to obtain a successful fermentation. Factors that influence inoculum quality include (Gershater *et al.*, 2009):

i. Seed media
ii. Seed culture incubation conditions
iii. Microbial growth
iv. Optimum transfer criteria
v. Inoculum level
vi. Number of seed-stage passages
vii. Assessing inoculum development

Large scale production usually requires increasing active biomass via multiple growth cycles (Figure 2.19). Studies on the complete inoculum preparation process, from initial inoculation to final stage productivity indicate that slant tubes are the most effective system for maintaining high growth concentration in the final stage (Gershater *et al.*, 2009).

2.9.2 Bioreactors

Batch Operation

Batch operation involves supplying all the media components, along with the inoculum at the beginning of the process and allowing microbial growth, along with product formation and substrate depletion. The microbial population goes through the typical lag, growth phases (Section 2.9.1).

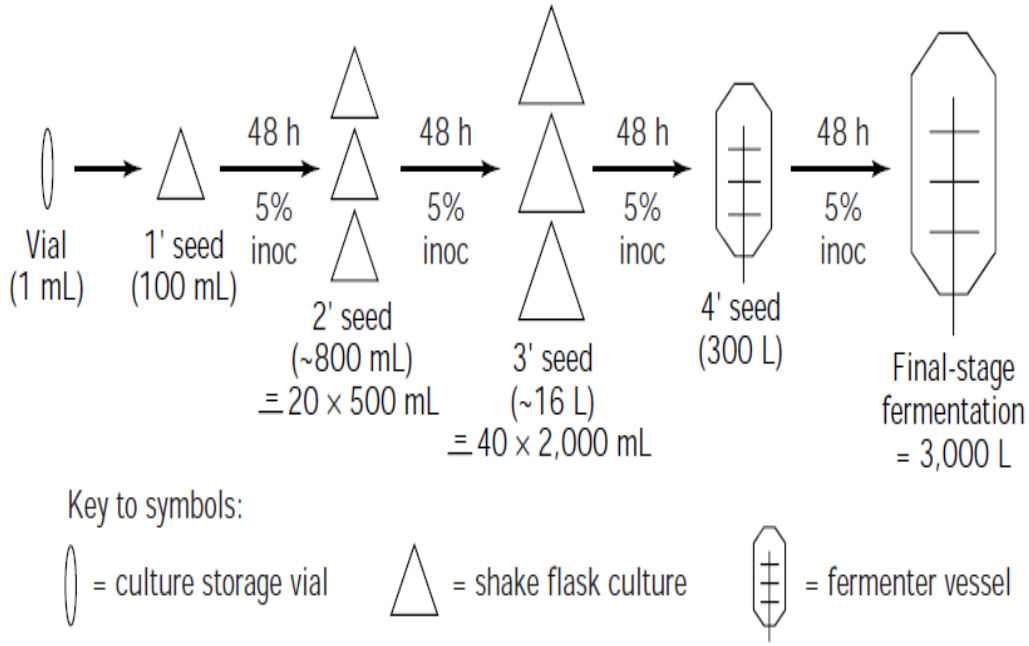

Figure 2.19: Multiple growth cycle fermentation inoculum train (Gershater *et al.*, 2009).

Most commercial fermentation for pharmaceutical production or brewing is batch (Williams, 2002) because it is efficient and good control is possible (Ghose and Tyagi, 1979; Cardona and Sanchez, 2007; Li *et al.*, 2011). Batch operation has the following advantages:

i. low risk of contamination due to relatively brief incubation period
ii. lower investment than continuous operation for the same bioreactor volume
iii. higher product conversion due to controlled culture conditions

However, nutrient depletion and product accumulation can limit batch operation (Li *et al.*, 2011; Li *et al.*, 2012). Other disadvantages include:

i. time-consuming because media needs to be prepared, sterilized, and cooled and the reactor has to be emptied and cleaned.
ii. preparing some subcultures for inoculation can be expensive
iii. laborious
iv. contamination risk in larger, industrial scale process is higher due to the potential contact with competing microbes or toxins.

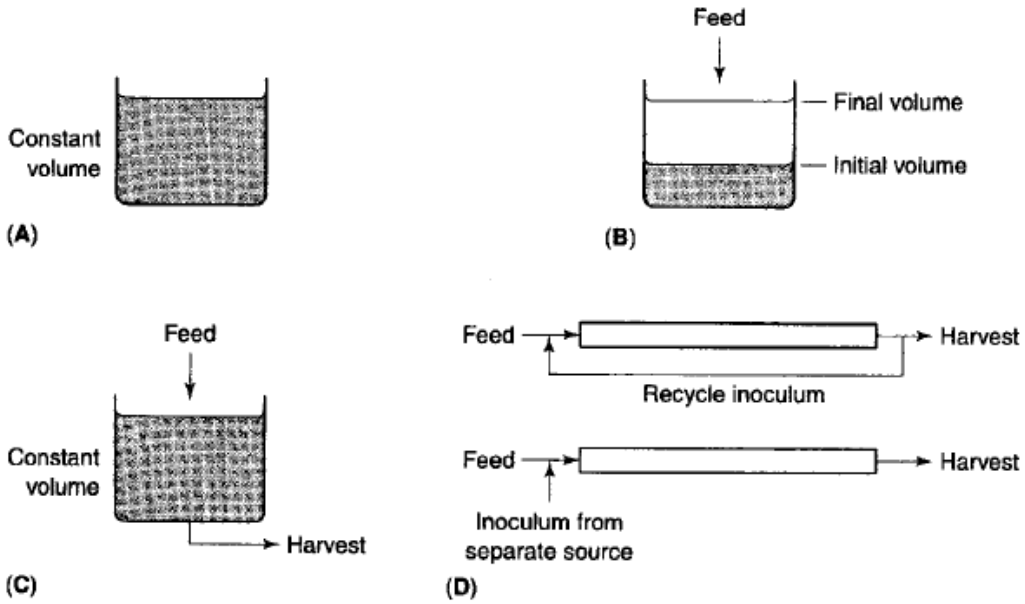

Figure 2.20: Batch (A), fed-batch (B), continuous (C), and continuous plug flow with/without cell recycle (D) fermentation modes (Chisti, 1999).

Continuous operation

Continuous operation is the other extreme and involves continuously feeding substrate to the culture media, with a corresponding flow of culture broth that is harvested to maintain the culture conditions constant. The system is at steady state and the time profile of continuous culture is shown in Figure 2.21.

Continuous conditions often increase productivity, are cheaper and more easily controlled. Other advantages include:

i. lower labour cost
ii. reduced toxicity risks because the process is automated
iii. stress on equipment due to sterilization is reduced
iv. less time is spent emptying, filling and sterilizing the fermenter

Continuous reactors are usually used for large-volume processes and growth rates can be maintained for a long time. Biomass concentration can be controlled by varying the dilution rate (Williams, 2002). The most commonly-used continuous reactors are continuous stirred tank reactors (CSTR) and plug flow reactors (Brethauer and Wyman, 2010).

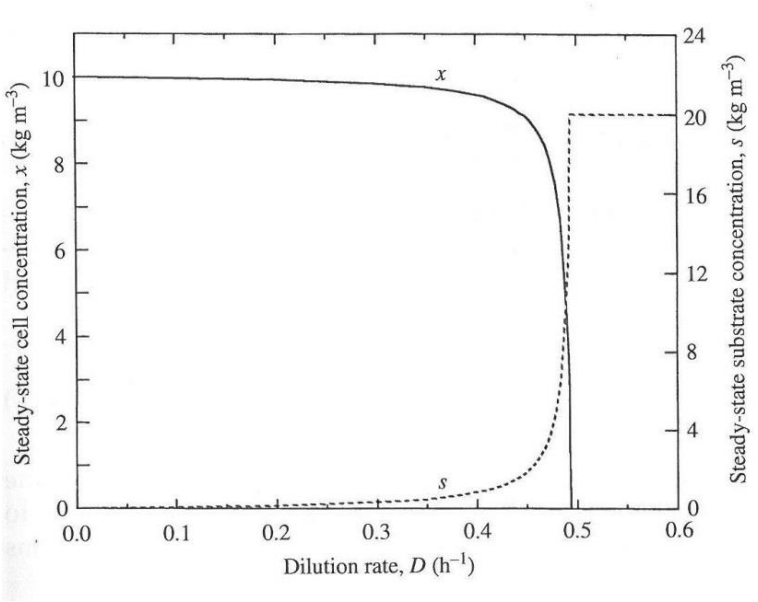

Figure 2.21: Effect of dilution rate on the steady state biomass and residual substrate concentration in continuous operation (Stanbury *et al.*, 2000).

Fed batch operation

Fed-batch operation is a modification to batch fermentation where fermentation is established in batch bode and then fresh media is added intermittently or continuously with or without removing fermenter contents at regular intervals (Stanbury *et al.*, 2000; Shuler and Kargi, 2002). Substrate concentration during this mode of operation is low and a high product formation rate is achieved. Fed-batch can prevent substrate inhibition and minimize catabolite repression. Fed batch operation is also suitable for secondary metabolite production (Enfors and Häggström, 2000; Saarela *et al.*, 2003).

The following strategies can be used in fed batch operation:

Fill and draw – intermittent removal of fermenter contents and replacement with equal volume of fresh broth. It also can be called 'cyclic fed-batch' or 'repeated fed-batch'. Operating volume is constant. This is also called cyclic or repeated fed batch.

Pulse feed **–** intermittent addition of media without removing contents so volume increases. This is also called variable volume fed batch operation.

Continuous feed – continuous addition of fresh media without removing contents so volume increases. This is also called variable volume fed batch.

As an extra factor in the operation, the added media can have the same or higher concentration of ingredients (or a limiting component) than the initial media.

Productivity

Microbes may be cultured in batch, fed batch or continuous mode (Section 2.9.2) with batch and continuous reactor operations representing the extremes. Productivity needs to be considered when selecting a reactor. Productivity in batch culture can be described as (Stanbury *et al.,* 2000):

$$R_{batch} = (x_{max}-x_0)/(t_i+t_{ii})$$

where,

R_{batch} = output of the culture in terms of cell mas concentration per unit time

x_{max} = maximum cell concentration obtained at stationary phase

x_0 = cell concentration at inoculation

t_i = the time during which organism grows at μ_{max}

t_{ii} = the time during which the microorganism is not growing at μ_{max}

For continuous operation, productivity is characterized as (Stanbury *et al.,* 2000):

$$R_{cont} = D\,\bar{x}\,(1-t_{iii}/T)$$

where,

R_{cont} = output of the culture in terms of cell concentration per hour

t_{iii} = time prior to formation of steady-state; includes time for vessel preparation, sterilization and operating in batch mode before continuous operation

T = time where steady-state conditions dominate

$\bar{x}$ = steady-state cell concentration

Maximum productivity in a chemostat system can be obtained by operating at the dilution rate giving the highest value of $D_{\bar{x}}$ (termed D_{max}.). Although a continuous operation steady-state system is beneficial, most fermentation industries operate in batch mode because most secondary metabolites are produced from mature cells; cell growth often inhibits product formation and the product will only be produced at very low dilution rates. Productivity of secondary metabolites in a batch operation may be significantly higher than achievable in a simple chemostat (Schuler and Kargi, 2002).

Types of Reactor

The three basic reactors for submerged fermentation are (Chisti, 1999): reactors with internal mechanical agitation (stirred tank); bubble columns (where gas sparging promotes agitation); and loop reactors (where mixing and liquid circulation are induced by gas motion and/or a mechanical pump).

Stirred tank reactors consist of cylindrical vessel with a central shaft that can support various impellers types (Figure 2.22), making it a reactor with internal mechanical agitation. This reactor can produce high volumetric mass transfer coefficient (K_La) values (Shuler and Kargi, 2002). The vessel has four equally-spaced vertically baffles. The impellers are about 8-10% of vessel diameter (Chisti, 1999). This arrangement provides enough agitation to disperse bubbles through the vessel and break larger bubbles into smaller bubbles (Shuler and Kargi, 2002).

Multiple axial, radial or a mix of impellers can be placed on the shaft (Chisti, 1999). Impeller type and shear effects on culture have been discussed in Section 2.7. Shear effects can also be caused by distribution and fragmentation of gas bubbles (Henzler, 2000; Frahm *et al.*, 2009).

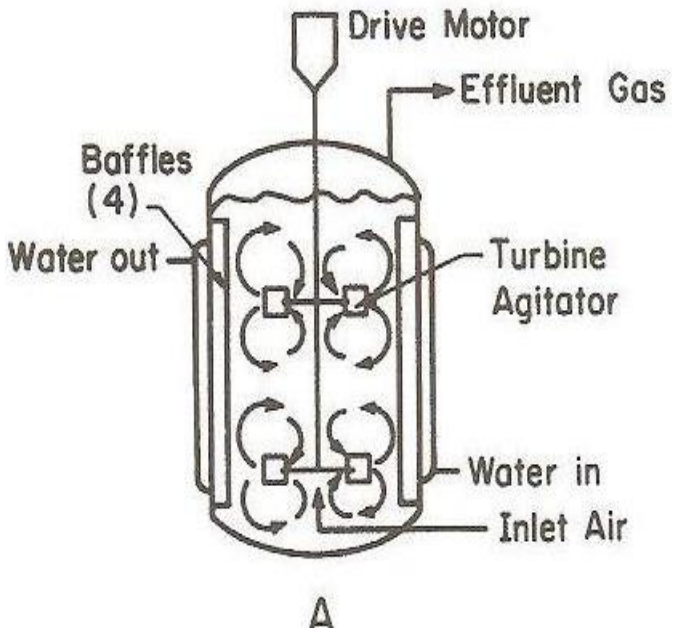

Figure 2.22: Schematic jacketed agitated vessel of stirred-tank reactor (Schuler and Kargi, 2002).

Small stirred tanks are usually made from glass vessels with stainless steel cover plates. They may be jacketed. These characteristics allow the reactor to be sterilized and also allow for the corrosive nature of some

fermentation media. However, glass vessel fermenters are not suitable for large scale fermentation (for instance, above 50 L). Sterility is the main concern as contaminants can affect production, time and cost (Shuler and Kargi, 2002). Foaming also affects stirred-tank reactors. It can be controlled by using mechanical foam breakers or by adding anti-foam solution to the culture media. Any excess foam escaping from the reactor can wet filters and decrease pressure and gas flow, and thus cause contamination.

The bubble column (Figure 2.23) is a simple reactor but is rarely used because it is less efficient than other reactors. It is unsuitable for high viscosity media or media containing large amounts of solids (Chisti, 1999). However, it is suitable for low-viscosity broths and has a higher oxygen transfer than stirred tank reactors. This reactor also has low-shear because there is no mechanical agitation. This reduces the risk of contamination and decreases cost. Besides having lower dynamic mixing capability than stirred tank reactor, bubble column reactors are limited by foaming and bubble fusion. This situation means bubble columns only work over a rather narrow range of gas flow rates, which varies with the nature of the fermentation broth. This problem can be reduced by using multistage columns, which redistribute gas flow. Overall, bubble columns are less flexible than stirred-tanks (Shuler and Kargi, 2002).

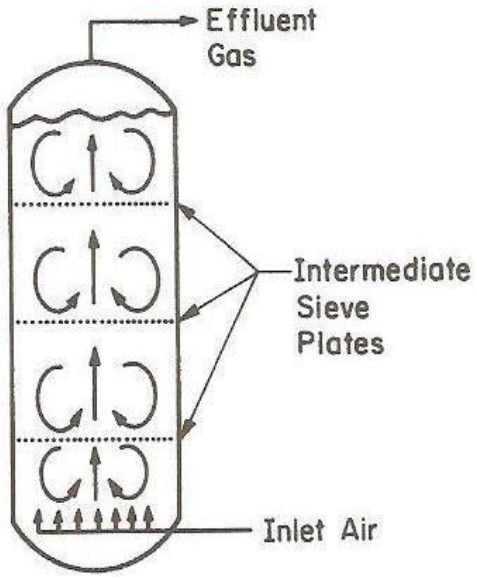

Figure 2.23: Bubble column (Schuler and Kargi, 2002).

In airlift fermenters, introduced air creates movement and mixing and creates loop circulation. There are internal-loop and external-loop designs (Figure 2.24). In the internal-loop configuration, a sparger is put into the riser.

The unsparged down comer is in the same vertical zones (Chisti *et al.*, 1988). In the external-loop reactor design, the riser and down comer are separate and joined near the top and bottom. These characteristics make the liquid circulate. As with bubble columns, loop reactors are unsuitable for high-viscosity broths. However, these reactors are good for suspending solids, transferring heat and air, and providing a low-shear environment (Chisti, 1999). Airlift reactors can handle more viscous broths than bubble column reactors. The largest airlift design reactor (1500 m^3) was developed by ICI (Imperial Chemical Industries) for single-cell protein production (Schuler and Kargi, 2002).

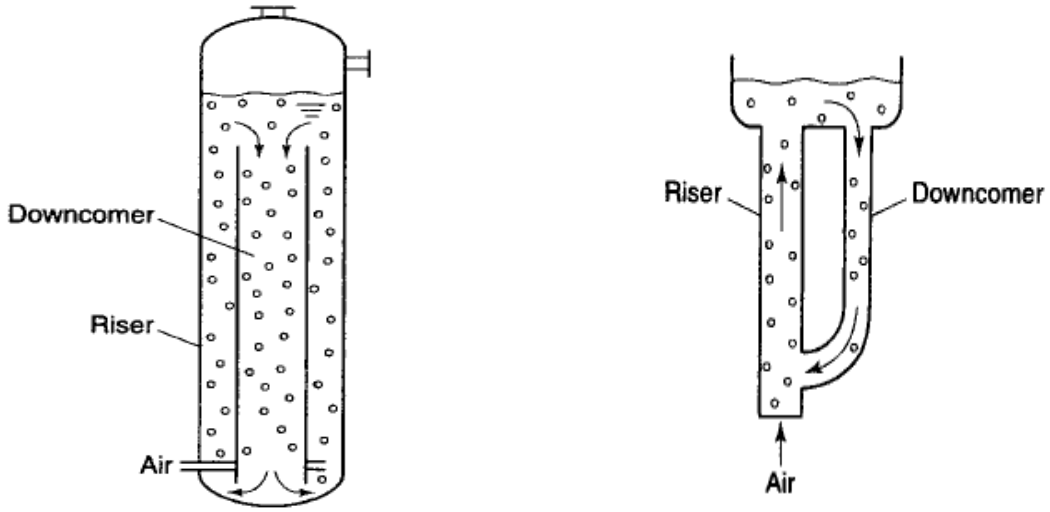

Figure 2.24: Internal (a) and external (b) loop airlift reactors (Chisti, 1999).

Reactors such as fluidized-bed reactors and hollow fibre reactors have also have been used as fermenters. The former incorporates a pump to circulate the media is suitable for small-scale animal cell fermentations and large-scale effluent treatment. Hollow fibre reactors, which are often used for culturing mammalian cells, have cartridges made of two different types of fibres that act as a separator membrane for gas and metabolite exchange. The cells are attached in the fibres (Allman, 1999).

Many different reactors, ranging from petri dishes to large stirred tank fermenters have been used in studies on BC production. The various types of fermenters used in the reported studies are summarised in Table 2.9.

Control systems

In Section 2.8, factors affecting growth and yield in fermentations such as pH, temperature, agitation and aeration were discussed. Reactors have a

control system to operate the heaters, pumps, and valves. Common measurements in a reactor system include agitation, temperature, pH, dissolved oxygen (DO) and aeration (Allman, 1999).

Table 2.9: Types of reactors used for BC production.

Static culture		
Tubes	4 x 12.5 cm	Ishihara *et al.*, 2002
Petri dishes	Unspecified	Keshk and Sameshima, 2006a
Plate	Unspecified	Mikkelsen *et al.*, 2009
Flasks	250 ml	Toda *et al.*, 1997; Yunoki *et al.*, 2004; Keshk and Sameshima, 2006b; Ha *et al.*, 2008; El-Saeid *et al.*, 2008; Coban and Biyik, 2011; Moosavi-Nasab *et al.*, 2011; Lu *et al.*, 2011;
Bottles	500 ml	Nguyen *et al.*, 2008
Beaker	1 L	Chavez-Pacheco *et al.*, 2011 ; Goh *et al.*, 2012;
Unspecified	5 L	Kongruang, 2008
Bioreactor	10 L, unspecified	Bae *et al.*, 2004; Goelzer *et al.*, 2009
Agitated culture		
Flasks (unbaffled)	500 ml, 500 ml, 500 cm^3, 250 ml, 250 ml, 250 ml, 250 ml	Son *et al.*, 2001 ; Ishida *et al.*, 2002 ; Joseph *et al.*, 2003 ; Son *et al.*, 2003, Park *et al.*, 2004; Zhou *et al.*, 2007 ; Dahman *et al.*, 2010
Flask (baffled)	500 ml, 250 ml	Seto *et al.*, 2006; Park *et al.*, 2004
Bioreactor/CSTR	2 L, unspecified, 5 L, 13 L	Joseph *et al.*, 2003 ; Park *et al.*, 2004 ; Zhou *et al.*, 2007 ; Hungund and Gupta, 2010a
Airlift	50 L	Ishida *et al.*, 2002

Mixing helps suspend the broth and substrate particles, control pH and temperature gradients, and also improve heat and mass transfer. Agitation enhances nutrients and substrate transfer from the fluid to the biocatalyst particles. It also favours removal or dilution of inhibitory metabolites that may be secreted (Chisti and Moo-Young, 2002).

Mixing is normally created by mechanical agitation or by sparging compressed air into the reactor. However, too much mixing/stirring may be detrimental and can damage cells and break biocatalyst pellets. Most microbes

are tolerant to shear that occur in bioreactors but cells such as animal cells, plant cells, protozoa and some microalgae can be destroyed (Chisti and Moo-Young, 2002). For effective fermentation, mixing needs to be considered and any negative effects minimized. Mizrahi and Moore (1970) studied several media additives that can minimize mechanical stress including (polyvinylpyrrolidone (PVP) and improved cell growth of human lymphocyte cell lines.

Effects of agitation on BC production were discussed in Section 2.7. High shear affects BC productivity and some researchers added water-soluble polysaccharides to the broth to reduce this problem (Iguchi *et al.*, 2002).

Mechanical agitation for mixing can produce up to 15 kW m^{-3} (Chisti and Moo-Young, 2002) so bioreactors require cooling systems to limit temperature rises, which may damage the culture. Temperature is controlled by circulating water in the cooling jacket on the bioreactor vessel or through an internal cooling coil. In some systems, cooling is controlled by recirculating culture broth through an external heat exchanger (Chisti and Moo-Young, 2002).

Controlling heat removal is complicated because there is only a small temperature difference between the cooling water and the culture broth. This means industrial-scale fermentations may be limited by the heat transfer capability. The three factors influencing heat removal are surface area for heat exchange, temperature difference between culture broth and coolant, and properties of the culture broth and coolant and their turbulence.

Bioreactor systems must also require heat transfer for sterilization, the subsequent cooling and for removing metabolic heat (Chisti, 1999). Usually, fermentation media and the bioreactor are sterilized together when heating the media. This heat may be supplied through the bioreactor wall. Sterilization involves high temperatures (usually 121^{o}C), which can cause unwanted reactions between media components. These may denature nutrients (due to charred media) or generate inhibitory compounds that can reduce product yields so some components are sterilised separately to minimise thermal damage. Overall, efficient temperature control is important to obtain the desired outcome.

Usually pH is controlled by adding acid or base to the culture broth (Allman, 1999) although initial adjustment may be done during media preparation. A combination of urea and ammonium sulphate (as nitrogen source) may stabilize pH in the media (Chisti and Moo-Young, 2002). The pH is detected with an electrode (pH probe), which must be calibrated before the

fermentation. Although probes can be autoclaved, they have a limited life of 20-50 cycles (Allman, 1999).

Pumps built into the bioreactor system supply acid and base to the culture broth. Reagent bottles are connected to the pumps by silicone tubes. Acid and base production in the fermenter will affect the amount required to control pH.

Dissolved oxygen (DO) is one of the most important factors in fermentation and good control is difficult. There are two types of DO probes. Galvanic probes are usually cheap, simple but not durable. Polarographic probes are complex, accurate but expensive. The latter are the most commonly used and require a voltage to polarize the anode and cathode**.** Polarization takes 2 to 6 hours.

Some microbes require oxygen to survive and insufficient oxygen will limit growth. Aerobic fermentations require oxygen throughout the process. Volumetric productivity of high density and high viscosity cell suspensions is proportional to cell concentration, which is limited by oxygen supply (Sajc *et al.*, 2000). Normally, air will be provided from a compressed air supply (Allman, 1999). The maximum air flow rate in small bioreactors is 1 volume of air per unit volume of culture broth (1 vvm). The maximum superficial aeration velocity in larger bioreactors such as bubble columns is 0.1 m/s (Chisti and Moo-Young, 2002). Airlift bioreactors have higher aeration rates than bubble columns as the gas is forced through perforated pipes located near the bottom of the bioreactor (Chisti, 1999).

Fermentation Economics

Product commercialization of biological products depends on integrating economic and technical factors (Figure 2.25). Usually three factors are considered: capital investment, operating cost, and a profitability analysis. In biopharmaceuticals, the average cost for developing new drug, including research and development costs, is $200 to $500 million. Hence, effective process design and methodologies are crucial in helping researchers evaluate and develop product (Petrides, 2000).

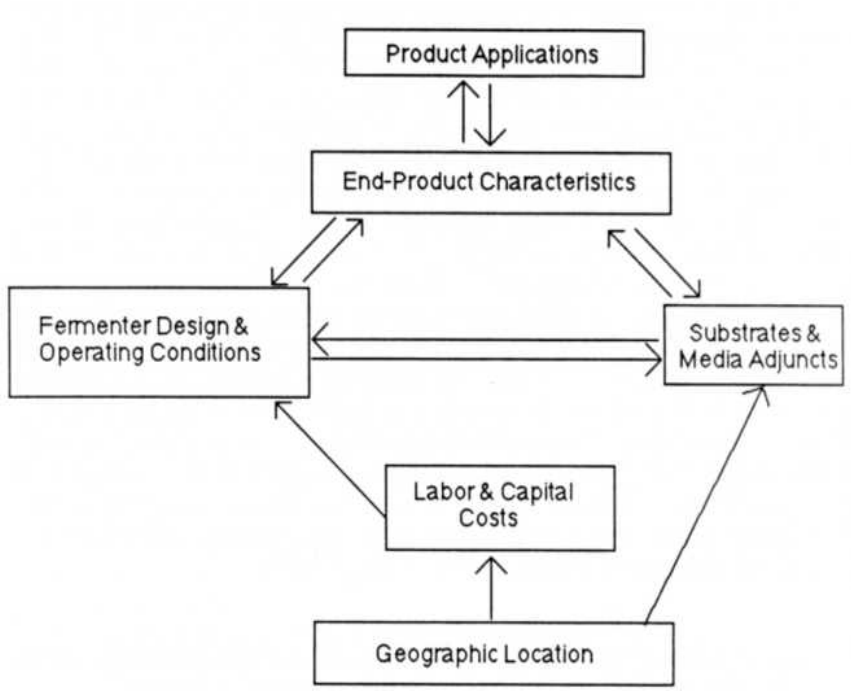

Figure 2.25: Major technical and economic elements involved in product commercialization (White and Brown, 1989).

Capital investment involves direct fixed capital (DFC), working capital, and start-up and validation costs. Bioprocessing plant operation involves the costs for raw materials, labour, utilities, waste disposal, etc. Estimates of DFC are based on total equipment purchase cost (PC) using several multipliers. As well as DFC, sufficient budget must also be available for raw materials, labour, utilities, waste treatment and other expenses (Petrides, 2000). White and Brown (1989) reported that the control system and suitable media are related for effective reactor operation and both influence end-product production. Media selection is affected by price and availability, which are affected by geographic location of the facility. Labour and capital costs affect the levels of capital and labour intensiveness of the fermentation process (White and Brown, 1989).

2.10 Choosing a Cultivation Method for BC Production

Productivity of BC production depends on cultivation conditions such as incubation period, carbon and nitrogen source, pH and sugar concentration (Embuscado *et al.*, 1994). Several production strategies have been investigated to increase BC production in different types of reactors. Yoshino *et al.* (1996) doubled BC production by using a cylindrical silicone membrane vessel with oxygen supplied from the bottom. Serafica *et al.* (2002) produced BC in a rotating disk reactor, which consisted of a cylindrical trough holding the

inoculated media. Flat, circular disks mounted on a rotating central shaft were dipped into the media. This rotating disk bioreactor was more efficient and reduced the fermentation time from 12-20 days down to about 3.5 days.

Hornung *et al*. (2007) developed an aerosol reactor where both glucose and oxygen were fed directly to the cells (Figure 2.26). This increased the oxygen-rich surface area to reactor volume ratio and improved BC production.

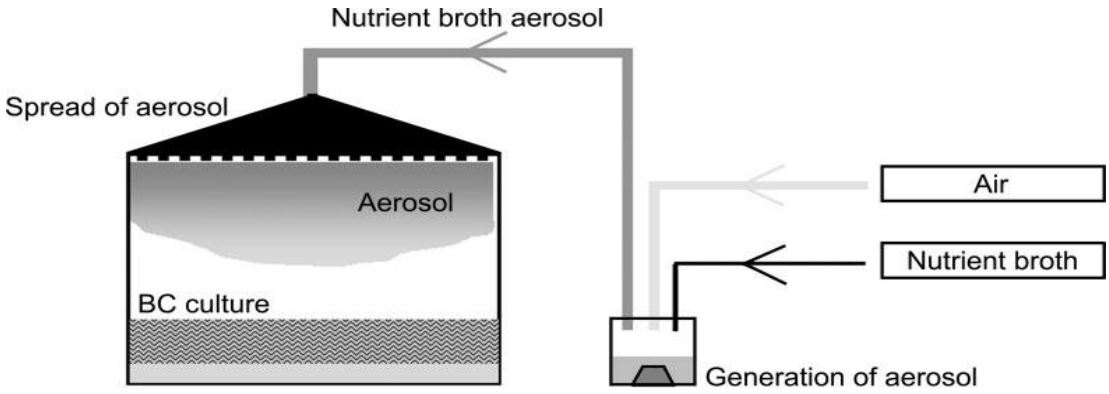

Figure 2.26: Direct supply of substrate (glucose) and oxygen (Hornung *et al.*, 2007).

Their previous study (Hornung *et al.*, 2007) showed that interactions between the wall of the culture vessel and the BC layer significantly affected BC production. Beaker cultivation created interactions between the wall and BC layer (wall effect) that hindered the BC layer from sinking. As the BC layer is slightly more dense (1030 kg/m^3) than the glucose media (1008 kg/m^3), this contributed a downward force. In conical flasks, the BC layer moves down into the culture broth and does not interact with the walls (Figure 2.27). They obtained a 10-cm BC layer in conical flasks compared with a 3-mm layer in funnel-shaped flasks and a 2-cm layer in beakers.

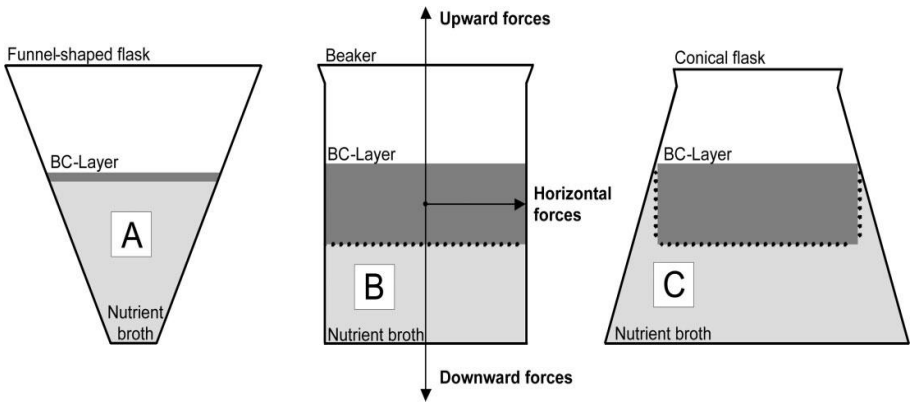

Figure 2.27: Different shapes of cultivation flasks (Hornung *et al.*, 2006).

Cheng *et al*. (2009) studied the effects of thirteen types of plastic composite support(PCS) biofilm reactors (Figure 2.28) on BC production by *G. xylinus* on various substrates including 50 g/L fructose. The best PCS, based on nitrogen per unit weight PCS, nitrogen leaching rate, BC production and biomass attached to the PCS, was made from soybean hulls, soybean flour, yeast extract and dried bovine RBC. A BC concentration of 7.05 g/L was obtained, which was 2.5 times higher than the control without PCS.

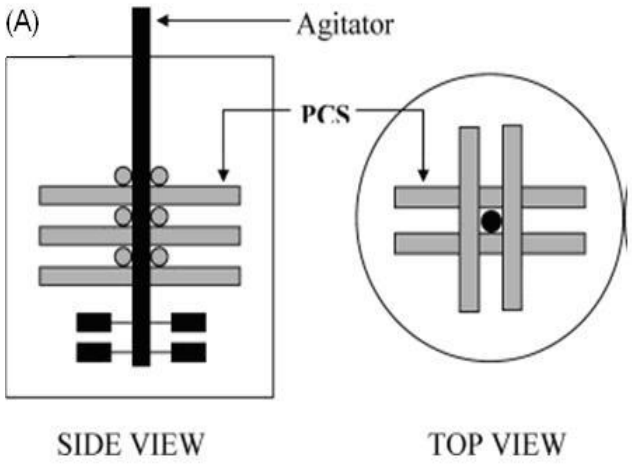

Figure 2.28: Diagram of the PCS biofilm bioreactor (Cheng *et al.*, 2009).

As BC production requires low shear stress and high oxygen transfer rates, Song *et al*. (2009) developed a modified airlift-type bubble column bioreactor (Figure 2.29)

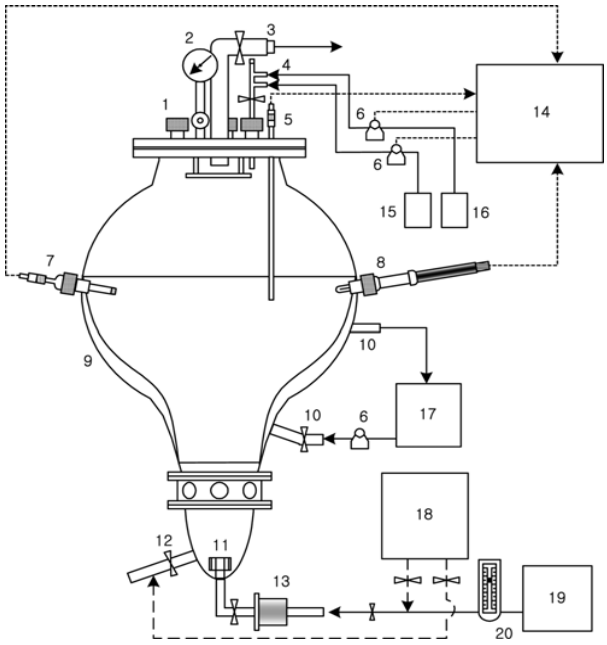

Figure 2.29: Schematic of 50-L spherical type bubble column bioreactor (Song *et al.*, 2009).

The fermenter has a cylindrical bottom and an upper spherical main reactor, which produced low shear and high oxygen transfer rates. Cel^- mutant (non-producing BC) strains were not produced. The BC production on 40 g/L saccharified food wastes increased from 5.0 g/L to 5.8 g/L if 4 g/L agar was added.

2.11 Summary and Thesis Objectives

Bacterial cellulose is a pure biopolymer with excellent mechanical strength, high biocompatibility, and high crystallinity. It is also non-toxic and bio-degradable. Various microbes have been used to produce BC but most researchers uses *G. xylinus*. Although BC produced under static growth conditions has the best physical properties (Suwannapinunt *et al.*, 2007), most research has been done under agitated condition because good aeration and mixing can be achieved (Watanabe and Yamanaka, 1995; Suwannapinunt *et al.*, 2007), which increases productivity.

Economic production relies on identifying high-yielding strains, and limiting processing costs. The latter affect the potential for developing uses for BC. Media costs affects BC production cost so there is increasing interest in developing growth media based on cheaper raw materials. Non-conventional cheap media such as agricultural organic wastes can be high in sugar and contain vitamins essential for BC synthesis (Castro *et al.*, 2011).

This study aims to identify ways to increase BC yield and productivity using low cost components such as glycerol and fish powder as carbon and nitrogen sources. Increased productivity via selecting the appropriate reactor and manipulating operating conditions is another way of minimising production costs. Production of BC in agitated culture on standard glucose media will be used for comparison. The microbe *G. xylinus* will be used because it is the most efficient BC producer in large scale and consumes wide variety of substrates (Moosavi-Nasab *et al.*, 2011).

The specific aims of the research are:

- To identify and/or develop low cost media for BC production. This will be done by identifying several types of wastes/substrates to grow *G. xylinus* and produce BC. Fish powder, fish hydrolysate, banana peels and glycerol will be supplied to the culture media as nitrogen and carbon sources. Commercial glucose will be used as a standard

carbon source for comparison. Substrate consumption, BC productivity and cell mass concentration will be investigated. This study will also briefly discuss the effect of low-cost ingredients on production cost.

- To identify the best reactor conditions (stirring and aeration) for growing *G. xylinus* for BC production. This will be done by identifying conditions that give rapid growth and high productivity in terms of reactor time. This study will investigate microbial production of BC using aerated and agitated cultivation. Growth of *G. xylinus* and production of BC, along with supply and consumption of substrate will be measured. The experiment will also investigate the effect of substrate concentration on bacterial growth and BC production. The BC productivity of optimized batch operation, fed-batch and continuous operation will be investigated and compared. Three methods of fed-batch operation will be used: fill and draw, pulse feed and continuous feed.

To support these aims, initial work will be done to investigate the composition of media suitable for maintaining viable *G. xylinus* cultures so they can rapidly acclimatize to the growth conditions in larger fermentation vessels and produce high BC yields. This will help increase overall productivity.

Chapter 3: Materials, Methodology and Trials

This chapter describes the materials, analytical methods, operating conditions and trials carried out to investigate production and optimisation of bacterial cellulose.

3.1 Materials

3.1.1 *Gluconacetobacter xylinus* DSM 46604

The bacterium *Gluconacetobacter xylinus* DSM 46604, from German Collection of Microorganisms and Cell Cultures, was used in this study. Stock cultures of the bacterium were grew at 30^{o}C on nutrient media agar slants containing the following media: 50 g/L D-glucose (UNIVAR), 5 g/L yeast extract (OXOID), 5 g/L ammonium sulphate (UNIVAR), 3 g/L disodium hydrogen phosphate (SIGMA), 0.05 g/L magnesium sulphate (M&B Laboratory Chemicals) and 20 g/L agar (Becton Dickinson). The growth media and seed media did not contain agar.

Preservation

To ensure viability, the bacteria were kept on solid agar slants. The agar contained 5 g yeast extract, 5 g ammonium sulphate, 3 g potassium hydrogen orthophosphate, 0.05 g magnesium sulphate, 50 g glucose and 20 g agar in 1 L distilled water. After being sterilized at 121^{o}C for 15 minutes, the media was transferred to a laminar flow cabinet equipped with a UV lamp and aseptically poured into tilted test tubes. After 5 days growth at 30^{o}C, the slants were stored at 4^{o}C for two weeks. Agar slants were refreshed every 4 to 5 weeks by transferring a loop full of bacteria to new agar slants. After the third subculture, the culture was tested for contamination by plating on a Petri dish containing growth agar. A decrease in colour of the agar in the slants indicates bacterial viability had decreased and they must be revived. Any contamination would show as colonies with a different appearance on the Petri dish.

3.1.2 Preparing Cells for Trials

Glucose media

To prepare the seed for trials, a loop full of bacteria was gently scraped from the surface of solid agar and transferred into 75 mL of 50 g/L glucose

growth media in a 200-mL conical flask. The flask was plugged with cotton wool and incubated on a shaking incubator (150 rpm) at 30°C for 5 days until white pellicles appeared.

Glycerol media

To prepare the seed for trials, a loop full of bacteria was gently scraped from the surface of solid agar and transferred into 75 mL of 20 g/L glycerol growth media in a 200-mL conical flask. The flask was plugged with cotton wool and incubated on a shaking incubator (150 rpm) at 30°C for 5 days until white pellicles appeared.

3.1.3 Culture Media

The following was added to 1 L of distilled water: 50 g D-glucose, 5 g ammonium sulphate, 3 g potassium hydrogen orthophosphate, 5 g yeast extract and 0.05 g magnesium sulphate. Media pH was adjusted to 6.8 with 6M sodium hydroxide and then sterilized at 121°C for 15 minute.

3.2 Media Substitutes

3.2.1 Fish Powder

Yeast extract in the media was replaced with fish powder made from the flesh of koi carp *(Cyprinus carpio*), a fresh-water pest fish, supplied by the Department of Biological Sciences, The University of Waikato. The fish powder was produced by mincing and drying carp flesh at 80°C for 24 hours. After drying, the dried material was ground to a fine powder in a Kenwood blender (Figure 3.1).

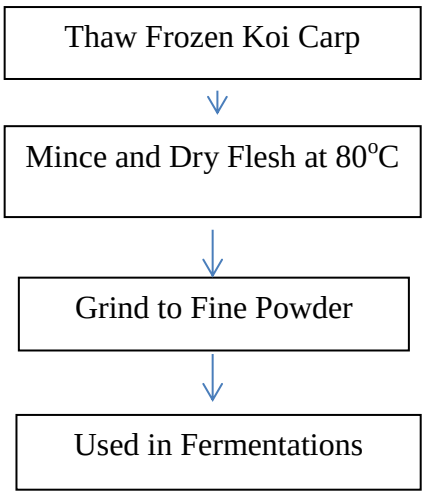

Figure 3.1: Fish powder preparation.

3.2.2 Fish Hydrolysate

Koi carp flesh was minced and mixed with an equal weight of distilled water. The slurry pH was adjusted to pH 1 and autoclaved for 15 minutes at 121^{o}C. The slurry was furthered centrifuged at 4000 rpm for 20 minutes in 50-mL graduated Falcon tubes. The precipitate was discarded and the supernatant used to replace yeast extract in the media. The soluble peptide content in the supernatant was determined by measuring density of a known volume and then converting this value to known mass using the following relationship:

$\rho = m/V$

where, ρ = density

m= mass

V= volume

3.2.3 Banana Peel Extract

Banana peels, wrapped in foil to retain moisture, were autoclaved for 15 minutes at 121^{o}C. An equal weight of distilled water was added and the banana peels were mashed with a mortar and pestle. Mashed peels were then filtered through No 1 Whatman filter paper. The filtrate was centrifuged in 50-mL graduated Falcon tube at 4000 rpm for 20 minutes and then filter-sterilized through a 0.2-µm Whatman filter. The filtrate was used immediately after being prepared. The content of banana peels extract was determined by measuring the density of a known volume of the banana extract, and also based on known mass of sugar concentration.

3.3 Analytical Methods

3.3.1 Sample preparation

Broth from shake flasks was harvested and homogenized at 100 rpm for 5 minutes, and two 5-mL samples were taken. When sampling from the bioreactor, two 5-mL samples were taken but they were not homogenized. Samples were centrifuged at 4000 rpm for 20 minutes, washed with distilled water and centrifuged again to remove culture broth. The washing procedure was repeated three times. Supernatant from the centrifugation was kept for glucose/glycerol concentration analysis while the washed pellets were used to determine biomass and BC concentration.

3.3.2 BC concentration

The washed pellet from one sample was treated with 1M NaOH at 90^{o}C for 30 minutes to dissolve cells. The BC obtained was centrifuged at 4000 rpm for 20 minutes, washed with distilled water, dried at 80^{o}C for 24 hours, and weighed.

3.3.3 Biomass concentration

The pellet from the second sample was suspended in 10 mL of cellulase (SIGMA) and 90 mL citrate buffer (pH 5.0), mixed for 5 minutes, then centrifuged at 4000 rpm for 20 minutes to remove suspended solids. The supernatant was kept at 50^{o}C for 30 minutes to hydrolyse the BC. The sample was then washed, centrifuged at 4000 rpm for 20 minutes and dried at 80^{o}C for 24 hours, and weighed until constant weight was achieved. The biomass concentration was expressed as gram dry cell weight per litre media (g DCW L^{-1})

3.3.4 Glucose analysis

Miller's method (1959) was used for determining glucose concentration in the culture broth. The 3,5-dinitrosalicylic acid (DNS) in hot alkaline solution reduces to 3-amino, 5–nitrosalicylic acid after being boiled for 5 minutes. The DNS reagent solution was prepared by dissolving 10 g NaOH and 40% sodium potassium tartrate (Rochelle's salt) in 1 L distilled water and then adding 2 g phenol and 10 g DNS reagent. Rochelle salt was added to prevent the reagent from dissolving oxygen, the phenol increased the colour intensity and sodium sulphite stabilized the colour obtained in the presence of phenol. The calibration curve was produced using 0.01 mg/mL to 1.0 mg/mL glucose. Three mL of DNS solution was added to 3 mL of the standard or sample, mixed and immersed in boiling water for 5 minutes. After being cooled at the room temperature, the absorbance was read at 575 nm.

Corrections were made to the glucose calibration curve, based on mg in a tube.

3.3.5 Glycerol Analysis

Glycerol concentration was determined by refractive index, which measures the change in angle of light. The applications follow Snell's law, which defines the refractive index as a constant ratio of the sine of the angle of incidence to the sine of the angle of refraction. A pipette was used to place a few drops of

sample at 20^{o}C placed on the prism and the lid was closed. The handheld device was pointed towards a light source and the refractive index was measured. This was converted to glycerol concentration using the standard curve (see Appendix) using a with regression equation (Stasiak-Różańska *et al.*, 2011).

3.3.6 Acetic Acid Analysis

To determine acetic acid in the broth, samples were collected during the fermentation. One-mL broth samples was taken at regular intervals and stored at -20^{o}C before chromatographic analysis. Before analysis, samples were thawed and then centrifuged at 12,000 rpm for 10 min. The supernatant was removed using a 1-mL syringe fitted with a 22G x 1 ½ sterile, single-use needle and then filtered through a 0.20-µm Minisart® -plus syringe filter. Forty microliters of 7% sulphuric acid was added to 1-mL filtered supernatant and mixed in a Minishaker for about 30 second. Adding the mineral acid shifted acid equilibrium of weak acids to the protonated form and helped maintain column functionality.

The supernatant was analysed in an HPLC system consisting of a Waters 515 HPLC pump, Waters column heater module, Waters 996 photodiode array detector (PDA), Waters 2414 refractive index (RI) detector, and Rheodyne 7725i manual sample injector with a 20-µL sample loop. The system was controlled with Waters Empower™ 2 chromatography software. The glucose was measured by a refractive index detector and the organic acids were measured with the UV detector. The mobile phase was 5 mmol L^{-1} sulphuric acid, which had been degassed in an ultrasonic bath under vacuum for 20 min.

The analyses were performed isocratically at a flow of 0.6 mL min^{-1} at 60^{o}C using a 300 x 7.8 mm i.d. cation exchange column (Aminex HPX.87H) with a precolumn (cation H^{+} microguard cartridge). The mobile phase was 5 mmol L^{-1} H_2SO_4 prepared with Milli-Q water and filtered through a 0.2 µm minisart®-plus syringe filter and degassed under vacuum for 20 min.

3.3.7 SEM (scanning electron microscope)

Scanning electron microscopy (SEM) was used to observe morphology and BC pellicles structure. Thin layers of samples were coated with platinum using an ion sputter. The coated samples were viewed and photographed at 20 kV using a Hitachi S 4700 SEM.

3.4 Shake Flasks Trials

Preliminary growth trials were done in shake flasks, which allow growth profiles, substrate consumption and metabolite production to be studied in a short time. The main drawback of shake flasks is that although reactor parameters such as temperature, agitation and initial substrate concentration can be controlled, other parameters, especially pH cannot be easily controlled. However, results obtained from shake flasks can be used to identify possible operating conditions in bioreactors.

3.4.1 BC Morphology

Unless stated otherwise, all trials were done at an initial pH of 6.8, 30^{o}C and using media described in Section 3.1.3. All vessels were inoculated with one loop full of bacteria that had been gently scraped from the surface of solid agar and transferred into the media (Section 3.1.3) containing 50 g/L glucose.

Static cultivation

Fermentations in 14.7-cm long, 1.2-cm diameter test tubes were done at 30^{o}C without agitation for 14 days. Thickness of the BC sheet was measured using a centimetre ruler. After 14 days, the BC layer was harvested and purified (Section 3.5) and dried at 80^{o}C in an oven for 24 hours. Morphology of the dried BC sheet was investigated using SEM.

Agitated cultivation

Sixty five mL of media (Section 3.1.3) was added to 200-mL shake flask. Fermentation was done with agitation at 150 rpm. Formed BC pellicles were harvested, purified (Section 3.5) and dried at 80^{o}C in an oven for 24 hours. Morphology of dried BC pellicles was investigated using SEM.

3.4.2 Carbon Source

Sixty five mL of media (Section 3.1.3) containing 50 g/L of glucose, sucrose or lactose was added to three different 200-mL shake flask. Fermentation was done at 30^{o}C, pH 6.8, 150 rpm for 5 days. The BC concentration was determined at the end of the fermentation (Section 3.5). The carbon source that produced the highest BC concentration was used in further trials.

Glucose concentration

To investigate the effect of substrate concentration, 40, 50, 60, 80 or 100 g/L glucose was added to media (Section 3.1.3) with a pH of 6.8. Fermentation was done at 30^{o}C, 150 rpm for 5 days in 200-mL shake flasks. The BC concentration (Section 3.5) was determined at the end of the fermentation. The optimal substrate concentration was used in further studies.

3.4.3 Nitrogen Source

To investigate the effect of nitrogen source on BC production, further trials were done in 200-mL shake flasks. Sixty five mL of media (Section 3.1.3) with a pH of 6.8 was used. Fermentations were done at 30^{o}C, 150 rpm for 5 days.

Yeast extract

The following concentrations of YE were used: 2, 3, 5, 7 and 9 g/L. The BC concentration was only determined at the end of the fermentation (Section 3.5). The optimal yeast extract was used in the further studies in bioreactor.

Fish powder

The following amounts of fish powder were used, along with the control (5 g/L YE): 5, 10, 15 and 20 g/L. The biomass, glucose and BC of samples taken every 24 hours were determined (Section 3.5). The optimal fish powder concentration was further studied in a bioreactor.

Fish hydrolysate

The following amounts of fish hydrolysate were used, along with a control (5 g/L YE): 5, 10, 15 and 20 g/L. The biomass, glucose and BC concentration of samples taken every 24 hours were determined (Section 3.5). The optimal fish peptide concentration was further studied in a bioreactor.

3.4.4 Carbon Source Substitutes

Banana peel extract

To study the effect of carbon substitutes, trials were done in 200-mL shake flasks using a combination of glucose and banana peel extract (Section 3.2) as the carbon source. Sixty five mL of media (Section 3.1.3) at pH 6.8 was used and fermentation done at 30^{o}C, 150 rpm for 5 days. Four different amounts of banana peel extract (10, 20, 30 and 40 g/L) were mixed with 40, 30, 20 and 10 g/L of glucose respectively. Biomass, sugar and BC concentration of samples

taken every 24 hours were determined (Section 3.5). Optimal combinations of media components was further studied in a bioreactor.

Glycerol

To study the effect of glycerol concentrations, trials were done in 200-mL shake flasks using 65 mL of media (Section 3.1.3) with a pH of 6.8. Fermentations were done out at 30^{o}C, 150 rpm for 5 days. Four different glycerol concentrations (10, 20, 30 and 40 g/L) were used. Glycerol concentration was determined using the density of a known volume and calculating mass. The biomass, glucose and BC concentration of sample was taken every 24 hours were determined (Section 3.5). The optimal concentration was further studied in a bioreactor.

3.5 Batch Bioreactor Trials

Further trials were done in a bioreactor, which provides better control over agitation and aeration and parameters such as pH can be controlled easily. Two different but similar bioreactors were used (Figure 3.2). Initial trials were done in a 5-L bioreactor (Inceltech LH, France) and a new, 3-L bioreactor (BioFlow Celligen 115, United States of America) was used for later research. Both reactors were fitted with an 8-blade impeller, temperature control, pH control, an aeration controller and a foaming sensor.

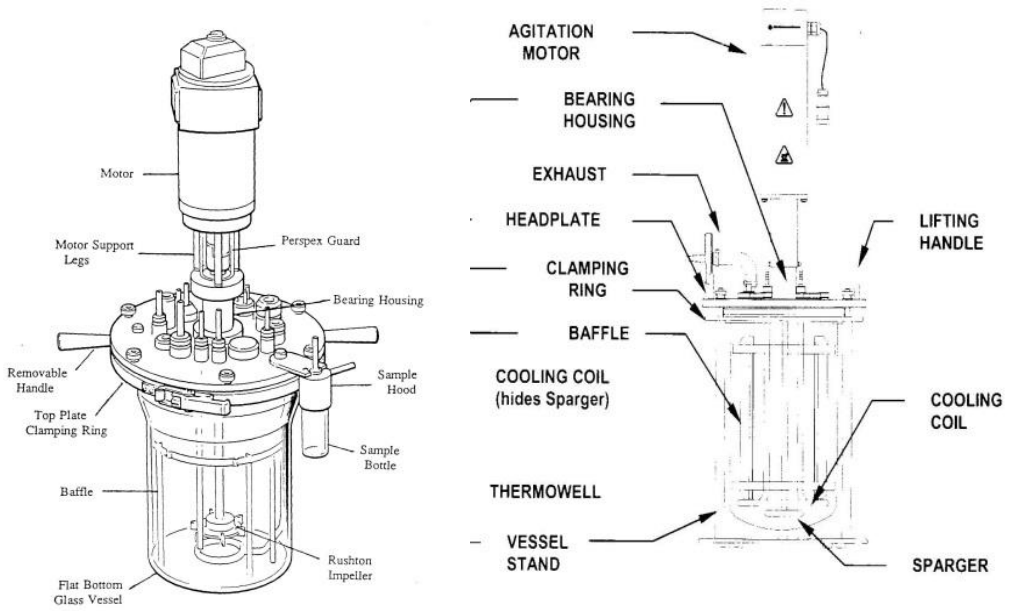

Figure 3.2: The 5-L Inceltech, LH Series 210 bioreactor (left) and the 3-L (BioFlo Celligen, 115 bioreactor (right).

Fermentation conditions

Agitated and aerated cultures were grown on 5% glucose standard media (Section 3.1.3) in the 5-L bench-top bioreactor (Inceltech, LH Series 210). Fermentations were done for 5 days, 30^{o}C, an aeration rate of 0.3 vvm with a working volume of 3 L. The pH was controlled at 6.8 using 1 M NaOH. The reactor was inoculated with 150 mL of seed culture (Section 3.1.2). Biomass, glucose and BC concentrations of samples taken every 24 hours were determined (Section 3.5).

The effect of the following conditions was investigated.

- Four different agitation rates (150, 200, 250 and 300 rpm) at a constant aeration rate of 0.3 vvm.
- Four different aeration rates (0.3, 0.6, 1.0 and 1.5 vvm) at a constant agitation rate of 150 rpm.
- Replacing YE in the media with 15 g/L fish powder). Agitation was at 150 rpm and aeration was 0.3 vvm.
- Replacing YE in the media with 15 mL/L fish. Agitation was at 150 rpm and aeration was 0.3 vvm.
- Combining banana peel slurry with glucose. Agitation was at 150 rpm and aeration was 0.3 vvm.
- Replacing glucose with 20 g/L glycerol. Agitation was at 150 rpm and aeration was 0.3 vvm.

3.6 Fermenter Operation

Further trials were done in a 3-L bioreactor (BioFlo, Celligen 115) using three different feed conditions (fill-and-draw, pulse feed, and continuous feed fed-batch). All trials were done at 30^{o}C, 150 rpm and 0.3 vvm aeration. Media composition is given in Section 3.1.3. Feeding media added for fill-and-draw fed-batch, pulse feed fed-batch and continuous feed fed-batch fermentations were double strength to study the BC profile with double strength media. The fermenter was inoculated with 150 mL seed culture for fill and-draw fed-batch. Seventy five mL seed culture was inoculated for pulse feed and continuous feed fed-batch. All operations started with a different working volume:

- Fill-and-draw fed-batch operation: working volume of 2 L.
- Pulse feed: initial volume of 1 L, followed by adding three lots of 250-mL double strength media to give a final volume of 1750 L.

- Continuous fed-batch: initial volume of 1 L, followed by adding 1-L of double strength media continuously at certain dilution rate to give a final volume of 2 L.

3.6.1 Fill-and-Draw Fed-batch Operation

Glucose

Standard media (Section 3.1.3) with 5% glucose was used. The fermenter was in operated in batch mode for three days and then switched to cyclic fed-batch operation on Day 3. This involved withdrawing 1 L of culture media and adding 1 L of double strength media on days 3, 5, 7 and 9. Fermentation continued until day 11. Biomass, glucose and BC concentrations in culture broth samples taken immediately before and after adding fresh media were determined (Section 3.5).

Glycerol

Standard media (Section 3.1.3) with 2% glycerol was used. The fermenter was operated in batch conditions for three days and then switched to fill-and-draw fed-batch operation on Day 3. This involved withdrawing 750 mL of culture media and adding 750 mL of double strength media on days 3, 5 and 7. Fermentation continued until day 9. Biomass, glucose and BC concentrations in culture broth samples taken immediately before and after adding fresh media were determined (Section 3.5).

3.6.2 Pulse Feed Fed-batch Operation

Glucose

The fermenter was operated under batch conditions using standard media (Section 3.1.3) with 5% glucose for three days and then switched to pulse feed fed-batch operation on Day 3. This involved feeding three lots of 250-mL of double strength culture media (section 3.1.3) on days 3, 5 and 7. Fermentation continued until day 11. Biomass, glucose and BC concentrations of culture broth samples taken immediately before and after adding new media were determined (Section 3.5).

Glycerol

The fermenter was in operated under batch conditions using standard media (Section 3.1.3) for three days and then switched to single fed-batch operation on Day 3. This involved feeding three 250-mL lots of double strength

culture media on days 3, 5 and 7. Fermentation continued until day 9. Biomass, glucose and BC concentrations in culture broth samples taken immediately before and after adding new media were determined (Section 3.5).

3.6.3 Continuous Fed-batch Operation

Glucose

The fermenter was operated under batch conditions for three days and then switched to continuous fed-batch operation on Day 3. This involved feeding double strength culture media continuously (Section 3.1.3) at dilution rates of 0.1 h^{-1} and 0.2 $h^{-1,}$ based on initial volume, until total volume was 2 L. The feed was then stopped and fermentation continued until glucose in the culture broth was depleted. Samples of culture broth were taken every 4 hours for the 0.1 h^{-1} dilution rate and every 2 hours for the 0.2 h^{-1} dilution rate, and used for biomass, glucose and BC concentration determinations (Section 3.5).

Glycerol

The fermenter was in operated under batch conditions for three days and then switched to single fed-batch operation on Day 3. This involved feeding double strength culture media continuously (Section 3.1.3) at dilution rates of 0.1 h^{-1} and 0.2 h^{-1} until the total volume was 2 L. The feed was then stopped and fermentation continued until glucose in the culture broth was depleted. Samples of culture broth were taken every 4 hours for the 0.1 h^{-1} dilution rate and every 2 hours for 0.2 h^{-1} dilution rate, and used for biomass, glucose and BC concentration determinations (Section 3.5).

3.7 Continuous Operation

The effect of substrate (5% glucose and 2% glycerol) and dilution rate (0.05 h^{-1}, 0.1 h^{-1}, 0.15 h^{-1} and 0.2 h^{-1}) were investigated. a schematic diagram of continuous operation is shown in Figure 3.4.

3.7.1 Glucose and Dilution Rate

The fermenter was operated under batch conditions for three days and then switched to continuous on Day 3. This involved constant feeding of 1 L of culture media with doubled concentration of standard media (Section 3.1.3) at four dilution rates (0.05 h^{-1}, 0.1 h^{-1}, 0.15 h^{-1} and 0.2 h^{-1}). The fermentation time continued until glucose depleted in the culture broth. The biomass, glucose and

BC concentration of culture broth samples taken every 2 to 6 hours were determined (Section 3.5).

3.7.2 Glycerol and Dilution Rate

The fermenter was in operated under batch conditions or three days and then switched to continuous operation on Day 3. This involved constant feeding of 1 L of culture media with doubled concentration of standard media (Section 3.1.3) at four dilution rates (0.05 h^{-1}, 0.1 h^{-1}, 0.15 h^{-1} and 0.2 h^{-1}). The fermentation time continued until glucose depleted in the culture broth. The biomass, glucose and BC concentration of culture broth samples taken every 2 to 6 hours were determined (Section 3.5).

3.8 Data Analyses and Regulations

Analyses were done in duplicate and reported as averages. All research work was done in Physical Containment Level 2 (PC2) rooms operated in accordance with section 39 of the Biosecurity Act 1993 (ERMA, 2007).

Chapter 4: Producing BC and Optimising Reactor Parameters

This chapter describes trials to identify optimal conditions for maintaining *G. xylinus* cultures on of agar slants. Trials were then done to investigate how incubation time affects BC pellicle formation and thickness. The effect of carbon sources and their concentration levels, yeast extract (YE) concentrations, and aeration and agitation on optimal for BC production by *G. xylinus* were then examined.

4.1 Maintaining *G. xylinus* cultures

4.1.1 Culturing and Maintaining *S*lants

Much research has been done to identify the quality and properties of BC as well as improving productivity in terms of cell growth and BC yield. Previous researchers have described the effect of physiological parameters and investigated metabolism of glucose in Hestrin-Schramm media or modified media. Bacterial strain, carbohydrate source, media pH, temperature and culture methods are important parameters for BC production by *Acetobacter xylinum* (Masaoka *et al.*, 1993; Geyer *et al.*, 1994).

The current research used a lyophilized strain of *G. xylinus* obtained from the German Collection of Microorganisms and Cell Cultures (Figure 4.1), without any further treatment. To prepare a new starter or broth seed, a colony of *G. xylinus* was gently collected from the surface of an agar slant (Figure 4.2) using a loop and transferred into sterile media in conical flasks.

Figure 4.1: Lyophilized *G. xylinus* DSM 46604 culture as received.

Results showed that *G. xylinus* slants could be kept for up to two months (Section 3.1.1), but fortnightly transfers helped maintain bacterial viability (Figure 4.2). Any change in colour of the agar slant indicated bacterial viability had decreased and sub-culturing was required.

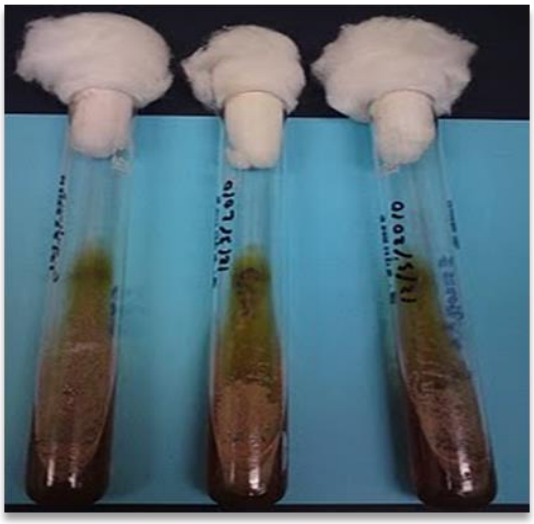

Figure 4.2: Colonies of *G. xylinus* DSM 46604 on GYM media agar slants.

4.2 Formation and Appearance of BC Pellicles

4.2.1 Effect of Cultivation Conditions on BC Production and Morphology

Culture conditions affect morphology of BC produced by *G. xylinus*. Static submerged cultures produced gelatinous mats while agitated submerged cultures resulted in BC sphere-like pellicles. Seed cultures were grown as described in Section 3.1.3 and inoculated into stationary shake flasks. For test tube cultures, a loop full of cells from the stock culture was directly transferred into test tubes containing sterile media and then incubated at 30^{o}C without agitation for 14 days. For agitated cultures, the shake flasks were incubated at 30^{o}C and 150 rpm for five days.

The pH of the culture media is the most important factor for cell growth. Optimum pH for cellulose production by *G. xylinus* is reported to be less than 7 (Hestrin and Schramm, 1952; Glasser *et al.*, 1958; Masaoka *et al.*, 1993). If pH changes, the strain can mutate into the gluconate-mutant and potentially become non-cellulose producers (Cel^{-}) (Hwang *et al.*, 1999). Mutagenesis also changes the BC structure from I_{α} to folded-chain cellulose II (Kuga *et al.*, 1993). In this study, the initial media pH was 6.

In static test tube cultures, thin white gelatinous mats were formed at the air-liquid interface (Figure 4.3), which gradually thickened to approximately 1.3-2.0 cm after 14 days (Figure 4.4). Several theories have been proposed to explain how the bacteria produce cellulose mats on the surface of the media (Section 2.4).

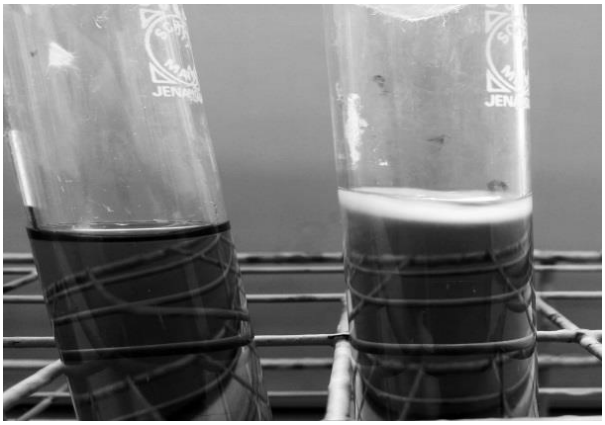

Figure 4.3: BC mats in static culture of *G. xylinus* DSM 46604 at (a) Day 0 and (b) Day 5.

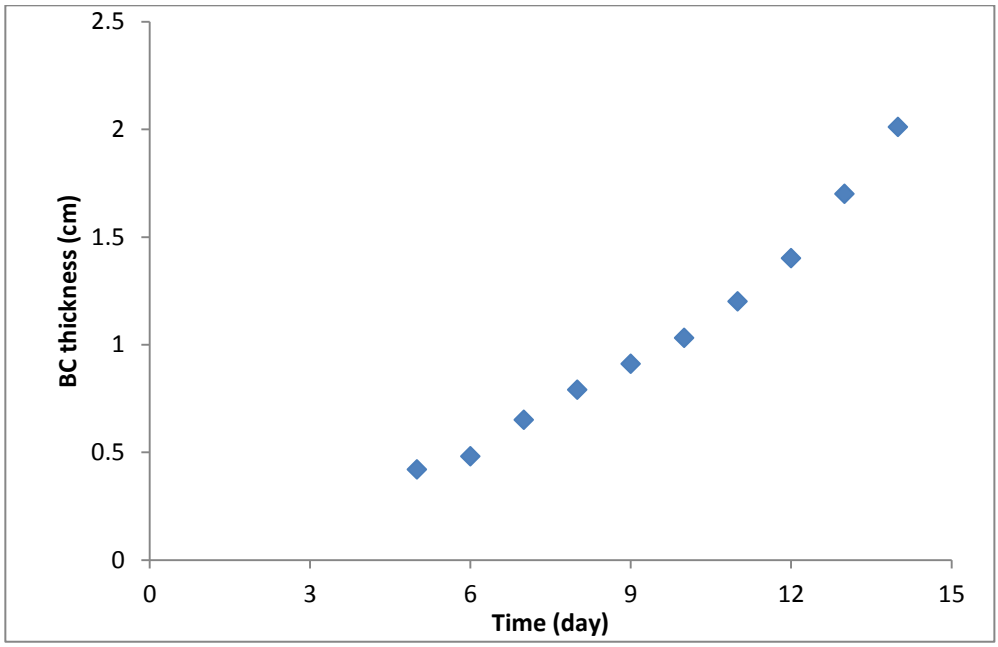

Figure 4.4: Effect of incubation time on thickness of BC mat produced by *G. xylinus* DSM 46604 in static culture.

The most accepted theory is that as the cells grow, they use the oxygen dissolved in the liquid and produce some cellulose, which is dispersed in the entire liquid phase. This is indicated by an increase in broth turbidity. When all the oxygen dissolved in the liquid phase is used up, the cells locate to the surface exposed to the gas phase and cellulose is formed as a mat on the surface of the

culture. Although the cells may divide, the population in the surface region does not increase but is in equilibrium because any extra cells are occluded and sink (Iguchi *et al.,* 2000). Because the bacteria secrete BC at the surface, productivity depends on surface area rather than volume of the culture vessel (Masaoka *et al.*, 1993).

Typical spherical pellicles formed in the broth after five days growth in agitated shake flask culture (Figure 4.5) and could be harvested (Figure 4.6). The cellulose pellicles harvested from the culture broth were light brown (Figure 4.6) due to contamination from components in the media.

Figure 4.5: Spherical cellulose pellicles in agitated shake flask culture.

Figure 4.6: Spherical cellulose pellicles harvested from agitated shake flasks before purification.

The initial glucose concentration in agitated shake flask cultures was 50 g/L. After five days, the residual glucose was 5 to 6 g/L, indicating the culture had used most of the glucose for growth and cellulose production. Productivity in stationary cultivation over the 14 days was 0.001 g BC/L/day, which is similar to that reported by Toda *et al.* (1997) under stationary growth conditions.

A low magnification SEM micrograph (Figure 4.7) of the cellulose pellicle surface shows a dense mat of BC fibrils ribbons, similar to that described by Czaja *et al.* (2004). Higher magnification SEM images of BC microfibrils produced under static (Figure 4.8) and agitated conditions (Figure 4.9) shows entangled fibres of cellulose, with a diameter ranging from approximately 35 nm to 90 nm, which is similar to that reported by Guhados *et al.* (2005). The SEM micrographs also showed that cellulose produced under static conditions tends to be more densely packed than when produced under agitated conditions. The SEM micrographs of BC produced in this study are similar to those reported by Czaja *et al.* (2004) and Suwannapinunt *et al.* (2007).

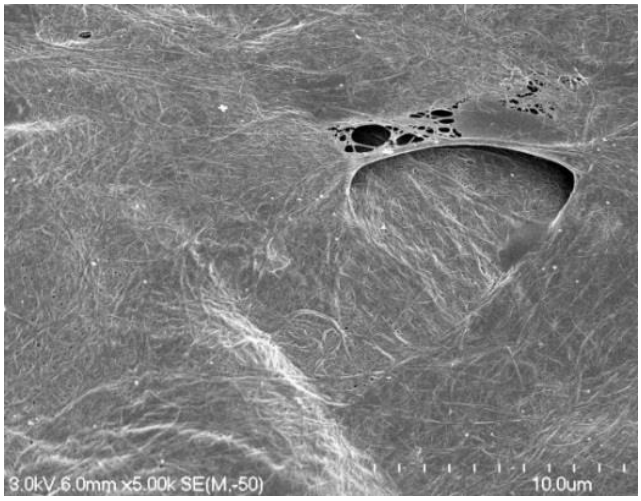

Figure 4.7: Low magnification SEM micrograph of the surface of purified cellulose pellicle formed in the agitated culture.

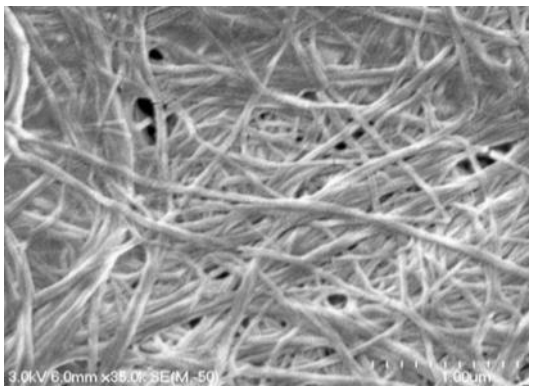

Figure 4.8: High magnification SEM micrograph of BC fibrils produced in static cultures.

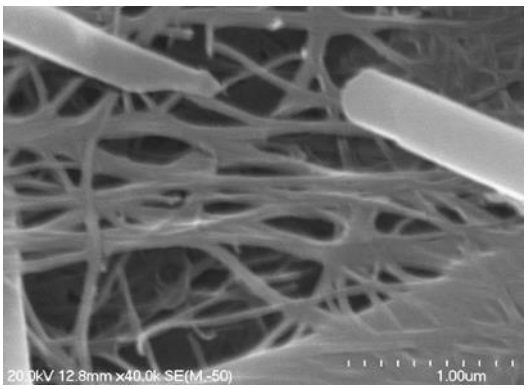

Figure 4.9: High resolution SEM micrograph of BC fibrils produced in agitated cultures.

The results show cultivation conditions influence the BC morphology. Static cultures are easier to perform than agitated cultures but static cultures require a longer cultivation period to obtain significant amounts of BC. Maintaining sterility during this long cultivation period is difficult because contamination may occur during the long incubation time.

4.2.2 Summary

This study showed that *G. xylinus* growing on glucose media could produce cellulose in static and agitated cultures. Culture conditions affected BC production and also morphology of the BC microfibrils. The BC obtained from static or agitated culture had different properties. The yield in static culture appeared to be higher so production could be cheaper than in agitated culture.

However, the risk of contamination in static culture is higher because of the long incubation time. Agitated culture will be better suited for industrial commercialization as aeration and agitation during growth will enhance mass transfer and BC production.

4.3 BC Production in Shake Flasks

4.3.1 Carbon Source

Agar slants and shake flasks were prepared using different carbon sources such as glucose, sucrose and lactose. Flasks were inoculated with fresh cultures of *G. xylinus* and incubated at 30^{o}C. Shake flasks were agitated at 150 rpm in a rotary incubator.

After five to seven days, many small colonies appeared on agar slants containing glucose but there was no growth on slants containing sucrose or lactose (Table 4.1).

Table 4.1: Effect of carbon source (50 g/L) on growth of *G. xylinus* DSM 46604 after 5 to 7 days on agar slants or in agitated shake flasks.

Carbon source	Agar Slants	Shake Flasks
Glucose	+	+
Sucrose	-	-
Lactose	-+	-+

+ : positively response -+ weak response -: no response

There was abundant growth in shake flasks containing glucose after five to seven days but no visible growth in shake flasks containing sucrose and lactose. The latter was used to see whether this microbial strain would produce BC on lactose. The result was an unexpected as other researchers had grown various strains of *G. xylinus* on sucrose (Pourramezan *et al.*, 2009), lactose (Mohite *et al.*, 2013) and mannitol (Nguyen *et al.*, 2008).

The differences between carbon sources could be due to the two major operative amphibolic pathways for oxidising carbohydrates: the pentose phosphate cycle and the Krebs cycle (Ross *et al.*, 1991). Mikkelsen *et al.* (2009) reported that BC production on sucrose was delayed by 12 hours compared with other carbon sources. The reason may be because sucrose could not be transported through the cell membrane and hydrolysed to glucose and fructose in

the periplasm (Velasco-Bedrán and López-Isunza, 2007). The BC production when *G. xylinus* DSM 46604 is grown on lactose agar slants and lactose media is also weak. Data in Table 4.1 are similar to those reported by Tsuchida and Yoshinaga (1997), who reported that the IFO 15237 and ATCC 35959 strains of *G. xylinus* did not grow on lactose and sucrose.

The outcome of the current investigated is that glucose was the most suitable sugar for BC synthesis. Many studies report that BC productivity could be enhanced by selecting the appropriate carbon source (Table 2.7/Section 2.9.1). For example, Heo and co-workers (2002) obtained the highest BC yield from *Acetobacter sp.* A9 using glucose as the carbon source. However, high concentrations of glucose can inhibit cell growth and BC production by *G. xylinus* due to accumulation of (keto) gluconic acids (Vandamme *et al.*, 1998).

Shake flasks cultures of *G. xylinus* DSM 46604 with varying glucose concentrations showed that BC production increased when glucose was increased from 40 to 50 g/L but then decreased slightly if higher glucose concentrations were used (Figure 4.10).

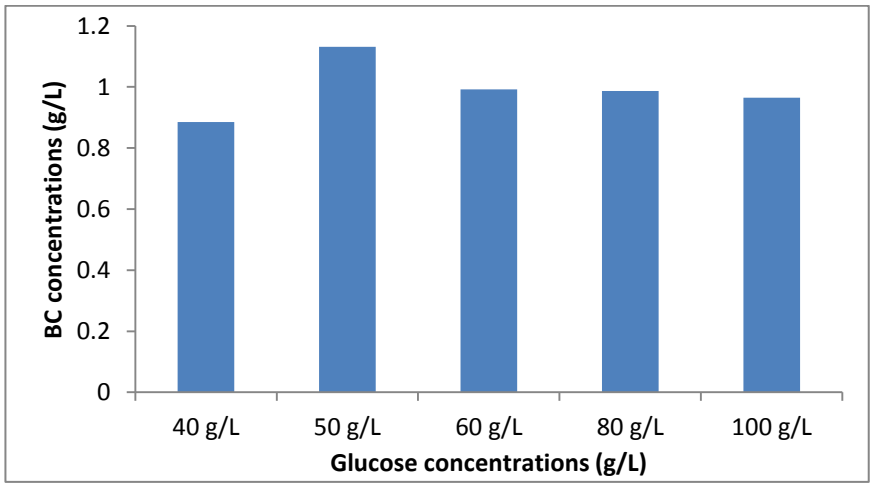

Figure 4.10: Effect of glucose concentration on BC production by *G. xylinus* DSM 46604 after seven days at 30°C, pH 6.8, and 150 rpm.

Masaoka *et al.* (1993) reported that BC production decreased if initial glucose concentration was greater than 40 g/L but other researchers have used higher sugar concentrations. For instance, Naritomi *et al.* (1997) used 70 g/L glucose, Ramana *et al.* (2000) used 50 g/L glucose, mannitol or sucrose and Chavez-Pacheco *et al.* (2005) used 50 g/L of glucose or sucrose (Appendix 1).

An initial glucose concentration of 50 g/L was used for the rest of the studies reported in this thesis, which is similar the initial sugar concentrations

most researchers used (Table 2.7/Section 2.9.1), even though Masaoka *et al*. (1993) reported that using glucose concentrations greater than 40 g/L decreased BC production.

The BC concentration of 1.13 g/L on 50 g/L glucose (Figure 4.10) obtained in this study is much lower than 3.5 g/L obtained by Ramana *et al.* (2000) growing *G. xylinus* NCIM in shake flasks on media with an initial glucose concentration of 50 g/L. Ishihara *et al.* (2002), using 20 g/L of D-glucose media at pH 5 in lidded glass tubes, obtained 623 mg BC, which was 35% production efficiency by *G. xylinus* ATCC53582.

4.3.2 Yeast Extract

Most microorganisms need a nitrogen source for growth. It is a major component of proteins, comprises 8-14% of the dry cell mass of bacteria (Chawla *et al.,* 2009). Several researchers have evaluated the effect of different nitrogen sources on BC production. Dudman (1960) used ammonium sulphate as a single nitrogen source and found both growth and BC production decreased. Ramana *et al.* (2000) found ammonium sulphate/ peptone or casein hydrolysate suitable for BC synthesis. Adding methionine for culturing *Acetobacter xylinum* reduced the lag time enhanced the growth rate during the early culture period and increased BC production rate (Matsuoka *et al.*, 1996).

Yeast extract (YE) contains many nitrogen compounds and many growth factors. Supplementing the growth media with yeast extract stimulates BC production (Son *et al.*, 2001). Many investigations reported in the 38 references on BC production (Appendix 1) used 5 g/L yeast extract in the growth media for producing BC. In this study, shake flask cultures with media containing 5 g/L ammonium sulphate $(NH_4)_2SO_4$ as the sole nitrogen source produced only 0.01 g/L BC after five days growth. This result is similar to that reported by Coban and Biyik (2011), who obtained 0.011 g/L BC using $(NH_4)_2SO_4$ in a glucose media.

Shake flask cultures using five different concentrations of YE along with ammonium sulphate as a nitrogen source showed that the highest BC concentration (5.2 g/L) was obtained by adding 5 g/L of YE.

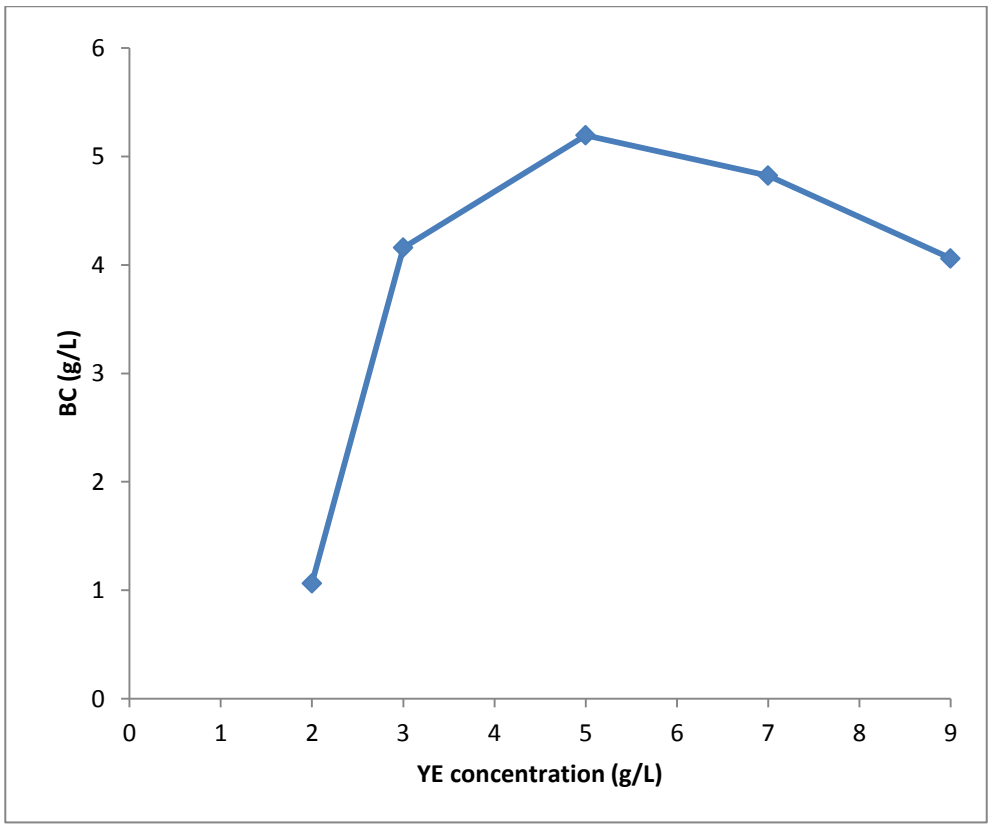

Figure 4.11: Effect of YE on BC production by *G. xylinus* DSM 46604.

The data indicate that adding both YE and $(NH_4)_2SO_4$ improved BC production, probably because organic nitrogen is necessary for cell growth and metabolism and has an important effect on BC synthesis.

4.3.3 Fish Hydrolysate

Fish hydrolysate contains amino acids such as glycine and tyrosine (Arasaratnam and Thayaananthan, 2010). Fish hydrolysate was manufactured with the aim of partially or wholly replacing expensive YE in the growth media for producing BC.

Media containing 5 g/L fish hydrolysate had the lowest BC production and culture on media with 5 g/L fish hydrolysate grew slowly (Table 4.2). The high residual glucose in the broth indicated limited growth.

The BC productivity increased with increasing fish hydrolysate concentration. The highest BC yield (g/g) was obtained using 20 g/L fish hydrolysate. The BC yields using 10 g/L and 15 g/L fish hydrolysate were similar (Table 4.2).

Table 4.2: Effect of fish hydrolysate on BC production by *G. xylinus* DSM 46604.

Parameter		Media component				
		5 g/L YE	5 g/L fish hydrolysate	10 g/L fish hydrolysate	15 g/L fish hydrolysate	20 g/L fish hydrolysate
Biomass	g/L	0.30	0.21	0.24	0.24	0.25
	g/g	0.05	0.03	0.03	0.03	0.04
BC	g/L	0.25	0.19	0.21	0.21	0.24
	g/g	0.04	0.03	0.03	0.03	0.04
Glucose	g/L	6.01	7.43	6.89	6.88	6.57

4.3.4 Fish Powder

The lowest BC production (0.19 g/L) was obtained on media with 5 g/L fish powder (Table 4.3). As with fish hydrolysate, the culture grew slowly in media this media and there was 7.9 g/L residual glucose in the fermentation broth after five days. Increasing the fish powder concentration did not significantly increase BC concentration. The highest BC yield of 0.04 g/g was produced using 15 g/L or 20 g/L fish powder. Growth on 5 g/L and 10 g/L fish powder produced BC yields of 0.02 g/g and 0.03 g/g respectively.

Table 4.3: Effect of fish powder on BC production by *G. xylinus* DSM 46604.

Parameter		Media component				
		YE (5 g/L)	5 g/L fish powder	10 g/L fish powder	15 g/L fish powder	20 g/L fish powder
Biomass	g/L	0.3	0.2	0.24	0.21	0.21
	g/g	0.05	0.03	0.03	0.03	0.03
BC	g/L	0.25	0.19	0.19	0.25	0.25
	g/g	0.04	0.02	0.03	0.04	0.04
Glucose	g/L	5.66	7.87	6.96	6.97	6.91

The effect of including 5 g/L yeast extract or varying concentrations of fish powder on BC yield demonstrate that fish powder has potential as a cheap nitrogen source for BC production by *G. xylinus* (Figure 4.12).

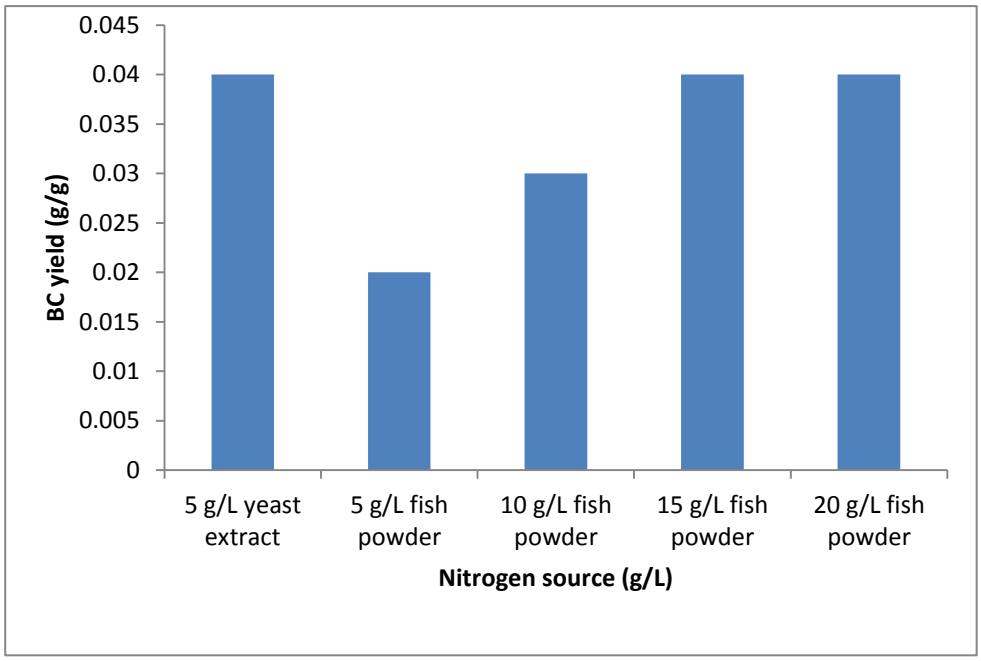

Figure 4.12: Effect of 5 g/L yeast extract media and fish powder on BC production by *G. xylinus* DSM 46604.

4.3.5 Banana Peel Extract

Banana peel is an abundantly available waste material containing fermentable sugars. Using banana peel extract as a media constituent for growth of bacteria and production of useful compounds has not been widely investigated. In an attempt to partially replace expensive glucose in the growth media, BC production in shake flask cultures using four different concentrations of glucose and banana peel extract were monitored (Table 4.4).

Table 4.4: Combination of banana peel extract and glucose used in the media for growing *G. xylinus* DSM 46604 in shake flasks.

Number	Combination
1	10 g/L banana peel extract + 40 g/L glucose
2	20 g/L banana peel extract + 30 g/L glucose
3	30 g/L banana peel extract + 20 g/L glucose
4	40 g/L banana peel extract + 10 g/L glucose

The highest BC concentration (0.43 g/L) as produced in media with the lowest banana peel extract content (Figure 4.13). The final pH in this media was also the lowest (pH 4.96). This low pH could be due to formation and accumulation of gluconic acid in the culture broth. The second media

combination produced a slightly less BC (0.37 g/L) and the fourth media combination produced only 0.17 g/L of BC.

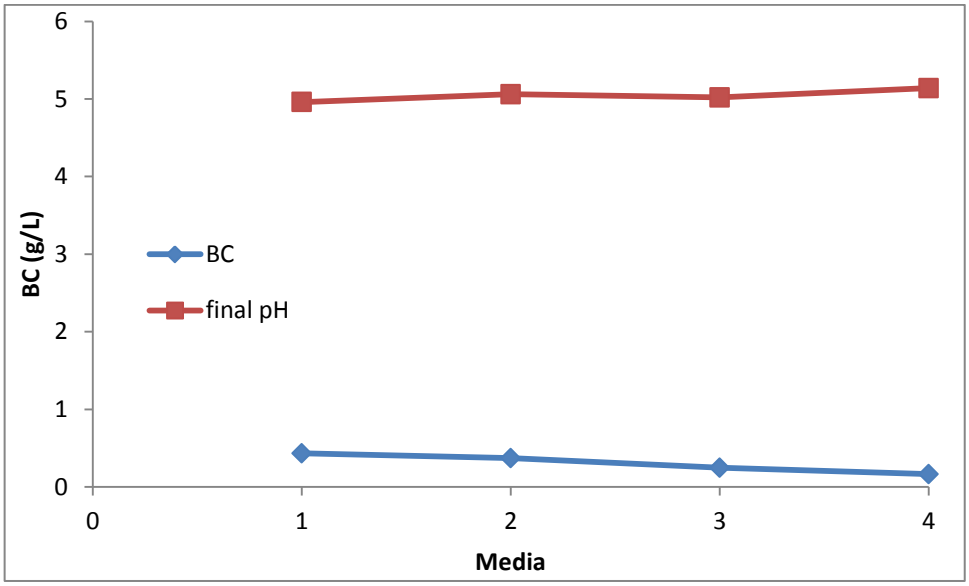

Figure 4.13: Effect of four different combinations of banana peel extract and glucose (see Table 4.4) on BC production and by *G. xylinus* DSM 46604.

Because *G. xylinus* can produce BC only between pH 4 and pH 7 (Bae *et al.*, 2004), the initial pH of the media used in this trial was pH 6.8 and it was not controlled. The data (Figure 4.13) indicated that combinations of glucose and banana peel extract could produce BC.

4.3.6 Glycerol

Glycerol is another cheap, readily available fermentation substrate. The suitability of glycerol as a component in media for BC production by *G. xylinus* was evaluated in shake flasks. Five glycerol concentrations between 10 and 50 g/L) were used. The BC concentration varied from 0.65 g/L when grown on 10 g/L glycerol to 1.43 g/L on 20 g/L glycerol (Figure 4.14). It then fell gradually to 1.39 g/L as glycerol was increased to 30, 40 or 50 g/L. The final pH of the media was similar, being between pH 5.43 and 5.68 from the initial pH 6.8. This differs from Jung *et al.* (2010), who reported maximum BC concentration on 30 g/L glycerol and a drop in concentration above this glycerol concentration. In the 38 investigations cited (Appendix 1), only three used glycerol as a carbon source (Kim *et al.*, 2006; Hungund and Gupta 2010a; Hungund and Gupta 2010b). They obtained BC concentrations of 4.5 g/L, 2.47 g/L and 1.2 g/L BC respectively on 15-20 g/L glycerol.

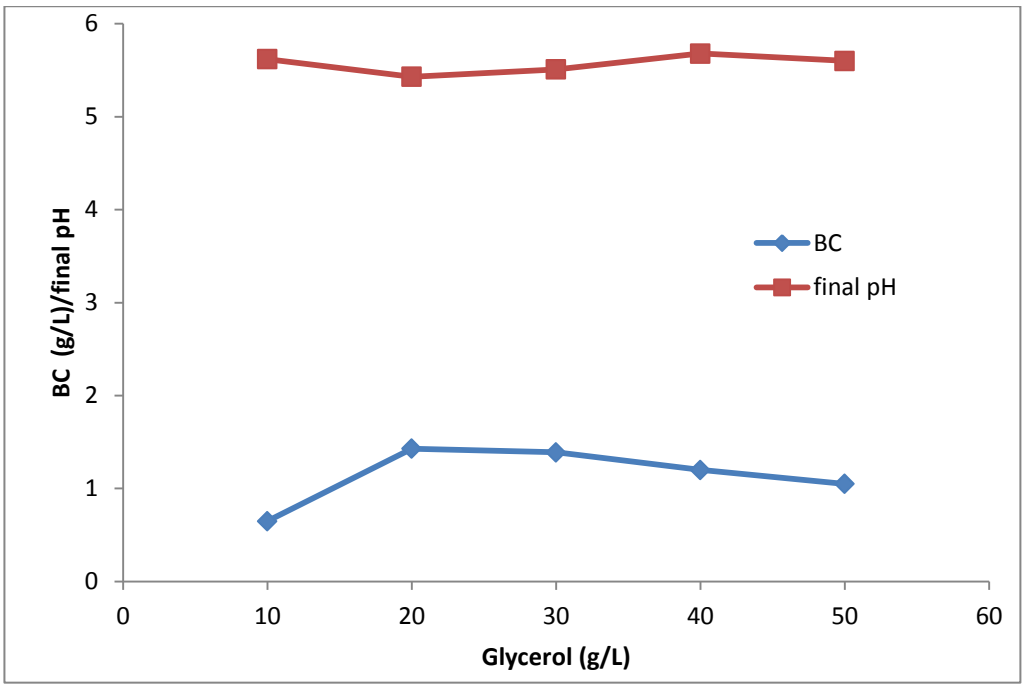

Figure 4.14: Effect of glycerol concentration on BC production by *G. xylinus* DSM 46604.

Glycerol is used by *G. xylinus* as carbon source for BC synthesis and cell growth (Weinhouse and Benziman, 1976). the glycerol is first converted to triose phosphate and then to BC without formation of gluconic acid. These metabolic pathways in *G. xylinus* could explain the high production of BC when growing on glycerol. The oxidation of triose phosphate is the primary reaction this organism uses to channel sugar carbon from the pentose cycle into the Krebs cycle. The results obtained in the present trials help develop a cost-effective fermentation method for BC production. Further study will be done in 3-L bioreactor operating under batch conditions.

4.3.7 Summary

The bacteria *G. xylinus* DSM46604 produced BC when grown on glucose or glycerol with conventional nitrogen sources or non-conventional nitrogen sources such as fish hydrolysate and fish powder. The fish powder and fish hydrolysate are cheaper nitrogen source than YE but these compounds need further characterization before they could be used to improve BC production. Glycerol showed potential as a low-cost carbon source for producing BC. Banana peel extract could be also be an alternative carbon source.

4.4 BC Production in a Batch Bioreactor

4.4.1 Effect of Aeration and Agitation

Aeration and agitation are both crucial factors for BC production in submerged cultures of *Gluconacetobacter* sp. In this study, the effect of aeration rates of 0.3, 0.6, 1.0 and 1.5 vvm on BC production was investigated. Both biomass and BC concentration increased with aeration rate but above aeration rates of 1.0 vvm, both BC and biomass concentrations decreased (Figure 4.15).

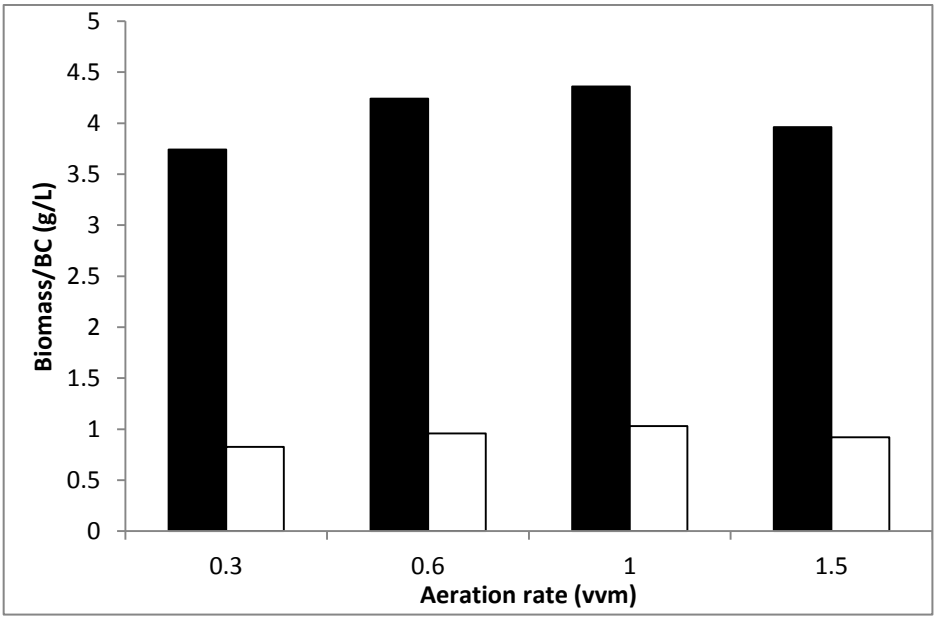

Figure 4.15: Effect of aeration rate on BC production (filled bars) and biomass (open bars) of *G. xylinus* DSM 46604 in batch fermentations.

Although biomass and BC concentrations increased with aeration, biomass yield was not affected by aeration rates (Table 4.5). The highest BC concentration and BC productivity was at 1.0 vvm so this aeration rate was used in further trials.

Most of the research under aerated and agitated cultivation conditions uses 0.4 to 1.7 vvm aeration rates to produce BC (Appendix 1). Hwang *et al.* (1999) reported that BC production decreased with increased dissolved oxygen tension (beyond 10% saturation). Although *G. xylinus* is a strict aerobe, it is possible limiting oxygen conditions existed at 0.3 vvm aeration because of a low K_{La}. Also, at the high aeration of 1.5 vvm, some fermentation broth was lost through the exhaust air line, compromising yield calculations.

Table 4.5: Effect of aeration rate on biomass and BC production by *G. xylinus* DSM 46604 in a batch bioreactor.

Parameter	Aeration rate (vvm)			
	0.3	0.6	1.0	1.5
BC (g/L)	3.4	4.2	4.4	4.0
Initial glucose (g/L)	50	50	50	50
Nitrogen source	YE + $(NH_4)_2SO_4$	YE + $(NH_4)_2SO_4$	YE + $(NH_4)_2SO_4$	YE + $(NH_4)_2SO_4$
Glucose consumed (g/L)	46.3	43.0	44.8	45.4
Biomass (g/L)	0.83	0.96	1.03	0.92
BC yield (g/g)	0.08	0.10	0.10	0.09
Biomass yield (g/g)	0.02	0.02	0.02	0.02
BC productivity (g/L day)	0.37	0.42	0.44	0.40
Biomass productivity (g/L day)	0.08	0.10	0.10	0.09

The effect of varying the agitation rate (150, 200, 250, 300 rpm) on BC production, biomass and glucose consumption when growing *G. xylinus* DSM 46604 in a batch bioreactor was investigated (Figures 4.16 to 4.19).

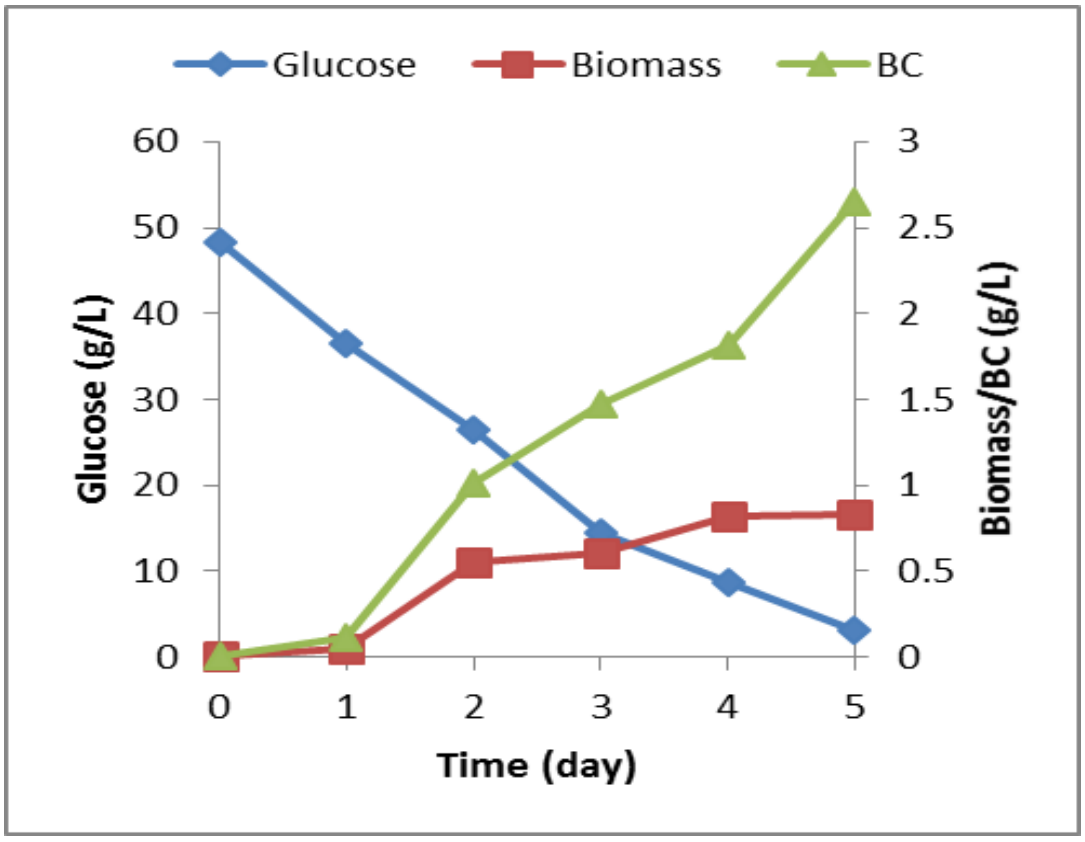

Figure 4.16: Glucose, biomass and BC profiles when growing *G. xylinus* DSM 46604 on 50 g/L glucose in a batch bioreactor at 150 rpm.

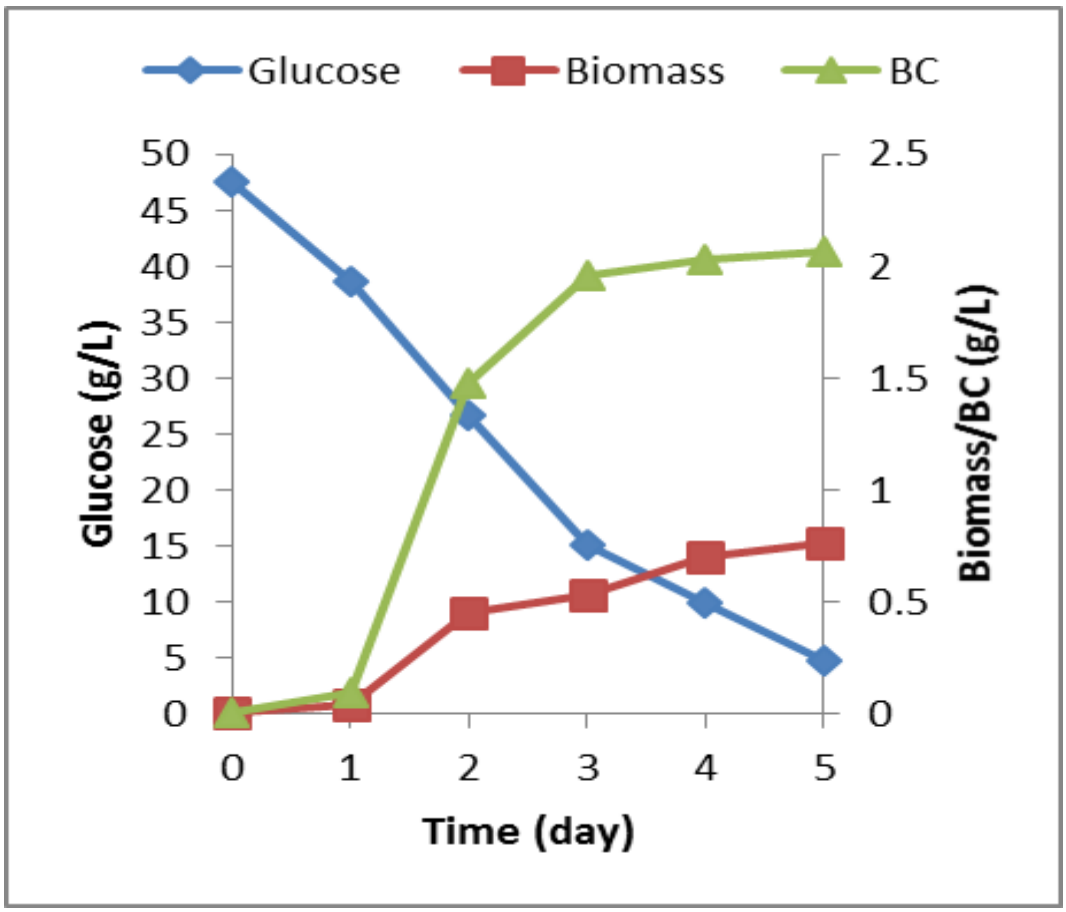

Figure 4.17: Glucose, biomass and BC profiles when growing *G. xylinus* DSM 46604 on 50 g/L glucose in a batch bioreactor at 200 rpm.

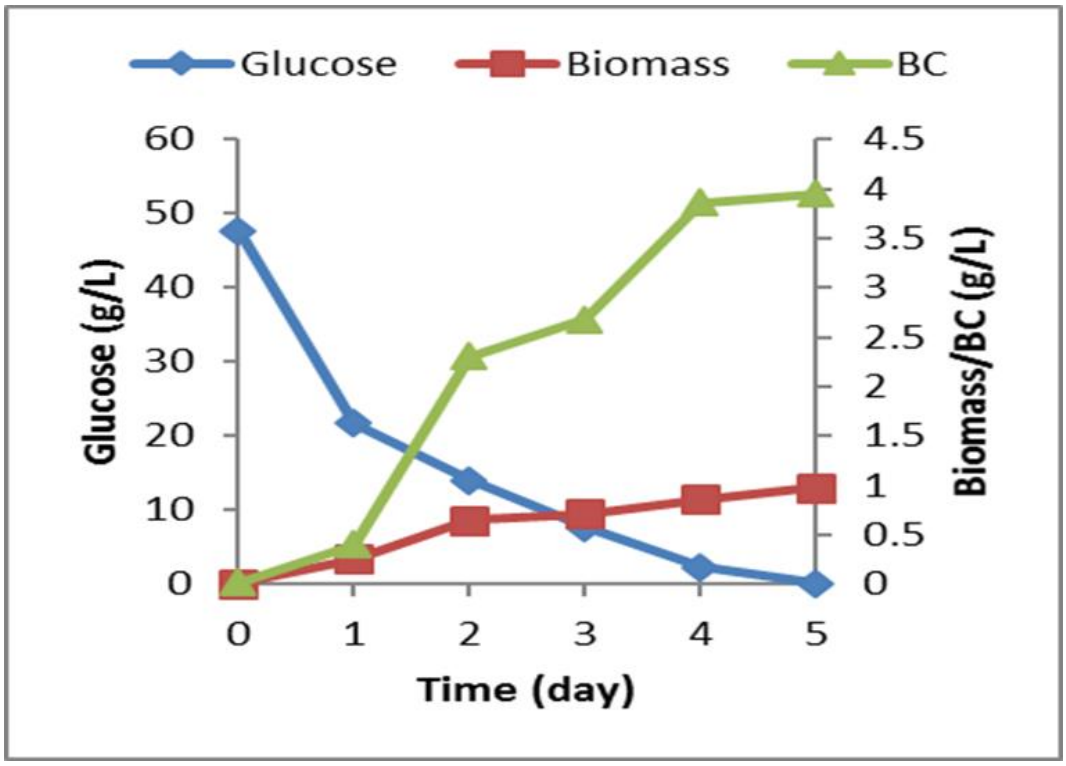

Figure 4.18: Glucose, biomass and BC profiles when growing *G. xylinus* DSM 46604 on 50 g/L glucose in a batch bioreactor at 250 rpm.

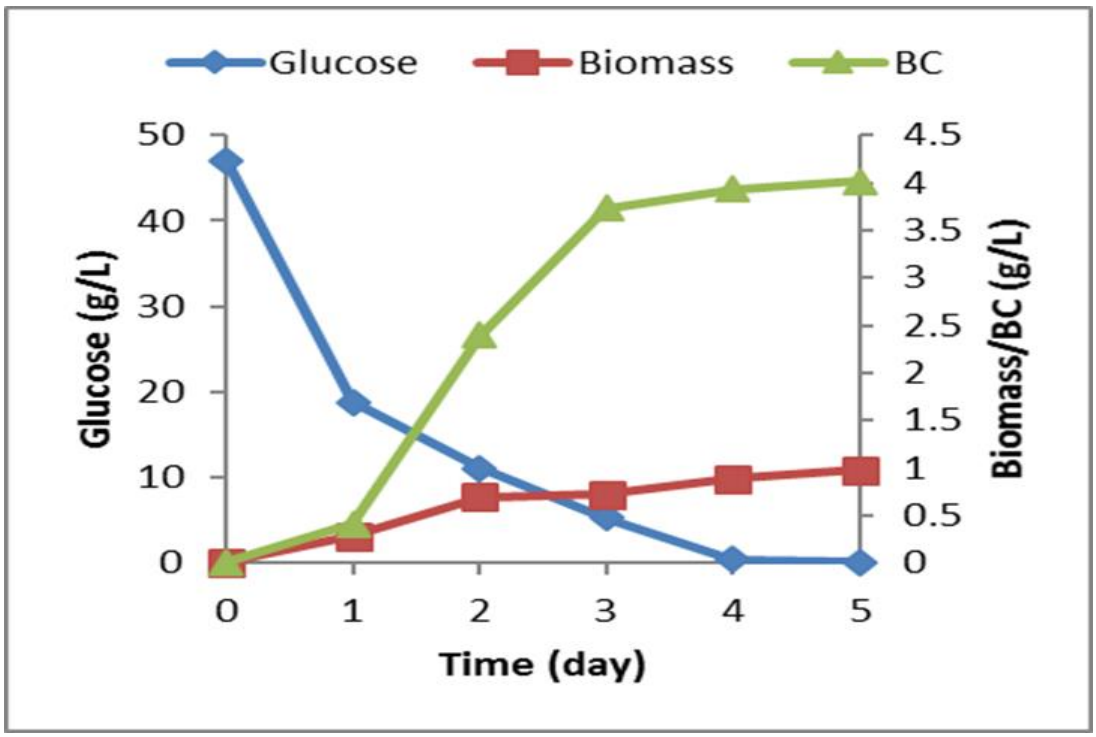

Figure 4.19: Glucose, biomass and BC profiles when growing *G. xylinus* DSM 46604 on 50 g/L glucose in a batch bioreactor at 300 rpm.

The BC production increased when agitation rate from 150 rpm to 300 rpm (Table 4.6). The highest BC concentration of 4.0 g/L occurred at 250 rpm. Because increasing agitation rate to 300 rpm did not significantly increase BC concentration (4.02 g/L BC), all further trials were done at 250 rpm.

Table 4.6: Effect of agitation speed on biomass and BC production by *G. xylinus* DSM 46604 on 50 g/L glucose in a batch bioreactor.

Parameter	Agitation speed (rpm)			
	150	200	250	300
BC (g/L)	2.1	2.7	4.0	4.0
Glucose consumed (g/L)	42.7	45.1	47.5	46.9
Biomass (g/L)	0.76	0.83	0.98	0.98
BC yield (g/g)	0.05	0.06	0.08	0.09
Biomass yield (g/g)	0.02	0.02	0.02	0.02
BC productivity (g/L/day)	0.21	0.27	0.405	0.40
Biomass productivity (g/L/day)	0.08	0.08	0.10	0.10

Jung *et al* (2005) cultured sp. in agitated fermentations and produced 1.2 g/L BC when growing *Gluconacetobacter hansenii* in a 5-L jar fermenter (3 L basal media) with 6 flat-blade turbine impellers at 300 rpm and aeration rate of 1.0 vvm. The productivity of 0.40 g/L/day is the same as the BC productivity obtained in this study of 0.40 g/L/day. Shear stress can affect BC production and can convert cellulose-producing cells into cellulose-negative mutants (Cel^-) that do not produce cellulose (Hestrin and Schramm, 1954; Valla and Kjosbakken, 1982). This will decrease productivity so there must be a balance between agitation to keep cells suspended and provide efficient oxygen and nutrient transfer and that which will adversely affect cell type.

4.4.2 Fish Hydrolysate

Shake flask studies indicated that media with 15 g/L fish hydrolysate, 50 g/L glucose, 3 g/L potassium orthophosphate, 5 g/L ammonium sulphate and 0.05 g/L magnesium sulphate is suitable. Batch fermentations in a 5-L bioreactor using 3 L of media showed that adding 5 g/L of YE produced 2.12 g/L BC (Figures 4.20), representing a BC yield of 0.05 g per g glucose used. Using 15 g/L fish hydrolysate increased BC concentration to 0.47 g/L BC (Figures 4.21), and represented a BC yield of 0.01 g per g glucose used.

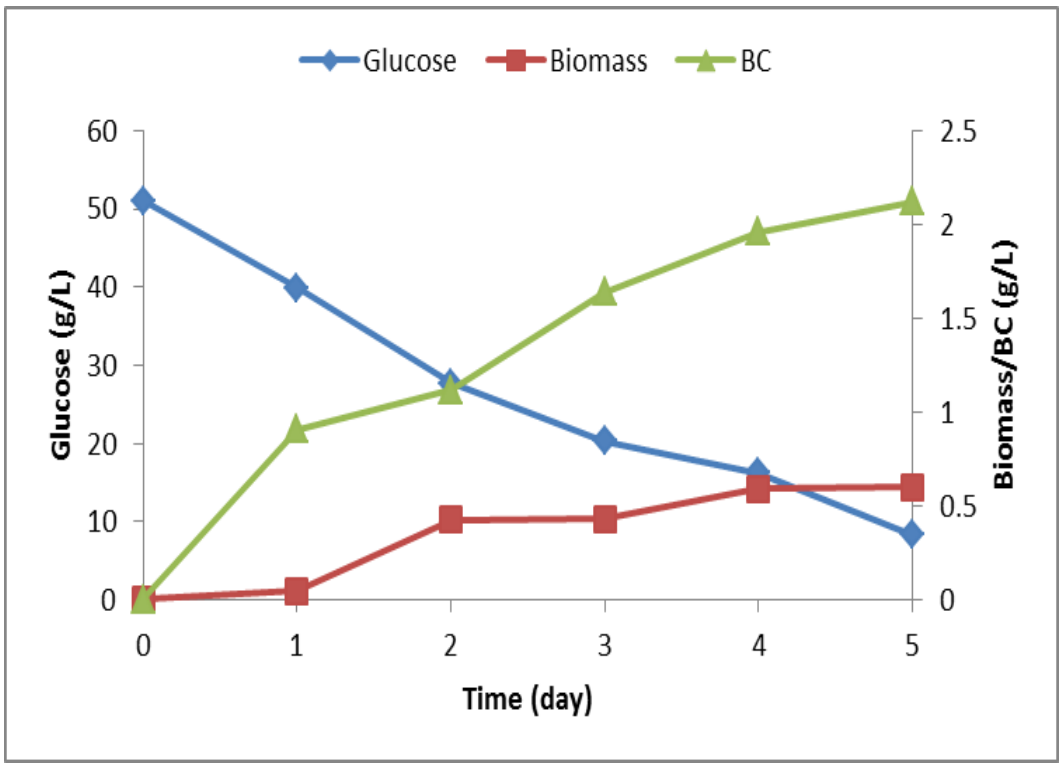

Figure 4.20: Glucose, biomass and BC profiles when growing *G. xylinus* DSM 46604 in a batch bioreactor with 5 g/L yeast extract.

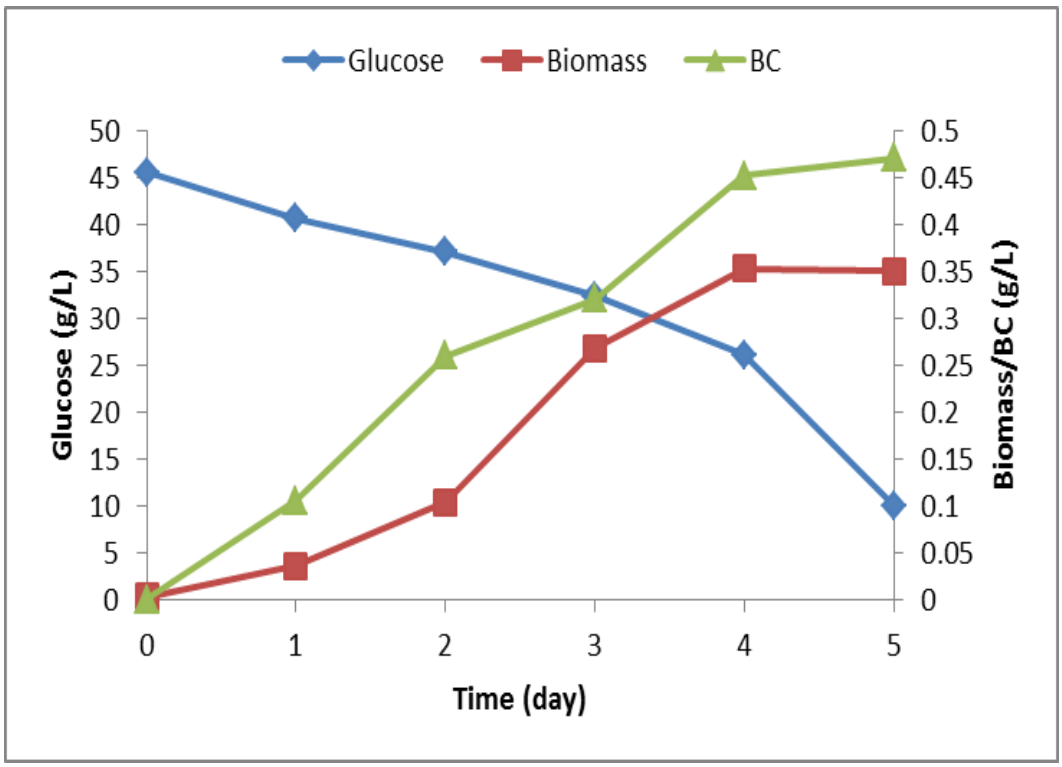

Figure 4.21: Glucose, biomass and BC profiles when growing *G. xylinus* DSM 46604 in a batch bioreactor with 15 g/L fish hydrolysate.

After five days, over 80% of the glucose was consumed when the media contained 5 g/L YE (Figure 4.20) and nearly 80% of the glucose was consumed when using 15 g/L fish hydrolysate (Figure 4.21). This meant there was a high residual glucose in the fermentation broth (8 to10 g/L).

Growth, biomass and BC production was lower on media with fish hydrolysate than media with YE. However, the biomass yield after five days was similar, being 0.01 g biomass per g glucose used.

The BC productivity when growing *G. xylinus* DSM 46604 on media with YE was four times higher (0.2 g/L/day) than on media with fish hydrolysate (0.05 g/L/day) (Table 4.7). Desipite the lower productivity, the data from the trials indicate that fish hydrolysate could be a cheap alternative for YE in growth media to produce BC even though there were some unknown compounds in the fish hydrolysate that inhibited growth and BC production. Further purification and characterization of the fish hydrolysate should be done to see if BC production on media with fish hydrolysate can be improved.

Table 4.7: Effect of 5 g/L YE and 15 g/L fish hydrolysate on glucose consumption, biomass and BC production by *G. xylinus* DSM 46604 grown in a batch bioreactor.

Parameter	Media component	
	5 g/L YE	15 g/L fish hydrolysate
BC (g/L)	2.1	0.5
Glucose consumed (g/L)	42.7	35.5
Biomass (g/L)	0.6	0.4
BC yield (g/g)	0.05	0.01
Biomass yield (g/g)	0.01	0.01
BC productivity (g/L/day)	0.21	0.05
Biomass productivity (g/L/day)	0.06	0.04

4.4.3 Fish Powder

Further trials with media containing 15 g/L fish powder, 50 g/L glucose, 3 g/L dipotassium orthophosphate, 5 g/L $(NH_4)_2SO_4$ and 0.05 g/L magnesium sulphate were done in a 5-L bioreactor. The pH was controlled at pH 6.8.

The glucose, biomass and BC profiles when growing *G. xylinus* on fish powder (Figure 4.22) were similar to those for fish hydrolysate (Figure 4.21). The BC productivity is four times higher (0.1g/L/day) when *G. xylinus* is grown on glucose with 5 g/L YE in the media than when using 15 g/L fish powder (0.05 g/L/day) (Table 4.8), due mainly to the fact that more glucose was consumed during the five-day fermentation on media with fish powder (43 g compared with 38 g).

In the fermentation with media containing 5 g/L YE, 84% of the initial glucose were used (Figure 4.20). However, only 77% of the initial glucose was used in media contained 15 g/L fish powder (Figure 4.22), so there as a large amount of residual glucose (11 g/L). The growth and biomass production when using 15 g/L fish powder was slightly lower than when using 15 g/L fish hydrolysate (Section 4.4.3). The slower growth and lower product concentrations could be due to the presence of some components in the fish powder. Although BC productivity with fish powder is lower than with YE, there is the potential to use fish powder as a low-cost nitrogen source.

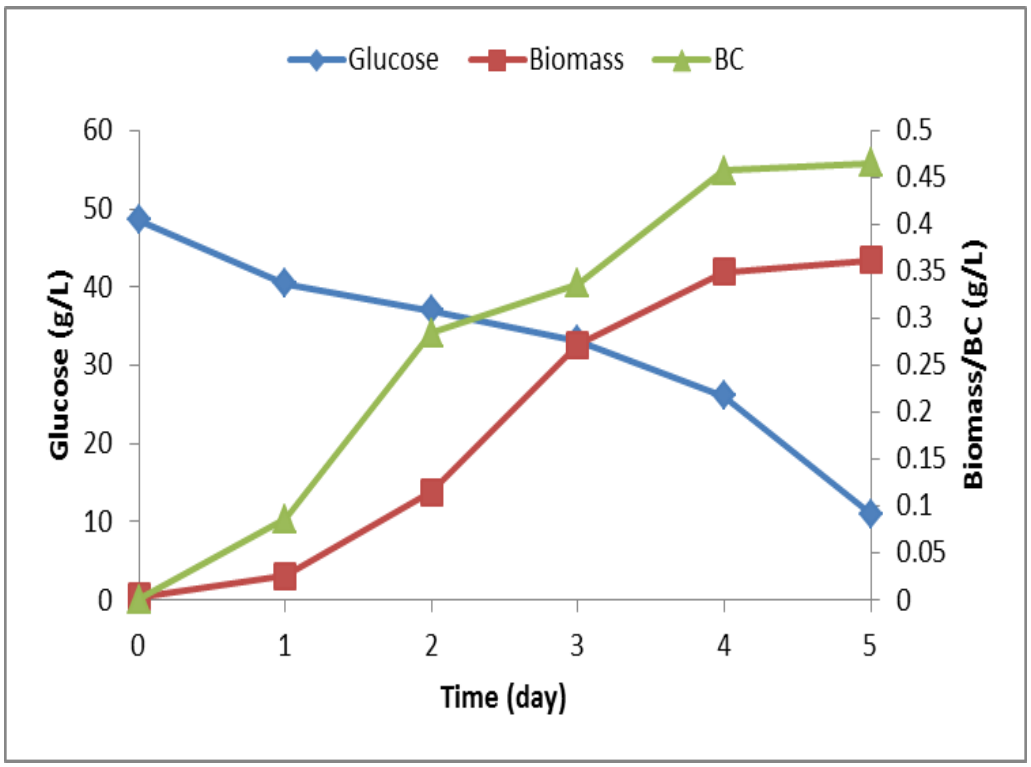

Figure 4.22: Glucose, biomass and BC profiles when growing *G. xylinus* DSM 46604 in a batch bioreactor using 15 g/L fish powder.

Table 4.8: Glucose consumption, biomass and BC production when growing *G. xylinus* DSM 46604 in a batch bioreactor with 5 g/L YE and 15 g/L fish powder.

Parameter	Media component	
	5 g/L YE	15 g/L fish powder
BC (g/L)	2.1	0.47
Glucose consumed (g/L)	42.7	37.6
Biomass (g/L)	0.6	0.36
BC yield (g/g)	0.05	0.01
Biomass yield (g/g)	0.01	0.01
BC productivity (g/L/day)	0.21	0.05
Biomass productivity (g/L/day)	0.06	0.04

The BC production in the YE media was slightly higher than in the fish hydrolysate media (Section 4.4.3) but BC production was better with YE than with fish powder in the media. After five days, the 5 g/L YE media had 0.6 g/L of *G. xylinus* biomass, corresponding to a yield of 0.01 g biomass/g glucose, which was similar to the biomass yield for 15 g/L fish powder (0.01 g/g). Results indicated fish powder could be an replacement for YE but it is recommended that the fish powder be further purified and also characterized.

4.4.4 Banana Peel Extract

Trials on the effect of banana peel extract on growth and BC production were scaled from the shake flask to the 3-L bioreactor. Media containing 50 g/L glucose was used as a control. Media containing 10 g/L banana peel extract and 40 g/L glucose produced encouraging results (Figures 4.24 and 4.25).

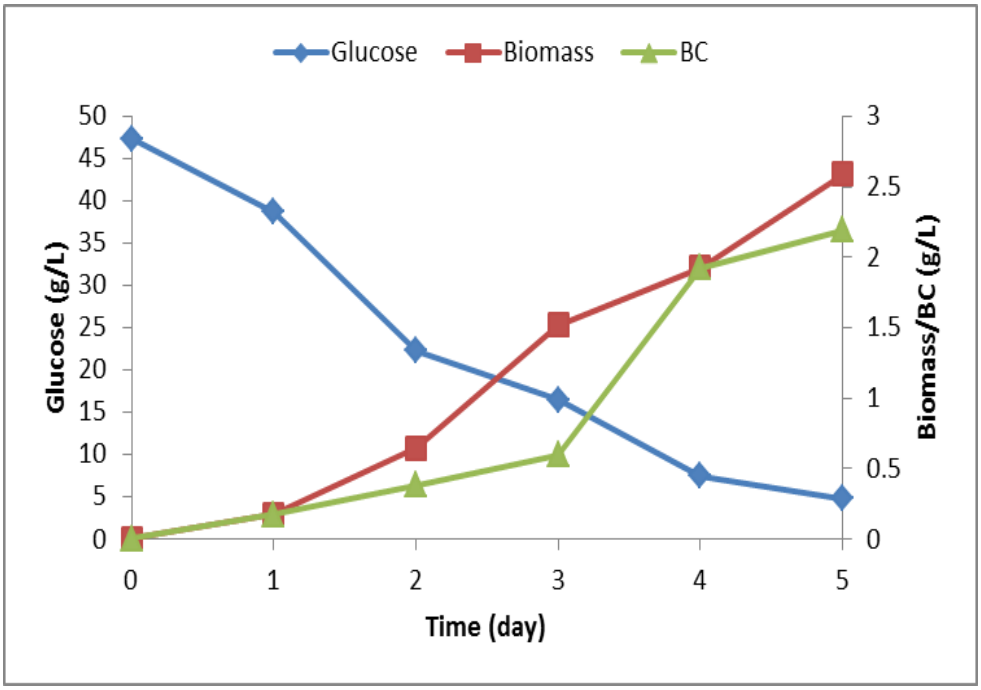

Figure 4.23: Glucose, biomass and BC profiles when growing *G. xylinus* DSM 46604 on 50 g/L glucose in a 3-L bioreactor.

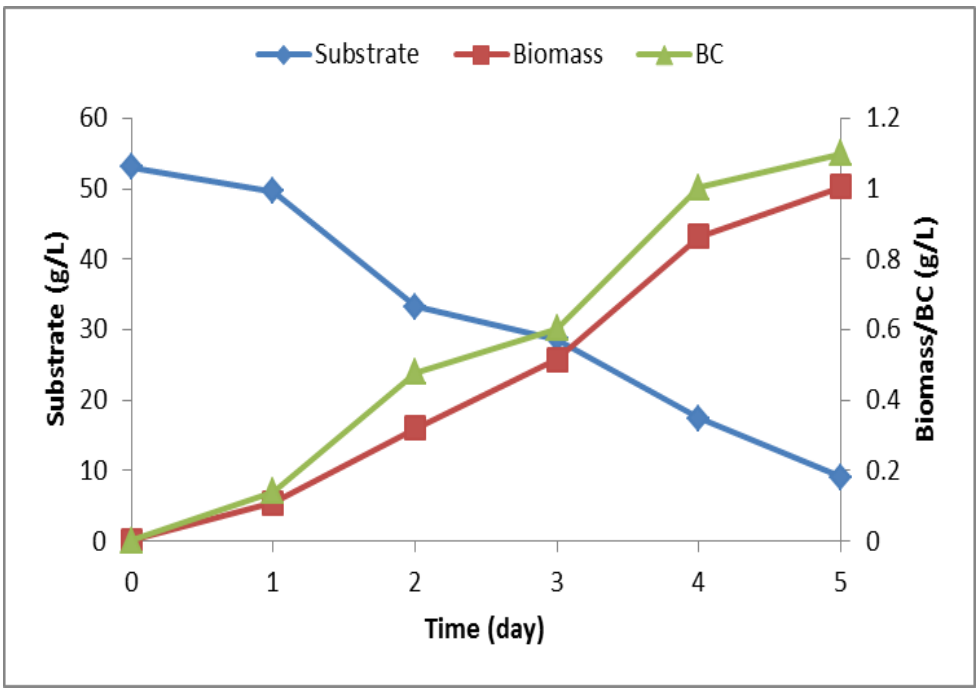

Figure 4.24: Glucose, biomass and BC profiles when growing *G. xylinus* DSM 46604 on media with 10 g/L banana peel extract and 40 g/L glucose in a 3-L bioreactor.

Growth of *G. xylinus* in banana peel extract supplemented media produced 1.1 g/L BC, representing 0.03 g BC/ g carbon consumed. When 50 g/L glucose was used, the BC concentration was 2.2 g/L, which represented 0.05 g BC/ g glucose. Productivity on glucose (0.21 g BC/L/day), was twice that achived on a mixture of 10 g/L banana peel extract and 40 g/L glucose (0.11 g BC/L/day) (Table 4.9).

Table 4.9: Effect of carbon source on *G. xylinus* DSM 46604 biomass and BC production in a 3-L bioreactor.

Parameter	Carbon source	
	Glucose (50 g/L)	Glucose (40 g/L) + banana peel extract (10 g/L)
BC (g/L)	2.2	1.1
Carbon source consumed (g/L)	42.5	44.0
Biomass (g/L)	2.6	1.01
BC yield (g/g)	0.05	0.03
Biomass yield (g/g)	0.06	0.02
BC productivity (g/L/day)	0.23	0.11
Biomass productivity (g/L/day)	0.26	0.10

The profiles for BC concentration when using glucose or a mixture of glucose and banana peel extract is shown in Figure 4.25. Both carbon sources yielded similar BC production until three days of growth. However, the culture grown using glucose the produced much higher BC concentrations than the culture grown using mixture of glucose and banana peel extract. The data show that expensive glucose could be partially replaced with banana peel extract, which is derived from an abundant waste material. The results also demonstrate that *G. xylinus* could be grown on waste-derived carbon sources to produce BC.

The lower BC yields obtained in this study show that the form of substrates made from waste materials does not support good cell growth and high BC production. Although banana peels contain considerable amounts of glucose, fructose, sucrose and maltose (Chandraju *et al.*, 2011), they also contain water-extractable phenolic compounds (Gonzalez-Montelongo *et al.,* 2010). Although, these sugars could be used by bacteria for growth, the water-soluble

phenolics have antimicrobial properties that alter membranes, especially in Gram negative bacteria (Lucchini *et al.*, 1990), leading to cell lysis.

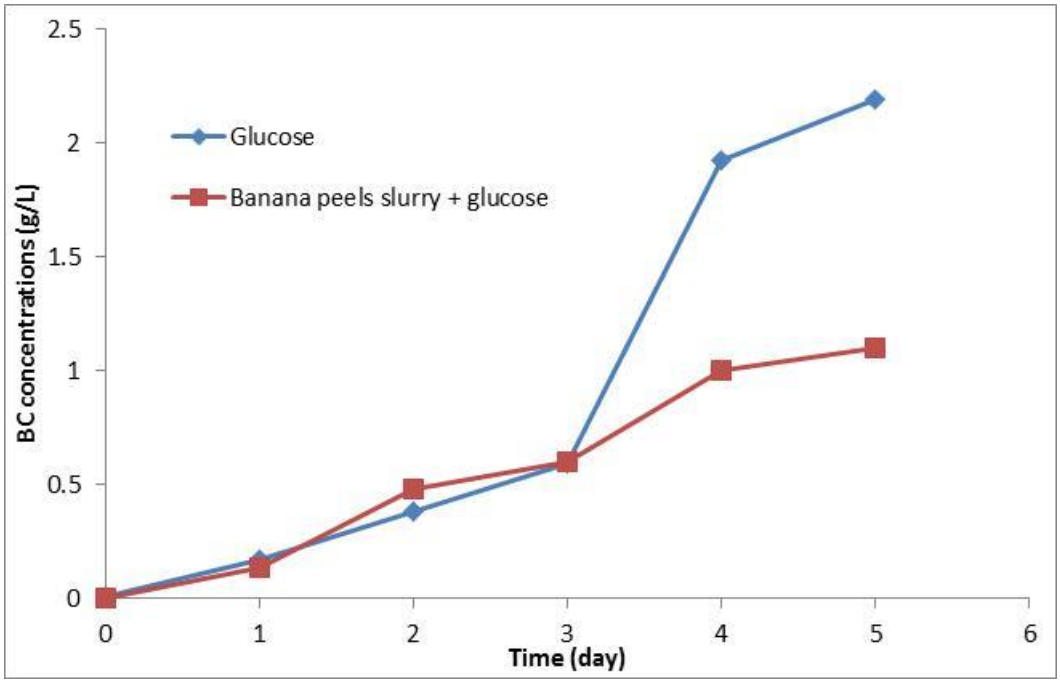

Figure 4.25: Effect of different carbon sources on profile for BC production by *G. xylinus* DSM 46604 in a 3-L bioreactor.

The major sugars in kiwifruit are glucose, fructose and sucrose (Beever and Hopkirk, 1990). Kiwifruit also contains some water-soluble phenolic compounds (Dawes and Keene, 1999) and proteolytic enzymes that disintegrate bacterial cell walls (Larocca *et al.*, 2010). Therefore, these non-conventional substrates for BC production need to undergo appropriate purification steps to remove any growth-inhibiting components and enhance bioavailability of the sugars before they are suitable as alternative components in the media.

4.4.5 Glycerol

Glycerol is another low-cost substrate for fermentation. Large quantities of crude glycerol will be generated by the growing biodiesel industry. Only a small proportion will be absorbed by the traditional sectors such as the pharmaceutical and other industries, so finding uses for the increased glycerol production could be a challenge. One way of using excess glycerol would be as a fermentation substrate for producing high value products such as BC.

Trials were done to investigate using glycerol as a carbon source to produce BC by *G. xylinus*. Shake flask trials showed that the highest BC concentration was produced on 20 g/L glycerol. The trials were scaled to growth

in a 3-L bioreactor using 2.5 L media containing 20 g/L glycerol. The control was 50 g/L glucose. Substrate consumption, biomass and BC production in the two media were measured and compared (Figures 4.26 and 4.27).

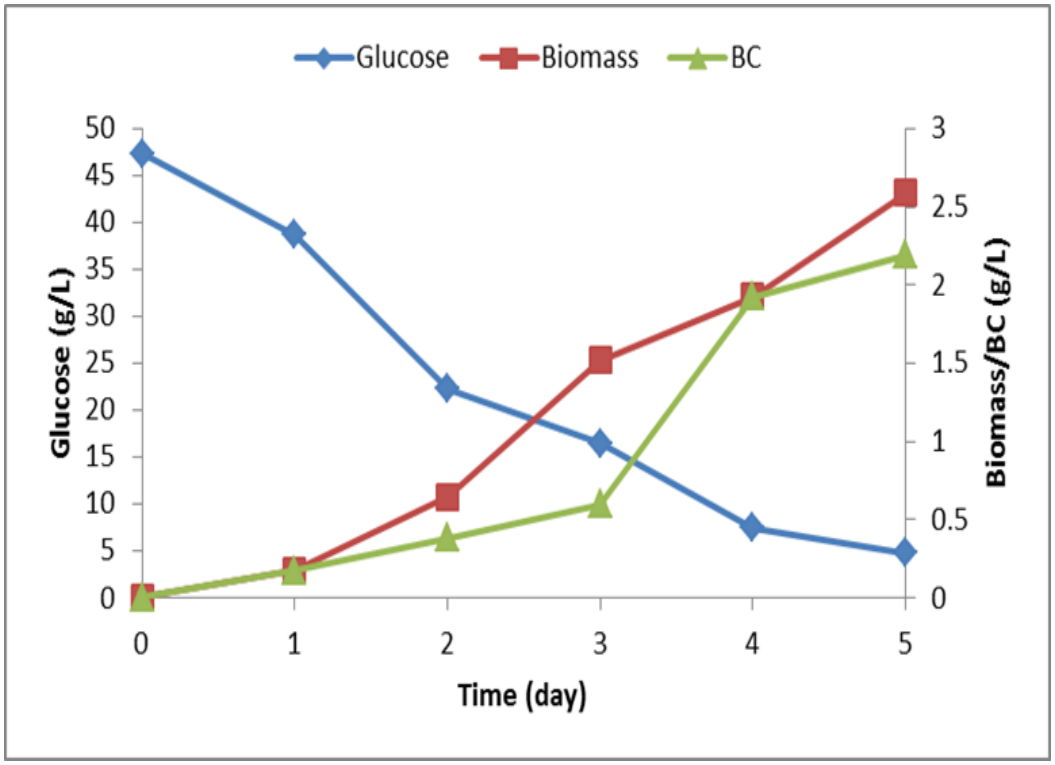

Figure 4.26: Glucose, biomass and BC profiles when growing *G. xylinus* DSM 46604 on 50 g/L glucose media in a 3-L bioreactor.

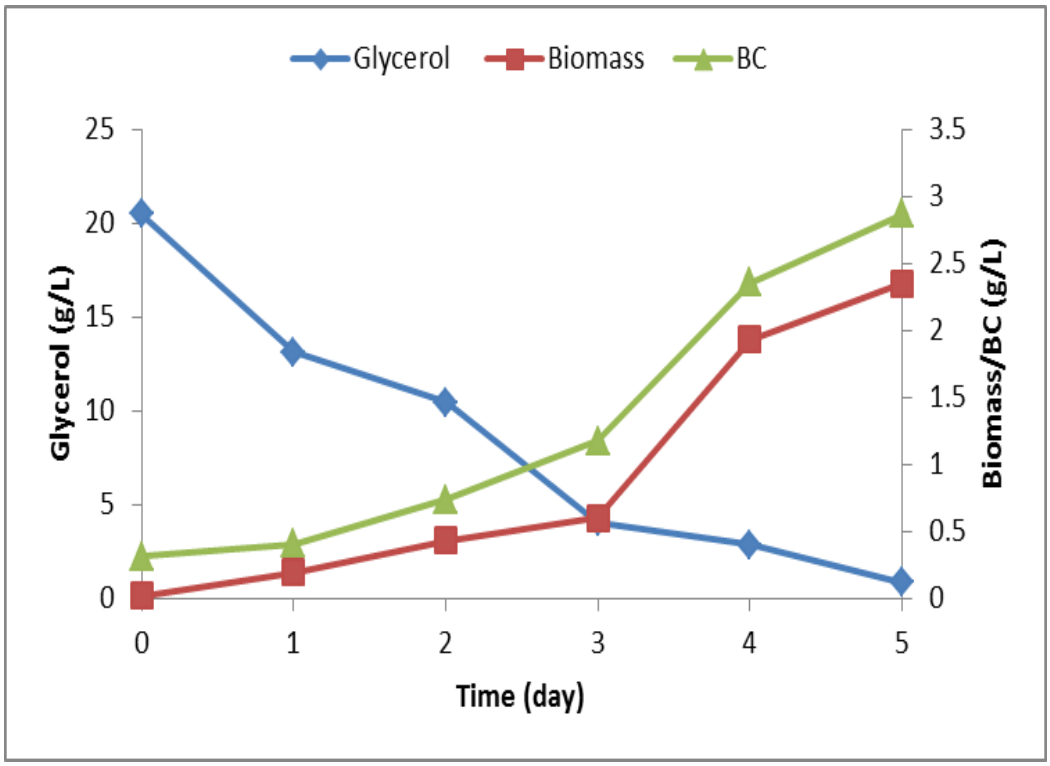

Figure 4.27: Glucose, biomass and BC profiles when growing G. *xylinus* DSM 46604 on 20 g/L glycerol in a 3-L bioreactor.

The BC concentration on 20 g/L glycerol after five days growth was 2.87 g/L, which was higher (2.19 g/L) than on 50 g/L glucose. The BC yield of 0.15 g/g glycerol obtained in this study (Table 4.10) is comparable to yields reported in other investigations. For example, Keshk and Sameshima (2006b), Kim *et al.* (2006) and Hungund and Gupta (2010a) obtained 0.12 -0.13 g BC/g glycerol in agitated cultures of *G. xylinus* using 20 g/L glycerol.

Table 4.10: Biomass and BC production by *G. xylinus* DSM 46604 growing on glucose or glycerol in a 3-L bioreactor.

Parameter	Carbon source	
	50 g/L glucose	20 g/L glycerol
BC (g/L)	2.19	2.87
Carbon source consumed (g/L)	42.52	19.66
Biomass (g/L)	2.59	2.35
BC yield (g/g)	0.05	0.15
Biomass yield (g/g)	0.06	0.12
BC productivity (g/L/day)	0.23	0.29
Biomass productivity (g/L/day)	0.26	0.24

Data from further trials on glucose and glycerol are shown in Figure 4.28.

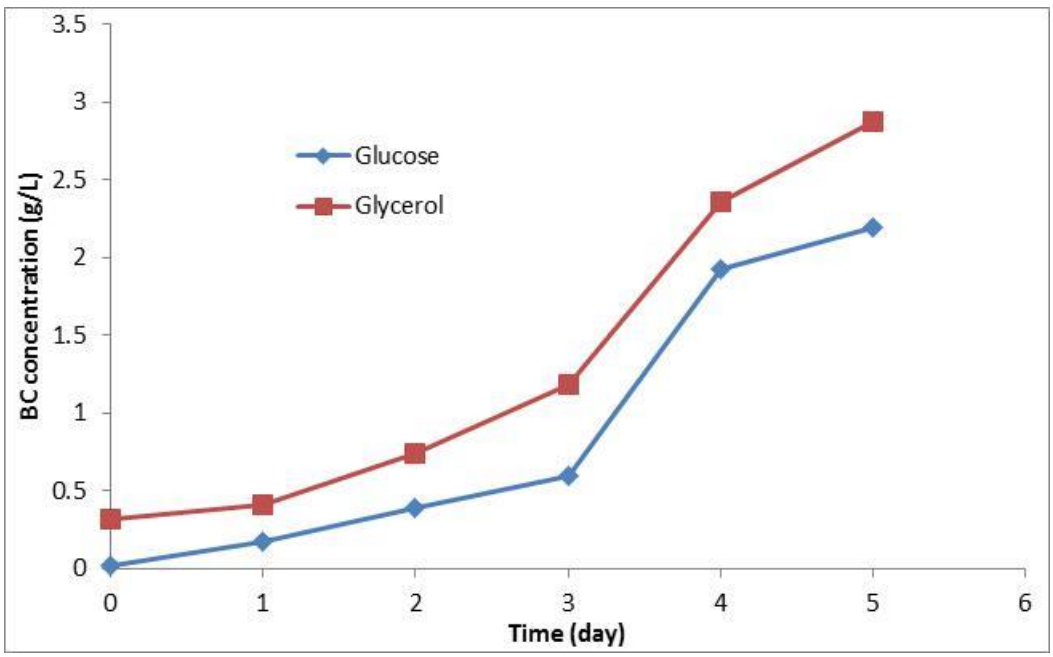

Figure 4.28: Profile for BC production when growing *G. xylinus* DSM 46604 on 50 g/L glucose or 20 g/L glycerol in a 3-L bioreactor.

When the carbon source is glycerol, BC biosynthesis occurs by first generating glucose via the gluconeogenesis metabolic pathway. This may become the rate limiting under the given physiological conditions (Ross *et al*., 1991). Glucose supported rapid initial synthesis of BC and it is assumed that the slower BC formation in initial stages growth on glycerol could be due to insufficient glucose being available. The second reason is that the media contained 50 g/L glucose but only 20 g/L glycerol, so the BC concentrations achieved on glucose should be higher than formed on glycerol. Lastly, glycerol can be channelled to form dihydroxyacetone (DHA) and small amounts of acetic acid via the TCA cycle (Schramm *et al.*, 1957).

4.4.6 Summary

When growing *G. xylinus* in stirred bioreactors, factors such as agitation and aeration influence BC production. Maximum BC production in the 3-L bioreactor was obtained at 250 rpm and increasing agitation did not significantly increase BC production. Providing aeration at 1.0 vvm improved BC production but greater aeration rates decreased BC production.

Studies on the effect of replacing yeast extract with either 15 g/L fish hydrolysate or 15 g/L fish powder as the nitrogen source in stirred bioreactors showed that similar BC yield (0.01 g/g glucose) were obtained. Supplementing 40 g/L of glucose with 10 g/L banana peel extract increased BC yield to 0.03 g/g carbon used. Replacing glucose with 20 g/L of glycerol increased BC yield to 0.15 g/g carbon source, which is three times higher than on glucose (0.05 g/g).

4.5 Overall Summary

Bacterial cellulose could be produced by growing *G. xylinus* in static and agitated cultivation. The two culture conditions affected BC yields and BC morphology. The SEM micrographs showed the arrangement of the BC microfibril structure. Shake flasks and bioreactor culture have advantages and disadvantages when producing BC. Shake flasks is cheaper but the system is not completely controlled (i.e. pH and aeration) and volume culture media can be processed. The system variables (e.g. pH, aeration, agitation) can be is controlled in a batch bioreactor and large volumes of culture media can be processed.

Studying the carbon source and nitrogen source effects on BC production allowed optimization of fermentation media, allowing fermentation cost and time to be more effective. Investigating the effect of carbon and nitrogen sources concentration gave a picture of how BC production is affected by these factors.

Using cheaper, non-conventional substrate to replace conventional media could produce BC and reduce fermentation cost. However, these non-conventional substrates need to be characterized to identify further information that is useful for research purpose, especially in BC production.

Chapter 5: Processing Strategies

This chapter describes growing *G. xylinus* DSM46604 on glucose and glycerol to produce BC using different fermentation processing strategies. The BC productivity and yield from batch, fed-batch and continuous cultures were determined and compared with batch culture.

5.1 Fed Batch Using Glucose as the Carbon Source

5.1.1 Fill-and-Draw Fed-Batch

To operate the fill-and-draw fed-batch cultures, fermentations were initiated as a batch culture using 50 g/L glucose and then switched to fill-and-draw culture (Section 3.6.1). When glucose concentration decreased to 10-15 g/L, 1000 ml of fermentation broth was withdrawn and 1000 ml fresh media with 100 g/L glucose was added to the fermenter which meant (taking Day 3 as the example) glucose concentration increased from 15 g/L to 52 g/L. Biomass and BC in the fermenter increased after every fill-and-draw cycle (Figure 5.1). Four cycles of broth withdrawal and fresh media addition were done and then fermentation conditions reverted to batch until glucose had fallen to below 5 g/L.

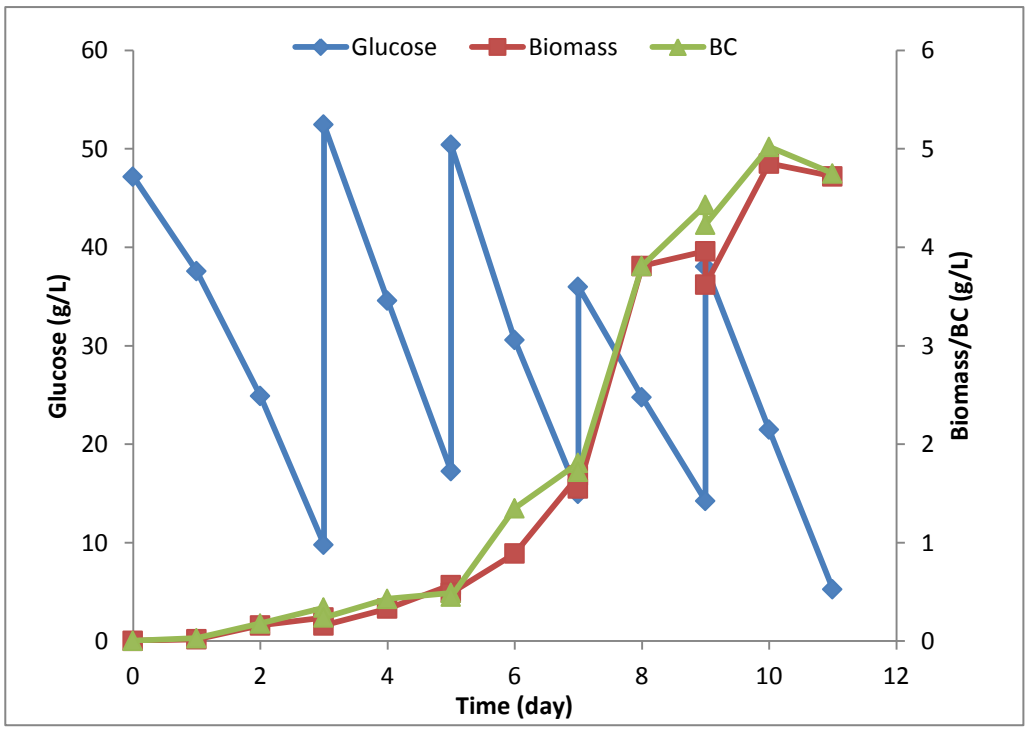

Figure 5.1: Glucose consumption, and biomass and BC profiles when growing *G. xylinus* under a fill-and-draw strategy.

This fill-and-draw strategy resulted in a BC concentration of 5.0 g/L, which is 2.3 times higher than that produced using the batch culture (Figure 4.20) because the fill-and-draw strategy ensures a high inoculum level when fresh media is added, reducing any lag phase. The carbon source is used more quickly and there is higher BC productivity (Table 5.1). Fill-and-draw processing also reduces costs associated with inoculum preparation for batch fermentation.

Table 5.1: Glucose consumption, BC yield and productivity when growing *G. xylinus* on glucose under a fill-and-draw fermentation strategy.

Parameter	Value
Maximum BC concentration (g/L)	5.02
Total glucose consumed (g/L)	323
Total BC formed (g/L)	17.2
BC yield (g/g)	0.05
BC productivity (g/L/day)	0.80

The BC yield under fill-and-draw conditions was 0.05 g/g glucose used and the BC productivity was 0.8 g/L/day (Table 5.1). Although the BC yield for fill-and-draw culture and batch fermentation were similar, BC productivity (0.8 g/L/day) was over three times higher for the fill-and-draw fermentation strategy than in batch culture (0.23 g/L/day).

Improvements in BC productivity by using different feed strategies for fed-batch processes have been reported. Bae and Shoda (2004) used intermittent addition of molasses as the carbon source in a 10-L agitated fermenter and obtained a BC concentration of 7.8 g/L. Hwang *et al.* (1999) doubled BC yield from 0.13 g/g glucose in batch culture to 0.25 g/g for fed-batch cultivation of *G. xylinus* on 40 g/L glucose. They obtained a final BC concentration of 10 g/L.

Apart from eliminating the detrimental effect of substrate and/or product inhibition, changing from batch culture to fed-batch culture reduces the non-productive downtime for cleaning, filling and sterilization, thereby increasing overall reactor productivity and reducing process cost. Also, the fill-and-draw strategy can be used for longer times than a simple batch process provided there is no contamination.

5.1.2 Fed-Batch with Pulse Feed

Fermentation on 50 g/L glucose was initiated as a batch process and then changed to fed-batch process with pulse feed (Section 3.6.2). When glucose concentration in the fermenter fell to about 15 g/L, fresh media with 100 g/L glucose was added. After pulse feeding fresh media on day 3 and day 5, glucose concentration in the fermenter increased to 51.9 g/L and 50 g/L respectively (Figure 5.2). However, a similar increase was not observed after fresh media was added on days 7 and day 9. After the fourth pulse feed, fermentation was changed back to batch mode to ensure all the glucose was consumed.

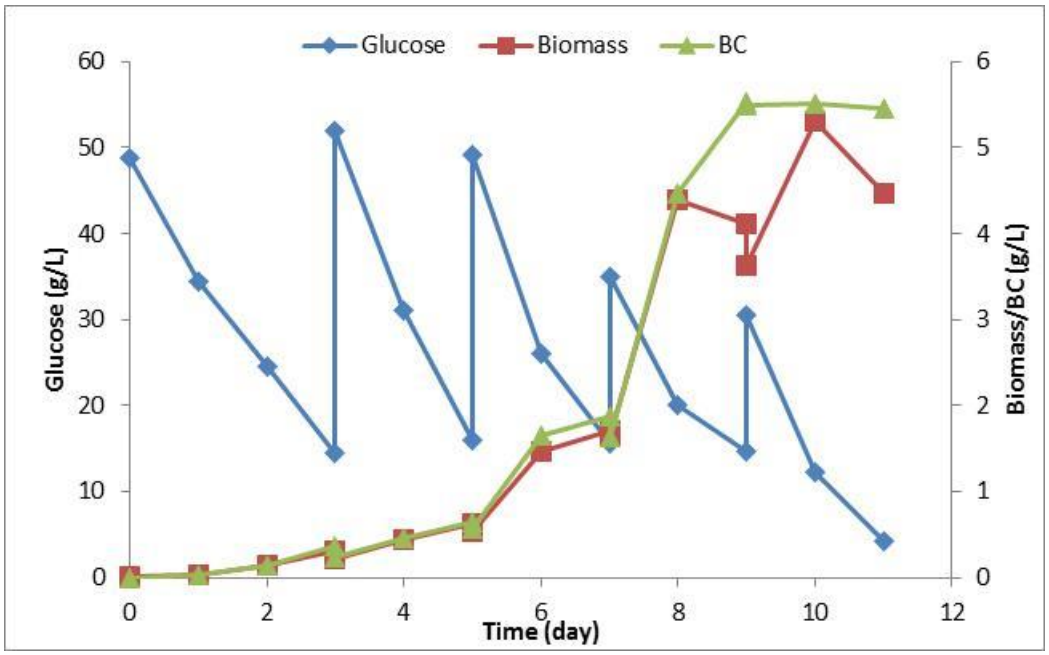

Figure 5.2: Glucose, biomass and BC profiles when growing *G. xylinus* under a fed-batch pulse feed strategy.

The BC concentration in media removed at the end of the first three cycles was 0.4, 0.6, 1.9 and 5.5 g/L respectively. The BC concentration in the pulse feed fed-batch fermentation was 2.5 times greater than in batch culture (2.2 g/L) and also higher than in fill-and-draw culture (Figure 5.1). Residual glucose (4.1 g/L) at the end of pulse feed fed batch fermentation was lower than in the fill-and-draw culture (5.3 g/L). The BC productivity in pulse feed strategy was 0.88 g/L/day with a yield of 0.03 g BC per g glucose used (Table 5.2). The BC yield from batch and from fill-and-draw fermentation strategies were similar but the BC yield using a pulse feed fermentation strategy (0.03 g/g) was lower than either fill-and-draw strategies. However, BC productivity of the pulse feed strategy (0.8 g/L/day) was slightly higher than the fill-and-draw operation (0.78

g/L/day) and over three times higher than that of the batch culture (0.23 g/L/day). These results illustrated that the pulse-feed fed-batch process could be a potential processing strategy for enhancing BC production.

Table 5.2: Glucose consumption, BC yield and productivity obtained in pulse feed fed batch culture.

Parameter	Value
Maximum BC concentration (g/L)	5.5
Total glucose consumed (g/L)	176.8
Total BC formed (g/L)	19.3
BC yield (g/g)	0.03
BC productivity (g/L/day)	0.88

5.1.3 Fed-Batch with Continuous Feed

Continuous Feed at D = 0.1 h^{-1}

The continuous fed-batch culture was started as batch fermentation on 50 g/L glucose and then switched to continuous fed-batch culture (Section 3.6.3). Continuous fed-batch fermentations were done at D= 0.1 h^{-1} (Figure 5.3) and 0.2 h^{-1} (Figure 5.4). When D is 0.1 h^{-1}, BC concentration increased gradually until 72 h and then increased rapidly to 4.2 g/L at 96 days. This BC concentration was almost double that obtained in batch culture (2.2 g/L) and higher than obtained in the fill-and-draw fermentation strategy (Figure 5.1). At the end of the fermentation, residual glucose was 2.8 g/L, which was lower than for the fill-and-draw fed-batch (5.3 g/L) and pulse feed fed-batch (4.1 g/L) strategies, indicating that glucose was quickly consumed. Productivity and BC yield under continuous fed-batch conditions with D of 0.1 h^{-1} were 0.53 g/L/day and 0.03 g BC per g glucose used respectively (Table 5.3).

The BC yields from batch (Table 4.9) and fill-and-draw fed-batch (Table 5.1) strategies were similar but higher (0.11 g/g glucose) under pulse feed fed-batch conditions (Table 5.2). The BC productivity of continuous fed-batch culture when D was 0.1 h^{-1} was over double that of batch culture (0.23 g/L/day), indicating continuous fed-batch operation enhanced BC volumetric productivity. Of the four strategies investigated, pulse feed gave the highest BC yield (0.11 g/g glucose). This yield was nearly four times higher than from continuous fed-batch fermentations at D= 0.1 h^{-1}.

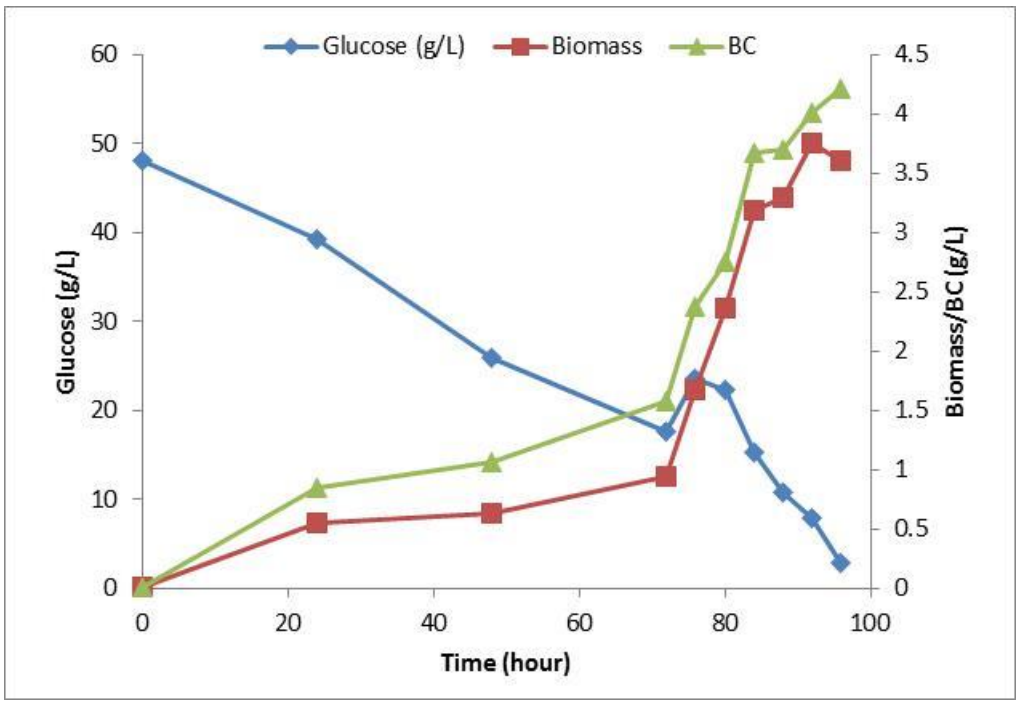

Figure 5.3: Glucose. biomass and BC profiles when growing *G. xylinus* in continuous fed-batch culture at D=0.1 h^{-1}.

Table 5.3: Glucose consumption, and BC yield and productivity when growing *G. xylinus* on 50 g/L glucose in continuous fed-batch at D = 0.1 h^{-1}.

Parameter	Value
Maximum BC concentration (g/L)	4.2
Total glucose consumed (g/L)	145.2
BC yield (g/g)	0.03
BC productivity (g/L/day)	0.53

Continuous Feed at D = 0.2 h^{-1}

On Day 3 of the batch culture, media with 100 g/L glucose was fed into the fermenter at D of 0.2 h^{-1}. The glucose fed was immediately consumed and the residual glucose gradually decreased to 3.1 g/L after 3.3 days (Figure 5.4), which was slightly higher than for D=0.1 h^{-1} (2.8 g/L) and indicates that operating at higher dilution rates may not be desirable. Unfortunately, fermentations were not run at higher dilution rates to ascertain wash-out.

Fed-batch with continuous feed at D of 0.2 h^{-1} produced 3.3 g/L biomass (Figure 5.4), which was slightly lower (3.6 g/L) than obtained for continuous fed-batch culture at D of 0.1 h^{-1} (Figure 5.3).

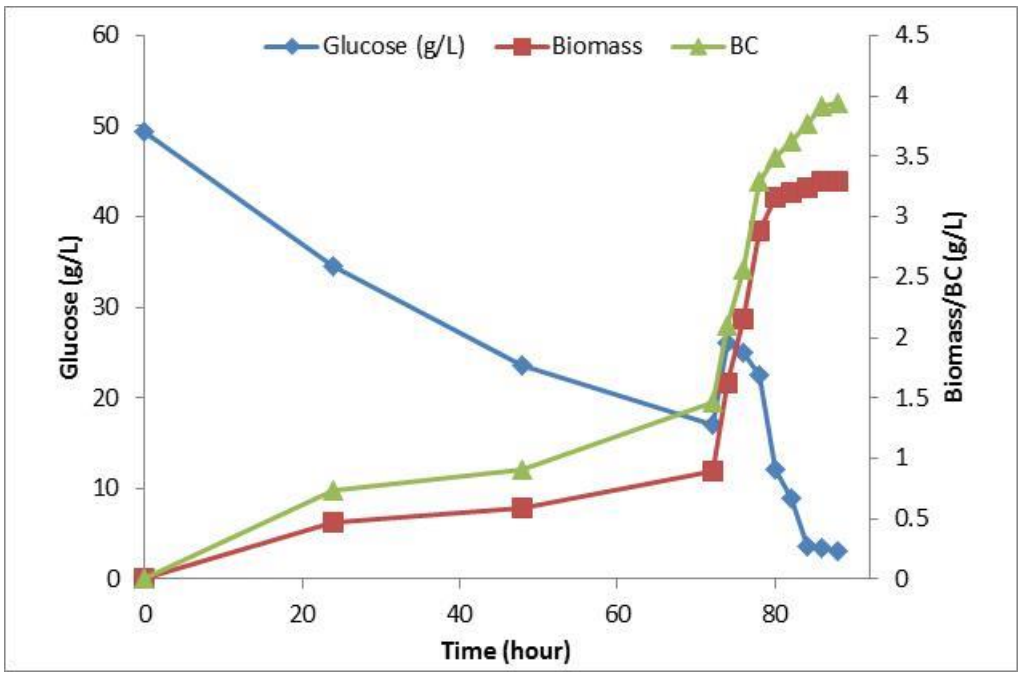

Figure 5.4: Glucose, biomass and BC profiles when growing *G. xylinus* in continuous fed-batch culture at D=0.2 h^{-1}.

At the end of the fermentation, BC was 4.0 g/L, which was higher than produced in batch culture (2.2 g/L). Continuous fed-batch at D of 0.2 h^{-1} gave a BC yield of 0.03 g per g glucose used and a BC productivity of 0.53 g/L/day (Table 5.4). These values are similar to the productivity and yields when D was 0.1 h^{-1}. Although total glucose consumed in continuous fed-batch at D of 0.2 h^{-1} was higher than at D of 0.1 h^{-1}, there was no improvement in BC yield compared with a batch process

Table 5.4: Glucose consumption, BC yield and productivity obtained in the continuous fed-batch culture at D = 0.2 h^{-1}.

Parameter	Value
Maximum BC concentration (g/L)	4.0
Total glucose consumed (g/L)	146.3
BC yield (g/g)	0.03
BC productivity (g/L/day)	0.53

5.2 Continuous Culture Using Glucose as the Carbon Source

The continuous culture was initiated as batch fermentation on 50 g/L glucose then switched to continuous culture at 72 h (Section 3.7). The effect of four different dilution rates (0.05 h^{-1}, 0.1 h^{-1}, 0.15 h^{-1} and 0.2 h^{-1}) on glucose

consumption, and biomass and BC production were investigated. The results were compared with those from the batch and fed-batch processes.

The profile of continuous culture with media containing 100 g/L glucose and D of 0.05 h^{-1} shows the culture reached steady state after about 105 h (Figure 5.5). These conditions were maintained for at least three days without any contamination. Continuous culture at D of 0.05 h^{-1} produced 5.6 g/L biomass with a biomass productivity if 0.3 g/L/h, which equals to 0.01 g/L/day. Biomass or BC productivity rate during continuous fermentation was defined as the biomass/BC concentration at a steady state multiplied by the dilution rate.

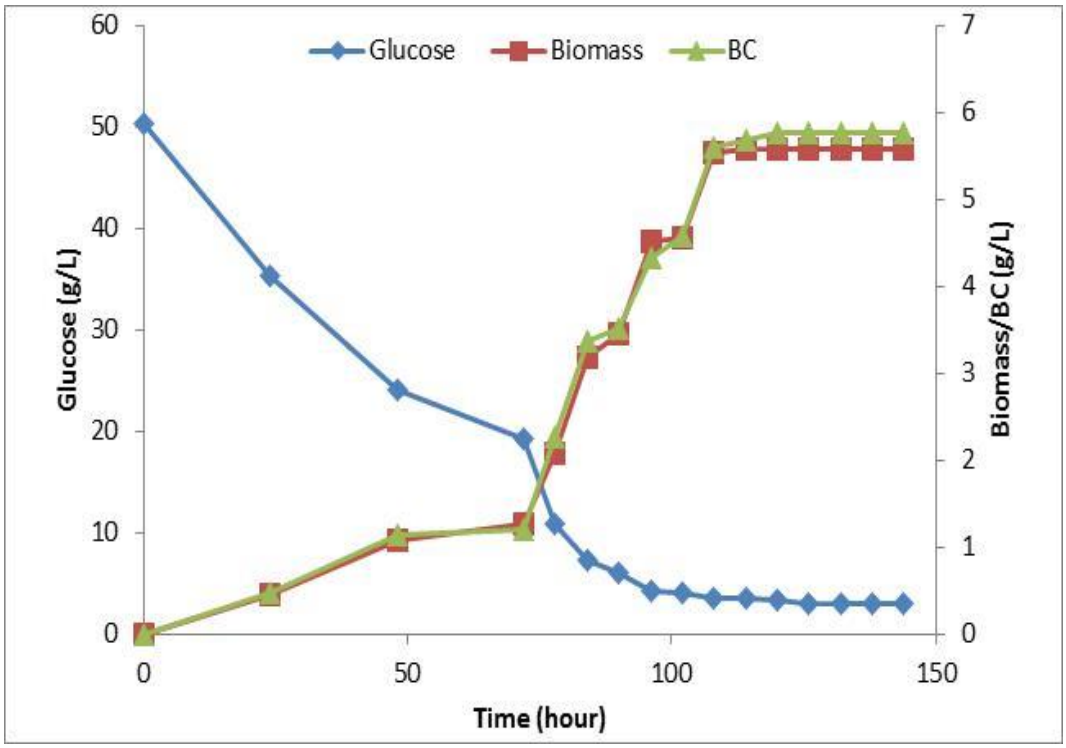

Figure 5.5: Glucose, biomass and BC concentration when growing *G. xylinus* in continuous culture at D=0.05 h^{-1}.

When dilution rate was increased to 0.1 h^{-1}, steady state was achieved after 120 h (Figure 5.6). The steady state biomass concentration for this dilution rate was 5.6 g/L, which corresponded to a biomass productivity of 0.55 g/L/h (0.02 g/L/day) and a BC yield of 0.13 g/g glucose used. Although BC yield at D of 0.1 h^{-1} of 0.13 g/g glucose (Figure 5.6) used was similar to the yield at D of 0.05 h^{-1}, BC productivity had doubled. When the dilution rate was further increased to D of 0.15 h^{-1}, steady state was reached after 88 h (Figure 5.7) and the steady state biomass was 5.9 g/L. The BC productivity increased to 0.63 g/L/h (0.03 g/L/day) but BC yield obtained was 0.09 g/g glucose used.

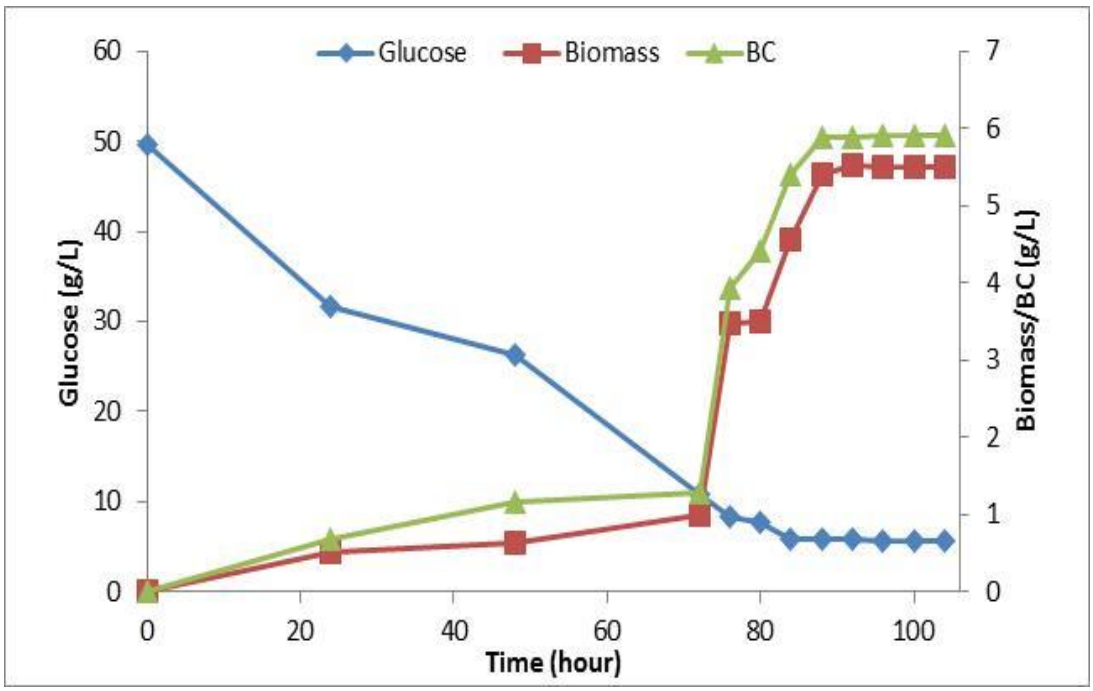

Figure 5.6: Glucose, biomass and BC profiles when growing *G. xylinus* in continuous culture at D=0.1 h^{-1}.

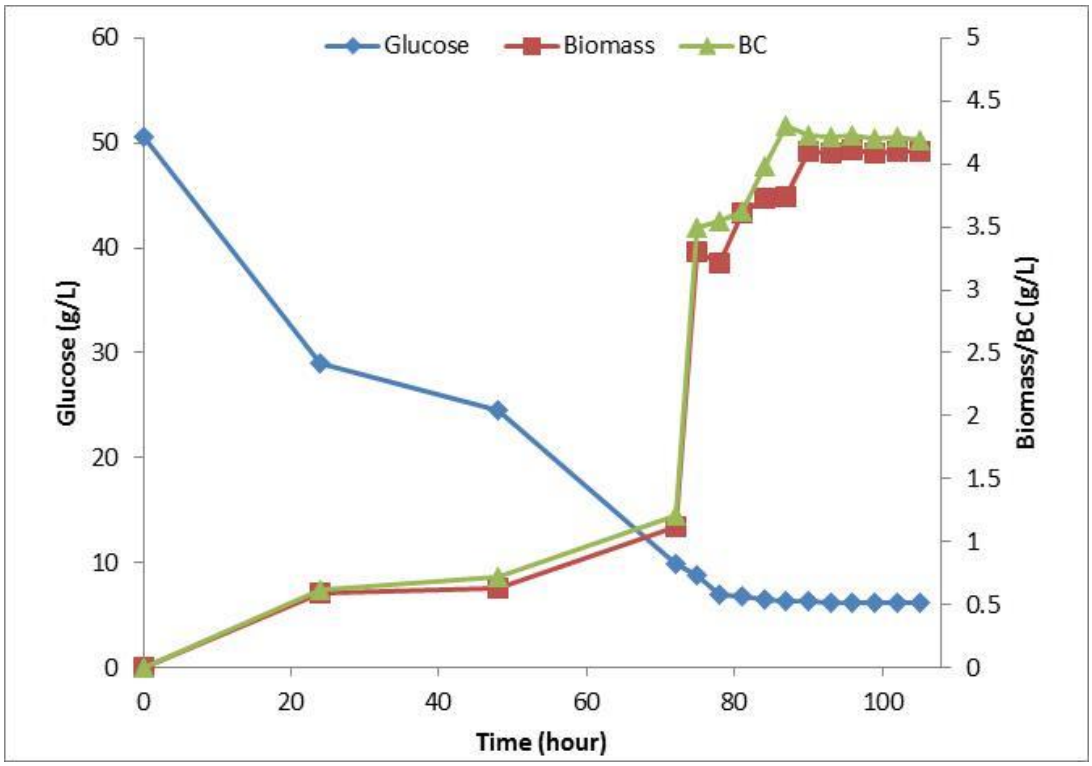

Figure 5.7: Glucose, biomass and BC profiles when growing *G. xylinus* in continuous culture at D=0.15 h^{-1}.

The BC productivity further increased to 0.7 g/L/h (or 0.03 g/L/day) when dilution rate increased to 0.2 h^{-1} but there was no significant change in BC yield (0.09 g/g glucose used) (Figure 5.8).

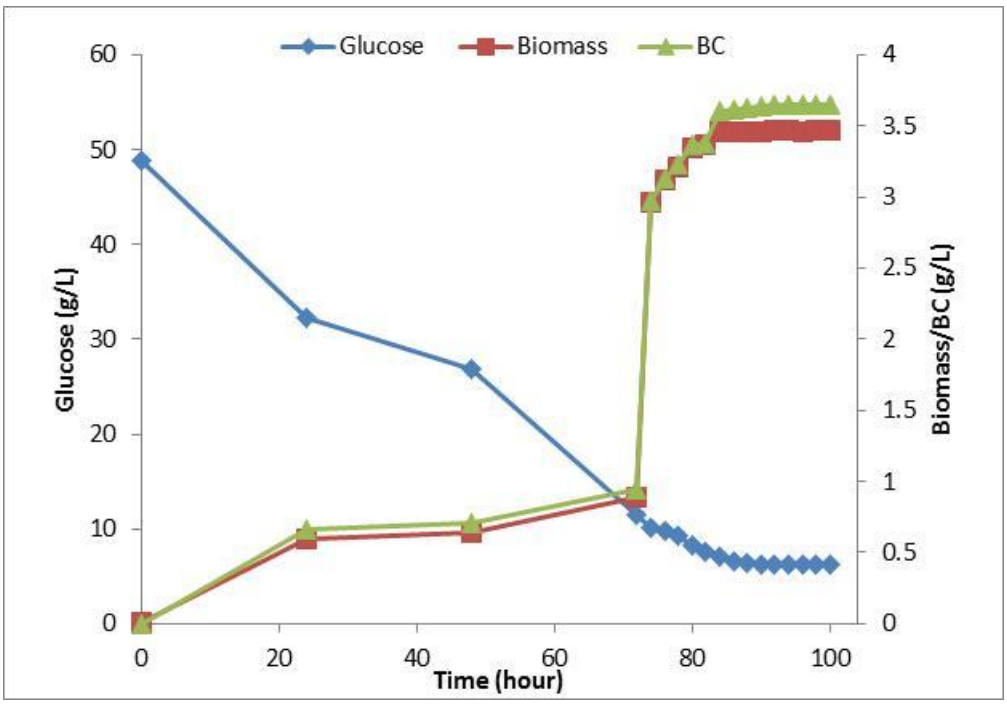

Figure 5.8: Glucose, biomass and BC profiles when growing *G. xylinus* in continuous culture at D=0.2 h^{-1}.

The effect of dilution rate on BC productivity and BC yield are summarised in Figure 5.9 and Figure 5.10 respectively. Doubling the dilution rate from 0.05 to 0.1 h^{-1} significantly increased BC productivity and there were continued to increase as dilution rate increased to 0.2 h^{-1}. The highest BC yield (0.13 g/g) was obtained at a D of 0.1 h^{-1} (Figure 5.10) and then it dropped significantly.

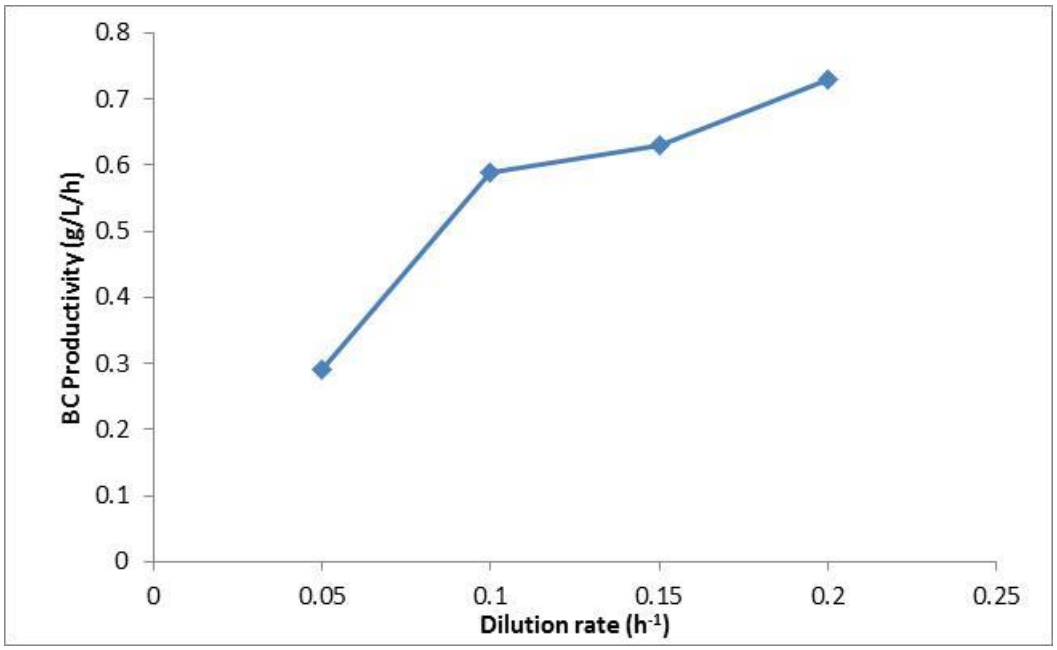

Figure 5.9: Effect of dilution rate on BC productivity when growing *G. xylinus* on 50 g/L glucose.

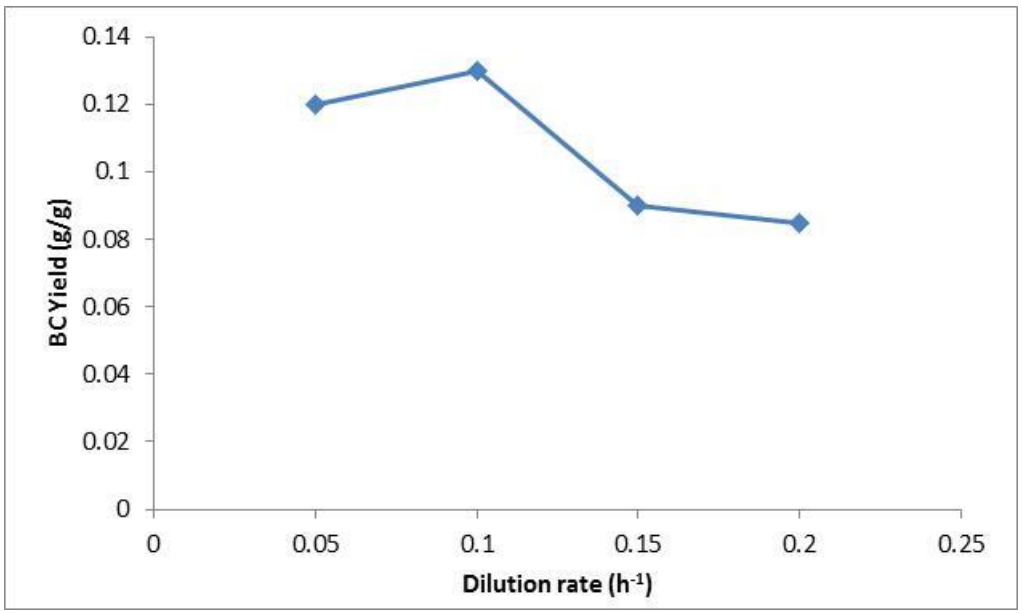

Figure 5.10: Effect of dilution rate on BC yield when growing *G. xylinus* on 50 g/L glucose in continuous culture.

The yield in continuous processes were higher than in batch culture indicating that continuous operation has potential for producing BC more quickly. However, BC yield decreased with increasing dilution rate and there was higher residual glucose. Therefore, glucose is wasted and BC collection, concentration and purifications costs would increase. Although operating at a low dilution rate or with low glucose concentrations in the feed allows complete consumption of glucose, BC productivity drops substantially.

Increasing cell concentration under steady state conditions is one way to increase glucose consumption and BC yields because BC concentration by *G. xylinus* is generally associated with cell growth (Marx-Figini and Pion, 1974; Naritomi *et al.*, 1998). A strategy that will help completely consume glucose is cell recycle or multistage culture. However, these processes were not investigated because it was considered that separating cells from the BC in the media or controlling the feed rate would be too difficult and/or complex (Naritomi *et al.*, 1998).

The conclusion from the trials is that dilution rate affects BC productivity and BC yield. The highest BC yield was obtained at a dilution rate (D) of 0.1 h^{-1}. Although increasing the dilution rate increased BC productivity, the BC concentration decreased.

5.3 Fed-Batch Using Glycerol as the Carbon Source

5.3.1 Fill-and-Draw Operation

The fermentation was initiated in batch mode with 20 g/L glycerol and then switched to fill-and-draw operation (Section 3.6.1). Four cycles of media withdrawal and fresh media addition were done when glycerol concentration fell to approximately 5 g/L (Figure 5.11). The fermentation was continued in batch mode after the fourth cycle to allow all the glycerol to be consumed.

The BC concentrations at the end of the four cycles were 1.09 g/L, 4.15 g/L, 6.34 g/L and 6.36 g/L (Figure 5.11). The fill-and-draw strategy produced nearly three times the BC concentration of batch culture (Figure 4.20).

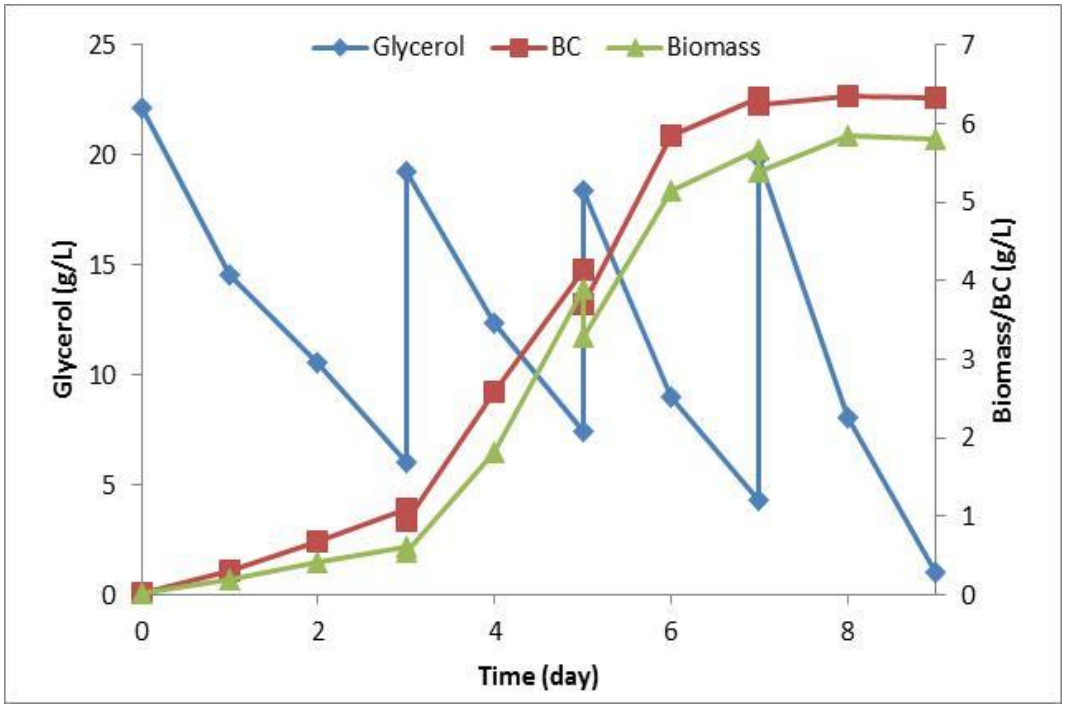

Figure 5.11: Glycerol, biomass and BC profiles when growing *G. xylinus* under a fill-and-draw strategy.

In comparison with fill-and-draw using glucose as the carbon source, growth on glycerol produced more 1.2 times as much biomass (5.8 g/L). Fill-and-draw fermentation conditions gave a BC productivity of 1.35 g/L/day and a BC yield of 0.2 g per g glycerol used (Table 5.5). This compares with fill-and-draw culture on glucose of a BC yield of 0.05 g/g glucose and a BC productivity of 0.78 g/L/day. In summary, growth on glycerol as the carbon source increased BC productivity by resulted in 1.73 and quadrupled the BC yield.

Table 5.5: Glycerol consumption, BC yield and BC productivity in fill-and-draw culture.

Parameter	Value
Maximum BC concentration (g/L)	6.4
Total glycerol consumed (g/L)	121.5
Total BC formed (g/L)	24.2
BC yield (g/g)	0.2
BC productivity (g/L/day)	1.35

5.3.2 Fed-Batch With Pulse Feed

The fermentation was initiated in batch mode with 20 g/L glycerol and then switched to pulse feed (Section 3.6.2). Fresh media (250 mL at each pulse) was added when glycerol concentration in the fermenter had decreased to about 5 g/L. Biomass concentration at the end of fermentation was 5.9 g/L, (Figure 5.12), which was higher than for fill-and-draw culture. Maximum BC concentration under pulse feed fed-batch culture was 6.4 g/L, which was nearly three times that produced using batch culture (Figure 4.20) and slightly higher than fill-and-draw operation (Figure 5.11). However, this operation did not improve BC yield, which was 0.10 g/g compared to 0.15 g/g for batch and 0.2 g/g for fill-and-draw. Almost all the added glycerol was consumed under both fill-and-draw (Figure 5.11) and pulse feed (Figure 5.12) fermentation mode.

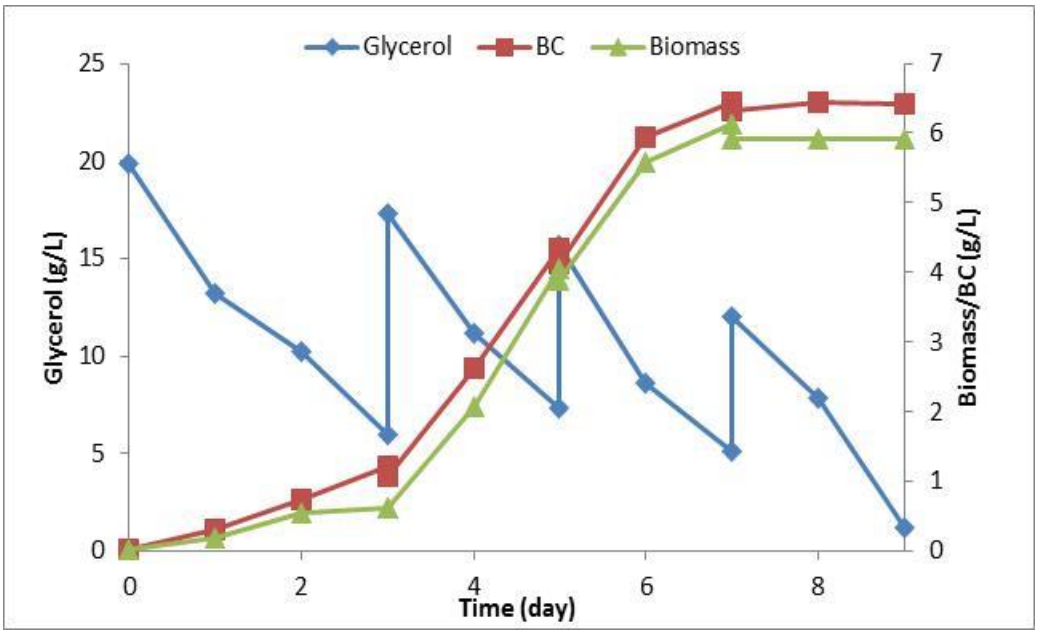

Figure 5.12: Glycerol, biomass and BC profiles when growing *G. xylinus* under a fed-batch pulse feed strategy.

The BC productivity in pulse feed fed-batch mode was 1.4 g/L/day and the BC yield was 0.54 g per g glycerol used (Table 5.6). This was higher than the values obtained on glucose, which produced a BC yield of 0.11 g/g and a BC productivity of 0.88 g/L/day (Table 5.2). The data demonstrated that using glycerol as the carbon source enhanced almost doubled BC productivity and almost quadrupled BC yield. Compared with a batch process on glucose, BC productivity increased nearly 500% and BC yield increased by over 250%.

Table 5.6: Glycerol consumption, BC yield and productivity in pulse feed fed-batch using glycerol as the carbon source.

Parameter	Value
Maximum BC concentration (g/L)	6.44
Total glycerol consumed (g/L)	63.38
Total BC formed (g/L)	24.38
BC yield (g/g)	0.10
BC productivity (g/L/day)	1.38

5.3.3 Fed-Batch with Continuous Feed

Continuous Feed at D = 0.1 h^{-1}

The culture was started in batch mode using 20 g/L glycerol and then switched to continuous fed-batch culture (Section 3.6.3) on day 3. Media containing glycerol was fed at dilution rates of 0.1 h^{-1} (Figure 5.13) or 0.2 h^{-1} (Figure 5.14). One litre of media containing 100 g/L glycerol was then fed at the required dilution rate for four days. The cultivation was terminated on day 4 when the reactor had filled to its working volume.

When the dilution rate was 0.1 h^{-1}, BC concentration increased with glycerol addition and reached 5.10 g/L after four days cultivation (Figure 5.13). The BC concentration for continuous fed-batch at D of 0.1 $h^{-1}$was higher for than produced in batch culture (2.2 g/L). Residual glycerol at the end of the process was 2.05 g/L, which was higher than for both fill-and-draw and pulse feed processes. This is reflected in the BC concentration, which was lower than for fill-and-draw and the pulse feed fed (5.10 g/L) processes.

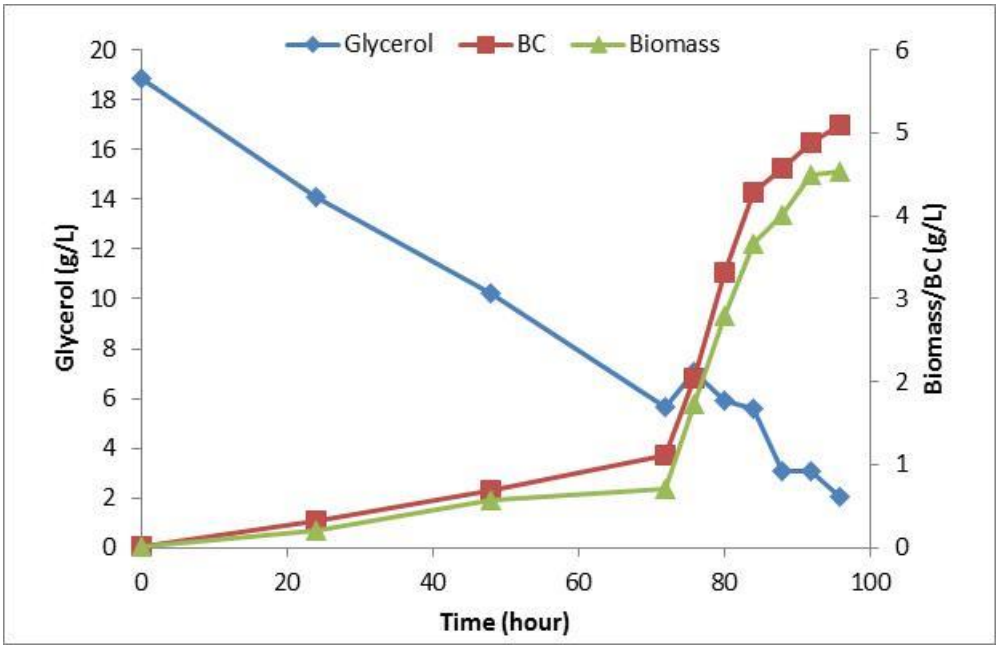

Figure 5.13: Glycerol, biomass and BC profiles when growing *G. xylinus* in continuous fed-batch culture at D = 0.1 h^{-1}.

The BC productivity (0.64 g/L/day) and BC yield (0.04 g per g glycerol used) achieved for continuous fed-batch at D of 0.1 h^{-1} is higher (Table 5.7) than obtained on glucose media, which produced a BC yield of 03 g/g and BC productivity of 0.53 g/L/day. This indicated that using glycerol as the carbon source enhanced BC productivity by 20% and BC yield by 30%. Compared to batch operation, BC productivity in continuous fed-batch operation more than doubled. However, BC yield for fed batch with continuous feed was lower obtained in batch culture (0.15 g/g).

Table 5.7: Glycerol consumption, BC yield and BC productivity for a continuous fed-batch process at D = 0.1 h^{-1}.

Parameter	Value
Maximum BC concentration (g/L)	5.10
Total glycerol consumed (g/L)	116.8
BC formed (g/L)	5.10
BC yield (g/g)	0.04
BC productivity (g/L/day)	0.64

Continuous Feed at D = 0.2 h^{-1}

The BC and biomass concentration at the end of continuous fermentation at a D of 0.2 h^{-1} were 3.7 g/L and 3.4 g/L respectively (Figure 5.14). The

residual glycerol (5.7 g/L) in the fermenter was higher (2.0 g/L) than for continuous fed-batch at D of 0.1 h^{-1} (Table 5.8), indicating that the dilution rate was above the optimum level for effective operation.

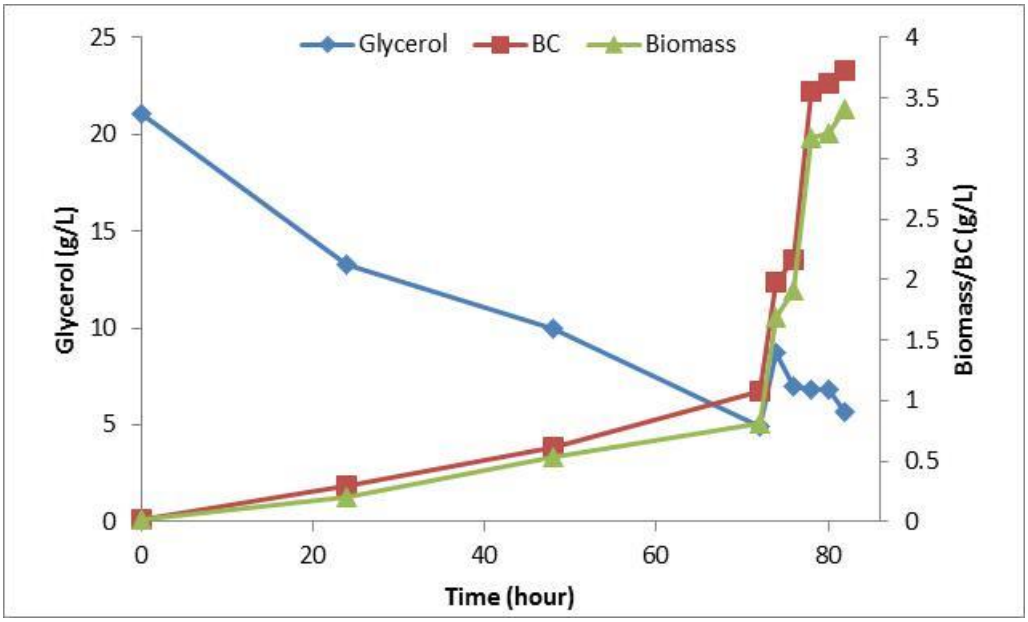

Figure 5.14: Glycerol, biomass and BC profiles when growing *G. xylinus* in continuous fed-batch culture at D = 0.2 h^{-1}.

The BC concentration (3.7 g/L) in continuous fed-batch operation at D of 0.2 h^{-1} was higher than produced in batch culture (2.2 g/L). Compared with both fill-and-draw and pulse feed fed-batch processes, the BC concentration for continuous fed-batch at D = 0.2 h^{-1} was lower (3.7 g/L). Glycerol consumption, BC yield and productivity are summarised in Table 5.8.

Table 5.8: Glycerol consumption, BC productivity and yield for a continuous fed-batch process at D = 0.2 h^{-1}.

Parameter	Value
Maximum BC concentration (g/L)	3.7
Total glycerol consumed (g/L)	115.4
BC formed (g/L)	3.7
BC yield (g/g)	0.03
BC productivity (g/L/day)	0.55

Although BC productivity (0.55 g/L/day) and BC yield (0.03 g/g for continuous fed-batch process at D = 0.2 h^{-1}were lower than when operating at a dilution rate of 0.1 h^{-1}, BC productivity was higher than for batch culture (0.3

g/L/day) on glycerol as the carbon source. Bae and Shoda (2004) reported BC concentration of 7.2 g/L of BC in fed-batch fermentations with continuous feeding of molasses that had been subjected to H_2SO_4-heat treatment, which was higher than the 5.10 g/L obtained on glycerol at D=0.1 h^{-1} (Table 5.7).

The results indicate glycerol can be used in fed-batch cultures with continuous feeding to obtain higher BC concentration and productivity. According to www.Alibaba.com (2014), crude glycerol costs USD 0.78 per kg and anhydrous glucose powder price costs USD 0.40 per kg. Using glycerol as the carbon source may increase the economic viability of BC production over other conventional purified carbon sources.

5.4 Continuous Culture with Glycerol as the Carbon Source

The fermentation was initiated in batch mode using media with 20 g/L glycerol and then switched to continuous culture with dilution rates of 0.05 h^{-1}, 0.1 h^{-1}, 0.15 h^{-1} or 0.2 h^{-1}after 72 h (Section 3.7.2).

When the dilution rate was 0.05 h^{-1}, steady state was reached within 12 h (Figure 5.15). This was then maintained at least for three days without any contamination. The BC yield (0.3 g/g) was twice as high as that obtained in batch culture on glycerol media.

When the dilution rate was increased to 0.1 h^{-1}, about 5.7 g/L of biomass was obtained at steady state and residual glycerol concentration was about 1.4 g/L (Figure 5.16). The BC productivity was 0.6 g/L/h (0.03 g/L/day) and the BC yield was 0.33 g/g glycerol used. These were both higher than obtained at the lower dilution rate of 0.05 h^{-1}. When the dilution rate was 0.15 h^{-1}, the culture reached steady state after 90 h and this was maintained until 112 h (Figure 5.17). The steady state biomass concentration was 5.1 g/L and the BC concentration was 5.13 g/L. This gave a BC productivity of 0.77 g/L/h (0.03 g/L/day) and a BC yield of 0.30 g/g. The BC productivity increased with dilution rate but BC yield did not. When the dilution rate was increased to 0.2 h^{-1}, the culture reached steady state after about 88 h and this was maintained until 100 h (Figure 5.18). The BC (3.8 g/L) and biomass (3.5 g/L) concentration at steady state were lower than at the lower dilution rates investigated and the residual glycerol was higher (5.5 g/L), indicating that feeding at higher glycerol concentrations inhibited growth. The BC productivity and yield were 0.76 g/L/h (0.03 g/L/day) and 0.33 g/g glycerol consumed respectively.

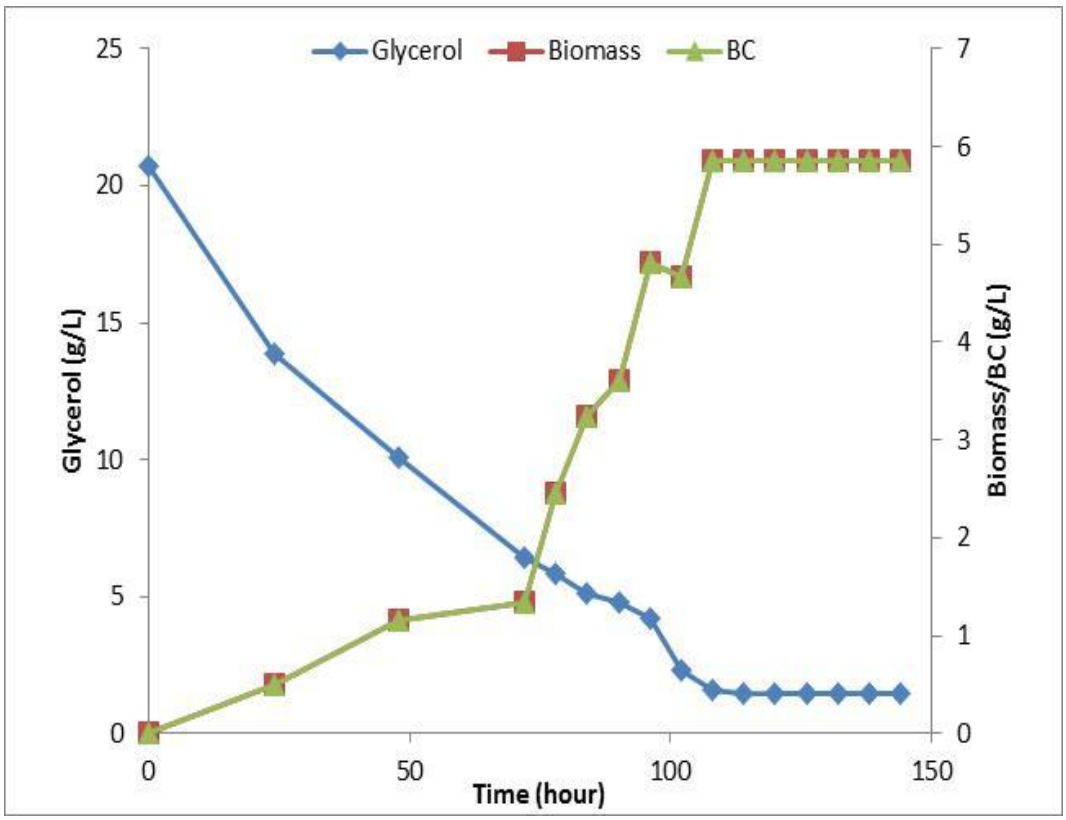

Figure 5.15: Glycerol, biomass and BC profiles when growing *G. xylinus* in continuous culture at D = 0.05 h^{-1}.

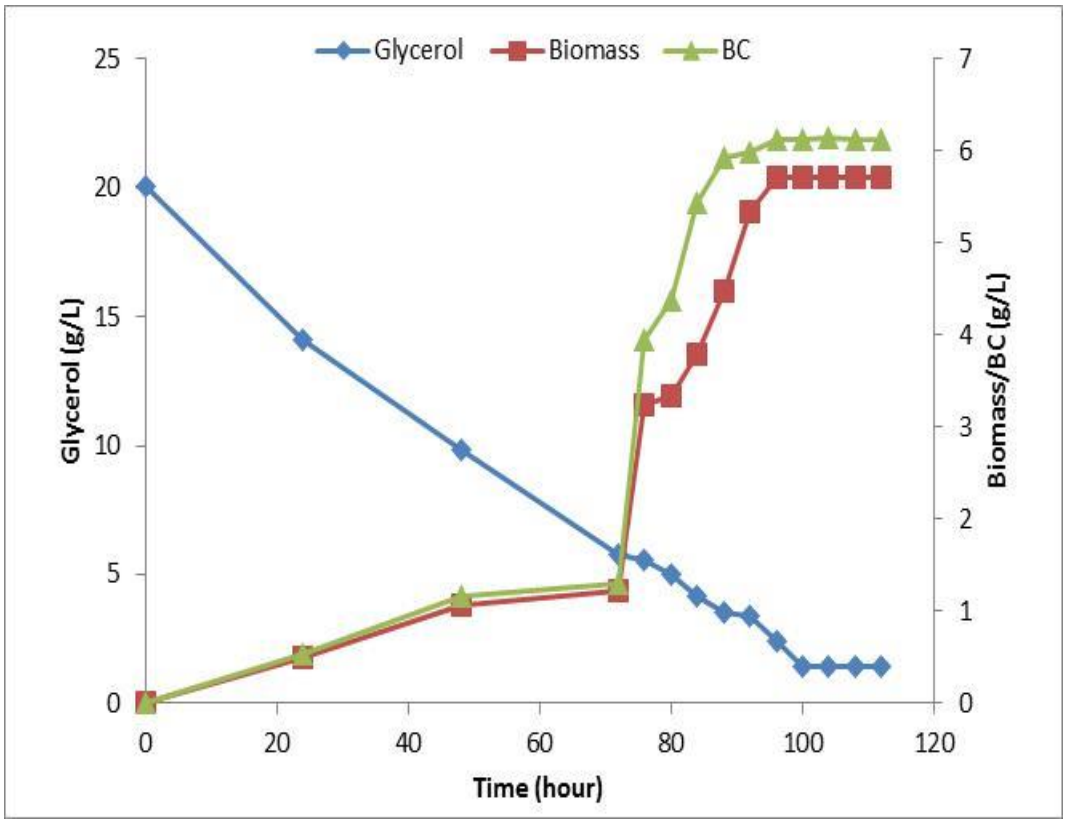

Figure 5.16: Glycerol, biomass and BC profiles when growing *G. xylinus* in continuous culture at D = 0.1 h^{-1}.

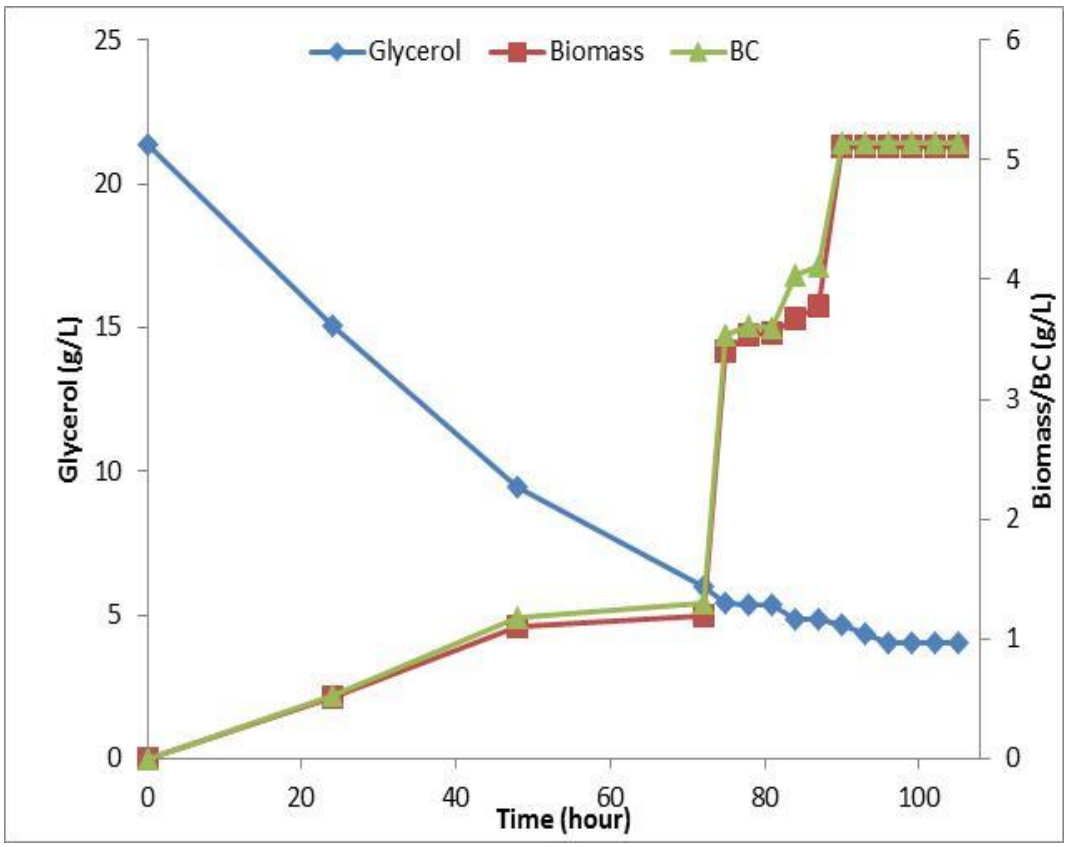

Figure 5.17: Glycerol, biomass and BC concentration when *G. xylinus* is grown in continuous culture at D = 0.15 h^{-1}.

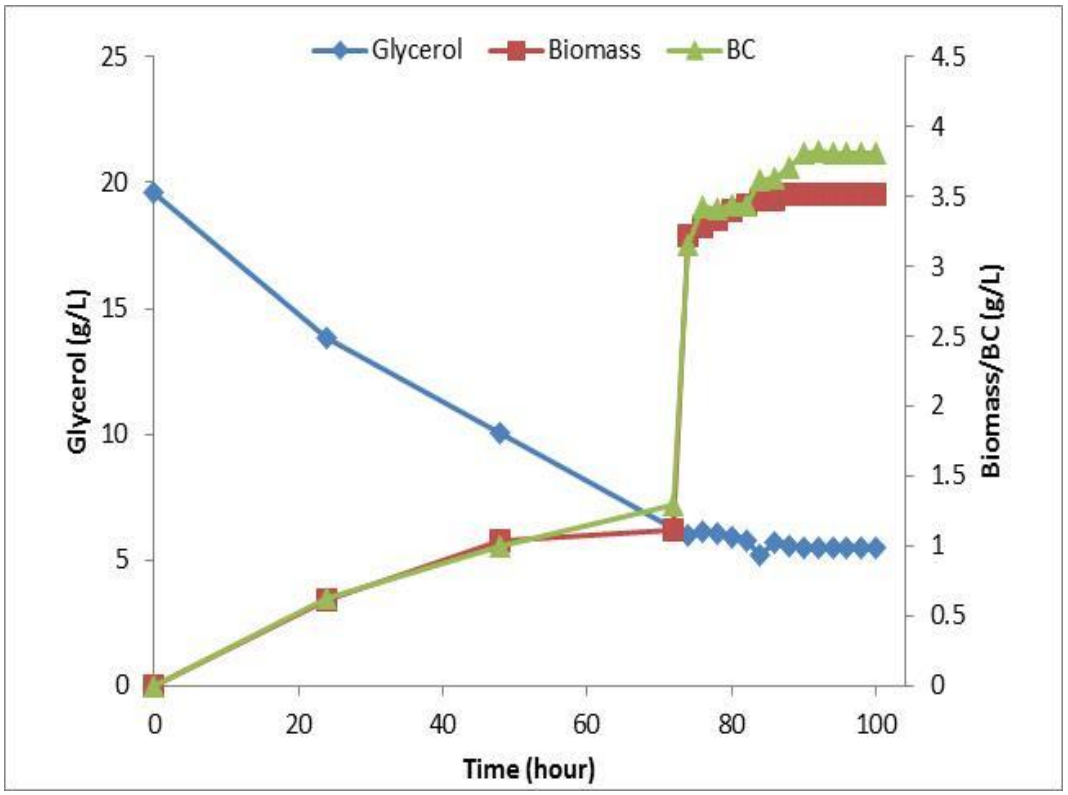

Figure 5.18: Glycerol, biomass and BC profiles when growing *G. xylinus* in continuous culture at D = 0.2 h^{-1}.

The effect of dilution rates on BC productivity and BC yield are shown in Figure 5.19 and Figure 5.20. The highest BC productivity (0.77 g/L/h) was obtained at a dilution rate of 0.15 h^{-1}. It then decreased slightly. The highest BC yield (0.33 g/g) was obtained at a dilution rate of 0.1 h^{-1}. It then decreased.

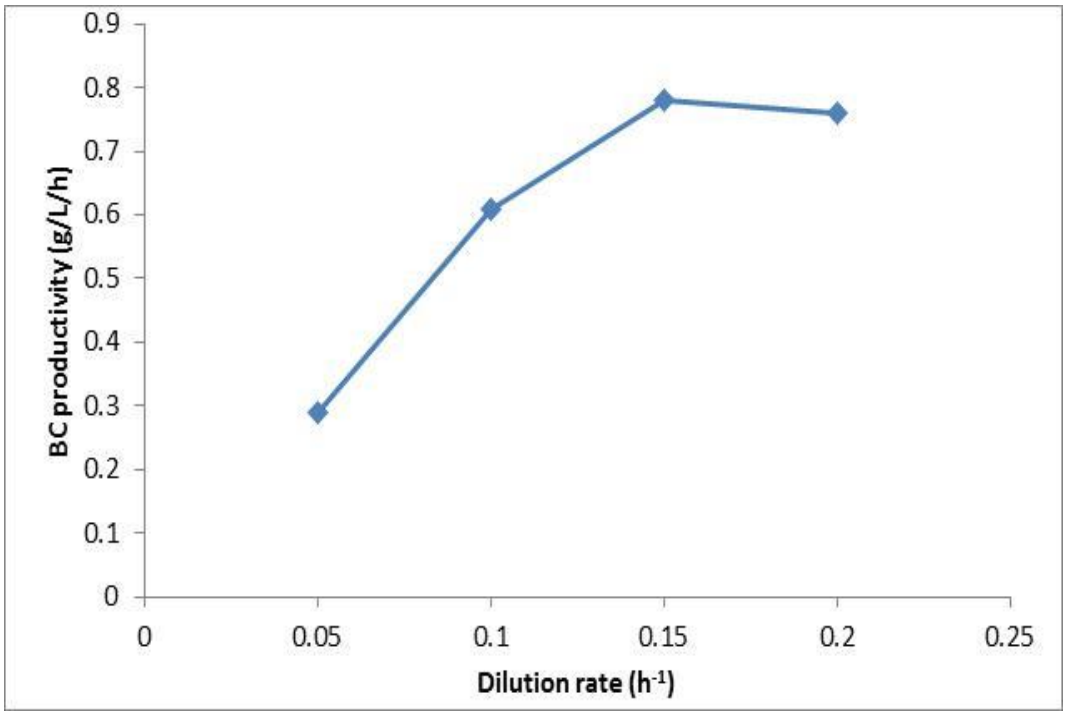

Figure 5.19: Effect of dilution rate on BC productivity when growing *G. xylinus* on glycerol.

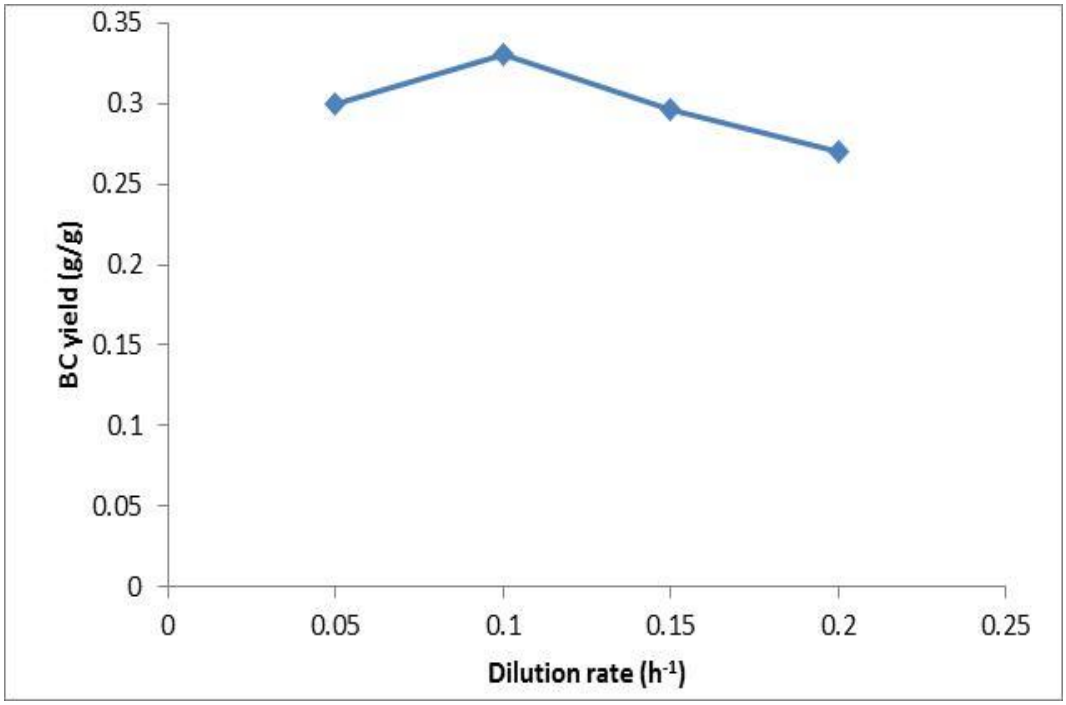

Figure 5.20: Effect of dilution rate on BC yield when growing *G. xylinus* on glycerol.

Dilution rate influenced BC productivity and BC yield in continuous culture on glycerol as the carbon source (Table 5.9). However, BC productivity and BC yield under continuous conditions were not higher than for batch culture on glycerol. For a cost effective continuous process, it is important to select the dilution rate/feeding rate that maximises product formation.

Table 5.9: Effect of dilution rate on BC productivity and BC yield in continuous culture using glycerol as the carbon source.

Dilution rate (h^{-1})	BC productivity (g/L/h)	BC yield (g/g)
0.05	0.29	0.3
0.1	0.61	0.33
0.15	0.77	0.3
0.2	0.76	0.27

Naritomi *et al.* (1998) reported that adding lactate can improve substrate consumption and hence cell growth and BC production. This suggests it is worth investigating the effect of adding additives to the feed media.

5.5 Summary of the Effect of Carbon Source and Bioreactor Operating Mode on BC Production

Changing from batch to fed-batch with pulse feed increased BC yield and productivity from 0.05 g/g and 0.23 g/L/day respectively for batch culture on glucose to 0.11 g/g and 0.88 g/L/day (Table 5.10). The BC yield in continuous fermentation on glucose was higher than for batch operation. Fill-and-draw fed-batch processes had similar BC yields to batch (0.05 g/g), but BC yields for continuous fed-batch slightly lower (0.03 g/g) than for batch. Overall, the fed-batch and continuous operations on glucose gave higher glucose consumption, cell growth, BC concentrations and BC yield.

Although glucose is the main carbon used for producing BC, glycerol can be also used. The BC productivity and yield on glycerol can improve under some operation modes (Table 5.10). For instance, three times as much BC was produced for batch growth on glycerol (0.15 g/g) than on glucose (0.05 g/g). The BC productivity also improved from 0.23 g/L/day to 0.29 g/L/day.

Table 5.10: BC productivity and BC yield from batch, fed-batch and continuous fermenter operation using glucose and glycerol as the carbon source.

Bioreactor operation mode	Parameter			
	BC yield (g/g)		BC productivity (g/L/day or g/L/h)	
	Glucose	Glycerol	Glucose	Glycerol
Batch	0.05	0.15	0.23 per day	0.29 per day
Fed-batch				
Fill-and-draw	0.05	0.2	0.78 per day	1.35 per day
Pulse feed (variable volume)	0.03	0.10	0.88 per day	1.38 per day
Continuous feed (variable volume)				
$D = 0.1\ h^{-1}$	0.03	0.04	0.53 per day	0.63 per day
$D = 0.2\ h^{-1}$	0.03	0.03	0.53per day	0.55 per day
Continuous				
$D = 0.05\ h^{-1}$	0.12	0.3	0.29 per hour	0.29 per hour
$D = 0.1\ h^{-1}$	0.13	0.33	0.59 per hour	0.61 per hour
$D = 0.15\ h^{-1}$	0.09	0.3	0.63 per hour	0.77 per hour
$D = 0.2\ h^{-1}$	0.09	0.27	0.73 per hour	0.76 per hour

Fed-batch operation on glycerol gave encouraging results with BC productivity nearly five times higher than that of batch operation (0.28 g/L/day). Compared to fill-and-draw operation on glucose, using glycerol almost doubled BC production rate. Pulse feed fed-batch operation on glycerol also increased BC productivity from 0.88 g/L/day on glucose to 1.4 g/L/day on glycerol. Continuous fed-batch operation on glycerol slightly improved BC productivity and BC yield compared with growth on glucose media.

Dilution rates also influenced BC productivity and BC yield for growth on glucose or glycerol media. The BC productivity and BC yield on glucose and glycerol were similar. This suggests that glycerol has the potential for improving BC production. Since glycerol can be used as a carbon source in BC fermentation, this high potential by-product adds value to the productive chain of the biodiesel industry, contributing to their competitiveness.

It was observed that BC production was closely associated with cell growth, which is demonstrated by the overlapping lines of BC concentration and biomass. Tsuchida and Yoshinaga (1997) investigated the relationship between BC production and cell mass and found that they are growth associated. Thus, enhancing cell growth could enhance BC productivity by *G. xylinus*. These results are increase the knowledge on BC synthesis in all processing strategies.

In conclusion, BC was produced under all processing strategies investigated but fed-batch and continuous operation on glycerol showed better potential for industrial BC production.

5.6 Overall Summary

Using different processing strategies affected cell formation, substrate consumption, and product formation. Using glucose and glycerol as the carbon source produced different substrate consumption and BC production profiles (Table 5.11). In a continuous operation at steady state, the specific growth rate equals dilution rate. This means specific growth rates could be maintained at 0.05 per hour, 0.1 per hour, 0.15 per hour and 0.2 per hour by feeding the substrates at the same rates. Overall, fed-batch and continuous operation improved BC production as did using glycerol rather than glucose as the carbon source.

Table 5.11: Summary effect of fermenter operating mode on BC concentration and BC yield.

Operation mode	Substrate	Conc (g/L)	Nitrogen source	Duration (days)	BC concentration (g/L)	BC yield (g/g)
Batch						
Agitated shake flask	Glucose	50	Yeast extract	5	0.25	0.04
	Glucose	50	Fish hydrolysate	5	0.24	0.04
	Glucose	50	Fish powder	5	0.25	0.04
	Glycerol	50	Yeast extract	5	1.43	not investigated
	Banana peel extract + Glucose	10 + 40	Yeast extract	5	0.43	not investigated
5-L Bioreactor	Glucose (control)	50	Yeast extract	5	2.1	0.01
	Glucose	50	Fish hydrolysate	5	0.5	0.01
	Glucose	50	Fish powder	5	0.47	0.01
	Glucose (control)	50	Yeast extract		2.2	0.05
	Glycerol (control)	20	Yeast extract	5	2.87	0.15
	Banana peel extract + Glucose	10 + 40	Yeast extract	5	1.1	0.03
Fed-batch						
Fill-and-draw	Glucose	50	Yeast extract	11	5.02	0.05
Pulse feed	Glucose	50	Yeast extract	11	5.5	0.03

(Table 5.11 cont.)

Operation mode	Substrate	Conc (g/L)	Nitrogen source	Duration (days)	BC concentration (g/L)	BC yield (g/g)
Continuous feed						
D=0.1 per hour	Glucose	50	Yeast extract	4	4.2	0.03
D=0.2 per hour	Glucose	50	Yeast extract	3.6	4	0.03
Fill-and-draw	Glycerol	20	Yeast extract	9	6.4	0.2
Pulse feed	Glycerol	20	Yeast extract	9	6.44	0.10
Continuous feed						
D=0.1 per hour	Glycerol	20	Yeast extract	4	5.1	0.04
D=0.2 per hour	Glycerol	20	Yeast extract	3.4	3.7	0.03
Continuous						
D = 0.05	Glucose	50	Yeast extract	6	5.76	0.13
D = 0.1	Glucose	50	Yeast extract	4.3	5.9	0.13
D = 0.15	Glucose	50	Yeast extract	4.4	4.2	0.09
D = 0.2	Glucose	50	Yeast extract	4.2	3.65	0.09
D = 0.05	Glycerol	20	Yeast extract	6	5.86	0.3
D = 0.1	Glycerol	20	Yeast extract	4.7	6.14	0.33
D = 0.15	Glycerol	20	Yeast extract	4.4	5.13	0.3
D = 0.2	Glycerol	20	Yeast extract	5.5	3.8	0.27

Chapter 6: Conclusions and Recommendations

6.1 Conclusions

Conditions to produce bacterial cellulose (BC) using the Gram negative bacterium *Gluconacetobacter xylinus* DSM46604 were investigated. Trials were done in flasks and in fermenters to investigate the effect of carbon source, nitrogen source, aeration and agitation. Various processing regimes such as batch, fed-batch and continuous fermentations were done in conventional 3- or 5-L fermenters.

Initial trials showed that the culture could be kept on GYM agar slopes for up to 2 months, but sub culturing was best done on a two-weekly basis or if there is any changes in agar colour.

Culture conditions and fermentation time affected BC production and morphology of the BC microfibrils. From SEM micrographs obtained, the BC pellicle produced from static culture shows a dense mat of BC fibrils ribbons, while BC pellicles from agitated culture shows entangled fibres of cellulose

Gluconoaceterbacter xylinus DSM46604 grew and produced BC on glucose in solid agar (slants) and in shake flask but did not grow on lactose and sucrose. Investigations using (40-100 g/L) glucose showed the highest BC concentration was obtained on 50 g/L glucose.

Nitrogen in the media can be supplied via inorganic sources such as ammonium sulphate or organic sources such as peptones, hydrolysates and yeast extract. The organic sources often contain unidentified growth factors but are more expensive than inorganic nitrogen sources. Trials using 0.2 %, to 0.9 % w/v yeast extract (YE) in agitated 200-mL shake flask indicated the highest BC production of (5.2 g/L) was achieved using 0.5% (w/v) YE. Fish hydrolysate and fish powder, which have the potential to be a cheap form of nitrogen in the media, were manufactured from waste fish. The results obtained indicated that fish hydrolysate and fish powder could be substitutes for YE, with the potential to reduce media cost. Trials using 0.5% to 2.0% w/v fish hydrolysate showed the highest BC production of 0.24 g/L was obtained using 2.0% w/v fish hydrolysate. The yield was comparable to that obtained on 0.5% w/v YE. Trials with 0.5% to

2.0% w/v fish powder in the media showed that the highest BC production of 0.25 g/L was obtained in 1.5% and 2.0% w/v fish powder.

The potential of using banana peel extract, both as a source of carbon and other nutrients was investigated in shake flask trials. Four combinations of banana peel extract and glucose, each producing about 50 g/L carbon media, were investigated. The highest BC production of 0.43 g/L was produced using 10 g/L banana peel extract + 40 g/L glucose.

Investigations using 1% to 5% w/v glycerol as a carbon source showed the highest BC production was achieved using 2% w/v glycerol. It is assumed that the higher glycerol concentrations produced substrate inhibition.

Productivity in large scale fermenters is affected by aeration and agitation. The optimal aeration rate in a 5-L bioreactor stirred fermenter for growing *G. xylinus* DSM46604 on 50 g/L glucose was 1.0 volumes air per volume media per minute (vvm). The BC concentration under these conditions was 4.4 g/L. The optimal agitation rate at 1.0 vvm was 200 rpm and a BC concentration of 4.0 g/L was obtained.

Batch fermentations using fish hydrolysate, fish powder, banana peel extract and glycerol were done in a 5-L and 3-L bioreactors. Optimum BC yields of 0.5 g/L, 0.47 g/L, 1.1 g/L and 2.87 g/L were obtained on 15 g/L fish hydrolysate + 50 g/L glucose, or 15 g/L fish powder + 50 g/L glucose or 10 g/L banana peel extract + 40 g/L glucose, or 20 g/L glycerol. The results showed that glucose and expensive nitrogen sources such as YE could be replaced by cheaper, non-conventional media components.

The BC concentration in the media and BC yields could be increased by growing *G. xylinus* DSM46604 under fed-batch or continuous conditions. The BC yield obtained on 50 g glucose per litre using a fill-and-draw fed-batch strategy or pulse-feed strategy were 0.05 and 0.11 g/g glucose used respectively compared with 0.05 g/g in batch operation. Maximum BC concentration obtained from fill-and-draw and pulse feed were 5.02 g/L and 5.5 g/L, respectively. These were compared with 2.2 g/L in batch operation. Using 20 g/L glycerol as the carbon source increased BC yield to 0.2 and 0.39 g/g glycerol used in fill-and-draw or pulse-feed strategies respectively. Using a continuous fed-batch strategy improved BC yield on 20 g/L glycerol to 0.3 g/g for a dilution rate of 0.1 h^{-1} dilution rate.

The highest BC yield in continuous operation on 50 g/L glucose or 20 g/L glycerol was obtained at a dilution rate of 0.1 h^{-1}. Obtaining BC yield at 0.13 g/g and 0.33 g/g from 50 g/L glucose and 20 g/L glycerol clearly showed that both growth media enhanced BC in continuous fermentation, compared to batch fermentation. In continuous operation, it is also demonstrated that various dilution rates affected BC productivity. It was found that by increasing dilution rate to 0.15 and 0.2 h^{-1} decreased BC yield on both 50 g/L glucose and 20 g/L glycerol media. This clearly showed that it is crucial to select the most suitable dilution rate for BC production. Also, by applying dilution rate at 0.1 h^{-1} improved BC yield on both 50 g/L glucose and 20 g/L glycerol media.

6.2 Recommendations

Trials using low cost waste materials such as fish hydrolysates and powders (for nitrogen) and banana peels (for carbon) did not significantly increase BC concentration in the fermentation broth or productivity It is recommended that further trials are done to investigate the potential for other low cost components such as waste from fruit and dairy processing

The pellicle form of BC is easier to harvest than collecting BC dispersed through the fermentation broth. Reactor conditions affect growth morphology. It is recommended that further trials are done to investigate the effect of varying fermentation conditions such as stirring rate, aeration rate and fermenter operation on BC yield. The trials showed that fed-batch and continuous fermenter operation had the potential to increase BC yield and productivity above that achieved in static or simple stirred tank reactor conditions. It is further trials be done to quantify this effect.

The effect of operating under controlled pH needs to be investigated. Although pH was monitored during the trials, it was not controlled so no conclusions can be drawn about the effect of pH on BC production or productivity. Trials should be done onto investigate the effect of dissolved oxygen on BC production. Under limited oxygen, the morphology of the BC changes as indicated by the growth pattern in stationary culture compared with stirred culture. Producing BC production under varying dissolved oxygen concentrations will identify the role oxygen has on cell growth and BC productivity. The dissolved

oxygen concentration was not monitored in the current trials as the instrument was not functioning properly. However, it is assumed aeration was adequate for the low cell mass.

Methods should be developed to reduce the amount of growth that adheres to the probes and walls of the fermenter. This growth habit makes it difficult to get representative samples, and hence model BC production.

This study also investigated the production of BC in static cultivation and agitated cultivation (shake flask and bioreactor) along with the effect of varying the reactor conditions on BC yield and BC productivity. More reliable assays should be used in any future research. Additional morphology analyses should be done to investigate the influence of reactor conditions and low cost media components on BC morphology and mechanical properties. This will help to produce desired, reliable BC pellicles.

Trials were also done in various fermentation modes to produce BC. More research is needed on operating modes as there are few reports on the effect of operation modes. This will help increase the understanding of how operation mode and conditions affect (and hence enhance) BC productivity. It was demonstrated that both fed-batch and continuous operation modes improved BC productivity.

Although pH was monitored in the current experiments, DO concentration was not monitored due to instrument malfunction and it was assumed that the aeration rate used was adequate for the low cell mass. Limited oxygen affects BC morphology, as indicated by the growth patterns in stationary culture. Trials on BC production under varying DO concentrations will identify the role of oxygen as a rate limiting factor in cell growth ad BC productivity.

Some samples obtained from fermentation were contaminated. The sampling vial and tube were identified as a contamination source. Using thorough aseptic techniques during sampling will minimize contamination. The BC pellicles tend to accumulate at the impellers in the reactor. Other procedures for mixing should be explored to minimize accumulation.

After high productivity conditions have been established, the physical properties and therefore potential uses of the BC can be determined. The next stage would then be obtaining accurate data for scale up and estimating production costs.

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Appendices

Appendix 1 (in the attached CD)

The Excel file in the CD included with this thesis contains a large file (28 A4 pages) of data obtained from literature on producing bacterial cellulose from microbes, covering the 1997-2012. The information includes:

- Organism (and strain)
- Media
    - Carbon sources, and initial concentration(s)
    - Nitrogen sources and concentration
    - Minerals, vitamins and other additives, and concentration(s)
- Reactor conditions
    - Growth temperature
    - Initial pH
    - Reactor type and working volume
    - Aeration and agitation conditions
    - BC concentration, g/L
    - BC yield, g/g substrate used
- Authors (see References for the complete citation)

Appendix 2. Calibration curves

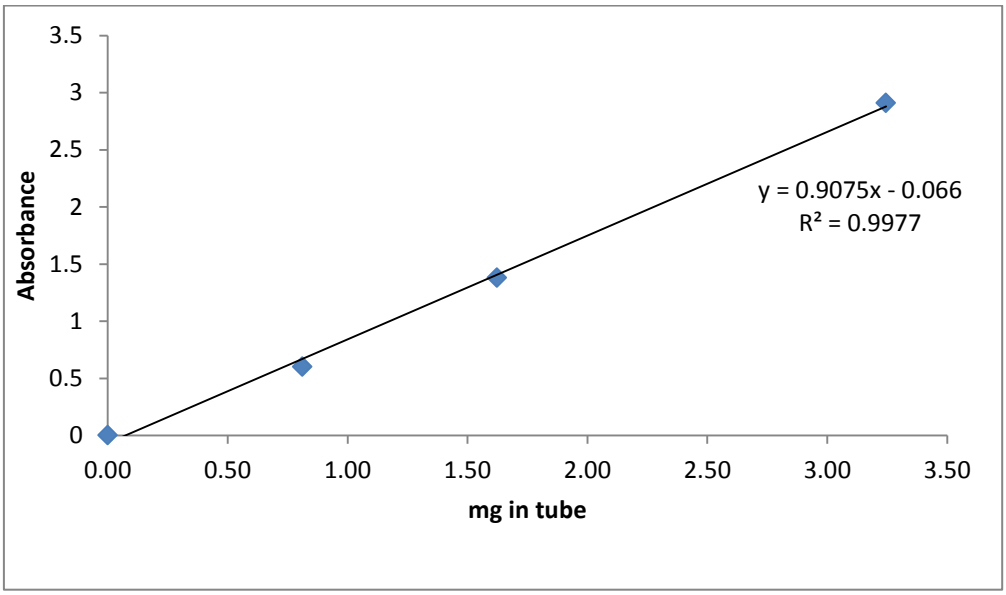

Figure A1: Glucose calibration curve at OD_{575}

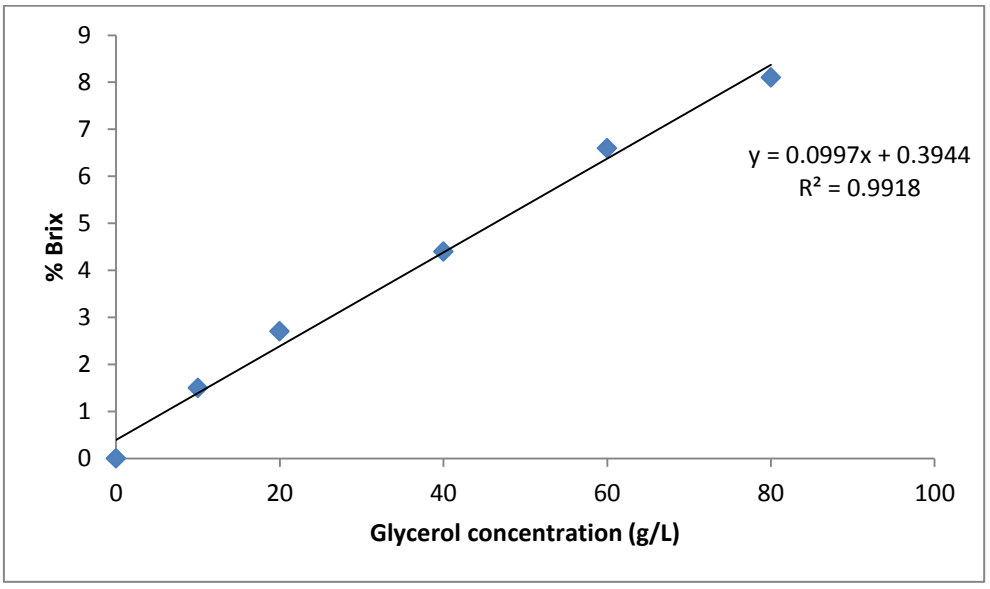

Figure A2: Glycerol calibration curve using a refractometer. Refractive index is measured in units of %Brix.

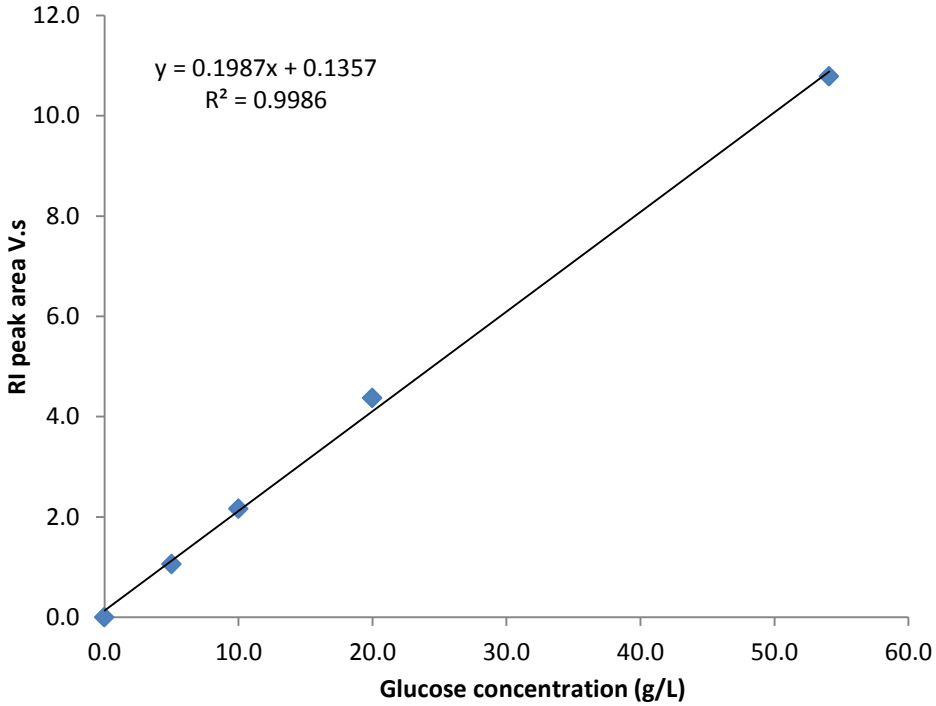

Figure A3: Glucose calibration curve from HPLC.

Appendix 3. List of Reagents and Instruments

A. Equipment

- Spectrophotometer (Ultrospec 2000, Pharmacia, Biotech)
- 5-L Bioreactor (Inceltech, LH Series 210)
- 3-L Bioreactor (BioFlo/Celligen 115, New Brunswick)
- High Performance Liquid Chromatography (Waters 515 HPLC) Pump, equipped with Waters 2996 Photoiodide Array Detector)
- Laminar Hood (Kendro, Hera Safe)
- Oven (Contherm, Thermotec 2000)

B. Reagents

i. DNS Assay

- Dinitrosalicyclic Acid Solution, 1% in 100 mL
  - Dinitrosalicyclic Acid: 1 g
  - Phenol: 0.2 g
  - Sodium sulphite anhydrous: 0.05 g
  - Sodium hydroxide: 1 g
  - Add water, mark up to 100 mL
- Potassium sodium tartrate solution (Rochelle Salt), 40%

ii. Media Compositions

- 50 g/L/20 g/L glycerol glucose
- 5 g/L Yeast extract
- 5 g/L Ammonium sulphate
- 3 g/L Potassium dihydrogen orthophosphate
- 0.05 g/L Magnesium sulphate
-

iii. Other reagents

- 6M NaOH for pH adjustment
- 1M NaOH for BC purification

Appendix 4. Calculations

A. Fermentation parameters:

Yield of BC = $g_{dry\ BC}/g_{glucose\ consumed}$

Yield of Biomass = $g_{biomass}/g_{glucose\ consumed}$

Rate of BC Production = BC concentration (g/L)/final volume*time

Rate of Biomass Production = Biomass concentration (g/L)/final volume*time

Time = the number of days of BC growth from the date of inoculation till the date of harvest and the day when the drop in glucose concentration was observed till day of harvest in bioreactor.

B. Dilutions for glucose standard curve using DNS Assay:

1. Standard solution of 54.09 g/L was prepared
2. Tube 1= A 100 µl of stock solution was extracted, 4900 µl of water added (1:50 dilution)
3. Tube 2 = A 100 µl of stock solution was extracted, 9900 µl of water (1:100)
4. Tube 3 = A 5000 µl of Tube 2 was mixed with 5000 µl of water (1:200)

C. Dilutions for glucose and acetic acid standard curve using HPLC:

1. Standard solution contained 10 mL of:
    - 50 g/L glucose
    - 15 g/L acetic acid
2. 5 mL of mixture was transferred to 5 mL of Milli-Q water
3. 5 mL of mixture from tube No. 2 was transferred to 5 mL of Milli-Q water

Acetic acid molecular weight: 60.05 g/mol

Glucose molecular weight: 180 g/mol

*All samples from fermentation broth was filtered, centrifuged and treated with 40 µl of 7% sulphuric acid to kill the bacteria before introduced to HPLC.