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**POTENTIALS OF UTILIZING RENEWABLE RESOURCES OF AGRICULTURE
RESIDUES FOR THE BIOSYNTHESIS OF BACTERIAL CELLULOSE NANOFIBERS**

by

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Amir Jalal Sani

Abstract

POTENTIALS OF UTILIZING RENEWABLE RESOURCES OF AGRICULTURE RESIDUES FOR THE BIOSYNTHESIS OF BACTERIAL CELLULOSE NANOFIBERS

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In the present study, production of bacterial cellulose nanofibers from single sugars and sugar mixtures in feedstock was investigated using different culture methods. This includes production in shake flask, stirred tank and draft tube airlift bioreactors. There has been no previous work done other than this reported work concerning production of bacterial cellulose nanofibers using sugar mixtures. Fructose was found to be the top producer with 5.65 g/L of bacterial cellulose nanofibers. The different compositions of sugar mixtures tested in this study were identical to acid hydrolyzates of agricultural residues. Maximum yield of 17.72 g/L was observed with mixture that resembles the acid hydrolyzate of wheat straw.

Modified stirred tank and airlift bioreactors showed higher production of bacterial cellulose nanofibers compared to shake flask. In general, results obtained in the present study demonstrate potential of improving production of nanofibers solely based on agricultural residues and using draft tube airlift bioreactors.

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Chapter 1

Introduction

Cellulose is the world's most abundant biopolymer. Pure cellulose is produced by the bacteria *Gluconoacetobacter xylinum*, which is called Bacterial Cellulose (BC). Unlike the cellulose from wood pulp, BC does not contain lignin or hemicellulose. In addition, BC has variety of unique properties, which makes it suitable for many different industrial applications. BC is a natural nanomaterial, which has a native dimension of less than 50nm in diameter. Cellulose fibers are relatively strong; have breaking strengths of up to 1 GN/m² (10,000 MPa). Its fiber has a high aspect ratio with a fiber diameter of 20-100 nm. As a result, it has a very high surface area per unit mass. This property along with its hydrophilic nature, results in very high liquid loading capacity. However, due to its high production cost, it has not been widely utilized.

Cellulose is one of the most abundant organic macromolecule on Earth, It is a great reservoir of carbon cycle and in plants, its biogenesis is important in cell growth and development (Fincher *et al* 1981; Green, 1980). Cellulose is composed of glucose monomers connected by β (1-4) glycosidic linkages, and its chemical formula is $(C_6H_{10}O_5)_n$. Cellulose forms the basic structural matrix of cell walls in almost all plants, in many fungi, and in some types of algae. However, this form of cellulose contains many impurities, including hemicellulose and lignin, and harsh chemical treatments are required to remove these impurities. The chemical treatments result in irreversible alterations of the structure of the cellulose, which permanently strip the polymer of its advantageous characteristics (J. D. Fontana *et al.*, 1990).

Nevertheless, such treatments are still used extensively by several industries. While plant-derived cellulose has been important in the production of paper and textile products, recent

evidence suggests that BC may be a better choice for manufacturing these products. Many species of bacteria, such as those in the genera of *Gluconoacetobacter*, *Agrobacterium*, *Achromobacter*, *Aerobacter*, *Azotobacter*, *Sarcina ventriculi*, *Salmonella*, *Escherichia* and *Rhizobium*, have been reported to produce solid, extra-cellular cellulose that has chemical and physical properties that are superior to the properties of plant-derived cellulose called Bacterial Cellulose (BC) (Ross *et al* 1991; Römling, 2002).

Brown was the first to verify the production of cellulose by bacteria (J. D. Fontana *et al.*, 1990). He has noticed that certain strains of *Gluconoacetobacter* produced a white gelatinous pellicle on the surface of a liquid medium. He further examined this substance and determined the chemical structure of this material was, in fact, identical to the chemical structure of plant-derived cellulose. It was also discovered that BC contains no lignin or other contaminants, as plant-derived cellulose does (Iguchi *et al.* 2000). Therefore, BC can be purified using less energy and without the use of structure-and property-altering chemicals, thereby maintaining the original characteristics of the cellulose.

As BC can be produced as a pure material, it has drawn much attention as a new functional material for industrial applications, and the discovery of numerous desirable features were later revealed. For instance, BC, when arranged in a form of a sheet, has high crystallinity and is comprised of ultra-fine fibers that possess a high Young's modulus (15-30 GPa). BC fibrils are inelastic and insoluble, and because of their molecular configuration, they have a tensile strength comparable to that of steel (Yamanaka *et al.*, 1989). These traits, along with its capacity for holding a quantity of water up to a hundred times of its weight, BC has been discovered as the perfect material for producing a wide variety of products, including high quality paper; high-fidelity acoustic speakers, and diet and dessert foods (Shoda *et al* 2005).

BC has been reported to have an excellent biodegradability, and becoming an ideal material for applications within the medical and drug-delivery industries. However, although BC is being considered as an alternative for plant-derived cellulose, it is still expensive, compared to other popular commercial organic products. Therefore, it is important to develop economical production methods to produce BC at low cost. However, one significant barrier, which has become the focal point for many researchers studying BC, is whether the culture should be grown under static or agitated conditions. The choice of a cultivation technique is dependent on the BC's anticipated commercial destination, since the structure, physical properties, and mechanical properties of BC are influenced significantly by the culture method employed (Ben-Bassat *et al.*, 2002; Galas *et al* 1999).

In 1990's the commercial production of the BC nanofibers was undertaken in Philippines, but it was labor-intensive, inefficient, and time consuming batch productions with minimum technical supervision. So, there is a need for efficient large scale production of BC with innovative fermentation methods (Gonzalo, 1997). Not only the innovative method is required for the large scale production of BC but also cheap feedstock is also necessary for low cost production of BC nanofibers. The production of wheat, cornfiber and DDGS (Dried Distillers Grains with Solubles) in North America was 25, 10.55, and 3.5 million tons per year respectively in 2007(The Canadian Encyclopedia, Statistics Canada, and University of Minnesota, 2007). Very small part of this agriculture wastes is used for the animal feed. These agricultural wastes can be used as a carbon source for the production of BC nanofibers. Using agricultural wastes as feedstock we can significantly reduce the cost of the BC nanofibers production. Single sugar mediums have been tested for the production of BC nanofibers by many researchers. However,

no work has yet been published in which mixtures of more than two sugars were tested as a carbon source for producing BC nanofibers.

In the present study, we investigated for the very first time production of BC nanofibers using sugar mixtures in feedstock. Production of BC using single sugars was also investigated to facilitate comparison and discussion. Individual sugars tested were glucose, fructose, xylose, galactose, arabinose, mannose and sucrose. Three sugar mixtures with compositions similar to that of the hydrolyzate from agriculture wastes of wheat straw, DDGS and corn fiber were mainly examined. In general, the sugar mixtures are composed of five individual sugars of glucose, xylose, arabinose, galactose and mannose in different proportions. As these agricultural wastes are abundant in Canada and in North America in general, we mimicked the proportions of sugars in these agricultural wastes and used them as the carbon source for the production of BC nanofibers as a preliminary study that will be expanded in future work with using real sugar hydrolyzates.

In addition to investigating different sugar compositions, the work was evolved to studying production of BC using different culture methods of the bacteria. Stirred tank reactor (STR) with modified internals and airlift bioreactors (ALR) with solid draft tube were further investigated and compared to what has been achieved in the preliminary study in shake flask culture.

Objectives

The main objective of this study is to improve BC production in fermentation using *Gluconoacetobacter xylinum* ATCC 700178 bacterial strain. In order to achieve that, different feedstock were examined that include single sugars and sugar mixtures. Single sugars were examined first to analyze the effect of each individual sugar on production of the nanofibers. For

all experiments conducted, results of BC production, sugar consumption and cell growth profiles were monitored, interpreted, and compared among individual sugars and sugar mixtures. Sugars that were tested during the single sugar fermentation experiments were glucose, fructose, xylose, galactose, arabinose, mannose and sucrose, while sugar mixtures were composed of the following individual sugars of glucose, xylose, arabinose, galactose and mannose.

In general, our main objectives of the present study can be summarized as follows:

- To study the effect of single sugar based feedstock on BC production in a preliminary study in shake flasks.
- To study the effect of sugar mixtures on production based on the knowledge developed from the single sugars production in shake flasks.
- To study the effect of different culture methods (i.e., STR and the draft tube ALR bioreactors) on the BC nanofibers production, utilizing the sugar mixture that achieved maximum production of BC in the shake flask experiments.

Chapter 2

Literature Review

2.1 Effect of Feedstock on BC Biosynthesis

Using the proper feedstock can reduce the cost of BC nanofibers production. Carbon and nitrogen sources in the feedstock are considered to be essential components to produce BC. Initially, the medium used for culturing BC, found to be too expensive and unsuitable for the commercial production of BC (Hestrin *et al.* 1954). Toyosaki *et al.* (1995) used corn steep liquor (CSL) and fructose based medium in shake-flasks and obtained high yields of BC compared to the conventional medium of yeast extract/peptone and glucose.

In the fermentation industry, molasses is the most popular carbon source used in general. Recently, using molasses and CSL as the carbon and nitrogen sources respectively gave high BC production at a low cost (S. Bae *et al.* 2004). Keshk *et al.* (2006) studied sugarcane molasses (MO), with and without the presence of lignosulfonate, as a sole carbon source for BC production in an HS medium. They used six strains of *Acetobacter xylinum*, found the strain IFO 13772 as the best BC producer, and strain IFO 14815 as the poorest BC producer. Keshk *et al.* (2006) concluded that MO is a better carbon source than glucose for most of the strains examined. There were no significant differences found in crystallinity among the BC produced from the different media.

Keshk *et al.* (2005) also studied different carbon sources for BC production. They used the *Gluconoacetobacter xylinum* (strain ATCC 10245) and found that glycerol gave the highest yield of BC, followed by glucose, fructose and inositol. They also studied the consumption rates

of these four substrates during and after the incubation period. They found consumption of glucose, fructose, inositol and glycerol to be 97.0%, 51.9%, 94.7% and 45.4% respectively. The cellulose yield was 8.4, 7.9, 7.4 and 13.0 respectively (calculated from the dry weight of BC and the weight of carbon source added (300mg/30 mL). The glycerol medium gave the highest yield (13.0) of BC.

Hong *et al.* (2008) studied a new carbon source, hydrolyzate of konjac powder with *Acetobacter acetii* subsp. *xylinum* (ATCC 23770) for producing BC. They found that hydrolyzate of konjac powder is an excellent carbon source for BC production. It is an abundant and commercially available in China. In addition, there are other species of konjac powder that have not yet been tested for BC production. Hong *et al.* (2008) also found that BC production was three times higher, when used hydrolyzates, than that when used mannose and five times higher than that when used a glucose-mannose mixture as the carbon source in static cultures. They also found that adding calcium cations could also improve the BC production in the static culture. The highest BC yield obtained with adding Ca^{2+} ions is only 1.5 times more than that without addition of Ca^{2+} ions.

J. D. Fontana *et al.* (1991) studied caffeine and related xanthines as potent stimulators for BC production by *G. xylinum*. These compounds are found in several plants infusions which are useful as supplements in culture medium for *G. xylinum*. The strain used was a wild-type strain of *G. xylinum*, named as strain (BF). The investigations focused on the positive effects of compounds from plants with high levels of xanthine-based substances. The examples are *Camellia* (commercial tea), *Paulinia* (Guarana), coffee (unroasted seeds), *Theobroma* (cacao), kola or *sterculia* (cola nut), and *Ilex* ("mate"), on the synthesis of cellulose by *G. xylinum*. These plants derived compounds are cheap and small quantities are enough for BC production. The

best BC yield was obtained at room temperature with tea infusions (*Camellia sinensis*) at longer cultivation times (>7d), and with Paulinia at shorter cultivation times of less than 5 days. They found that growth for 15 days at room temperature was 310.4 mg % dry weight (per 100 mL of liquid and non agitated culture medium) for tea. And 295.5 mg % dry weight (per 100 mL of liquid and non agitated culture medium) for Paulinia.

Ciechańska *et al.* (1999) found that aeration is important in an agitated culture, because the metabolism of the bacterial cells depends on oxygen available. They obtained 0.16 g/L of BC in the shaking culture without aeration, while, the productivity increased to 1.4 g/L in aerated shaking cultures. To improve the capacity of the agitated culture, a ring with polypropylene fishing line was placed in the bioreactor. They obtained 4.2 g of BC with regulated pH 6.0 under the polypropylene ring, at shaking and aerated conditions. H. Son *et al.* (2003) reported that *Acetobacter* sp. V₆ is insensitive to agitation at 200 rpm. They found that adding several inorganic salts to the culture medium could increase the production of BC nanofibers.

The synthetic medium developed in reference of HS medium contained glucose, inorganic salts and ethanol in distilled water. Eight days of cultivation produced 4.16 g/L of BC at 200 rpm in the synthetic medium. This value is roughly 2.6 times higher than the yield obtained in traditional HS medium (1.58 g/L). They also tested the affect of Nicotinamide concentration on producing BC and found that a medium that contained 0.00005% Nicotinamide (by volume) produced the highest amount of BC, while lower concentration caused low BC production.

Toda *et al.* (1997) studied the effects of acetic acid on abilities of *Acetobacter* strain DA to produce BC. Without acetic acid in the GPY medium (GPY medium consists of 20 g/L of glucose, 5 g/L of polypepton and 5 g/L of yeast extract) the BC produced was 0.03g cellulose/g

of glucose. However, the yield increased to 0.19 by adding the optimal initial acetic acid concentration of 20 g/L. In contrast, using 20 g/L of acetic acid, strain A.P (*Gluconoacetobacter pasteurians*) reduces BC production. Toda *et al.* (1997) confirmed their findings by using two kinds of buffer solutions, i.e., a 0.1 M acetate buffer and a 0.04 M phosphate citric acid buffer. They found the yield of BC was higher (0.125-0.15) when the pH of the culture was at 3.5-4.5 using the acetate buffer.

In contrast, citric acid buffer gave the low BC yield (0.02-0.03) at the same pH. These results show the increase in BC production was because of presence of acetic acid and that it did not depend on the pH (Toda *et al.*, 1997). Kouda *et al* (1998) considered the inhibitory affects of the partial pressure of carbon dioxide ($p\text{CO}_2$) on BC production. The study was done using *Gluconoacetobacter xylinum* subsp. *sucrofermentans* (BPR3001A). The study included a 50-L jar fermentor that was purged with air containing $p\text{CO}_2$ (0.15-0.20 atm) and found that $p\text{CO}_2$ decreased the BC yield, volumetric production rate, and viable cells concentration

Vandamme *et al.* (1998) produced BC in a static culture and in an agitated submerged fermentation process using micro particles with precise control of pH. Glucose and sucrose are used as carbon sources, and acetic acid as the energy source. They found the yields of BC were high. They found that BC formation could be increased by adding insoluble microparticles, such as diatomaceous earth, silica, small glass beads, to the submerged, agitated/aerated *Gluconoacetobacter* culture. The combined use of fructose (70 g/L), glucose (35 g/L), and acetic acid (7.5 g/L) in a static culture produced 28.4 g/L of cellulose. The cellulose yield in the media to which microparticles were added was more than tripled (1.1-3.6 g/L) compared to the level normally obtained in the similar reference cultures (without micro particles).

The micro carrier-enhanced cellulose synthesis was believed to be the result of developing an oxygen-limiting biofilm around these particles. Zhou *et al.* (2007) studied the affects of sodium alginate in shake flasks and in a stirred-tank reactor. The strain used was *Acetobacter xylinum* Nust 4.1. They found the maximum BC production of 6.0 g/L occurred in the shake flask at 0.04% (w/v) of sodium alginate and the BC production by *G. xylinum* Nust 4.1 without sodium alginate was 3.7 g/L. However, the trend in improving the BC production by adding more sodium alginate was not observed. This trend suggests that higher concentrations of sodium alginate may impede BC production because of an increase of broth viscosity. Without sodium alginate, the BC was produced as clumps of different sizes, fibrous shapes, and was entangled with the internals of the reactor, however with 0.04% (w/v) sodium alginate, these problems did not occur.

The maximum yield of BC reached in the STR was 1.89 g/L for 60 h at a sodium alginate concentration of 0.04%. This yield was 1.7 times greater than the 1.09 g/L produced in the control medium without sodium alginate (Zhou *et al.*, 2007). The BC production by *G. xylinum* Nust 4.1 in the stirred-tank reactor was much lower than that in shake flasks, because of the higher viscosity of the culture broth in the reactor. A. Krystynowicz *et al*, (2002) reported the strain *E₂₅* of *A. xylinum* showed higher stability in BC production under stationary culture condition, whereas intense agitation and aeration reduce cellulose synthesis drastically.

Gluconoacetobacter xylinum has been investigated by a number of people. It has been found the biosynthesis pathway of this organism ends at good amount of cellulose in the presence of corn steep liquor (Matsuoka *et al* 1996; Oikawa *et al* 1995; Ramana *et al* 2000; Zaar *et al* 1979). The synthesis of cellulose from 412 strains was observed and the most suitable *Gluconoacetobacter*

strain for cellulose production for agitated culture was *Gluconoacetobacter* sp. BPR 2001 (Toyosaki *et al.* 1995).

2.2 Effect of Culture Methods on BC Production

A bioreactor is a device that supports the biologically active environment for a living organism growing in it. The most commonly used bioreactors are the STR and ALR. Chemical processes, involving organisms and biochemically active substances in aerobic or anaerobic conditions, are conducted in bioreactors. The fermentor (bioreactor) must provide a controlled environment for the growth of microorganisms to get the desired product (Stanbury *et al.* 1995). The aeration and the agitation are the components of the bioreactor and include (1) Agitator (impeller) (2) Baffles and (3) Aeration system (Sparger). Maxblend and the Gate with Turbine impellers are found to be suitable for the BC nanofibers production (Kouda *et al.* 1997). Interesting hydrodynamic parameters in the bioreactor design are the gas holdup, the magnitude of induced liquid circulation, liquid-phase dispersion coefficient in different areas of the reactor (Gavrilescu *et al.* 1998a).

Among many types of fermentors described in the literature few showed satisfactory results for industrial aerobic fermentations. The chemical and the biochemical process industries are turning towards the use of ALR reactors because of the gas-liquid contacting operations. The ALR bioreactors can also be called pneumatic bioreactors that exploit hydrostatic pressure differences to circulate the fluid (Dahman *et al.* 2008). The idea of the ALR reactor came from that of bubble column (Gavrilescu *et al.* 1998c). ALR are similar to bubble-column reactors with a slight difference of draft tube. An ALR that contains an inner draft tube is called as ALR with

an internal loop, whereas, an ALR that contains an external draft is called as ALR with an external loop. The draft tube improves circulation and oxygen transfer, and it equalizes shear forces in the reactor.

ALR has a baffled riser (liquid rising) tube and a downcomer tube (liquid descending). In normal operation, the mixture of gas or the air is sparged at the base of the riser column. The medium in the vessel is circulated by the driving force which is generated by the difference in the density between the liquid column in the riser (the air bubbles are more in the riser) and the down comer column (it has low air bubbles as they release at the top of the loop). These fermentors are ideal for cell growth because of less or no shear compared to the stirred vessels (Dahman *et al.* 2008). The fluid dynamics of the ALR is used to determine the mass and the heat transfer (Gavrilescu *et al.* 1998b).

Bioreactors commonly used for mixing are stirred tank, bubble column, airlift and fluidized bed reactors. Agitation in a stirrer tank reactor is accomplished by mechanical stirring. They provide good mixing and appreciable values of mass and heat transfer coefficients. Disadvantages of the stirred vessels are mechanical issues related to the moving parts (e.g. shafts and impeller) which can introduce contamination while operating cost is also higher due to high energy consumption and cost of maintenance (Gumery *et al.* 2009).

Figure 1&2 shows typical ALR and STR respectively.

Figure 1. Some typical ALR fermentors are shown in the above figure (a) bubble column ALR (b) ALR with net draft tube (c) ALR fermentor with solid draft tube.

Figure 2. STR with one multi-bladed impeller.

Production of BC had been investigated in static as well as in agitated conditions (Sakairiet *al.* 1998; Watanabe *et al.* 1998). In general, static conditions are more favourable than agitated conditions. However, the static condition needs more area and workforce, resulting in the industrial scale production more expensive. Therefore, in commercial production of BC, the agitated condition is more precise. Different types of culture conditions such as shake flasks, STR, ALR, rotation disk reactors, and modified ALR have been tested for BC production in agitated conditions.

The design of the ideal reactor increases the synthesis of BC. Sakairi *et al.*(1998) and Watanabe *et al.*(1998) described several BC production techniques. Some of these techniques show a potential tool for economic and commercial BC production, such as; stationary culture (in plastic trays), agitated culture (jar fermentors), cultivation in horizontal fermentors. While others have tested internal loop ALR (Budhiono *et al.* 1999; Kouda *et al.* 1996; Sattler *et al.* 1990).

A high rate of BC production needs a high oxygen transfer rate within the agitated culture. This needs a higher oxygen transfer coefficient (k_{La}) and, subsequently, higher agitation power, which needs a large motor, resulting in higher energy costs. Producing BC in an agitated and aerated culture increases the viscosity and reduces the homogeneity of the broth, which in turn reduces the oxygen level in the broth and reduces the rate of production of BC. As a result, the agitator configuration has been studied extensively. Screening strains suitable for agitated culture and the influence of the culture medium ingredients on the BC productivity have been reported (Matsuoka *et al.*, 1996; Toyosaki *et al.*, 1995).

Kouda *et al.* (1997) investigated the effect of agitator configuration on BC production and reported on the mixing and oxygen transfer capacity of various impellers. Measurements of the k_{La} values gave information on oxygen transfer capacity of each agitator. Impellers with large k_{La} values mix culture broth well and are suitable for BC fermentation for examples Maxblend and gate with turbine impellers. Kouda *et al.* (1997) summarised the configuration characteristics suitable for BC fermentation as follows: distance between any area of the culture broth and the impeller should be small. This feature reduces the high viscosity of the culture broth where the shear speed is low.

An impeller should have a configuration which has a high shear region around the sparger. This is important for dispersion of bubbles and the increase of k_{La} . These characteristics should be adapted in scaling-up the impellers, since mixing and oxygen transfer are both important for BC production in aerated and agitated culture. Figure 3 shows the Maxblend and the gate with turbine impellers used for the BC production.

Figure 3. Agitators' configurations. The gate with turbine and maxblend impellers used in BC production.

Naritomi *et al.* (2002) investigated *Acetobacter xylinum* subsp. *Sucrofermentans* (BPR3001A) in various broth exchange ratios (α), (the volume of drawn broth per total volume of broth) and found that at $\alpha=0.9$ the maximum BC production rate was 0.43 g/L/h. The values of $\alpha > 0.9$ resulted in lower BC production rate due to inadequate energy for cell growth and BC production. However, a value of $\alpha < 0.9$ lead to oxygen limiting conditions and resulted in decreased BC yield because of increased metabolic maintenance. Chao *et al.* (2001) successfully used a 50-L, internal-loop ALR provided with an oxygen-enriched gas supply to improve the oxygen transfer rate and obtain a high cellulose production rate. The strain used was *Gluconoacetobacter xylinum* subsp. *Sucrofermentans* (BPR2001). It has been reported that in air, at initial fructose concentration of 40 g/L the BC production rate was 0.059 g/L/h. With oxygen-enriched air, the BC production increased to 0.093 g/L/h.

Chao *et al.* (2000) studied a 50-L internal-loop, ALR to improve the oxygen transfer rate and achieved a high cellulose production rate using *Gluconoacetobacter xylinum* subsp. *Sucrofermentans* (BPR2001). The bioreactor gave 3.8 g/L of BC after 67 h with a normal air

supply. However, with oxygen enriched air the production rate of BC increased significantly. The concentration of BC becomes doubled and the production rate was 0.116 g/L/h, which was two times higher than that of air-supplied culture and comparable to that in a mechanically agitated stirred-tank fermentor. The BC produced by the ALR formed a unique, elliptical pellet, different from the traditional fibrous form produced using the stirred-tank reactor.

Chao *et al.* (2001) studied the 50-L internal-loop ALR and found that adding 0.1% (w/w) agar (water-soluble polysaccharide) increases the BC production. The BC production reached to 8.7 g/L compared with 6.3 g/L in the control medium using *Gluconoacetobacter xylinum* subsp *Sucrofermentans* (BPR2001). The cultivation period to reach the maximum BC concentration was roughly half of that in CSL-fructose medium without adding the polysaccharide. Adding polysaccharide hindered forming large clumps of BC in the broth, which resulted in increase in the production rate of BC. Since, smaller pellets are advantageous for transferring nutrients and oxygen into bacterial cells found inside and on the surface of the cellulose matrix in the pellets, faster growth rates could be achieved. On the other hand, at high agitation speed in a stirred-tank reactor, the turbines and the baffle plates destroyed cellulose clumps, dispersed fibrous BC to keep efficient uptake of nutrients. Under this condition, adding a polysaccharide was not effective to increase the production rate of BC.

Wu *et al.* (1990) were the first to propose a modified, ALR consisting of a bubble column and a conventional internal loop ALR. The proposed reactor included a circular, wire-mesh draft tube. When the gas velocity exceeded 2 cm/s, the proposed reactor's k_La would always be higher than that of the original bubble column or ALR (J. Y. Wu *et al.* 1991; W. T. Wu *et al.* 1992). At a superficial gas velocity of 5.02 cm/s, the k_La value of the proposed reactor with a mesh draft tube was double than that of the ALR. The value of the volumetric oxygen transfer coefficient,

k_{La} , increased as diameter ratio of the riser and downcomer decreased at higher airflow rates. Correlation between volumetric oxygen transfer coefficient with the true superficial air velocity under different reactor pressures was determined. The k_{La} decreased with an increasing reactor pressure. As a result, the proposed reactor produced an increase in cell mass of *Saccharomyces cerevisiae* in fed-batch culture compared to the fed-batch culture of ALR or bubble reactors at the same operating conditions.

Tung *et al.* (1997) further modified the ALR by adding two concentric wire-mesh draft tubes with a sparger located between the tubes. The draft tubes had a significant affect on breaking bubbles into smaller ones. As a result, the modified reactor had a higher gas holdup, a higher volumetric oxygen transfer coefficient, and a lower mixing time than the bubble column. In addition, the reactor showed significantly shorter cultivation periods to reach maximum cell concentrations when applied to fermentation. Cheng *et al.* (2002) were able to develop a rectangular, wire-mesh draft tube that was easier to scale up and set up, since it allowed bubble slicing and subdivision. The modified ALR was also found to have a higher volumetric oxygen transfer coefficient and mixing capability. Because of the use of the modified ALR, the final BC concentrations produced after 72 h of cultivation were three times higher than those produced in the conventional bubble column reactors.

The modified ALR formed a unique elliptical BC pellets that was different from the fibrous form produced when used the STR. The dissolved oxygen in the modified ALR was maintained above 35% throughout the cultivation and elliptical pellets with an average diameter of 10 mm were obtained in this reactor. Owing to the mild shear stress and homogeneous field of hydrodynamic forces in the reactor, pellets that were different from those of the fibrous form were obtained. However, the size of the pellets varied strongly on dissolved oxygen in the broth.

Results showed that BC in pellet form showed a more compact and dense structure than that in fibrous form. This showed that some mechanical properties of the BC in pellet form might surpass those of the fibrous form. Figure 4a & 4b shows the morphology of BC in STR and ALR respectively.

Figure 4. Morphology of BC produced in (a) STR and (b) ALR (Cheng *et al.*, 2002).

Oxygen is important for aerobic growth and BC formation by *Gluconoacetobacter xylinum* (*G. xylinum*). Cellulose usually occurs only at the upper film and air interface (Borzani *et al.* 1995; Schramn *et al.* 1954). Hornung *et al.* (2006) identified the causes that affect in increase in the rate of BC production, e.g., the affect of the rate of mass transfer of substrate on the microbial process. They developed a model for diffusion of glucose through the growing cellulose layer and solved the model. The model clearly showed there are other factors affecting the BC production besides the glucose diffusion. Therefore, it was assumed that the BC that was produced was pushed down gradually as the new BC product is formed on top of it. The aerobic

zone is rather thin, approximately 1 mm, and only those bacterial cells, which are immobilized within this aerobic zone, can synthesize BC.

The cells in the deeper zone are inactive and they cannot produce BC due to immobilizing the bacteria between the cellulose fibers and sinking the BC product. They concluded the total cell count is not important. The only important cell count is in the aerobic zone, because product formation and cellulose production vary on the conditions in the aerobic zone. For the active cells within the aerobic cellulose matrix, nutrients have to diffuse from the substrate solution below the cellulose through the oxygen-limited cellulose.

Jung *et al.* (2007) developed fermentation equipment with a 6-flat-blade-turbine impeller and a spin filter consisting of a cylinder surrounded by stainless steel mesh, the bottom of which was attached to the agitator shaft. The used strain was *Gluconoacetobacter hansenii* PJK (KCTC 10505 BP). They found that, in a 2-liter-jar fermentor without a spin filter, rotating at 500 rpm, suppressed the occurrence of mutants. The BC production was also studied in a jar fermentor with the spin filter operating at 500 rpm at pH 5, and it was observed that the viable cell concentration was increased. The high mass of viable cells produced much BC during the cultivation.

The BC in the jar fermentor equipped with a spin filter was 0.044 g dry BC/h obtained at 68 hours of cultivation. It was greater than 0.031 g dry BC/h obtained at 51 h in a conventional jar fermentor. However, the production rate of BC in a jar fermentor equipped with a spin filter decreased with culture time and reached 0.033 g dry BC/h at 140 hours, even though the cell density in the jar fermentor increased with culture time. The high density of BC after 68 h of cultivation hindered the high stress generated at the impeller tip. The fraction of mutants

increased with culture time, reaching 0.451 at 92 h of cultivation and 0.810 at 140 h. Therefore, in order to increase the production rate later, they suggested increasing the rotating speed of the impeller with the culture time.

The rotating disc reactor of Bungay and Serafica gave one gram (1g) of cellulose (dry mass) after five days of cultivation (Bungay *et al.* 1997). A. Krystynowicz *et al.* (1997) produced 12.6 g dry mass in seven days of cultivation. Kim *et al.* (2007) used *Gluconacetobacter* sp. RKY5 in a rotary-film contractor (bioreactor) for improved BC production. They found that up to eight rotating discs in a bioreactor worked best and that, beyond that number, the disks had negative impacts on the BC production. The rotational speed was maintained at the optimum value of 15 rpm, and the BC produced and the cell concentrations were 5.14 g/L and 5.15 g/L, respectively. They also studied the effect of aeration on BC production and found that an aeration rate of 1.25 vvm (i.e., gas volume flow per unit of liquid volume per minute) gave the highest quantity of BC. The amount of BC that they obtained at this volume of air was 5.67 g/L, and the cell concentration was 5.35 g/L. The optimal pH was 6.0.

Joseph *et al.* (2003) studied the shake flask cultures added with polyacrylamide-co-acrylic acid using *Gluconoacetobacter xylinum* BPR2001 (ATCC No 700178). As a result, an increase in the production of BC was observed. For polyacrylamide-co-acrylic acid concentrations up to 3 g/L and a 7-day incubation period, the cellulose production increased monotonically from 2.7 ± 0.8 to 6.5 ± 0.5 g/L at a shaker speed of 175 rpm and from 1.7 ± 0.01 to 3.7 ± 0.5 g/L at a shaker speed of 375 rpm. Addition of polyacrylamide-co-acrylic acid also changed the morphology of the biomass from morphous/stringy forms to spheroidal particles with diameters ≤ 2 mm.

Under static conditions, the BC forms as pellicles at the air/liquid interface (Okiyama 1992; Schramm *et al.* 1954). Although it has been proven to produce successful commercial cellulose products on a small scale, such as *nata de coco*, transducer diaphragms, wound-care and dressing materials, it is not suitable for large-scale production, since it is labour intensive and requires large surface areas (Budhiono *et al.*, 1999). Under agitated conditions, BC can be produced in the form of a fibrous suspension, irregular masses, pellets, or spheres (Dudman, 1960). The agitated conditions are considered more suitable for commercial production of BC due to the higher production rates that potentially can be achieved (Nishi *et al.*, 1990; Ring *et al.* 1986).

Previous work has shown that higher yield of BC can be obtained by using CSL and molasses as nitrogen and carbon source respectively. Molasses is found to be better carbon source compared to glucose. Molasses is about 50% sugar by dry weight, predominantly sucrose, but also containing significant amounts of glucose and fructose. The non-sugar content includes many salts such as calcium, potassium, oxalate, and chloride. The supplements like Glycerol, Acetic acid, hydrolyzate of konjac powder, plant infusions such as tea (*Camellia sinensis*), and the combined use of fructose, glucose and acetic acid give higher yields of BC.

Generally, the agitated culture conditions, which are suitable for commercial production, found to produce irregular masses, fibrous suspension, pellets and spheres of BC. The BC produced in the ALR has unique elliptical pellets. The results showed the BC in pellet form has more compact and dense structure than that in fibrous form. In STR bioreactors, maxblend and the gate with turbine impellers showed good mixing properties and produces high volumetric oxygen transfer coefficient in the broth. On the other hand, the internal-loop ALR produce higher BC yield. Modified ALR with a circular wire mesh draft tube has more oxygen transfer

coefficient. The modification of ALR having concentric wire-mesh draft tubes has a high gas hold up and lower mixing time than the conventional bubble column. It also requires a shorter cultivation period to reach the maximum cell concentration. Table 1 summarizes the BC production using different culture methods.

Table 1. BC production under different culture conditions

Strain	Apparatus	Carbon Source	Contributions	BC Production (g/L)	Time (hr)	Reference
<i>A.xylinum</i> BPR2001	Jar fermentor, Shaken Flask (180 rpm)	Fructose + Corn steep liquor	-----	7.7, 4.4	73, 72	(Toyosaki <i>et al.</i> , 1995)
<i>A.xylinum</i> SpV6	Aeration, shaking, ring	Glucose	Ethly Alcohol	1.4	49	(Ciechańska <i>et al.</i> , 1999)
<i>A.xylinum</i> Strain DA	Shake flask (200 rpm)	Glucose	Without inorganic salts, with inorganic salts	1.58 , 4.16	192	(H. -. Son <i>et al.</i> , 2003)
<i>A.xylinum</i> Nust4.1	Shake Flask, STR (200 rpm)	Glucose + Sucrose + Corn Steep Liquor	Sodium Alginate (0.04%)	6.0, 1.89	60	(Kouda <i>et al.</i> , 1996)
<i>A.xylinum</i> subsp <i>sucrofermentans</i> BPR 2001	50-L internal loop ALR	Fructose + Corn Steep Liquor	Air, oxygen enriched air	3.8, 8	67	(Chao <i>et al.</i> , 2000)
<i>G.xylinum</i> subsp <i>sucrofermentans</i> BPR 2001	50-L ALR, STR	CSL-Fru	Agar (0.1w/w)	8.7, 6.9	65	(Chao <i>et al.</i> , 2001)
<i>A. xylinum</i> BPR2001	Shake Flask (175rpm), (375 rpm)	Fructose + CSL	Polyacrylamide-co-acrylic acid (3g/L)	(2.7-6.6), (1.7-3.7)	49	(Joseph <i>et al.</i> , 2003)
<i>A. xylinum</i> BPR 2001	Bioreactor (stirrer speed (700 rpm)	Fructose + CSL	Polyacrylamide-co-acrylic acid (2g/L)	5.30±0.74	72	(Joseph <i>et al.</i> , 2003)
<i>GluconGluconoacetobacter hansenii</i> PJK (KCTC 10505 BP)	Jar fermentor with spin filtr (500 rpm)	Glucose	-----	4.57	140	(Jung <i>et al.</i> , 2007)
<i>GluconGluconoacetobacter</i> sp. RKY5	Rotary Film Contractor with eight discs aeration rate 1.25 vvm and at (15 rpm discs speed)	Glucose	-----	6.17	96	(Kim <i>et al.</i> , 2007)
<i>A. xylinum</i> sp. A9	Shaking Flask	Glucose	Ethanol	15.2	192	(H. J. Son <i>et al.</i> 2001)
<i>A.xylinum</i> subsp. <i>sucrofermentans</i> BPR3001A	Stirred Tank	Fructose	Ethanol	-	-	(Naritomi <i>et al.</i> 1998b)
<i>A. xylinum</i> BRC5	Jar fermentor	Glucose	Ethanol, Oxygen	15.3	50	(Hwang <i>et al</i> 1999)
<i>B. xylinum</i> BPR 2001	Jar fermentor	Molasses	Molasses H ₂ SO ₄ heat treatment	7.8	72	(S. Bae <i>et al</i> 2004)
<i>A. xylinum</i> BPR 2001	Jar fermentor	Fructose	Non, (Agar & Oxygen), Agar	5.3, 14.1, 12	72, 72, 56	(S. Bae <i>et al.</i> 2004)
<i>A. xylinum</i> subsp. <i>sucrofermentans</i> BPR2001A	ALR	Fructose	Oxygen	10.4	52	(Chao <i>et al.</i> , 2000)
<i>G.xylinum</i> subsp. BPR3001A	ALR	Fructose	Agar, Oxygen	8.7	44	(Chao <i>et al.</i> , 2001)
<i>G.xylinum</i> E ₂₅	Rotating	Glucose	No	3.5	168	(A. Krystynowicz <i>et al.</i> , 2002)

Chapter 3

Materials and Methods

3.1 Preparation of Bacterial Strain and Culture Conditions

Gluconoacetobacter xylinum (ATCC 700178) was purchased from the American Type Culture Collection (ATCC), Manassas, VA 20108, USA. The YGC 459 medium, glucose 50.0g, yeast 5.0g, CaCO_3 12.5g, agar 15.0g (for solid medium only) and 1 L distilled water were used to activate the bacteria. Liquid stock of the culture was prepared by transferring the freeze-dried culture into the liquid YGC 459 medium (without Agar) and then stored at -80°C . The solid YGC 459 medium (with Agar) was used to prepare Agar plates. The bacteria from the liquid medium were transferred aseptically onto the Agar plates. Agar plates were placed at 28°C in the Incubator, (Symphony, 5.3 A, VWR), for bacterial growth.

3.2 Medium Used

The medium reported by Chao *et al.*, (2000) has all the essential components required for the bacterial metabolism. All chemicals: KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $(\text{NH}_4)_2\text{SO}_4$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, Inositol, Nicotinic acid, Pyridoxine hydrochloride, D-pantothenic acid calcium, Riboflavin, Folic acid, D-biotin, thiamine hydrochloride, fructose, glucose, sucrose, L(+) arabinose, D-(+)-xylose, mannose, and D-(+) galactose; were purchased from Sigma-Aldrich and were used as received from the supplier. Corn steep liquor (CSL) was supplied by Casco, London, Ontario, Canada, upon request and used as received. Media composition was as follows: 20 mL/L of CSL (nitrogen source), sugars (carbon source: single or mixtures; Table 2) 40 g/L, 1 g/L of KH_2PO_4 , 0.25 g/L

of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.3 g/L of $(\text{NH}_4)_2\text{SO}_4$, 3.6 mg/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 14.7 mg/L of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.42 mg/L of $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 1.73 mg/L of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.39 mg/L of $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 0.05 mg/L of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2 mg/L of Inositol, 0.4 mg/L of nicotinic acid, 0.4 mg/L of pyridoxine hydrochloride, 0.2 mg/L of D-pantothenic acid calcium, 0.2 mg/L of riboflavin, 0.2 g/L of folic acid, 0.2 $\mu\text{g/L}$ of D-biotin and 0.4 g/L of thiamine.

3.3 BC Production in Shake Flasks with Different Feedstock

Initially, ten shake flasks of 500 mL were prepared with either single sugars or sugar mixtures. The total initial concentration of sugar (single sugar or mixture of sugars) in the feedstock was always kept at 40 g/L with pH 5.0. Single sugars examined were arabinose, xylose, glucose, mannose, fructose, galactose, and sucrose. The compositions of the sugar mixtures that were examined as feedstock were identical to the sugar compositions of acidic hydrolyzate extracts of WS, CF and DDGS as previously reported in the literatures. These sugar mixtures were named Mix_1_WS, Mix_2_CF and Mix_3_DDGS respectively. Table 2 summarizes the composition of the different feedstock examined in the present study.

Table 2. Approximate composition of sugars (g/L) in acid hydrolyzate of agricultural residues.

Equivalent Agriculture Residue	Wheat Straw	Corn Fiber	DDGS
Feedstock	Mix_1_WS	Mix_2_CF	Mix_3_DDGS
Glucose	19.2	21.35	17.95
Xylose	13.3	10.1	12.7
Arabinose	3.3	6.45	7.8
Galactose	2.3	2.05	0.9
Mannose	1.75	0.0	0.6
Reference	Qureshi <i>et al.</i> (2007)	Grohmann <i>et al.</i> (1997)	Ezeji <i>et al.</i> (2008)

The sugars were sterilized separately as recommended by Solomons, (1969) because the sugar may react with the ammonium ions and amino acids and produces black nitrogen containing compounds which may have negative impact on the growth of many micro-organisms. CSL was warmed separately to reduce viscosity. Warmed CSL was then added into the flasks that contain solution of sugars and medium components. The distilled water was then added to bring the volume up to the total volume of 200 mL. The acidity was adjusted to pH 5.0 by using 2N NaOH and 2N H₂SO₄. The medium was then sterilised in the autoclave (MLS-3780, Labo autoclave, Sanyo) for 20 min at 121⁰C. The medium was then left to cool down to room temperature. After cooling down to room temperature, the sterilized flasks were aseptically

inoculated with 2 mL (1 mL of inoculums/ 100 mL of the medium) of the inoculums suspensions of *G. xylinum* (per flask). The top of all the flasks were then covered with sponge and the aluminum foil. These flasks were then placed on the shaker (MaxQ 2000 CO₂, Thermo Scientific) at a shaker speed of 175 rpm at 29°C for 7days. Samples were taken periodically from the culture broth for the analysis of the amount of BC, sugar concentration, pH and viable cell number.

3.4 BC Production in Bioreactors (stirred tank and airlift)

In this study, a 2 litre Biostat. B reactor of B. Braun Biotech Inc. was used. The bioreactor has PID control and processes the data using highly integrated microprocessor-board (DFC-2) with 80C188 processor. The temperature was measured by temperature sensor Pt 100. It has Ingold pH electrode with pasty electrolyte and Ingold pO₂-Electrode. It had dosing counter for acid/alkali agent pump. Figure 5 shows schematic diagram of the bioreactor used in this study. Production of BC in bioreactors was examined using Mix_1_WS as the feedstock (see Table 2 for compositions). A working volume of 1.5L was employed throughout all fermentation experiments. Figure 5&6 show the schematic diagram and the experimental set up of the bioreactors used in this study.

Figure 5. Schematic diagram of the bioreactor used in this study.

Figure 6. (a) Experimental set up of (a) ALR and (b) STR.

3.4.1 Stirred Tank Bioreactor

In order to examine BC production in STR, gate with turbine impeller was utilized. According to the literature, gate with turbine impeller (See Figure 3) was the most suitable for BC production as it increases the dissolve oxygen concentration in the medium and increases the aeration capacity of the fermentor, k_La (Kouda *et al.*, 1997). Table 3 shows the specification of this impeller. Figure 7 shows the schematic diagram of the STR with the “Gate with the Turbine” impeller.

Table 3. Sparger and agitator sizes.

Agitator	d/D	h (mm)	L (mm)	ds (mm)	hs (mm)
Gate with Turbine	0.5	30	86	45	15

d/D is the ratio of impeller diameter (d) to vessel diameter (D); h is the distance between vessel bottom and impeller; L is the height of the impeller; ds is the diameter of the ring sparger; hs is the distance between vessel bottom and the ring sparger.

Figure 7. STR equipped with the “Gate with the turbine” impeller.

The bioreactor charged with the medium and the sugar (i.e., Mix_1_WS) was autoclaved at 121⁰C for 20min. The CSL was autoclaved separately, which was added to the bioreactor contents later after autoclaving. The bioreactor was then inoculated aseptically at room temperature with 10 mL of the inoculum prepared as explained above (1mL of inoculums / 100 mL of the medium). Then, 2-3 drops of Anti foam were also added. Operating conditions of the bioreactor were: pH 5, impeller speed 175rpm and the temperature 29⁰C and the air flow rate of 3 l/min were also set. To maintain the pH at 5.0, solutions of 2N NaOH and 2N H₂SO₄ were used. Samples were taken periodically from the culture broth for the analysis of the amount of BC, sugar concentration, pH and viable cell number.

3.4.2 Draft tube Airlift Bioreactor

To examine production of BC using ALR, a draft tube was designed and mounted inside the bioreactor. Literature review showed that d/D ratio (d/D is the ratio of the draft tube diameter (d) to vessel diameter (D)) of 0.5 gave high production of BC (Chao *et al.*, 2000; Kouda *et al.*, 1997). The height of the draft tube was 950mm. Figure 8 shows the schematic diagram of ALR. Table 4 shows the specifications of the draft tube used in this study.

Table 4. Dimensions of the solid draft tube used in the ALR.

Tube	d/D	h (mm)	L (mm)	ds (mm)	hs (mm)
Soild draft tube	0.5	30	950	45	15

d/D is the ratio of the draft tube diameter (d) to vessel diameter (D); h is the distance between vessel bottom and draft tube; L is the height of the draft tube; ds is the diameter of the ring sparger; hs is the distance between vessel bottom and the ring sparger.

Figure 8. Schematic diagram of the ALR with solid draft tube.

Working volume of 1.5 litres was employed for this experiment. At first, 1500 mL of the medium with sugar mixture of Mix_1_WS was prepared. The procedure for sterilizing and setting up the bioreactor was same as was in the STR. The ALR is then inoculated with 15 mL of inoculums (1mL of inoculums / 100 mL of the medium) using pipette. Then, 2-3 drops of Anti foam were also added. The jacket around the reactor filled with circulating water from the controller was used to control the temperature at 29⁰C during the cultivation. To maintain the pH at 5.0, solutions of 2N NaOH and 2N H₂SO₄ were used. The air flow rate was set at 3 l/min. Samples were taken periodically from the culture both for the analysis of the amount of BC, sugar concentration, pH and viable cell number.

3.5 Sampling

In all fermentation experiments, samples of 4 mL were first taken after 24hr of inoculation and thereafter, at equal intervals of 48 h. The sampling procedure was done in the biosafety hood, which was previously cleaned with ethanol and was left in the UV light for 10 min. All other tools such as pipette and the burner were also cleaned with ethanol and left under the UV light for 10min. The samples were taken one by one from each flask. A flask at a time is taken off from the shaker and was shaken vigorously by hand in the lamina flow for homogenizing the medium. In STR and ALR, sample of 4 mL was taken after 24 hr of inoculation and thereafter, at equal intervals of 48h. Samples were kept at -80°C in the freezer until analyzed for cell count and sugar concentration.

3.6 Analytical Techniques

3.6.1 Flow Cytometer

In this research the number of cells/mL of the medium was measured by using flow cytometer Guava EasyCyte Mini, (Guava Technologies). This flow cytometer has a blue laser beam (75mW) of a single wavelength (532 nm wavelength) and is directed on stream of fluid. It has three detectors with the band pass filter ratio of (525/30 nm) for Green, (583/26 nm) for Yellow, and (690/50) for Red. These are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter, FSC) tells the relative size and several perpendiculars to it (Side Scatter, SSC) tell about the cellular granularity and complexity. Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the light in some way. The fluorescent chemicals found in the particle or attached to the particle may be

excited into emitting light at a longer wavelength than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and by analysing fluctuations in brightness at each detector, one for each fluorescent emission peak, it is then possible to derive various types of information about the physical and chemical structure of each individual particle. For example, number of viable cells in medium. Figure 9 shows the flow cytometer used in this study.

Figure 9. Flow cytometer guava easy cyte mini

At first, 1 mL of the sample was centrifuged at 4000 rpm. The supernatant was then filtered using filter of pore size 0.8 μm , Whatman, USA, to get rid of debris larger than bacteria in the medium. Then 5 μL of the supernatant is mixed with 95 μL of distilled water (5% v/v). The sample is then vortexed for 3 min to separate any bacterial cell clots adhering together. Thereafter, samples were separately fed into the flow cytometer using its automated feeding system. Before running each sample, the cleaning procedure was performed according to the manufacturer's guidelines using the prescribed cleaning solution. All the readings were analyzed

by the Guava® Express Plus software Module (built-in software), and graphs were plotted using Microsoft Excel.

3.6.2 Gravimetric Method

The production of cellulose was quantified gravimetrically after 7 days of incubation. The total biomass including the medium in shake flask, STR and ALR was autoclaved separately with 4N NaOH (the volume of NaOH used was 40% of the total medium volume) at 121°C for 20min to kill the bacterial cells. Thereafter, the biomass is centrifuged at 4000 rpm for 20 min using accuSpin™ 400 (Fisher Scientific). The extracted cellulose was repeatedly washed with distilled water and centrifuged three times. The extracted cellulose was homogenised in a grinder (Kenmore) for 15 seconds. Thereafter, 1mL of the sample was pipette out in a previously weight crucible and dried in an oven up to a constant weight for 15min at 80°C. The weight of the cellulose produced in 1mL is then converted to per litre. The pH values of all the flasks were measured before the inoculation of the *Gluconobacter xylinum*. The final pH values were measured after 7 days of incubation. Easy Seven, Mettler Toledo was used for pH measurement. This equipment was calibrated, according to the manual instructions provided by the manufacturer, using buffers of pH values 4.0, 7.0 and 10.0. The pH in STR and in the ALR was maintained at 5 by the controller.

3.6.3 High Performance Liquid Chromatography (HPLC)

In this research the change in the sugar concentration with time was determined by HPLC (High Performance Liquid Chromatography). The gel column used in this study was Aminex HPX-

87H, Biorad, Hercules, USA). The samples were taken by the auto sampler equipment (series 200, Perkin Elmer). The retention time was measured by refractive detector (Refractive Index Detector 2414, Waters). The flow rate of the mobile phase was 0.6 mL/min. The temperature of the column was maintained at 60⁰C. The pressure was maintained at 1150 psi. The mobile phase of 5 mM H₂SO₄ was moved by pump (200 Pump, Perkin Elmer). Data was processed by the computer software (Turbochrom Navigator). Figure 10 shows the HPLC used for sugar concentration analysis.

Figure 10. Perkin Elmer HPLC

Sample of 3 mL from fermentation broth was centrifuged at 4000 rpm for 20 min. The supernatant was filtered through a 0.45 µm filter (Whatman, USA). The samples were then diluted 20 times with distil water before placing in HPLC.

3.6.4 Fourier Transform Infrared Spectroscopy

The samples were air dried completely in the form of thin film which was then placed on a magnetic holder (Czaja *et al.* 2004). The FTIR spectrum was obtained using Perkins Elmer's Spectrometer 100 series, Model Spectrum one. The software used for measuring spectra was also Spectrum one. A mid-range Deuterated triglycine sulfate (FR-DTGS) infrared detector processes signals with a 68340 integrated chip. The spectra were recorded at a resolution of 2 cm⁻¹ with the accumulation of 32 scans in the range of 4000 to 400 cm⁻¹.

3.7 Determination of Volumetric Oxygen Transfer Coefficient k_La Values

Volumetric oxygen transfer coefficient (k_La) was calculated for STR and ALR. The k_La is used to measure the aeration capacity of the fermentor. The larger the k_La the larger is the aeration capacity of the fermentor. More dissolved oxygen in the medium usually translates into BC production (Kouda *et al.*, 1997). The oxygen transfer rate from the air bubble to the microbe is described by the following equation.

$$\frac{dC_L}{dt} = k_La (C^* - C_L)$$

where, C_L is the concentration of the dissolved oxygen in the fermentation broth (m moles/ dm³), t is the time (hours), $\frac{dC_L}{dt}$ is the rate of change in oxygen concentration over a time period, i.e the oxygen transfer rate (m moles O₂ /dm³ h¹), k_La is the volumetric oxygen transfer coefficient (cm/ h¹), a is the gas / liquid interface area per liquid volume (cm²/cm³), C^* is the saturated dissolved oxygen concentration (m moles/ dm³). The rate of oxygen transfer at any given time is

equal to the slope of the tangent to the curve of values of dissolved oxygen concentration in the fermentor against the aeration time (Stanburg *et al.* 1984).

The k_La was measured by the static gassing-out method under atmospheric pressure as described by Chao *et al.*, (2000). After purging out the dissolved oxygen by nitrogen, air was supplied and the transient dissolved oxygen profiles were measured at 29°C by dissolved oxygen probe. The $\ln(C^*-C)$ was plotted as a function of time. The slope of this straight line is $-k_La$. The k_La is then plotted as a function of airflow rate. The k_La , was monitored in suspension solutions containing BC nanofibers at different concentrations. The BC produced in STR and ALR was washed with tap water three times and then suspended in tap water in STR and ALR with proportions of 0.25%(wt/wt), 0.50% (wt/wt) and 0.75% (wt/wt). The agitation speed in the STR was maintained at 175 rpm. The air flow rate was maintained at 1, 2, 3 and 4L/min in both reactors.

Chapter 4

Results and Discussion

4.1 Effect of Different Feedstock on BC Production

Production of BC nanofibers was investigated in shake flask using different feedstock of single sugars and sugar mixtures. Mainly, fructose, sucrose, glucose, xylose, arabinose, mannose, galactose, Mix_1_WS, Mix_2_CF and Mix_3_DDGS (see Table 2) were examined.

4.1.1 Measurement of BC Production

Table 5 shows the BC production, yield (g of cellulose produced/g of sugar consumed), sugar consumption during fermentation, final bacterial cell concentration and final pH of different single sugars and sugar mixtures feedstock for BC production in shake flask culture. Examining Table 5 shows that among all the sugars tested in shake flask culture, fructose gave the highest production of 5.65 g/L. Final pH (~ 4.59) of fructose is closest to the optimum pH value of 5.0 (see Table 5), and this would be the reason behind the higher production of BC nanofibers in fructose medium (Verschuren *et al.*, 2000). Lower pH values and higher productions were obtained in case of sugar mixtures. Good production was obtained in Mix_1_WS although the pH was reduced below the optimum value.

This result shows that pH is not the only factor that affects the BC production as it also depends on the capacity of *G. xylinum* to polymerize the polysaccharide to cellulose (Keshk *et al.* 2005).

Table 5 Results of fermentation of acetobacter xylinum in shake flask.

Feedstock	Average Cell Proliferation Rate (10 ⁷ cells/mL h) ^b	BC Production (g/L)	Sugar consumed %	Yield (g of BC produced/ g of sugar consumed)	BC productivity (g/L hr)	Final pH
Fructose	0.29	5.65	95.58	14.77	0.033	4.59
Sucrose	0.53	4.7	97.75	12.02	0.027	4.13
Glucose	0.36	1.75	94.22	4.64	0.010	3.45
Galactose	0.41	2.45	90.20	6.79	0.014	3.66
Mannose	0.30	2.00	90.76	5.50	0.011	3.54
Xylose	0.48	1.1	82.28	3.34	0.006	3.33
Arabinose	0.26	4.1	88.97	11.52	0.024	3.78
Mix_1_WS	0.29	5.2	73.33	17.72	0.030	3.41
Mix_2-CF	0.29	2.4	86.08	6.96	0.014	3.31
Mix_3-DDGS	0.45	3.75	80.30	11.67	0.022	3.41

^b Calculated based on the hourly increase in cell count.

Apparently, the high fructose consumption may have stabilized pH around 4.5, favouring cell growth and BC synthesis (Verschuren *et al.* 2000) (see Table 5). Weinhouse *et al.* (1972) found that *G. xylinum* produces acetates, pyruvates or succinates in fructose media that promote BC synthesis.

Xylose was found to be the lowest producer of BC nanofibers resulting with only 1.1 g/L of the product. This is in agreement with previously published results, that explained the least importance of xylose in BC production by *G. xylinum* (ATCC 700178) apparently due to the complexity of metabolizing xylose (S. Bae *et al.* 2004; Ishihara *et al.* 2002). Using glucose produced only 1.75 g/L of BC nanofibers. This may be related to most of glucose being metabolized for producing energy (Matsuoka *et al.*, 1996). To date, no work has been published in which arabinose was used alone as carbon source for BC production. Surprisingly, arabinose gave the highest yield of 11.52 among all the seven sugars tested. It appears that arabinose was easily metabolized to produce cellulose. Figure 11 shows the BC yield obtained in shake flask using different feedstock, final pH values of the medium and production of BC nanofibers in g/L.

Figure 11. BC yield obtained in shake flask culture using different feedstock, final pH of the medium and the BC nanofiber production in g/L.

The BC yield using galactose was 6.79 in our study, this is 70% higher than reported Ramana *et al.* (2000). Ramana *et al.* (2000) used HS (Hestrin – Schramm) medium and peptone as the nitrogen source. While, we have focussed on CSL (corn steep liquor) in this study. Corn steep liquor contains proteins, peptides, and amino acids and may have strengthened the buffering capacity of the medium, which may have improved of BC in our galactose medium (Stanburg *et al.* 1984).

Table 5 also shows BC nanofiber production in a shake flask using the mixtures of sugars with composition similar of the agricultural waste residues. These mixtures produced 5.2 g/L, 3.75 g/L and 2.4 g/L of BC nanofibers respectively. Mix_1_WS produced the highest amount of BC nanofibers. Although, glucose forms the major portion of the mixture in Mix_1_WS, the BC nanofibers production from the sugar mixtures of wheat straw, corn fiber and DDGS were found to have increased. Glucose produced only 1.75 g/L of BC nanofibers when used as the sole feedstock. The sugar mixtures (Mix_1_WS, Mix_2_CF, and Mix_3_DDGS) have produced 66.34%, 27.08% and 53.33% more BC nanofibers compared to when pure glucose medium was used. Wheat straw, DDGS and corn fiber produced 7.964%, 33.62% and 57.52% less BC nanofibers respectively as compared to fructose. Based on these results Mix_1_WS was found to be the best producer of BC nanofibers among all three-sugar mixtures tested. Final pH values were recorded after 7days of fermentations.

4.1.2 Cell Growth Analysis

Table 5 shows the average cell proliferation rate in shake flask culture with individual sugars and sugar mixtures. The highest cell proliferation rate increase was observed for sucrose. The

lowest increase was observed for fructose. The cell concentration has been increasing at a constant rate in fructose medium. The cell concentration in Mix_3_DDGS is the second highest among all the sugars tested but it produced 33.62% less BC compared to the top producer fructose. The exponential increase in cell concentration was observed after three days of cultivation in all sugars and sugar mixtures. Among the three sugar mixtures, Mix_3_DDGS had the highest cell concentration at the end of the 7 days of fermentation. The cell concentration in Mix_1_WS and Mix_2_CF were close to each other. However, Mix_1_WS produced 2.8 g/L more BC nanofibers compared to the Mix_2_CF. In comparison to fructose, Mix_1_WS had equal cell concentration and produced only 0.45 g/L less BC nanofibers. In comparison to glucose, the Mix_1_WS had less cells but produced 3.45 g/L more BC nanofibers (see Table 5). This shows that Mix_1_WS was a good mixture of sugars that favours the metabolism of *Gluconoacetobacter xylinum* and produced a good amount of BC nanofibers. Appendix A contains values obtained from cell count measurements (cells/mL) using different feedstock in shake flask culture.

4.1.3 Sugar Consumption

Total fraction of sugar consumed during shake flask fermentation are given in Table 5. At the end of the 7-day fermentation, the xylose concentration was highest, while sucrose concentration was the lowest. Maximum amount of sugar was consumed during the first day of fermentation. The most consumed sugar was sucrose with 39 g/L of sucrose consumed and a yield (gram of cellulose produced/gram of sugar consumed) of 4.7 of BC nanofibers was obtained. Arabinose was also found to be a good substrate for production of cellulose nanofibers and this feedstock

resulted in a production of 4.1 of BC nanofibers. Among galactose, xylose and mannose, galactose was found to be the best producer of the BC nanofibers followed by mannose and xylose. Among the sugar mixtures, Mix_1_WS had the highest concentration of sugars and Mix_3_DDGS had the lowest concentration of sugars at the end of 7th day of fermentation. The BC yield was calculated and Mix_1_WS was found to be the best producer of BC nanofibers (Table 5). The yield of the three sugar mixtures: Mix_1_WS, Mix_2_CF and Mix_3_DDGS was found to be 17.72%, 11.67% and 6.96% respectively. The yield of BC using Mix_1_WS was 16.64% higher than the top producing substrate fructose. These results strongly support the idea of using the mixture of sugars resembling their proportion in wheat straw as a cheap carbon source for production of BC nanofibers. Appendix B and B.1 show the retention times of different sugars obtained by HPLC and the calibration graphs for each sugar respectively.

4.2 Effect of Different Culture Methods on BC Production

Based on the results obtained in the previous section, maximum yield was observed with the sugar mixture Mix_1_WS. In order to improve the production of the BC nanofibers, production was further investigated in STR and ALR using the sugar mixture, Mix_1_WS in the feedstock.

4.2.1 Measurement of BC production

Figure 12 shows comparison for the BC production obtained in shake flask, STR and ALR using the sugar mixture resembling the sugar composition of the agricultural residue, wheat straw. According to the literature there are only two publications in which ALR has been used for BC nanofibers production. Cheng *et al.* (2002) and Chao *et al.* (2000) reported that they obtained BC production of 7.72 g/L and 3.8 g/L by using glucose and fructose respectively in ALR. In

their studies, BC nanofibers production in ALR was further increased by using oxygen enriched air. As shown in Figure 12, in our study maximum BC production of 20 g/L was obtained in ALR. Oxygen enriched air was not used which could have further increased the BC nanofibers production. Normal air supply was solely used in this study. The production in ALR was 1.29 times higher than the STR and 3.8 times higher than the shake flask culture. In STR, 22.6 % less BC was produced compared to the ALR, and 66.40% more cellulose was produced compared to the shake flask culture. Production of BC nanofibers in STR was smaller compared to the ALR because of the shear produced by the impeller in the STR. The low production of BC nanofibers in shake flask can be related to less amount of dissolve oxygen in the medium as no air was supplied during shake flask fermentation. In case of the ALR there was less shear and more dissolve oxygen compared to STR and thus it produced more cellulose (Cheng *et al.*, 2002).

Figure 12. BC production in different reactors using sugar composition which resembles sugar composition of wheat straw.

4.2.2 Cell Growth Analysis

Figure 13 shows the change in cell concentration when wheat straw sugar composition was used in shake flask, STR and ALR.

Figure 13. Changes in cell concentrations of *G. xylinum* as monitored during the 7-day cultivation period in shake flask, STR and ALR.

Figure 13 shows exponential growth in cell number after third day of fermentation in shake flask. The concentration of cells in the STR and in ALR increased at a steady rate. Despite the fact that there is no great difference in number of cells between the two reactors, 6.1 g/L more BC nanofibers were produced in ALR compared to STR. In comparison to shake flask ALR had 50 million less cells on final day of fermentation. However, the final cellulose production was 14.8 g/L more in ALR compared to shake flask. The higher production of BC nanofibers in STR and ALR shows that most sugar was metabolised for BC production instead of reproducing the cells in these reactors. Whereas, most of the sugar consumption in shake flask was utilized for

cell reproduction. Appendix A shows the numerical values obtained in cells count measurement (cells/mL) using Mix_I_WS as a feedstock in shake flask, STR and ALR. These values were used to plot Figure 13.

4.2.3 Sugar Consumption

Figure 14 shows that total consumption of sugar was higher in ALR than STR and shake flask. The total sugar consumption in ALR was 9.23 g/L higher compared to shake flask and 2.28 g/L higher compared to STR. The trend of sugar consumption in all the reactors was similar. On day one, consumption of sugars was higher in ALR followed by shake flask and STR reactors.

Figure 14 Changes in sugar concentration in different reactors.

From day two, the total consumption of sugar in STR increases compared to total consumption of sugars in shake flask. As BC is produced, the viscosity of the broth increases and dissolved oxygen concentration in the medium decreases. This increase in broth viscosity reduces the consumption of the sugars in the ALR compared to STR and shake flask. Increase in broth viscosity would be a possible reason of low consumption of sugars in airlift reactor than other reactors on the 7th day of fermentation. At the end of fermentation, more amount of sugar remained in shake flask compared to STR and ALR. In shake flask, 86.74% more sugar remained compared to ALR, and 21.34% more sugar remained compared to STR. Yield obtained for ALR was the highest among the other two culture methods. The yield was slightly lower than that for STR and much lower for shake flask. Figure 15 shows the BC yield in different reactors. These data indicate that ALR and sugar composition, which resembles acid hydrolyzate of wheat straw, is a good feedstock for BC nanofibers production as it gave the highest yield of 51.84% among other reactors.

Figure 15. Yield (%) (g of BC produced/ g of sugar consumed).

In shake flask, glucose was the most consumed sugar among the five individual sugars of Mix_1_WS. The least consumed sugar was arabinose. The BC nanofibers produced by Mix_1_WS was comparable to the BC produced by the fructose that had produced 5.6 g/L of BC nanofibers in shake flask culture.

Figure 16 shows the changes in concentration of individual sugars of Mix_1_WS in shake flask.

Figure 16. Changes in sugar concentrations of the individual sugars in Mix_1_WS in shake flask

Figure 17 shows the changes in concentration of individual sugars in STR using Mix_1_WS. Among five sugars of Mix_1_WS, glucose was the most consumed sugar. Total glucose consumed at the end of the fermentation was 93.8% followed by the total consumption of xylose, galactose and mannose (82.45%) and arabinose (81.81%). Table 6 shows the average sugar consumption for Mix_1_WS in shake flask, STR and ALR.

Table 6. Average sugar consumption calculated for Mix_1_WS during the BC production in different culture methods.

Medium	Sugar Consumption			Total sugar consumed	Remaining Sugar %
	Glucose	Xylose, Galactose and Mannose	Arabinose		
	g/L day	g/L day	g/L day	g/L day	g/L day
Shake Flask	16.58	9.13	2.73	28.44	28.90
STR	16.96	14.35	2.65	33.96	15.10
ALR	18.60	16.45	3.22	38.27	4.325

The total sugar concentration of Mix_1_WS at the end of the fermentation was 78.71% less in STR than in shake flask. Only 31.61 g/L of sugars out of 40 g/L were consumed in STR. This sugar consumption is 7.21% higher compared to shake flask fermentation.

Figure 17. Changes in sugar concentrations of the individual sugars of Mix_1_WS in STR.

In ALR, same trend of sugar consumption was observed. Figure 18 shows that glucose was the most consumed sugar in the air ALR, followed by the three sugars; xylose, galactose, and mannose. The least consumed sugar was arabinose. The consumption of sugars in ALR was 27.49% higher than in shake flask on the first day of fermentation (see Table 6).

Figure 18. Changes in sugar concentration of Mix_1_WS in ALR.

With individual sugars, glucose was the most consumed sugar among the five sugars of Mix_1_WS in air ALR. Only 9.39% more glucose was consumed in ALR than in shake flask. The total consumption of glucose was 3.89% higher in ALR than in STR. The total consumption of xylose, galactose and mannose is 9.08% higher in ALR than in STR, and 42.99% higher than in shake flask (see Table 6).

Arabinose was the least consumed sugar in all the three reactors. However, 0.49 g/L and 0.56 g/L more arabinose was consumed in ALR compared to shake flask and STR respectively. These results show that oxygen and agitating the medium is needed for high productivity of the

BC nanofibers. Second, Mix_1_WS sugars were found to be a good carbon source for producing BC nanofibers in STR. The Gate impeller is found to be a good design for agitating the medium which increases the dissolved oxygen level in the medium. Lastly, ALR with solid draft tube also found to be a good bioreactor for BC nanofiber production.

4.2.4 Morphological Differences.

The morphology of the BC nanofibers produced was investigated using SEM. Figures 19 (a1,a2 and a3) show the digital pictures of the BC obtained in STR, shake flask and ALR respectively. Whereas, Figure 19 (b1,b2 and b3) shows the SEM pictures of the BC produced in STR, shake flask and ALR respectively.

Figure 19. Morphology of BC produced in (a1 & b1) Shake Flask, (a2 & b2) STR and (a3 & b3) ALR using Mix_1_WS.

Figure 19 shows that BC was produced in fibrous form in STR. While it was produced in pellets form in ALR that are more compact and dense than the fibrous form of BC as it can be seen from the SEM picture of BC pellet (see figure b3). These results are in complete agreement with the previous results reported by Chao *et al.*, (2000) and Cheng *et al.*, (2002). The morphology of the BC depends on the fermentation equipment, for example, static or agitated. It is known that

gelatinous membrane of BC formed at the air liquid interface of static culture (Cheng *et al.*, 2002; Hestrin *et al.* 1954). On the other hand, STR produces fibrous form of BC (Cheng *et al.*, 2002; Watanabe *et al.*, 1998). ALR produces pellets of BC but the sizes of the pellets dependent on the amount of the oxygen supplied (Chao *et al.*, 2000; Cheng *et al.*, 2002). Only sparging air in the fermentation medium greatly reduces the size of pellets (Cheng *et al.*, 2002). BC application in membrane and dried form has already been proposed (Vandamme *et al.*, 1998; Watanabe *et al.*, 1993; Yoshinaga *et al.* 1997). Chao *et al.* (2000) has reported that degree of polymerization of BC pellets was higher than that of cellulose fibers. This high degree of polymerization and thickness of BC pellets would allow its use in new applications e.g. as a carrier of microbial cells (Chao *et al.*, 2000). Figure 20 shows the production of BC in (a) ALR and (b) STR reactors respectively. Examining this figure shows obvious morphological difference of BC nanofibers in different culture methods.

Figure 20. Production of BC in (a) ALR and (b) STRs.

4.2.5 Crystallinity of BC produced using different culture methods

Figure 21 shows the FTIR spectra of the BC produced in shake flask, STR and ALR respectively.

Figure 21. FTIR spectra of cellulose synthesised in shake flask ALR and STR reactors.

Peak assignments of spectra shown in Figure 21 are listed in Table 7. Nelson *et al.* (1964) proposed a ratio of infrared absorption (Abs at 1372/2900 cm^{-1}) as a measure of crystallinity. To calculate the f_a fraction, $f_a = 2.55f_{\alpha}^{IR} - 0.32$ equation was used where f_{α}^{IR} is equal to $\frac{A_{\alpha}}{A_{\alpha} + A_{\beta}}$, A_{α} & A_{β} are the absorbencies at 750 and 710 cm^{-1} , respectively (Yamamoto *et al.* 1996). Where

α and β represents cellulose I alpha (α) and cellulose I (β) respectively. Bacteria and many algae produce cellulose I (alpha) predominantly while cellulose I (beta) is derived from plants.

Table 7. Peak assignments of FTIR bands as shown in Figure 21.

Bands	Absorbance		
	Shake Flask	STR	ALR
OH Stretching vibration at 3346 cm^{-1}	0.780169	0.325157	0.545829
C-H Stretching Vibration at 2900 cm^{-1}	0.768764	0.297733	0.501800
C-H Bending vibration at 1372 cm^{-1}	0.690745	0.271488	0.465577
C-O-C and C-O-H Stretching vibration of sugar ring at 1060 cm^{-1}	0.674479	0.27115	0.451246

However, all native cellulose contains quantities of both allomorphs (Sugiyama *et al.* 1991). Cellulose I (alpha) consists of β -1,4 glucan chains aligned in parallel. This cellulose produced in pellicle form by *A. xylinum*. Cellulose I (beta) consists of anti-parallel β -1,4 glucan chains, found in shaken cultures of *A. xylinum* (Ross *et al.*, 1991). Table 8 shows the I_α and I_β content and the crystallinity of the cellulose produced in shake flask, STR and ALR as calculated from Figure 21.

Table 8. Cellulose I_{α} and I_{β} content (%) and crystallinity of BC from different culture conditions determined by FTIR measurement.

Cellulose sample	I_{α}	I_{β}	% Crystallinity (Abs at 1372/2900 cm^{-1})
Shake flask	88.68	11.32	89
STR	89.62	10.38	91
ALR	90.72	9.28	92

The FTIR spectra of the cellulose produced in different culture conditions are in agreement of the previously published results (Czaja *et al.* 2004). The I_{α} content and the crystallinity of cellulose produced in ALR is more than that produced in STR and shake flask. The I_{α} content of cellulose and the crystallinity also depends on the medium used (Keshk *et al.* 2006). The crystallinity and the I_{α} cellulose content from sugarcane molasses medium were 87 and 41% respectively, and the crystallinity and I_{α} cellulose content for the BC produced from the HS medium were 88 and 42% respectively (Keshk *et al.* 2006). This is the first time when sugar mixture of more than two sugars was used in a medium. The crystallinity obtained using the sugar mixture is comparable to the crystallinity reported by Mikkelsen *et al.* (2009) using fructose, glucose, glycerol and mannitol individually as a carbon source. The crystallinity obtained in the present study is more than reported by Czaja *et al.* (2004) using HS medium (Hestrin and Schramm). The BC produced using sugar mixture shows that it had abundant of I_{α} allomorph, in all three reactors, which is more than what has been reported by Imai *et al.* (1998). This amount is comparable to reported by Zhou *et al.* (2007).

Table 9 shows the general results of the three reactors used for the production of BC nanofibers using sugar mixture Mix_1_WS.

Table 9. General results of fermentation of *Gluconoacetobacter xylinum* in different types of reactor.

	Shake flask	STR	ALR
Initial sugar concentration (g/L)	40	40	40
Final cell concentration (cells/mL)	77,677,144	29,541,297	27,461,285
Residual sugar concentration (g/L)	10.667	8.39	1.414
Sugars consumed (g/L)	29.33	31.61	38.58
Final cellulose concentration (g/L)	5.2	15.48	20.0
Yield by weight (%; g of cellulose produced/g of sugar consumed)	17.72	48.97	51.84
BC Volumetric Productivity (g/L hr)	0.0309	0.0827	0.1190
Crystallinity	0.89	0.91	0.92

4.3 Volumetric Oxygen Transfer Coefficient (k_{La})

Volumetric oxygen transfer coefficient k_{La} in STR and ALR were calculated in suspension solutions that have different concentrations of biocellulose nanofibers. Figures 22a, b and c represent the k_{La} values calculated in STR and ALR with concentrations of 0.25% (wt/wt), 0.50% (wt/wt) and 0.75% (wt/wt) BC fibers and pellets suspension respectively.

Figure 22. Change in k_{La} as a function of Time in STR and ALR with different concentration of BC pellets and fibrous suspension (a) 0.25% (wt/wt) (b) 0.50% (wt/wt) and (c) 0.75% (wt/wt).

Figure 22 a,b and c shows that k_{La} was high in case of lower BC content. This is similar to what was reported by Cheng *et al.*, (2002). Table 10 shows that k_{La} increases with time and air flow rates, for the two culture methods. In general, this increase was higher in ALR, as clearly seen from the slopes of the best fit lines in Figures 22a, b and c.

Table 10. Volumetric oxygen transfer coefficient (k_{La}) values for STR and ALR reactors using different air flow rates.

Airflow rate (l/min)	k_{La} (hr ⁻¹)					
	ALR BC pellets	ALR BC pellets	ALR BC pellets	STR BC fibers	STR BC fibers	STR BC fibers
	0.25%(wt/wt)	0.50%(wt/wt)	0.75%(wt/wt)	0.25%(wt/wt)	0.50%(wt/wt)	0.75%(wt/wt)
	t)	t)	t)	t)	t)	t)
1	9.762	9.372	6.84	8.004	5.394	6.384
2	13.48	13.53	6.69	13.23	8.004	8.286
3	14.904	14.67	8.646	14.676	8.94	9.018
4	18.522	17.916	15.384	15.144	9.978	9.756

As concentration of BC nanofibers increases, the viscosity of the medium also increases which decreases dissolve oxygen concentration in the medium. Chao *et al.*, (2000) found that k_{La} decreases when medium viscosity increases. In this case to increase the dissolve oxygen concentration in the medium air or oxygen enriched air supply is necessary. The high production of BC nanofibers in the ALR is because of the high dissolve oxygen concentration in the medium. The k_{La} in the ALR was 36.58% higher than in STR at airflow rate of 4l/min. The

values of k_La in STR decrease as the BC concentration increases. At constant airflow rate, increasing the agitation speed can increase the dissolve oxygen concentration in the medium (R. C. Fontana *et al.* 2009). However, increasing the agitation speed would need more energy to maintain a homogeneous system that would help the transfer of oxygen to the culture (Hess *et al.* 2002; W. Wu *et al.* 2002). The airflow sets up and maintains the hydrodynamic flow of the medium in the ALR. Increasing airflow rate in the ALR would increase the dissolve oxygen concentration, which enhance BC production.

4.3 Statistical Analysis

As the final cellulose concentration was higher in the ALR. A second replicate of ALR experiment was also performed to confirm the results. The results of the second experiment are compared with the first experiment and are shown in Table 11. A minor error was observed in total sugar consumption and final cellulose concentration between experiments. The increase in the final cellulose production and the sugar consumption is may be due to more number of bacterial cells in the second experiment. The other possible errors are human errors, which include the inoculation, and the error in maintaining the optimum conditions of the bioreactor. The other errors are may be due to analytical equipments e.g. HPLC and flow cytometer.

The yield of the BC increased in the second experiment and the production of BC was 4.26% higher in the second experiment compared to the first experiment. Similarly, 1.17% more sugar was consumed in the second experiment. The final cell concentration was 7.01% higher than the first experiment on the final day of fermentation.

Table 11 Comparison of the results of ALR experiments 1 and 2.

	ALR Exp-1	ALR Exp-2	% Error
Final cell concentration (cells/mL)	27,461,285	29,531,486	7.01
Sugars consumed (g/L)	38.58	39.04	1.17
Final cellulose concentration (g/L)	20.0	20.89	4.26
Yield by weight %; g of BC produced/g of sugar consumed)	51.84	53.50	3.10
BC Volumetric Productivity (g/L hr)	0.1190	0.1243	4.26

Results of the individual sugar consumption and the errors are shown in the Table 12. An error of up to 3% was observed in different sugars consumptions.

Table 12 Comparison of individual sugar consumption in repeated experiments of ALR.

Sugar	Time (Days)	ALR Exp-1	ALR Exp-2	% Error
Glucose	1	18.378	18.023	1.933
	3	18.563	18.195	1.982
	5	18.722	18.479	1.297
	7	18.752	18.907	0.819
Xylose+ Galactose + Mannose	1	16.270	15.777	3.030
	3	16.400	15.851	3.347
	5	16.561	16.475	0.519
	7	16.594	16.911	1.874
Arabinose	1	3.183	3.154	0.911
	3	3.221	3.180	1.272
	5	3.239	3.159	2.469
	7	3.240	3.230	0.308

The instrumental errors of the Biostat. B fermentor from B. Braun Biotech International USA is given in Table 13.

Table 13 Instrumental errorr of Biostat.B fermentor.

Probes	Relative Error (%)
pH	± 0.05 of pH
Dissolve oxygen	$\pm 0.5\%$
Temperature	$\pm 0.25^{\circ}\text{C}$

The overall results show that the second fermentation gave us more than 98.83% accuracy of the sugar consumption and 95.74% accuracy in the final cellulose production that were obtained in the first experiment.

Chapter 5

Conclusion and Future Work

5.1 Conclusion

In the present study, several individual sugars and sugar mixtures were initially tested in shake flasks for producing BC nanofibers. Sugar mixtures investigated had compositions identical to the acid hydrolyzates of several agricultural wastes (wheat straw, DDGS and corn fiber).

Results showed that fructose was the best producer of the BC nanofibers among all feedstock tested in shake flask culture. Among the three sugar mixtures, wheat straw sugar composition was found to be the best feedstock producing 5.2 g/L of BC nanofibers.

The wheat straw sugars composition (Mix_1_WS) was further investigated as the carbon source in STR and in the ALR. Gate impeller was used for agitation of the medium, with 175 rpm, in the STR, as it is known that Gate impeller increases the dissolve oxygen concentration in the medium. For ALR, internal loop air ALR with solid draft tube was used in this study. A pH of 5 and the aeration rate of 3 l/min was maintained in both the reactors. The production of BC nanofibers in STR and ALR was 15.48 g/L and 20.0 g/L respectively. The best yield of 51.84 was obtained in ALR followed by 48.97 in STR and 17.72 in shake flask. This high production of the BC nanofibers in both reactors is because of more dissolve oxygen in the medium and the controlled pH. More cell growth was observed in the STR than in ALR. Higher sugar consumption rate was observed in ALR.

So, Mix_1_WS sugar mixture, which resembles the sugar composition of wheat straw, was found to be the best out of all three sugars mixtures tested. The design of the Gate impeller in STR and solid draft tube in the ALR found to have good effect on producing BC nanofibers.

5.2 Future work

As it is known that, the production of BC can be increased by increasing the surface area of the air/liquid interface (Masaoka *et al.* 1993; Okiyama *et al.* 1992). Rotary disc biological contractor can be used in future to see its effects on producing BC nanofibers. Some important features of this reactor are that it provides more surface area in the form of discs. Medium conditions can be controlled during the fermentation and it is easy to use. Other agricultural wastes sugars compositions for example, rice straw and Soya bean straw can be used to check their suitability for producing BC nanofibers. Actual acid hydrolyzate of wheat straw residue and SSF (simultaneous saccharification fermentation) can also be studied to examine their effects on BC nanofibers production. It is also important to examine the metabolism of *Gluconoacetobacter xylinum* in sugar mixtures. Fed-batch and the continuous systems can also be studied to see their effects on the yield of BC nanofibers.

Appendices

Appendix A

Following are the values obtained in cell count measurements (cells/mL) using different feedstock in shake flask culture.

Days	Glucose	Fructose	Xylose	Sucrose	Arabinose	Mannose	Galactose	Mix 2 CF	Mix 3 DDGS
0	15,321,405	15,321,405	15,321,405	15,321,405	15,321,405	15,321,405	15,321,405	15,321,405	15,321,405
1	11,450,892	15,589,154	62,616,936	46,287,91	36,509,460	18,150,730	10,536,821	64,850,82	13,482,525
3	22,487,332	23,786,280	64,543,608	12,659,9576	41,635,924	50,679,200	17,809,672	15,058,417	21,031,856
5	86,369,504	33,197,180	71,940,368	13,009,7872	50,522,092	73,372,952	21,574,730	28,004,016	82,309,400
7	92,902,608	46,594,324	77,848,392	14,252,566,4	53,704,536	84,728,816	10,892,7432	73,730,432	11,979,8920

Following are the values obtained in cell count measurements (cells/mL) using Mix_1_WS as feedstock in shake flask, STR and ALR.

Days	Shake flask	STR	ALR
0	15,321,405	15,695,345	13,693,303
1	52,927,06	17,984,735	13,981,676
3	95,036,28	21,483,199	18,184,198
5	49,167,880	25,673,591	22,033,546
7	77,677,144	29,541,297	27,461,285

Appendix B

Retention Time of different sugars in High Performance Liquid Chromatography (HPLC)

Sugar	Retention Time [min]
Glucose	9.12
Fructose	9.87
Mannose	9.67
Galactose	9.71
Sucrose	8.60
Xylose	9.75
Arabinose	10.63

Appendix B.1

Following are the calibration graphs for each sugar using a solution of three different concentrations 10, 50 and 100 ppm. The straight line equation was used to calculate the final sugar concentration in the medium.

Appendix C

Following graphs show the % DO (Dissolved Oxygen) with respect to time in STR and ALR having different concentrations of BC fibers and pellets.

Airlift reactor

STR

Appendix C.1

Following Figures show the plot of $\ln(C^*-C)$ mg/L with respect to time at different airflow rates in ALR and STR.

Airlift Reactor

For BC pellets 0.25% (wt/wt)

For BC pellets 0.50% (wt/wt)

For BC pellets 0.75% (wt/wt)

Stirred tank reactor

BC fibers 0.25% (wt/wt)

BC fibers 0.50% (wt/wt)

BC fibers 0.75% (wt/wt)

Appendix C.2

Following are the plots of k_{La} as function of airflow rates.

Airlift reactor

Appendix C.3

STR

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