ENHANCEMENT OF BIOCELLULOSE PRODUCTION IN MIXED MEDIUM CULTURE

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SIEW KAH WENG

Thesis submitted to the Faculty of Chemical and Natural Resources Engineering in partial fulfillment of the requirements for the degree of Bachelor of Engineering

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SUPERVISOR'S DECLARATION

I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Engineering.

Signature:
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STUDENT'S DECLARATION

I hereby declare that the work in the thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

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Special dedication to my family members,
My friends, my fellow colleague
and all faculty members

For all of your support and belief in me

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ABSTRACT

In this research, the results for biocellulose production by *Acetobacter xylinus* in mixed culture medium were reported. Biocellulose production was determined by utilizing different feedstocks of single sugars and sugar mixtures which were applied according to certain glucose to fructose ratio. Data for pH changes and the biocellulose production from every medium culture were thoroughly analyzed. In this experiment, it was ensured that all the samples had the initial temperature and pH of 30ºC and pH 5.5 respectively. The temperature was kept constant throughout the whole experiment while the changed pH value was taken as final pH at the end of the experiment for every sample. The highest production using sugar mixtures of 1:9 glucose to fructose ratio was 1.57g/L. The final pH values recorded in the different sugar mixtures were in the range of 4.0–5.5. The lowest final pH of 4.56 was determined in the medium that contained a single carbon source of glucose, as most of the glucose was converted into gluconic acid and lead to lowest biocellulose production of 0.69g/l. In contrast, the highest pH value of 5.3 was determined in the medium that contained a single carbon source of fructose and lead to the higher biocellulose production of 0.9g/l. Analyzing profiles for final pH and biocellulose production for the medium with higher glucose concentration showed that the glucose was preferable to be converted to gluconic acid rather than biocellulose synthesize. Besides, it was also determined that biocellulose production rate in mixed culture medium was higher than in culture medium that only consist of a single carbon source and this had proved that the experiment of enhancing biocellulose production with mixed medium culture was applicable. Results reported in this study demonstrated that the production of biocellulose can be enhanced by using carbon sources mixture with a suitable ratio. This not only represented that biocellulose would be a renewable source of cellulose in the future, but also might lead to major improvements in production if proper supplements and control were utilized in the fermentation process.

ABSTRAK

Kajian ini memberikan laporan tentang keputusan penghasilan biosellulosa yang dihasilkan oleh *A. xylinum* di dalam medium kultur campuran. Biosellulosa yang dihasilkan dengan menggunakan gula tunggal dan gula campuran mengikut nisbah glukosa kepada fruktosa yang telah ditetapkan sebagai bahan mentah di dalam kultur media. Data untuk perubahan nilai pH dan penghasilan biosellulosa dalam setiap kultur media dianalisis. Di dalam eksperimen ini, suhu dan pH medium kultur dipastikan dalam 30°C dan pH 5.5. Selepas itu, suhu kultur media dipastikan malar sepanjang eksperimen dijalankan. Untuk pH pula, nilai pH yang telah berubah untuk setiap kultur media telah dicatatkan pada akhir eksperimen. Penghasilan biosellulosa yang paling tinggi didapati daripada kultur media yang mengandungi campuran gula dalam nisbah 1:9 glukosa kepada fruktosa iaitu sebanyak 1.57 g/L. Semua nilai pH untuk pada akhir eksperimen untuk setiap sampel ialah dalam lingkungan pH 4-5.5. Walau bagaimanapun, nilai pH yang terendah didapati pada akhir eksperimen ialah pH 4.6 yang didapati dari kultur media yang hanya mengandungi satu sumber karbon glukosa sahaja. Selepas itu, kultur media tersebut menghasilkan jumlah biosellulosa yang terendah iaitu sebanyak 0.69 g/L. Sebaliknya, nilai pH yang paling tinggi didapati dari kultur media yang hanya mengandungi satu sumber karbon iaitu fruktosa sahaja. Kultur media ini menghasilkan jumlah biosellulosa yang lebih banyak iaitu sebanyak 0.9 g/L. Dengan mangkaji dan memperbandingkan semua semua hasil biosellulosa daripada setiap kultur media, adalah didapati bahawa penghasilan asid gluconic lebih diutamakan daripada penghasilan biosellulosa apabila kandungan glukosa dalam media kultur semakin bertambah. Tambahan lagi, ia juga didapati bahawa kadar penghasilan biosellulosa di dalam kultur media campuran yang mengandungi campuran glukosa dan fruktosa sebagai sumber carbon adalah lebih tinggi daripada kultur media yang hanya mengandungi satu jenis sumber karbon. Ini telah membuktikan bahawa usaha untuk meningkatkan penghasilan biosellulosa dengan menggunakan kultur media campuran adalah berjaya. Keputusan eksperimen menunjukkan penghasilan biosellulosa boleh ditingkatkan dengan menggunakan kultur media campuran dan nisbah campuran sumber carbon yang sesuai. Ini bukan hanya menunjukkan biosellulosa sebagai sumber sellulosa yang boleh diperbaharui pada masa hadapan, tetapi juga menunjukkan bahawa jumlah penghasilan biosellulosa boleh ditingkatkan lagi dengan menggunakan bahan mentah dan pengawalan yang sesuai dalam proses penapaian.

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LIST OF SYMBOLS

°C	Degree Celsius
%	Percentage
×	Times
Wt%	Weight Percentage
β	Beta

LIST OF ABBREVIATIONS

A.	*Acetobacter*
BC	Biocellulose
CO2	Carbon Dioxide
DA	Dalton
FTIR	Fourier Transformed Infrared Spectrocopy
IR	Infrared
M	Molarity
O2	Oxygen
SEM	Scanning Electron Microscopy
WAC	Water Absorption Capacity
Wh	Hydrated Weight
Wd	Dried Weight
3-D	Three Dimensional

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

In this chapter, an overview of the study will be introduced. The title of my study is the 'Enhancement of Biocellulose Production in Mixed Medium Culture'.

1.2 BACKGROUND OF STUDY

Anselme Payen was the French Chemist who found out the existence of cellulose in 1838. Samples are taken from the plant matters and the chemical formula of cellulose was designed (Klemm *et al.*, 2005). Thermoplastic polymer (celluloid) was the first cellulose production by Hyatt Manufacturing Company in 1870 (Raymond, 1986). The polymer structure of cellulose was determined by Herman Staudinger in 1920. It was first chemically synthesized in 1992 by Kobayashi and Shoda (Klemm *et al.*, 2005).

Biocellulose is a form of cellulose product, produced by a specified bacteria. It is also called microbial cellulose. It was first recognized as cellulose in 1886 (Kuga and Brown, 1988). The bacteria which can produce cellulose are from the genera *Aerobacter, Acetobacter, Achromobacter, Agrobacterium, Alacaligenes, Azotobacter, Pseudomonas, Rhizobium* and *Sarcina* synthesize cellulose (Ross *et al*., 1991). However, Acetobacter is the only species which can synthesis enough cellulose for commercial purposes. *Acetobacter xylinum* is the mostly used bacteria species for commercial interest. *A. xylinum* is recently renamed as *Glucoacetobacter xylinus* (Ross, 1991).

In a survey, it shows that the industry produces 200000 tons of biocellulose in 2006 and is targeted to be reaching 5000000 tons during 2015 align with the increasing world demand for biocellulose products as is estimated that the world market will face the problem of inadequate fossil feedstock in the next 10 to 20 years (Williams, 2006). Hence the aim of this research is to enhance and improve the production of biocellulose align with the rapid increasing demand of biocellulose products.

1.3 PROBLEM STATEMENT

Most of the industrial polymer productions such as plastic products are giving negative impacts towards our mother nature. Many of the synthetic polymers nowadays are manufactured from petrochemicals and are non-biodegradable (Gautam, 2009). Plastics are a product of polyethylene polymer (from fossil resources) that is unable to be biodegraded. The continuous usage of non-biodegradable products will lead to environmental pollutions. A research done in 2009 has determined that about eight billion plastic bags are used in Malaysia (Rajeswary and Himanshu, 2010). Even there is recycling system, there is not much plastic is being recycled and pollutions still going on, contributes to greenhouse effect. There are over 380 billion plastic bags are used annually but only 5.2% is sent for recycling, so the others plastics are left on earth forthousands years (Thangham, 2007).

Moreover, fossil carbon source is limited and will out of stock one day (Steinbüchel, 2005)**.** Hence, cellulose which is biodegradable is the next polymer that will replace the non-biodegradable polymer. However, cellulose is gained form plants and will damage the Mother Nature too if trees are cut off to obtain cellulose. Therefore, biodegradable biocellulose that is produced by bacteria (*A. xylinum*) is preferred to reduce the consumption of trees. In the future, degradable polymers will be replacing the today's commercialized plastic products in market (Gautam, 2009). Therefore, ways for enhancement of biocellulose production are significant as the bio cellulose is the alternative polymer which will be used worldwide in the future and using mixed medium culture is one of the biocellulose enhancement efforts.

1.4 RESEARCH OBJECTIVE

The main objective of this study on Enhancement of Biocellulose in Mixed Medium Culture is to determine for the most suitable carbon sources mixture composition to enhance the production of biocellulose in mixed medium culture.

1.5 SCOPE OF STUDY

This research is based on experimental studies of biocellulose production, using mixed medium culture. To achieve the objectives mentioned above, three scopes have been identified:

i. To produce biocellulose using fructose and glucose which are used to prepare the mixed medium culture.

ii. To analyze the properties of biocellulose using FTIR.

iii. To characterize the morphology of the produced biocellulose by using Scanning Electron Microscope.

1.6 RATIONALE AND SIGNIFICANCE OF STUDY

Normally, single sugar is used in preparing the medium for biocellulose production. For example, biocellulose is produced from medium culture containing glucose with *Acetobacter xylinum* (Masaoka *et al.*, 1992)**.** In this research, the medium culture is prepared using fructose glucose mixture in different ratio. The ratio of the two components in the mixture that will produce the highest amount of biocellulose is determined in the end of the research and will be recommended for the usage in the real polymer industry. It will be a new era for polymer industry.

In the previous research which was done by Yaser Dahman, Kithsiri E. Jayasuriya and Magdalina Kalis, the biocellulose production rate in mixed medium culture was higher than the biocellulose production rate in single culture medium because the analyzed data proving that big sum of the metabolized sugars are mostly synthesized for bacterial cell growth and maintenance but not for biocellulose

production in medium culture with single sugars, causing low cellulose production. On the other hand, sugar was consumed was for synthesizing biocellulose production with sugar mixtures (Dahman *et al.*, 2010). This is very helpful information for the effort to enhance the biocellulose production in this era. Hence in order to be more advance in the effort of biocellulose production, this research has been done by using the mixed culture medium too but this research is a further on project as the optimum ratio of two carbon sources in the mixed culture medium which will produce the maximum amount of biocellulose is the main target to be determined.

As biocellulose is the alternative polymer in the future, it has a high potential to be commercialize in a big scale. Hence, the enhancement of biocellulose production is for the increasing demand for biocellulose products (Joong, 2001). Biocellulose can be used to produce biodegradable plastic products, facial mask, biopaper and and even used in medical field (Rainer and Farah, 1998**).** The market of bioprocess product is developing rapidly.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

A review of literature is performed to identify studies relevant to the topic. The main source for the literature search is the Science Direct website. The review is organized chronologically to offer insight to how past research efforts have laid the groundwork for subsequent studies, including the present research effort. The review is detailed so that the present research effort can be properly tailored to add to the present body of literature as well as to justly the scope and direction of the present research effort.

2.2 BIOCELLULOSE

Cellulose is a polymer that we can be determined in most of plants. On the other hand, biocellulose is the cellulose produced by bacteria; Acetobacter species called *Acetobacter xylinum* or recently is called *Gluconacetobacter xylinus* (Ross *et al.*, 1991). Similar to cellulose, biocellulose is a biodegradable polymer which is used to produce environment friendly products. Biocellulose is mainly designed to replace the usage of cellulose to produce things such as paper and also to replace the usage of non-biodegradable polyethylene polymer for plastic products. This is because the production of papers needs a lot of trees to be cut off and this is proved to be harmful to environment since long time ago (Sangok and Shoda, 2005). Moreover, the usage of non-biodegradable polymer products can be on Earth for thousands of years as pollutants (Gerald, 2008). Therefore, an end must be put on this after the usage of biocellulose products are introduced worldwide and this will absolutely reduce the

pollution rates. This is significant as the pollution on our earth nowadays has reached its critical level.

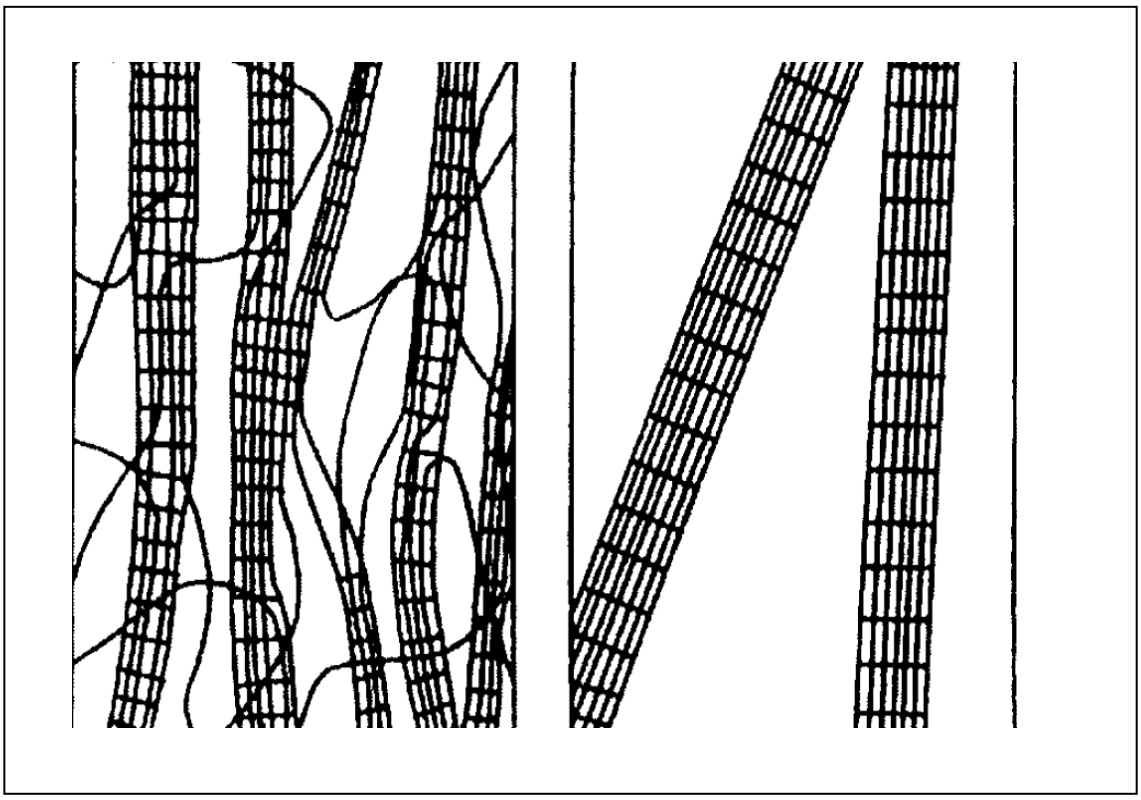

Figure 2.1**:**Schematic model of biocellulose micro fibrils (right) drawn in comparison with the ‘fringed micelles’ of plant cellulose (left) fibrils

Source: Iguchi *et al.* (2000.)

(a)

(b)

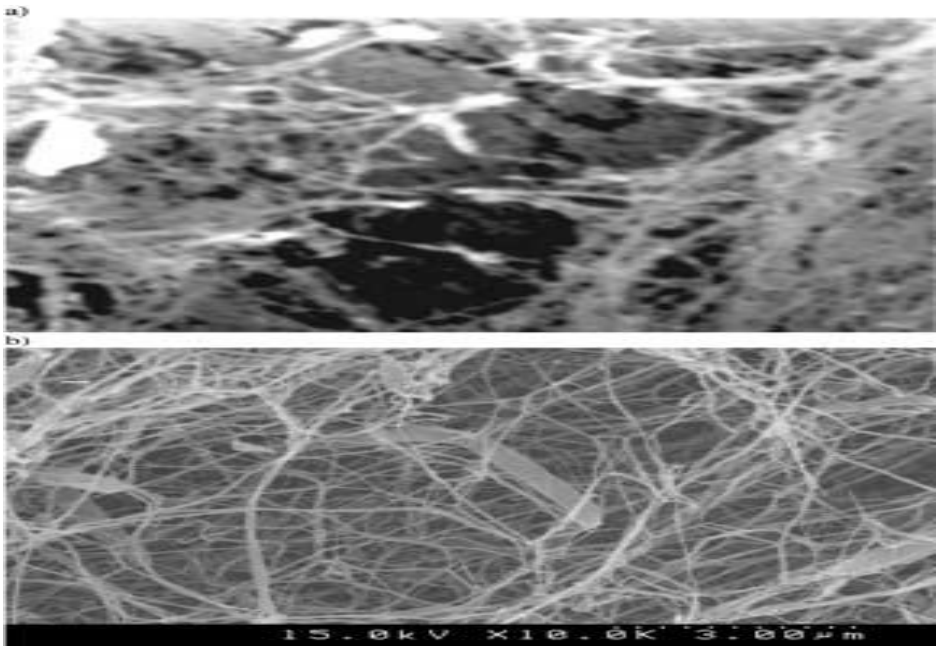

Figure 2.2: Scanning Electron Microscopy images of BC membrane from static culture of *A. xylinum* (a) and bacterial cells with attached cellulose ribbons (b)

Source: Stanislaw *et al.* (2010)

From Figure 2.1, we can observe the structure differences between biocellulose and Plant Cellulose. It shows that the plant cellulose is associated with naturally formed lignin and hemicellulose and they are hard to be removed. This means that plant cellulose is harder to be purified than biocellulose. Then from Figure 2.2, we can notice that the Biocellulose is in the form of fibrils on the surface of the medium culture via the microscopic image. The second microscopic image shows that the rod-shaped Acetobacter *xylinum* is within the biocellulose fibrils.

The biocellulose structure begins to be formed in its actual biosynthesis when the carbon compounds within the culture medium are utilized by *A. xylinum*. After that, it is polymerized and becomes single, linear b-1,4-glucan chains and is excreted into the surrounding medium via a linear row of pores, situated on the outer membrane. The assemble of the b-1,4-glucan chains in the outer part of the cell is a precise, hierarchical process. Then, they start to form subfibrils that consist of 10–15 nascent b-1,4-glucan

chains, microfibrils, and lastly becoming bundles of microfibrils, containing loosely wound ribbon which is consisting of an estimated amount of 1000 individual glucan chains (Ross *et al.*, 1991). Consequently, a thick, gelatinous membrane is formed in the static culture conditions as we can see in Figure 2.3. It is characterized by a 3-D structure comprising of an ultrafine network of cellulose nanofibres (3–8 nm) that are very uniaxially oriented (Czaja *et al.*, 2004).

Sucha 3-D structure which cannot be found in plant cellulose can bring about high cellulose crystallinity (60–80%) and powerful mechanical strength for biocellulose. Biocellulose is stronger than plant cellulose but the biocellulose fibrils are about 100 times smaller than that of plant cellulose. Hence, this unique nano-morphology gives rise to a bigger surface area which enables biocellulose to keep a larger amount of water (up to 200 times of its dry mass). In addition to that, biocellulose also performs great elasticity, high wet strength, and conformability. The small size biocellulose fibrils is determined to be the main factor that bring about its incredible compound for wound healing system. Moreover, the unique property of the biocellulose makes it to be a never-dried cellulose membrane which is a very nano-porous substance. For example, biocellulose enable antibiotics to be transferred in to the wound and be the protecting barrier against any external infection (Bielecki *et al.,* 2002). Different from plant cellulose, biocellulose is thoroughly free of lignin and hemicelluloses which can be observed in Figure 2.1 above.

2.2.1 Chemical Structure

Considering the chemical structure of cellulose, it is a homopolymer comprising of glucose glycosidically attached in a b-1_4 conformation while the repeating unit of the polymer synthesis comprises of two glucose molecules linked together under 180 degrees rotation with each other. Biocellulose has a similar chemical structure to that of plant cellulose but differs from their degree of polymerization (Jonas and Farah, 1998). A long straight unbranched polymer chain is produced by the bonding among glucose units in cellulose and the capacity to produce intermolecular hydrogen bonds between adjacent glucan chains is very high. Ribbons of microfibrils are synthesized at the surface of *A. xylinum* cellulose. The dimensions of the ribbons are 3–4 nm thick and 70–80 nm wide and the hydrophobic bonds are used to maintain the shape of the microbial

cellulose sheet (Chawla *et al.,* 2008). Experiment has been brought out and it is concluded the occurring of the initial development of inter- and intramolecular hydrogen bonds in cellulose sheet lead to the formation of cellulose crystalline structure (Bielecki *et al.,* 2005). The existence of tunnels as observed by scanning electron microscope (SEM) argues for some kind of coordination during the pellicle formation and a random formation of cellulose microfibrils (Shibazaki *et al.,* 1995).

X-ray diffraction can be used to distinguish two common crystalline forms of cellulose designated as I and II (Kuga *et al.,* 1993). The microbial cellulose observed under SEM showed a significant difference in appearance of the external and internal surfaces of the pellicles. The external surfaces had irregular clusters of fibrils, whereas internal surfaces were organized in fractured sections. The relative reactivity of the OH groups in the glucose residues has been determined to decrease in the order of 6'OH>2'OH>3'OH. Furthermore, the nitration rate is highly depends on the concentration of nitric acid in the process. The 6'OH groups in the crystalline and disordered components are subjected to nitration at nearly the same rate during lower concentrations (Chawla *et al.,* 2008). These two components are randomly spread in the entire partition of each microfibril. On the other hand at higher concentration, all OH groups go through nitration very fast. There is no regioselective reactivity being identified among the three kinds of OH groups in solid-phase acetylation and this may because of the characteristic reaction which precedes in a very thin layer between the acetylated and nonacetylated regions in each microfibril (Yamamoto *et al.*, 2006*)*).

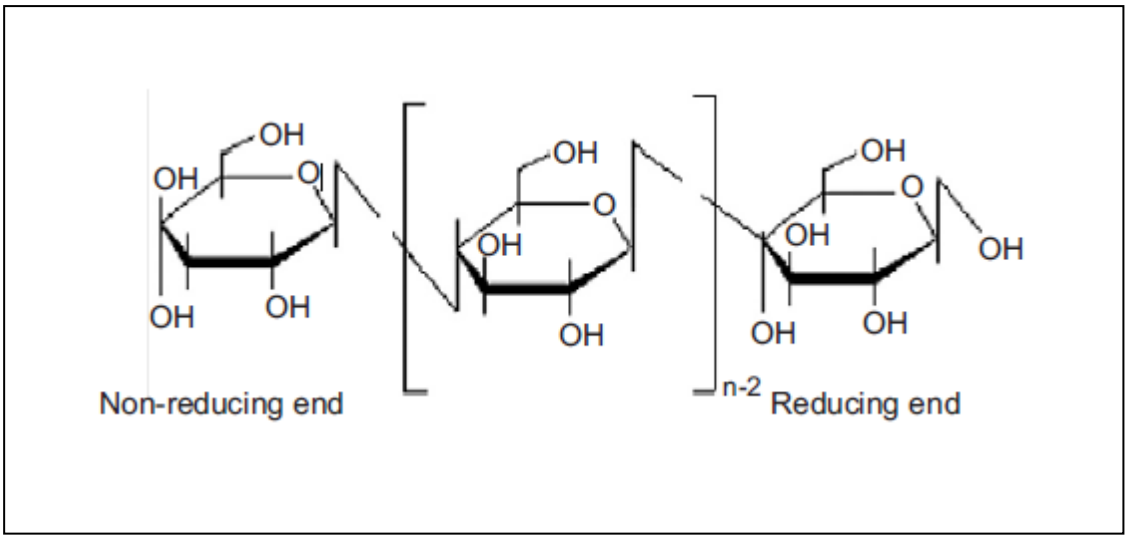

Figure 2.3: Repeating units of cellulose

Chawla *et al.* (2008)

2.2.2 Biocellulose Properties

Biocellulose has high tensile strength, crystallinity, moldability and high degree of polymerization as well as insolubility in most of the solvents (George *et al.*, 2005). Normally, biocellulose fibrils are 0.1–10 mm thick which is a hundred times thinner than that of plant cellulose fibrils with good shape retention. Moreover, its water adsorption capacity is over 100 times higher by mass and it is also much stronger than plant cellulose (Schrecker *et al*., 2005). Macroscopic morphology of cellulose strictly depends on the culture conditions, which can easily be tailored for the physicochemical properties. It has been reported that cellulose fibre has molecular weight of approximately 142.73 kDa and possesses the degree of polymerization of 793 (Wanichapichart *et al*., 2002). It can be dissolved in concentrated acids such as nitric, sulphuric or hydrochloric acid as well as soluble in 8.5 % NaOH solution. The solubility of cellulose in the alkali can be increased by adding 1 % of urea to the solution (Laskiewicz, 1998)). Despite of the higher stability of alkali-treated cellulose membrane which can withstand temperature in the range of between 343 and 370 °C, it can be degraded at a higher temperature above 300 °C. Composites prepared by adding bacterial cellulose and microfibrillated cellulose (MFC) processed through fibrillation of kraft pulp were compared for mechanical properties and it is determined that the

bending strength increased up to 425 Mpa, while the Young's modulus increased from 19 to 28 Gpa, nearly retaining the modulus of the bacterial cellulose sheets (Nakagaito *et al*., 2005).

The uniform nano-scalar network structure is the special characteristic of the biocellulose that lead to its mechanical properties which oriented bi-dimensionally when compressed. The swelling property of cellulose under different conditions has been studied and it has been observed that. NaOH at lower concentration brings about greater swelling in fibres comparing with other alkalis at the same concentrations (George *et al*., 2005). From the observation, it is determined that the percentage mass gain by the cellulose membranes after soaking in different alkaline solution is following the order of NaOH>KOH>Na2CO3>K2CO3. The pervaporation characteristics of deproteinated microbial cellulose membrane are investigated over a wide range of water-ethanol feed composition and it has been found to be promising for dehydration of azeotropes of ethanol and it has a high selectivity towards water at a reasonable flux (Dubey *et al.,* 2002). The cellulose membrane as a molecular separation medium is its basic characteristic in aqueous conditions and it is well defined with the modification with chemical treatments to control its molecular permeation characteristics (Shibazaki *et al.,* 1999). Biocellulose possesses an interesting character that is the ability to control and modify not only the physical characteristics but also the chemical composition of the cellulose fibre (Shirai *et al.,* 1994). Direct dyes such as amide black and trpan red, fluorescent brightening agents or derivatives like carboxymethyl cellulose can be applied to alter the structure of the cellulose assembly (Cousins *et al.,* 1997). *A. xylinum* which is cultivated in Hestrin-Schramm (HS) medium that comprises of acetyl glucomannan avoids the assembly of cellulose microfibrils and the crystal structure of cellulose is changed (Shakairi *et al.,* 1998). In addition to that, loose bundles of cellulose microfibrils also can be observed when *A. xylinum* is cultivated in Hestrin-Schramm medium which consists of glucuronoxylan because glucuronoxylan in the medium prevents the assembly of cellulose microfibrils and changes the crystal structure of cellulose too (Chawla *et al.,* 2008*).* In contrast, pectin which presents in the HS medium helps in assembly of cellulose fibrils but no obvious effect is observed (Tokoh *et al.,* 2002).

2.3 BACTERIA THAT SYNTHESIZE CELLULOSE

Table 2.1: Biocellulose producers

Genus	Cellulose structure
Acetobacter	extracellular pellicle composed of ribbons
Achromobacter	fibrils
Aerobacter	fibrils
Agrobacterium	short fibrils
Alcaligenes	fibrils
Pseudomonas	no distinct fibrils
Rhizobium	short fibrils
Sarcina	amorphous cellulose
Zoogloea	not well defined

Source: Jonas and Farah (1998)

Table 2.1 above shows a variety of bacteria which can synthesize cellulose. The table also indicates that the produced cellulose structure is different for each type of bacteria.

2.3.1 *Acetobacter xylinum*

*Acetobacte*r *xylinum* is the most suitable bacteria used for the production of commercialized biocellulose. It is also known as *Acetobacter xylinum* or *Gluconacetobacter xylinus. Acetobacter* bacteria are normally found to have symbiotic relationships with various plants like sugarcane and coffee plants (Muthukuramasamy *et al., 2002*). *Acetobacter xylinum* is a gram-negative, aerobic bacterium which has been used as a model organism for the study of bacterial cellulose synthesis since long time ago; mainly because *Acetobacter xylinum* is able to produce a large quantity of biocellulose compare with other bacteria (Mayer *et al.*, 1991). For instance, a single *A. xylinum* cell is able to polymerize 200 000 glucose molecules per second into β-1,4 glucan chains that are then secreted to the surrounding medium, forming biocellulose in bundles-like shape (Ross *et al.*, 1991). The biocellulose fibres are synthesized in the membrane by cellulose synthase and are excreted through a row of 50 – 80 pore-like

synthetic sites (Delmerl and Amor, 1995). The formation of this floating cellulose matrix is thought to allow *A. xylinum,* an obligate aerobe, to grow in the higher oxygen tension at the surface of the medium. The cellulose synthase operon (*asc*) has been characterized, showing that the operon contains three genes, acsAB which codes for a 168 kDa polypeptide which is the cellulose synthase and acsC as well as acsD which are involved in cellulose production and crystallization (Saxena *et al,* 1994). The figure 2.4 below obviously shows that the *Acetobacter xynilum* is a rod-shaped bacteria. The biocellulose fibres also can be noticed in the surrounding of the bacteria in the medium.

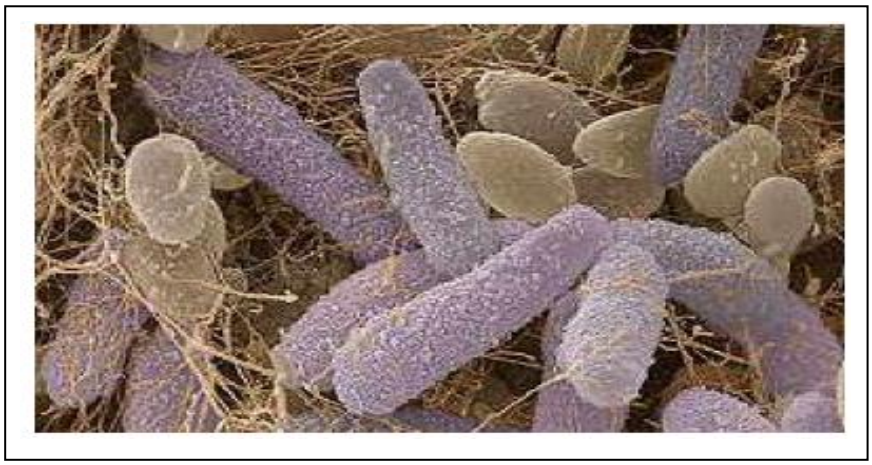

Figure 2.4: *Acetobacter xylinum* within the biocellulose fibre

Source: Norhayati (2009)

2.4 BIOCELLULOSE BIOSYNTHESIZE PATHWAYS

There are 2 types of biological pathways which are single step and multi-step process. The biosynthetic pathway of biocellulose is regulated precisely and specifically under multi-step process and an amount of both individual enzymes and complexes of catalytic as well as regulatory proteins are involved but the supramolecular structures of those components are not well defined yet (Chawla *et al*., 2008).

The pathway comprises of uridine diphosphoglucose (UDPGlc) synthesis and glucose polymerization process. Researches are still being brought out to explore the molecular mechanisms of glucose polymerization into long and unbranched chains as it is still new but the pathways and mechanisms of uridine diphosphoglucose (UDPGlc) synthesis are already well defined. Documentation on biochemical reactions of cellulose synthesis by *A. xylinum* is done extensively (Delmer and Amor, 1995). The process involves the formation of precursor, referred as UDPGlc which is needed to synthesize cellulose, polymerization of glucose molecules into the b-1_4 glucan and nascent chain that produces ribbon-like structure of cellulose chains which comprises of hundreds or even thousands of individual cellulose chains, followed bycell extrusion, and the fibrils assembly by themselves (Bielecki *et al.,* 2005). The cellulose formation is highly related to the catabolic processes of oxidation in *A. xylinum* where a total of 10 % of the energy derived from catabolic reactions is consumed. Formation of biocellulose will not interrupt the synthesis route of protein production or other anabolic processes. *A. xylinum* follows either pentose phosphate cycle or the Krebs cycle coupled with gluconeogenesis (Tonouchi *et al.,* 1996).

2.4.1 Synthesis of the Cellulose Precursor

Many types of carbon compounds like hexoses, glycerol, dihydroxyacetone, pyruvate, and dicarboxylic acids can be synthesized by *A. xylinum* to produce cellulose with an efficiency that reaches about 50. UDPGlc which is a product of a conventional pathway that includes glucose phosphorylation to glucose-6-phosphate (Glc-6-P) which is catalyzed by glucokinase, followed by isomerization of this intermediate to Glc-a-1-P which is catalyzed by phosphoglucomutase, and conversion of the latter metabolite to UDPGlc by UDPGlc pyrophosphorylase, is the direct cellulose precursor (Chawla *et al*., 2008). Different *A. xylinum* strains will lead to different pyrophosphorylase activity and the highest activity has been identified in the most effective cellulose producers such as A. *xylinum* ssp. *Sucrofermentans* BPR2001 (Bielecki *et al.,* 2005). *A. xylinum* contain cellulose synthase which is strongly attached to its cytoplasmic membrane has a molecular weight of 400–500 kDa but it appears to be very unstable. It can be isolated from membranes by using digitonin or detergents and is treated with trypsin followed by its entrapment on cellulose (Chawla *et al.*, 2008).

Cellulose synthases contain conserved amino acids and other glycosyl tranferases which appeared in a large globular region and it is being actively analyzed. The globular region of bacterial and plant cellulose synthases are varies in size while the vascular plant cellulose synthases having at least two insertions. According to the analysis of the transmembrane segments and UDPGlc which presents in cytosol, the globular region is estimated to be cytoplasmic (Brown *et al.,* 2000). Normally, UDPGlc-forming cellulose synthase is the catalyst for cellulose biosynthesis in higher plants or in the prokaryotes. Basically, this is a 4-b-glucosyl transferase process as it converts consecutive glucopyranose residues from UDPGlc to the polysaccharide chain which is newly determined. Oligomeric cellulose synthase complexes are usually referred to as terminal complexes and they are the components that mainly involve in b-1_4 glucan chain synthesis. There are three types of subunits with molecular weights of 90, 67 and 54 kDa in purified cellulose synthase but there are researchers who declare that it only comprises of two polypeptides with molecular weight of 83 and 93 kDa respectively (Chawla *et al.,* 2008). It has been determined that the 83-kDa polypeptide is a catalytic subunit which shows high affinity towards UDPGlc via the study of photolabelling affinity (Richmond *et al.,* 1991).

Usually, the cultivation of *Acetobacter xylinum* uses acetylated carbohydrate derivatives as carbon sources instead of glucose and the biocelluloses produced are analyzed by FTIR to determine the possible incorporation of these substrates into the b-1_4-glucan chains. However, it is impossible to use acetylated glucose as feed for the production of acetylated cellulose as it will lead to complication of active transport phenomenon, specificity of cellulose synthase, or the possibility for the formation of UDPGlc (Eindfeldt *et al.*, 1993).

2.4.2 Mechanism of Biosynthesis

Acetobacter xylinum or any other cellulose-producing organisms including plants follows two immediate steps for cellulose production. First of all is the formation of b-1_4 glucan chain with polymerization of glucose units and secondly, is the assembly and crystallization of cellulose chain (Chawla *et al.*, 2008). The rate of assembly and crystallization limits the rate of polymerization.

i. Formation of b-1_4 Glucan Chain

Cellulose biosynthesis is catalyzed by cellulose synthase when the glucose units are polymerized into the b-1_4 glucan chain. Figure 2.5 below shows the formation of cellulose fibrils and the precellulosic polymer is formed in the cytoplasmic membrane. There are a couple of hypotheses for this mechanism in *A. xylinum*. First of all, it is indicated that the lipid intermediate is not involved in the polymerization of the b-1_4 glucan. The non-reducing end of the polysaccharide is added with the glucose residues while the reducing ends are nascent polymer chains that located away from the cells (Brown *et al.*, 2000). The cellulose molecule has 180° of torsion angle between the two adjacent glucose residues and the twofold screw axis of the b-1_4 glucan is maintained by the growing chain. In contrast, the second hypothesis states that a lipid intermediate is involved in the polymerization of b-1_4 glucan. It has been proved that the synthesis of acetone which is a soluble polysaccharide is involving in the lipid intermediate (Chawla *et al.*, 2008). The purified cellulose

synthase in *A. xylinum* subunits that does not contain the lipid composition catalyzes polymer synthesize.

ii. Assembly and Crystallization of Cellulose Chains

Cellulose has unique structure and properties which is due to the course of extrusion of chains and their assembly at the external part of the cells. The protofibril of approximately 2–4 nm diameter is produced by spinning the precellulosic polymer molecules which is formed inside the bacterial cell, out of the 'cellulose export components' and the protofibrils are gathered in structure of a ribbon-shaped microfibril which has 80′4 nm in diameter (Iguchi *et al.,* 2000).

The presence of some 50 to 80 pore-like sites arranged in a regular row along the long axis of the cell is indicated by electron micrographs of the surface of the cell envelope and is observed to combine with the extracellular cellulosic ribbon (Chawla *et al.,* 2008). It is assumed that these discrete structures of the lipopolysaccharide layer are the sites of extrusion for precellulosic polymers. The cellulose production is initiates by the aggregation b-1_4 glucan chains. The existence of such tactoidal aggregates and of analogous structures in algal preparations shows that the synthesis of a big amount of b-1_4 glucan chains simultaneously at a spatially limited site is a common character of the assembly of cellulose microfibrils in both higher and lower organisms (Chawla *et al.,* 2008). Though the process of assembly and crystallization of cellulose chains occurs in the extracellular space, the mutual orientation and association of glucan chains, aggregates, microfibrils, bundles, and ribbon are apparently governed by the original pattern of extrusion sites and this process is normally defined as cell directed because, (Ross *et al.,* 1991). Stronger aeration or the presence of some particular components which are unable to penetrate through the cells membrane, but can produce competitive hydrogen bonds with the b-1_4 glucan chain, lead to significant changes in the supramolecular organization of cellulose chains. Then, the thermodynamically more stable amorphous cellulose II is produced instead of cellulose I which is a ribbon-like polymer.

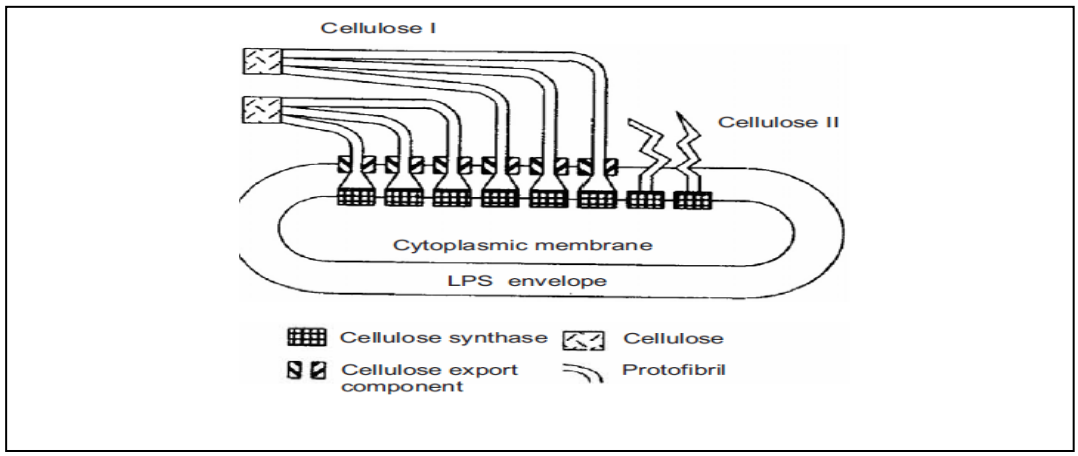

Figure 2.5: Assembly of cellulose microfibrils by *A. xylinum*

Chawla *et al.* (2008)

The figure 2.5 above has shown the differences in the assembly of cellulose I and cellulose II outside the cytoplasmic membrane (Chawla *et al.*, 2008). The speed of 2 mm/min which is the typical chain elongation rate brings about polymerization of more than 108 glucose molecules in the b-1_4 glucan (Bielecki *et al.*, 2005).

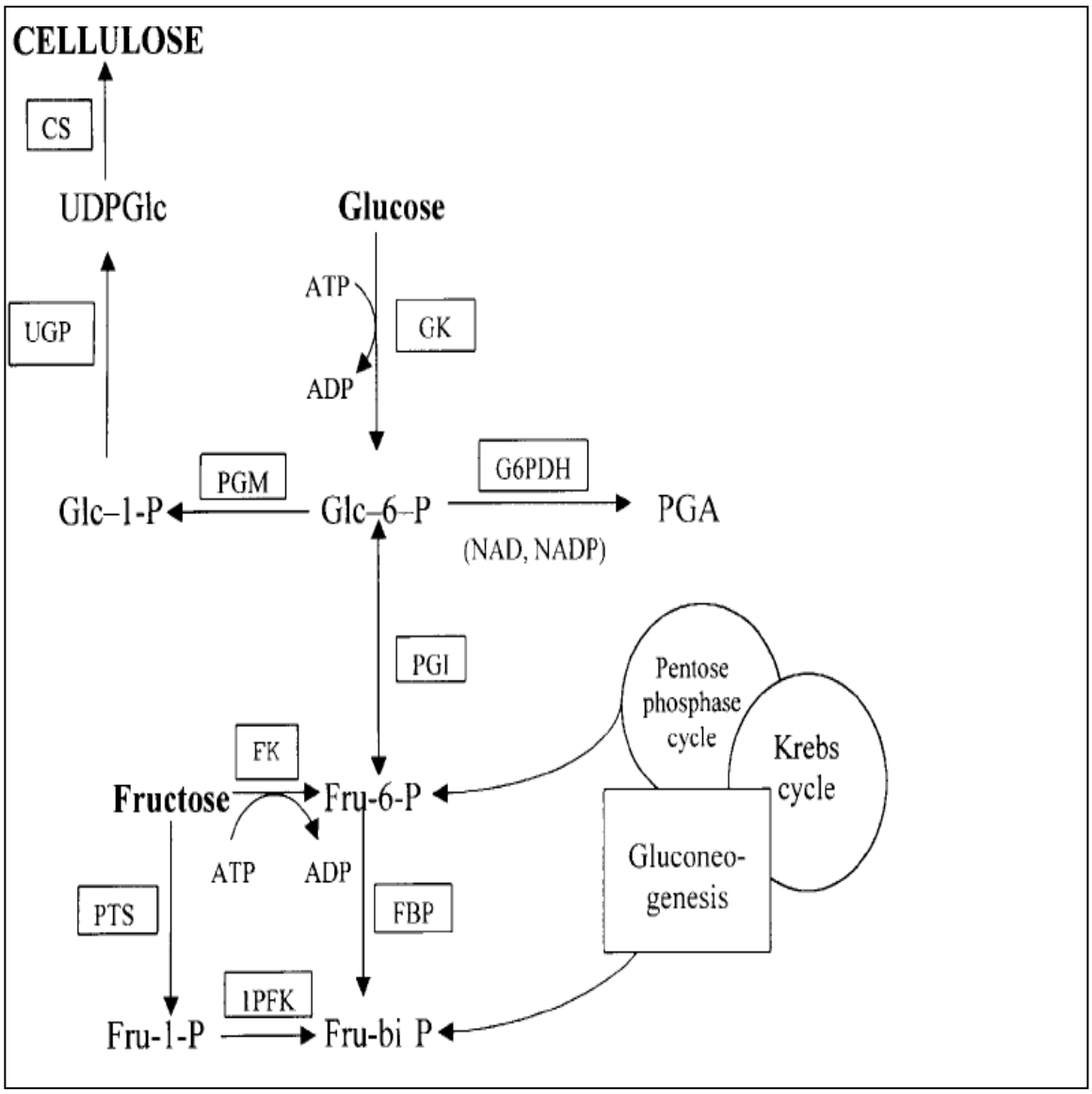

Figure 2.6: Pathways of Carbon Metabolism in *A. xylinum*.

Source: Stanisla, B. *et al.* (2010)

According to the figure 2.6, biocellulose which is synthesized by *A. xylinum* is a final product of carbon metabolism, which depending on the physiological state of the cell involves either the pentose phosphate cycle or the Krebs cycle, coupled with gluconeogenesis (Tonouchi *et al*., 1996).

2.5 THE APPLICATIONS OF BIOCELLULOSE IN INDUSTRIES

Relatively few industries using microbial cellulose are organized; however, as the product becomes more well-known and its properties are further exploited, this undoubtedly will change. A few representative product areas and potential products are (Brown, 2009);

i) Healthfood Industries - Nata de Coco

The Philippine desert, Nata de Coco, has been a cottage industry for the past seventy years in the Philippines (Lapuz *et al*, 1969).

ii) Healthfood Industries - Kombucha Elixir or Manchurian Tea

Kombucha is a fermented product which is consumed by a growing number of individuals for improved health needs. It is a product where the *Acetobacter* along with yeasts are cultured in a medium containing tea extract and sugar (Chang *et al.*, 2011)

iii) Audio Components

Sony Corporation, in conjunction with Ajinomoto developed the first audio speaker diaphragms using microbial cellulose (Martins *et al.*, 2009).

iv) Wound Care Products and Medical Industry

Biocellulose could provide non-woven, shaped objects in medicine such as artificial arteries, vessels, skin, and so on. Certainly its unique water absorptive properties of microbial cellulose would suggest a large potential market in wound care and drug delivery. Because of its high water absorption capacity, 25

wet celluloses can be used as a temporary artificial skin to treat severe skin burns. Moreover, cellulose somehow seems to enhance the growth of human skin cells (Vandamme *et al.*, 1997).

v) Paper and Paper Products

Microbial cellulose has been investigated as a binder in papers, and because of it consists of extremely small clusters of cellulose microfibrils, this property greatly adds to strength and durability of pulp when integrated into paper. Ajinomoto Co. along with Mitsubishi Paper Mills in Japan is currently active in developing microbial cellulose for paper products (Filho, 2001).

2.6 MEDIUM CULTURE

Medium culture is also known as growth culture. There are various types of bacterial medium culture which are used to culture bacteria in the laboratory. Some media formulations require specific recipes, in which certain ingredients must be presented in specific amounts, depends to the specific bacteria to be cultured (Bauman, 2007). These specific media is also defined as synthetic media and they are used for bacterial growth for those bacteria which have very particular needs. Medium can be made in two forms that is in solid agar form or in liquid broth form (Perry and Stanley, 1997).

The medium used in this research is one of these types of growth culture. The *Acetobacter xynilum* requires medium with suitable carbon sources, pH, nitrogen supply, oxygen supply, nutrient supply temperature and so on to be well grown as well as enable the bacteria to produce sufficient amount of biocellulose. Hence, the medium used in the research is the Hestrin and Sehramn medium in liquid form, also called as HS Medium. It contains carbon sources, citric acid, yeast extract which is the nutrient supply, peptone which is the nitrogen supply and dibasic sodium phosphate which act as buffer in the medium (Byrom, 1991). It is the most suitable medium to be used for biocellulose production from *A. xylinum*. The medium applied in the research is known as mixed medium culture because it contains two types of carbon sources which

comprise of glucose and fructose instead of just using a single carbon source in common.

2.6.1 Culture Medium Condition

Medium culture is very important for bacteria cultivation in laboratory or industrial production. It is important to know the optimum range pH and temperature to be applied in the medium for particular bacteria in order to get the optimum amount of production. Different species of bacteria will need different types of surrounding or medium condition in order to live actively.

It is suggested that pH 4 and 4.5 brings about better results for the industrial production in order to avoid contaminations (Farah, 1998). This is because many bacteria cannot adapt to low pH medium condition except acidic bacterium such as *Acetobacter xylinum*. Besides, it is also said that the highest water binding capacity of cellulose has been identified in Hestrin-Schramm (H-S) medium at pH 4.8 to 6.0 (Wlochowicz, 2001). This water binding capacity is an important characteristic of biocellulose to be a wound treatment product's raw material. Furthermore, a high level of cellulose production has been determined within a wide pH range of between 4.5 and 7.5, and is maximum at pH 6.5 (Son *et al.*, 2001). Normally, the optimal pH range for cellulose production by *A. xylinum* is accepted as 4.0 to 7.0 (Delmer and Amor, 1995). A studied has been brought out where the effects of various pHs have been determined using HS medium. As a result, the highest yield obtained is at pH 7 and the lowest yield is obtained at pH 4 (Pourramezan *et al.*, 2009). Besides the pH of the medium culture, temperature is also an important parameter to be observed in the medium culture. This is because the yield of bacterial cellulose is also temperature dependent. It has been revealed that the optimal growth temperature for cellulose production is 25 to 30°C (Cannon and Anderson, 1991).

However, many scientists have used the temperature range from 28 to 30°C in their experiment (Geyer *et al*., 1994). Different medium temperature will cause different degree of polymerisation of the biocellulose and lead to different water binding capacity. Compared to the biocellulose that produced at 25 and 35°C, the biocellulose synthesized at 30°C has a lower degree of polymerization that is about 10,000 and a

higher water binding capacity about 164% (Schmauder *et al.,* 1992). There has been experiment brought out to examine the effects of various temperatures (20 to 40°C) using HS medium. As a result, the determined optimum temperature for cellulose production is at 30°C (Son *et al.,* 2001). There is no significant difference in cellulose production at 25°C but cellulose production reduced above 35°C (Son *et al.,* 2001). Maybe, the bacterium is deactivated at the temperature above 35°C. According to another source, the optimum temperature has been obtained at 30°C too and the lowest yield was obtained at 45°C (Pourramezan *et al*., 2009). Therefore, it can be concluded that the optimum temperature for the growth of *Acetobacter xylinum* is at 30°C and the bacterium cannot live under the medium condition above 45°C. In term of membrane permeability, it has been proved that the permeability of biocellulose obtained at 25°C is less permeable than the one obtained at 30°C (Pineda *et al*., 2007). This may be due to culture temperature that affects and changes the metabolism of the bacteria which influences the rate of biocellulose production as well as the rate of fibril formation.

This is the reason that the pH and temperature chosen to be constant for the medium culture of the project is pH 5.5 and 30°C respectively. Although the pH that gives result in the highest biocellulose production is determined to be 6.5 but considering the contamination issue, a lower pH of 5.5 is selected. Anyway, it is still in the range of optimum pH for biocellulose production.

2.7 NUTRITION AND GROWTH OF BACTERIA

The substances required for energy generation and cellular biosynthesis for every organism must be determined in the surrounding environment itself. The chemicals and elements from the environment which are synthesized for bacterial growth are referred to as nutritional requirements. Many bacteria can be grown the laboratory in culture media which are designed to provide all the essential nutrients in solution for bacterial growth (Todar, 2011).

In every medium culture for microorganism incubation, a suitable environment must be provided for the species under cultivation requires. The basic elements required are oxygen, water, nitrogen source, carbon source, energy source, minerals, vitamins, and trace biochemical. Most bacteria synthesize glucose for energy and carbon sources. Some bacteria are able to utilize light as energy source and others can use sulfur as energy source. As nitrogen source, most bacteria require protein, peptides, or amino acids, but many can use ammonia, nitrates or nitrogen molecules (Eddlemen, 1999).

The genera physiological functions are as shown in the table 2.2. The table 2.2 shows the nutrients that should be applied in a culture medium and their function in the medium. The nutrients applied depend to the microorganism being cultured.

Table 2.2: Major elements, their sources and functions in bacterial cells

Element	% of dry weight	Source	Function
Carbon	50	organic compounds(CO_2)	Main constituent of cellular material
Oxygen	20	H_2O, organic compounds, CO_2, and O_2	Constituent of cell material and cell water; O_2 is electron acceptor in aerobic respiration
Nitrogen	14	NH_3, NO_3, organic compounds, N_2	Constituent of amino acids, nucleic acids nucleotides, and coenzymes
Hydrogen	8	H_2O, organic compounds, H_2	Main constituent of organic compounds and cell water
Phosphorus	3	inorganic phosphates (PO_4)	Constituent of nucleic acids, nucleotides, phospholipids, LPS, teichoic acids
Sulfur	1	SO_4, H_2S, S^o, organic sulfur compounds	Constituent of cysteine, methionine, glutathione, several coenzymes
Potassium	1	Potassium salts	Main cellular inorganic cation and cofactor for certain enzymes
Magnesium	0.5	Magnesium salts	Inorganic cellular cation, cofactor for certain enzymatic reactions
Calcium	0.5	Calcium salts	Inorganic cellular cation, cofactor for certain enzymes and a component of endospores

Source: Todar (2011)

2.7.1 Carbon and Energy Sources for Bacterial Growth

A bacterium must have an energy source, a source of carbon and other required nutrients, and a permissive range of physical conditions such as O_2 concentration, temperature, and pH in order to grow in nature or in the laboratory. Therefore, bacteria are referred to as individuals or groups based on their patterns of growth under various nutritional or physical conditions (Todar, 2011). For example, phototrophs are organisms that use light as an energy source while anaerobes are organisms that grow without oxygen and thermophiles are organisms that grow at high temperatures. All living organisms require a source of energy. Organisms that use radiant energy are called phototrophs. Organisms which oxidize an organic form of carbon are called heterotrophs or chemoheterotrophs such as the *Acetobacter xylinum*. Organisms that oxidize inorganic compounds are called lithotrophs. Among the carbon sources, glucose gave the highest yield, followed by fructose, sucrose and ethanol (Halil and Biyik, 2011).

The carbon requirements of organisms must be met by organic carbon which is a chemical compound with a carbon-hydrogen bond or by CO_2. Organisms which utilize organic carbon are called heterotrophs and organisms that use CO_2 as a sole source of carbon for growth are referred to as autotrophs (Todar, 2011). Hence, four major nutritional types of procaryotes may be defined on the basis of carbon and energy sources for growth as shown in the table 2.3.

Table 2.3: Major nutritional types of prokaryotes

Nutritional Type	Energy Source	Carbon Source	Examples
Photoautotrophs	Light	CO_2	Cyanobacteria, some Purple and Green Bacteria
Photoheterotrophs	Light	Organic compounds	Some Purple and Green Bacteria
Chemoautotrophs or Lithotrophs (Lithoautotrophs)	Inorganic compounds, e.g. H_2, NH_3, NO_2, H_2S	CO_2	A few Bacteria and many Archaea
Chemoheterotrophs or Heterotrophs	Organic compounds	Organic compounds	Most Bacteria, some Archaea

Source: Todar (2011)

However, almost all eucaryotes are either photoautotrophic such as plants and algae or heterotrophic like animals, protozoa and fungi (Todar, 2011). Lithotrophy is unique to procaryotes and photoheterotrophy but common in the Purple and Green Bacteria which is determined only in a very few eucaryotic algae. Phototrophy has not been found in the Archaea, except for nonphotosynthetic light-driven ATP synthesis in the extreme halophiles.

2.7.2 Carbon Sources in Medium Culture for Biocellulose Production.

Table 2.4: The example of carbon sources for biocellulose synthesize in medium culture with *A. xylinum*

	Yield (%)*(Reference)		
Carbon Source	**Keshk & Sameshima (2005)**	**Jonas & Farah (1998)**	**Yield (%)**
Monosaccharides			
Gkucose	100	100	100
Fructose	24	15	
Arabinose		14	21.26
Xylose	38	11	15.87
Disaccharides			
Lactose	22	16	53.17
Maltose	25	7	24.45
Sucrose	69	33	187.41

Source: Pourramezan (2009)

The table 2.4 above shows the carbon sources which are usually used in industry to produce biocellulose. Single carbon source is usually used in the medium culture.

i) Glucose

By far, glucose is the most common carbohydrate on earth. It is identified as a monosaccharide, an aldose, a hexose, and is a reducing sugar. Besides, it is also referred as dextrose as it is dextrorotatory (Charles, 2003). In other words, it has an optical isomer that rotates plane polarized light to the right and also an origin for the D designation. Furthermore, glucose is normally synthesized by chlorophyll in plants using carbon dioxide from the air and sunlight as an energy source. Then it is further converted to starch for storage.

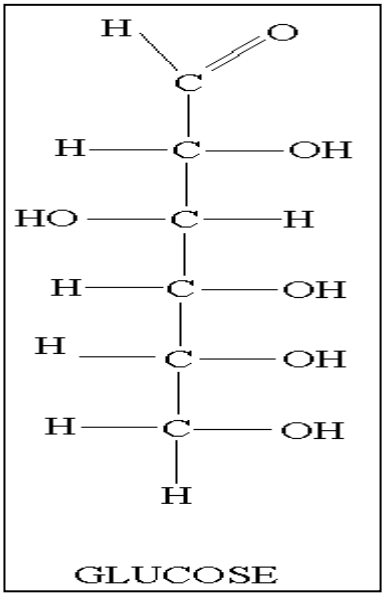

Figure 2.7: Molecular structure of d-glucose

Source: Charles (2003).

However, there is a problem when using glucose as a carbon source in the medium culture of biocellulose production from *Acetobacter xylinum.* In the medium culture with high glucose concentration, it is preferable to be converted to be gluconic acid by *Acetobacter xylinum* rather than biocellulose synthesize (Masaoka, S. *et al.,* 1993). This will reduce the efficiency of cellulose production in the medium. The production of gluconic acid in the medium also will cause the pH to drop till 4.0 and the bacteria is deactivated when it keep decreasing (Young *et al*., 1997). The bacteria growth will be stopped when the acidity of the medium is too high. Due to this fact, applying a high initial glucose concentration in order to produce higher cellulose production is a failure as the excess glucose is converted into (keto)gluconic acids (Vandamme *et al.,*1997). This process is presented in the following figure 2.8. In other words, higher concentration of glucose in a medium culture will decrease the pH of the medium and reduce the biocellulose production after an optimal concentration as shown in the figure 2.9. Hence in the project, mixed culture medium of glucose and fructose is used for biocellulose synthesize enhancement and the optimal ratio is determined.

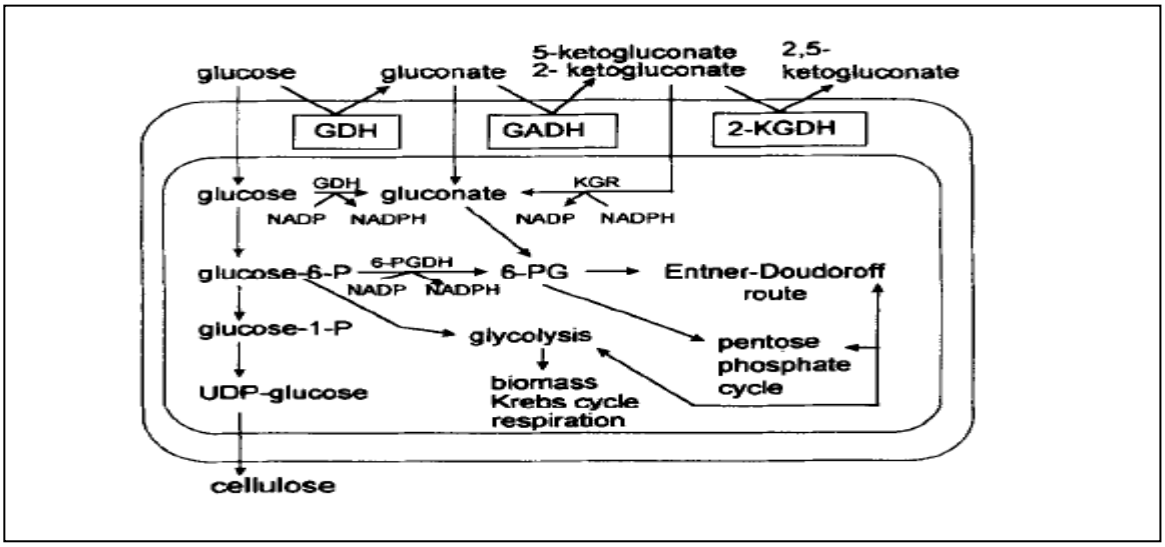

Figure 2.8: Pathways of carbon metabolism in *Acetobacte*r *xylinum*

Source: Wulf *et al.* (1996)

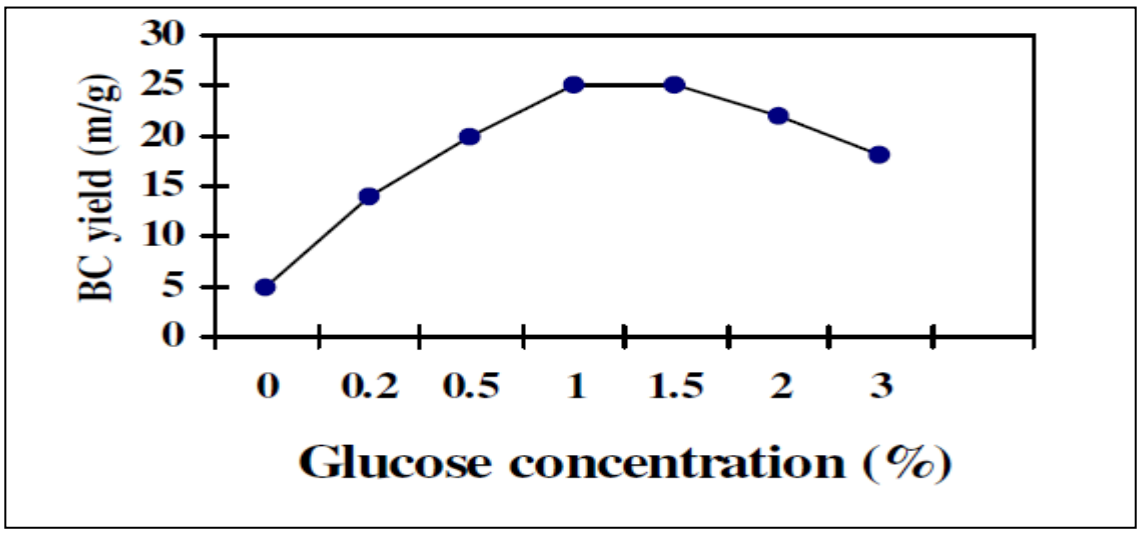

Figure 2.9: Biocellulose yields from glucose culture at various percentages

Source: Keshk and Shameshima (2005)

ii) Fructose

Fructose is the other carbon source which is applied in the mix medium culture with the glucose. When fructose is added in a medium, it will stimulate the biocellulose production (Matsuoka *et al.,* 1996). Fructose is usually determined together with glucose and sucrose in honey and fruit juices. Fructose and glucose is the monosaccharides that can be determined in disaccharide, sucrose (Charles, 2003). Fructose is identified as a monosaccharide. It is also referred as the most important ketose sugar and a hexose. Similar with glucose, fructose is also a reducing sugar. An older common name for fructose is levulose, after its levorotatory property of rotating plane polarized light to the left (Charles, 2003). This condition is in contrast to glucose which is dextrorotatory with the rotating plane polarized light to the right.

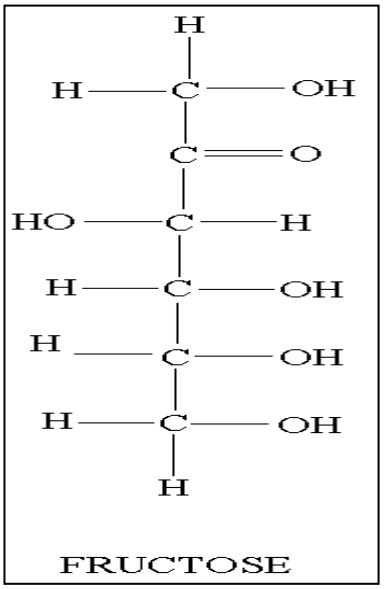

Figure 2.10: Molecular structure of d-fructose

Source: Charles (2003)

2.7.3 Growth Factors

Ignoring the possibility that an organism whether it is an autotroph or a heterotroph, they will need small amounts of certain organic compounds for growth either organic carbon or $CO_{2,}$ because they are essential components that the organism is not able to synthesize from available nutrients. Such compounds are called growth factors (Todar, 2011). Growth factors are required in small amounts by cells because they fulfil1 specific roles in biosynthesis. The need for a growth factor results from either a blocked or missing metabolic pathway in the cells (Ikonomou *et al*., 2001). Normally, yeast extract are mainly used in bacteria cultivation within laboratory to provide growth factors in the culture medium. Growth factors are organized into three categories.

i) purines and pyrimidines: required for synthesis of nucleic acids (DNA and RNA)

ii) amino acids: required for the synthesis of proteins.

iii) vitamins: needed as coenzymes and functional groups of certain enzymes

2.7.4 Yeast Extract

Yeast extract is used in most media for microbes from plants and animals. Some of these microbes require vitamins and other growth factors from their plant or animal hosts and yeast extract is rich in vitamins, minerals, and digested nucleic acids (Chan,L. *et al.,* 1998). However, yeast extract is very hydroscopic and is difficult to keep dry in the classroom and is sometimes difficult for students to obtain (Todar, 2011). At a certain temperature, health of the cell is damaged and the intracellular of the cell degrades. Then, the enzyme activities begin at the cell from within. This is referred as autolysis or self lysis. This process of self lysis occurs in all cells at death (Eddlemen, 1999).

2.7.5 The Process of Autolysis

Death of the cell stimulates the process of autolysis. First of all, the membranous systems that consist of cytoplasmic membrane and other organelles of the cell disorganized. This allows the enzymes to react with cellular components which are degraded and rendered soluble. The peptide bonds of the proteins are broken down into smaller constituent units, such as peptides and amino acids by the proteolytic enzyme which is referred as protease (Friedli, 1996). Similarly, RNA and DNA are degraded and give yield to compounds such as nucleosides, mononucleotides, and polynucleotides by enzyme nuclease. Besides that, the enzymes glucanase and proteinase degrade the cell wall constituents such as glucans and mannoproteins (Dharmadhikari, 2001). This is the main cause that makes the cell wall to become porous. Then, the mixture of degraded cellular components which is called as the autolysate, leaks via the cell wall into the extracellular surrounding medium.

The process of degradation of the cellular components continues to occur in the surrounding medium. Yeast autolysis is strongly influenced by temperature and pH. For instance in wine production process, it occurs at low pH which is in the range of 3 to 4, and at relatively low temperatures which is as low as 15 to 18°C (Dharmadhikari, 2001). There two important aspects of yeast autolysis. Firstly, it is the degradation and solubilization of cellular components that is referred as proteolysis. Secondly is the degradation of the cell wall that is responsible for the shape of the yeast cell (Eddlemen, 1999). Hence, the concentrated form of solubilized cell constituents resulting from autolysis is referred to as yeast extract. The significant pathway is the proteolysis**.** This is the process where the yeast cell contains a variety of protein degrading enzymes which are mostly located in a vacuole. When the cell death, its cellular matrix is disorganized and the enzymes mix with their substrate. The proteolytic enzymes hydrolyze peptide bonds and yield. The protein breakdown products such as peptides and amino acids are produced by peptide bonds that are hydrolyzed by proteolytic enzymes. The nitrogenous compounds released at the beginning consisted mostly of large protein fractions. At a later state, the protein fragments were further degraded, and an increase in amino acid concentration was noted (Dharmadhikari, 2001). This is how the yeast extract provides the peptides and amino acids to be synthesized by bacteria in the culture media.

2.8 BIOCELLULOSE TREATMENT

In order to purify the synthesized biocellulose from its impurities that is on the surface or within the biocelluose strands, washing or soaking with just using distilled water is not enough. The purification treatment showed that when only distilled water is used, some components of the culture medium remain, and it can allow the microbiological attack of the membranes. This is the main reason NaOH treatment is introduced in biocellulose purification process. Normally, the harvested biocellulose is soaked in alkaline solution which contains NaOH overnight (Lee *et al.,* 2004). Then only the distilled water or deionized water is used to wash the treated biocellulose. After cultivation, the biocellulose synthesized on the surface of each medium culture are harvested and washed with water, 1% NaOH at 90°C for 15 min and washed with distilled water successively (Keshk and Sameshima, 2005).

Recently, a two steps biocellulose treatment process is being promoted. Figure 2.11 shows the difference between the biocellulose before and after two-stage purification. Unlike plant cellulose, which is bound with lignin, hemicellulose and other chemicals which occur naturally with cellulose, BC is not bound to other chemicals and is of relative high purity (Klemm *et al.,* 2006). Hence, the purification process applied in biocellulose treatment is also differs from plant cellulose purification because it is aimed to remove only the remaining organic material which is a nutrition source for the bacteria in the culture medium. Proteins, nucleic acids as well as vitamin B are the impurities which come from either yeast extract or produced by bacteria during fermentation process. These impurities responsible for the yellowish colour of the culture medium too. The aim to remove the remaining rod-shape bacteria of *A. xylinum*, as shown on the surface of untreated biocellulose is the other main reason of applying alkaline treatment. There are two stages in the double step purification process. First of all, biocellulose is washed in 2.5 wt% NaOH solution and then soaked with 2.5 wt.% NaOCl overnight (Sharman *et al.,* 2011). The concentration of NaOH is fixed to be 2.5 wt.% solution in order to maintain the cellulose I polymorph of biocellulose and avoid the synthesis of cellulose II which is characterized by lower mechanical properties (Lee *et al.,* 1994). It has been proven and reported that the NaOH concentrations which over 6% will have the potential to change the crystal structure of biocellulose from cellulose I to cellulose II (Gomes *et al*., 2007).

From a research, it can be observed that the changes in structure from cellulose I to cellulose II due to the alkalization process is followed by the breaking of many inter- and intra-molecular hydrogen bonds which are naturally formed in cellulose (Laszkiewicz, 1997). Non-cellulose materials such as protein and nucleic acids derived from bacterial cells and the culture broth are removed from the pellicle by using a two-step purification process. This allows the formation of strong inter- and intra-fibrillar hydrogen bonds. In the process, an alkaline solution of NaOH followed by a NaOCl solution is the proper sequence to be followed. As shown in figure 2.11c, the biocellulose is being free from impurities and it is because of this two-step purification method used (Saharman *et al.,* 2011). Moreover, the biocellulose treated by the two-step method was odourless with a neutral pH. The two-step treated biocellulose also can go through a longer storage period without experiencing a change in colour and quality. Furthermore, the three dimensional structure of biocellulose can be maintained, allowing the internal side of the biocellulose to be easily observed.

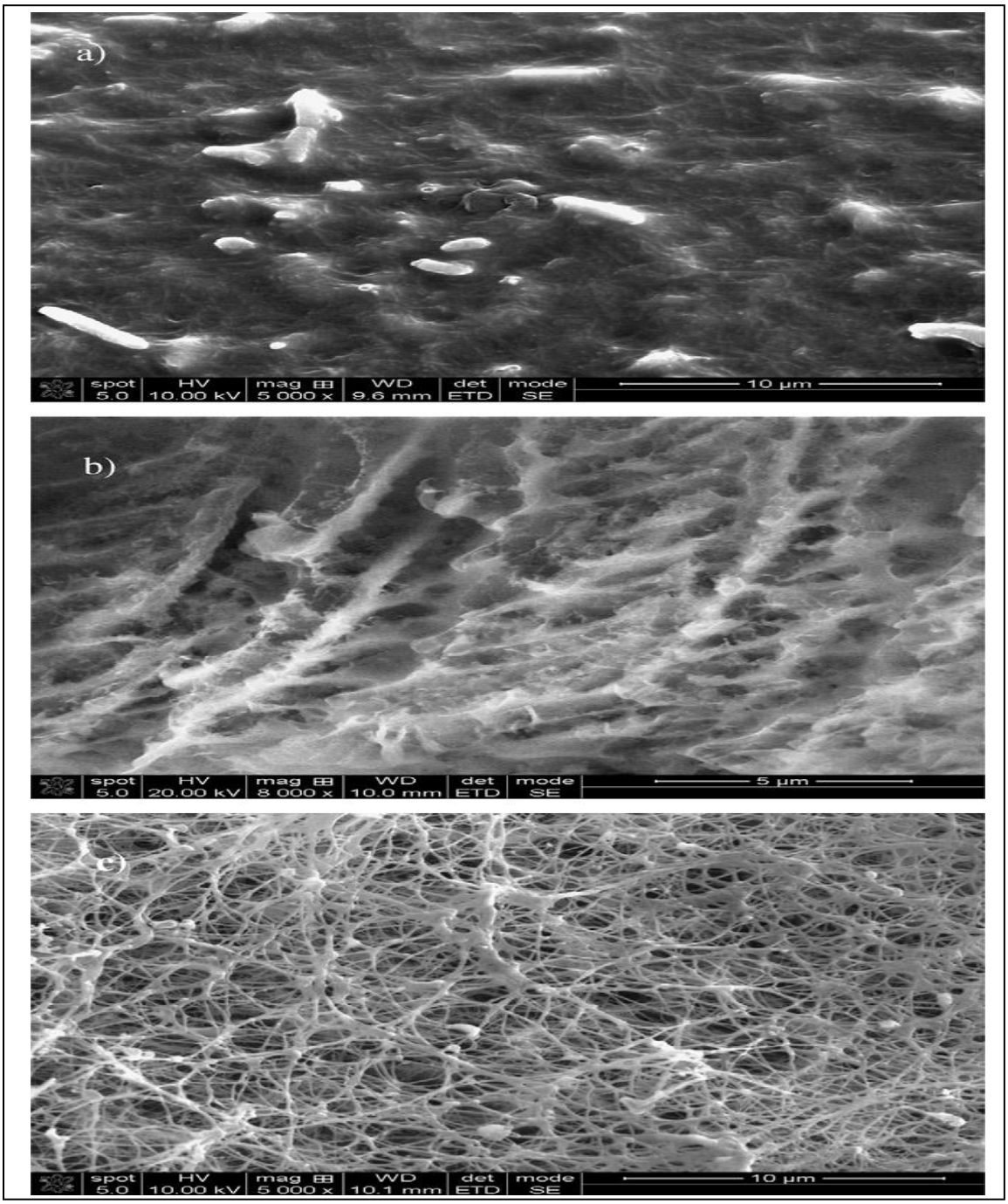

Figure 2.11: SEM image of (a) untreated BC, (b) single-step purified BC and (c) two-step purified BC gel

Source: Saharman *et al.* (2011)

2.9 FOURIER TRANSFORM INFRARED (FTIR)

FT-IR is the Fourier Transform InfraRed which is the preferable method of infrared spectroscopy. Figure 2.12 shows the FT-IR system while figure 2.13 shows the process flow within the FT-IR. IR radiation is passed through a sample in infrared spectroscopy. However, not all the infrared radiation is being absorbed by the sample. Hence, some of it is just passed through. A molecular fingerprint of the sample is created by the resulting spectrum which represents the molecular absorption and transmission. Scanning electron microscopy of cellulose and cells were prepared (Chávez-Pacheco *et al.*, 2005). Similar to a fingerprint, there are no two unique molecular structures which will produce the same infrared spectrum and this makes infrared spectroscopy useful for a few types of analysis. It can identify unknown materials, determine the quality or consistency of a sample as well as determine the amount of components in a mixture.

Figure 2.12: FT-IR system

Source: Chávez-Pacheco *et al.* (2005)

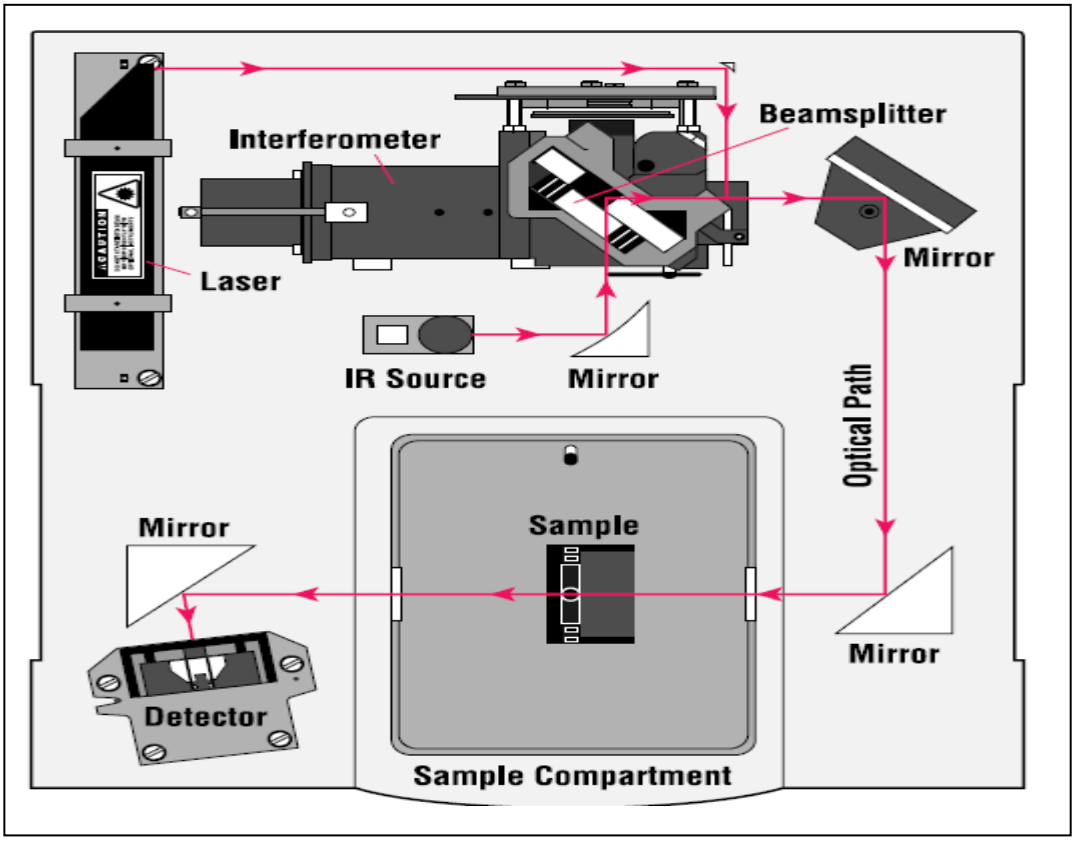

Figure 2.13: Fourier-Transform Infrared Spectroscopy process

Source: Madison (2011)

FTIR has been a workhorse technique for materials analysis in the laboratory for over seventy years. An infrared spectrum acts as a fingerprint of a sample with absorption peaks which is due to the frequencies of vibrations between the bonds of the atoms that are the material made from. C-O-C and C-O-H are the stretching vibration of the sugar ring (Sun *et al.*, 2007). Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. So, infrared spectroscopy can result in a positive identification of every different kind of material. Every distinct hydroxyl group leads to a single stretching band at a frequency which reduces with increasing strength of hydrogen bonding (Sturcova *et al.*, 2004). That is how the sample's finger print is formed. Furthermore, the size of the peaks in the spectrum is a straight determination of the amount of material present. Infrared is an excellent tool for quantitative analysis with modern software algorithms.

FTIR can be used for biocellulose determination. In order to indicate the chemical composition of the produced cellulose or PVA nanocomposites, a calibration

model has been developed with FTIR according to a known mixture of PVA and microcrystalline cellulose (Brown, 2007). FT-IR spectroscopy has been applied mainly to determine the chemical structure of the membrane. The infrared spectra of membranes are usually measured at wave numbers ranging from 4000 to 400 cm−1 using a FT-IR spectrum RXI (Sheykhnazari, 2011).

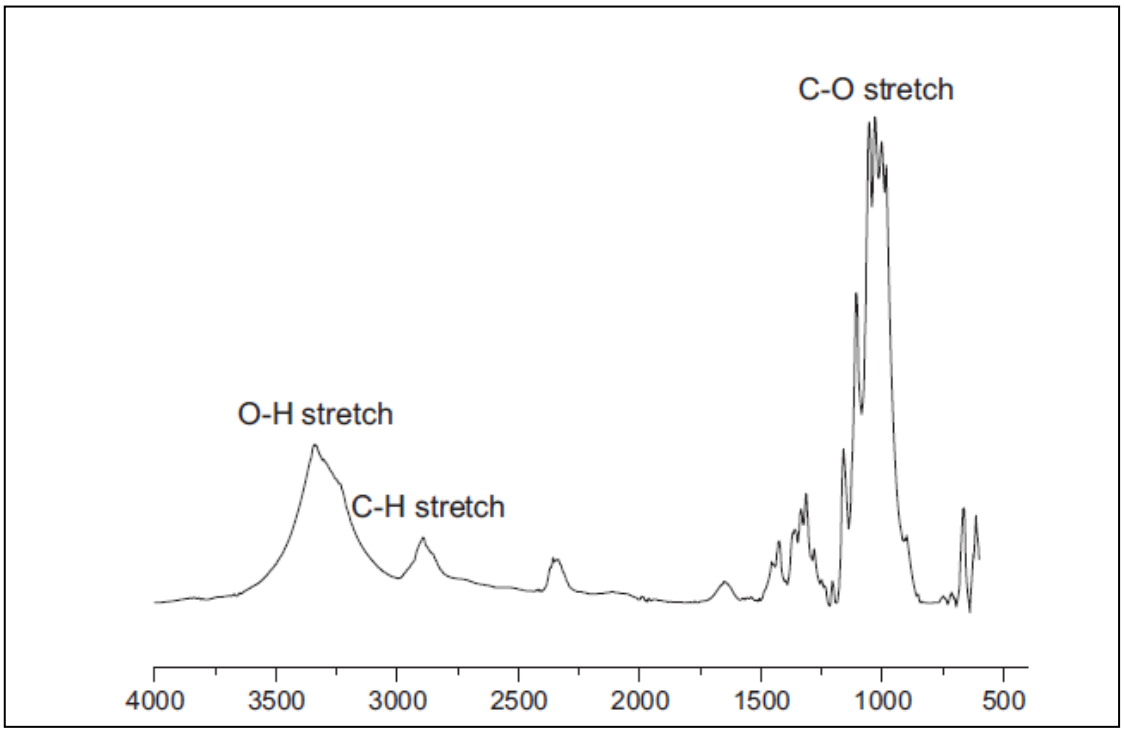

Figure 2.14: FT-IR spectra for biocellulose produced by Acetobacter *xylinum*

Source: Trovatti *et al.* (2011)

The figure 2.14 above shows the spectra the cellulosic substrates of biocellulose which is produced by *Acetobacter xylinum*. It indicates that the biocelluose is having strong bands at around 3300, 2880 and 1100 cm−1 which are related to the vibrations of the O–H, C–H and C–O–C groups of cellulose, respectively, typically reported for this material obtained from *Acetobacter xylinum* (El-Saied *et al.*, 2008**).**

2.10 SCANNING ELECTRON MICROSCOPE

Scanning Electron Microscopy (SEM) is the suitable equipment to be used for the determination of surface structures of mollicutes. Using SEM, a wide area of the sample can be observed in focus at a time (Fregler *et al.,* 1993). It also provides a wider range of magnification that enables the researchers to focus in easier on an interested area of a specimen, comparing with the samples being scanned under a lower magnification microscope. Moreover, SEM also gives out images in three-dimensional structure which are more suitable for the visibility of human's naked eye comparing to the two-dimensional images obtained via transmission electron microscope (Chawla *et al.,* 2008). Hence, the images form SEM can be easily interpreted and observed by researchers. Furthermore, it is also easier to operate with SEM as it just requires a few operation steps and so, it not time consuming as well. Anyway, a SEM user has to be cautious that there are a few risks during sample preparation for SEM testing as the integrity and ultrastructure of the mollicutes can be easily distorted. The basic steps in preparing the specimens for SEM testing includes surface cleaning, stabilizing the sample with a fixative, rinsing, dehydrating, drying, mounting the specimen on a metal holder, and the sample is coated with a layer of an electric conductor component (Bozzola and Russell, 1992).

A focused beam of high-energy electrons is used in the scanning electron microscope (SEM) to produce a various signals at the solid specimens' surface. The data about the sample which comprising of external morphology or texture, chemical composition, and crystalline structure and orientation of materials making up the sample are revealed by the signals, that are retrieved from electron-sample interactions (Susan Swapp, 2011). Normally, data are collected over a selected area of the surface of the sample in most applications. Then, a 2-dimensional image which shows the spatial variations in these properties is produced. A scanning mode using conventional SEM techniques with a magnification ranging from 20X to approximately 30,000X, spatial resolution of 50 to 100 nm can generate images for areas ranging from approximately 1 cm to 5 microns in width (Susan Swapp, 2011).

Figure 2.15: Scanning Electron Microscope

Source: Swapp (2011)

Figure 2.16 below shows the SEM images of a biocellulose membrane for its surface and cross section. It has been observed that the biocellulose membrane does not possess a porous morphology. Its surface shows that it is a homogenous structure with some crevasses which may be caused by the culture stage. In the meanwhile, the cross section of the biocellulose shows that the membrane is a layer formation (Klemm *et. al*., 2001). Pores can not be observed via the cross section.

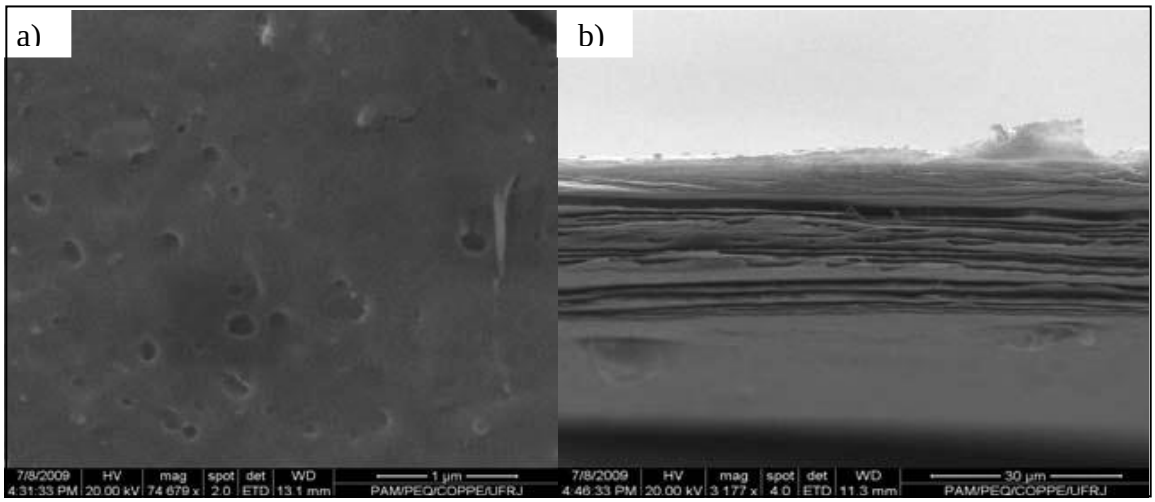

Figure 2.16: SEM images of biocellulose: a) surface b) cross section

Source: Pineda *et al.* (2007)

2.10.1 SEM Sample Preparation

Surface cleaning, stabilizing the sample with a fixative, rinsing, dehydrating, drying, mounting the specimen on a metal holder, and coating the sample with a layer of a material that is electrically conductive are the basic steps involved in SEM sample preparation (Bozzola and Russell, 1992). The steps are explained in more detail, individually due to the fact that each of these steps is significant and the outcome of the study will be affected.

i) Cleaning the Surface of the Specimen

It is important to have a proper surface cleaning of the sample as it may retain various impurities that includes dust, silt, detritus, media components, and so on. The type of unwanted particles is depending on the source of the biological material as well as the experiment which may have been brought out for SEM specimen preparation. These unwanted particles may be fixed permanently to the surface of the specimen surface and it will be difficult to remove them later if these impurities are not removed prior to fixation (Bozzola and Russell, 1992). It is also has been suggested that the sample should be rinsed

as fast as possible in an appropriate buffered solution of the suitable pH, osmotic strength, and temperature.

ii) Stabilizing the Specimen

There are many ways to stabilize a biological sample. It is usually conducted with fixatives. For instance, fixation can be achieved by microinjection, perfusion, immersions, or with vapours by using a variety of fixatives such as thiocarbohydrazide, tannic acid, osmium tetroxide, or aldehydes (Dykstra and Reuss, 2003). For sample such as mollicutes, it can be immersed in a 1.5% glutaraldehyde solution which is prepared in 0.1 M cacodylic acid buffer with pH 7.3 and incubated at 4 °C overnight for chemical fixation. The bulk conductivity of the sample can be improved by applying a postfixative. However, it does not guarantee that the sample has a better stabilization which is prepared for SEM.

iii) Rinsing the Specimen

The following step is sample rinsing. The sample must be rinsed after the fixation step in order to remove the excess fixative. For example, the sample can be rinsed in 0.1 M cacodylic acid buffer of pH 7.3 for four times. It is begun with one time for 10 min, and the rest for 20 min at 4 °C. The sample may able to be stored in this EM buffer for a few months as it contains arsenic and so, the growth of unwanted microorganisms in the storage container is inhibited (Hobert and Holzenburg, 1998). Anyway, it is suggested that the cacodylic acid buffer is changing at least one time per month in order to keep the sample in the container for a longer duration.

iv) Dehydrating the Specimen

The dehydration process of a biological sample needs to be done very carefully. It is typically performed with either a graded series of acetone or ethanol. The protocol that proved most suitable for dehydrating mollicutes for SEM includes the immersion of the specimens in 50% acetone for 5 min, 70% acetone for 10 min, 80% acetone for 10 min, 90% acetone for 15 min, and 100% acetone (dried with CaCl2) twice for 20 min at 4 °C. This process allows the water in the samples to be slowly exchanged through liquids with lower surface tensions (Stadtländer, 2007).

v) Drying the Specimen

The operation of the scanning electron microscope is done under a vacuum condition. Therefore, the samples must be completely dried or it can be damaged in the electron microscope chamber. There are electron microscopists who have considered to apply a procedure which is referred as the Critical Point Drying (CPD) as the best method to dry a SEM sample. After the transition of carbon dioxide from the liquid to the gas phase at the critical point, it is removed and the specimen is dried without destroying its structure (Hobert and Holzenburg, 1998). It is very significant to follow properly the instructions of the CPD manufacturer or the ultrastructure of the sample will be totally damaged.

vi) Mounting the Specimen

The sample must be mounted on a holder which can be inserted into the scanning electron microscope after it has been cleaned, fixed, rinsed, dehydrated, and dried via a proper procedure. Normally, the sample is mounted on the holder which is made of metallic (aluminium) by using a double-sticky tape. It is significant for the electron microscopist to decide on the best orientation of the sample on the mounting stub before it is being attached (Stadtländer, 2007). This is due to the fact that, a re-orientation is hard to be done and will seriously damage the sample.

vii) Coating the Specimen

Last but not least is the coating of the sample. The sample needs to be coated in order to increase its conductivity in the scanning electron microscope and to avoid the production of high voltage charges on the sample by grounding the charge (Bozzola and Russell, 1992). Normally, the sample will be coated with a thin layer of approximately 20 nm to 30 nm of a conductive metal such as gold, gold-palladium, or platinum.

2.11 WATER ABSORPTION CAPACITY (WAC)

Biocellulose is actually formed as a thick, gelatinous membrane under the static culture conditions. It has some standardized characteristics such as its water content and density. Biocellulose contains 96.28% moisture and has an apparent density of 990 g/l (Rezaee *et al.,* 2008). The shape and size of the synthesized biocellulose which is a gelatinous membrane, depends to the fermentation technique and condition used. *Acetobacter xylinum* is a simple gram negative bacterium which has an ability to synthesize a large quantity of high-quality cellulose organized as twisting ribbons of microfibrillar bundles.

Various carbon compounds of the nutrition medium are utilized by the bacteria, then polymerized into single, linear β-1-4-glucan chains and finally secreted outside the cells through a linear row of pores located on their outer membrane during the process of actual biosynthesis (Rezaee *et al.,* 2008). Hence, biocellulose has a totally different structure compare to the plant cellulose. The structure of the biocellulose itself leads to its high cellulose crystallinity (60–80%) and an enormous mechanical strength. In additional to that, the size of biocellulose fibrils is about 100 times smaller than that of plant cellulose which is an impressive fact (Shirai *et al.*, 1997). Instead, this is also the unique nano morphology that brings about a large surface area which gives the biocellulose the ability to hold a large amount of water and, at the same time, displays great elasticity, high wet strength, as well as conformability. Indeed, the key factor that gives to its remarkable performance as an effective adsorbent is the small size of microbial fibrils. Unlike celluloses of plant origin, microbial cellulose is entirely free of lignin and hemicelluloses (Lynd *et al.,* 2002). The maximum amount of water that is

able to be absorbed and maintain in the biocellulose is the Water Absorption Capacity (WAC).

In order to determine the Water Adsorption Capacity, the dried membranes are immersed in deionized water at room temperature until equilibration. After that the membranes are removed from the water and excess water at the surface of the membranes is blotted out with Kimwipes paper. The weights of the swollen membranes were measured, and the procedure was repeated until no further weight change was observed (Saibuatong and Phisalaphong, 2009).

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

This chapter focuses on the achievement of the conceptual study, laboratory work, analyzing and completion of the project. The detailed experimental procedure will be discussed via this chapter.

3.2 MATERIAL AND APPARATUS

The apparatus list in the research includes incubator, cone flask, autoclave, FTIR, SEM, and Electronic Weighing Machine. On the other hand, the material used consists of carbon sources (glucose:fructose), 5g/l yeast extract, 5g/l peptone, 2.7g/l dibasic sodium phosphate(buffer) and 1.15g/l citric acid.

3.3 MEASUREMENT AND ANALYSIS

The properties of biocellulose are determined using Fourier Transform Infrared spectroscopy (FTIR). Then, the bio cellulose in every different medium is weighed with electronic weighing machine and is compared and the production efficiency of every culture medium containing different ratio of mixed carbon sources is determined. Besides, the surface and cross section properties of biocellulose produced are observed by Scanning Electron Microscope (SEM).

3.3.1 FT-IR

Before everything biocellulose sample is located across a hole in a magnetic holder, the sample is air-dried on a glass slide in the form of a thin film. FT-IR spectroscopy is applied mainly to study the chemical structure of the biocellulose. The infrared spectra of the biocellulose samples are measured at wavelengths ranging from 4000 to 400 cm−1 using a FT-IR spectrum RXI.

3.3.2 Water Absorption Capacity

In order to determine the WAC, the dried biocellulose samples are immersed in DI water at room temperature till it reaches equilibrium. Then, the samples are taken out from the water and excess water left on the biocellulose surface is blotted out with Kimwipes paper. After that, the weights of the hydrated biocellulose samples are measured, and repeat the procedure till no observation of further weight change. The water content is calculated using the following formula:

$$WAC\ (\%) = [(Wh - Wd)/Wh] \times 100\% \quad (1)$$

Wh and Wd indicate the weight of the hydrated and dried biocellulose samples respectively.

3.3.3 Scanning Electron Microscope

The morphology of the biocellulose samples is captured and observed by using. A CamScan model MV2300 scanning electron microscope which operates at 12 kV is used. Before the samples are covered with a 1 nm gold layer on copper supports, they need to be dried.

3.4 THE METHOD OF BIOCELLULOSE PRODUCTION

Carbon sources (glucose and fructose) which are the experiment's main materials are consumed from Sigma & Aldrich. They provide high quality chemicals. Figure 3.1 below is the overall methodology flow chart of the biocellulose production.

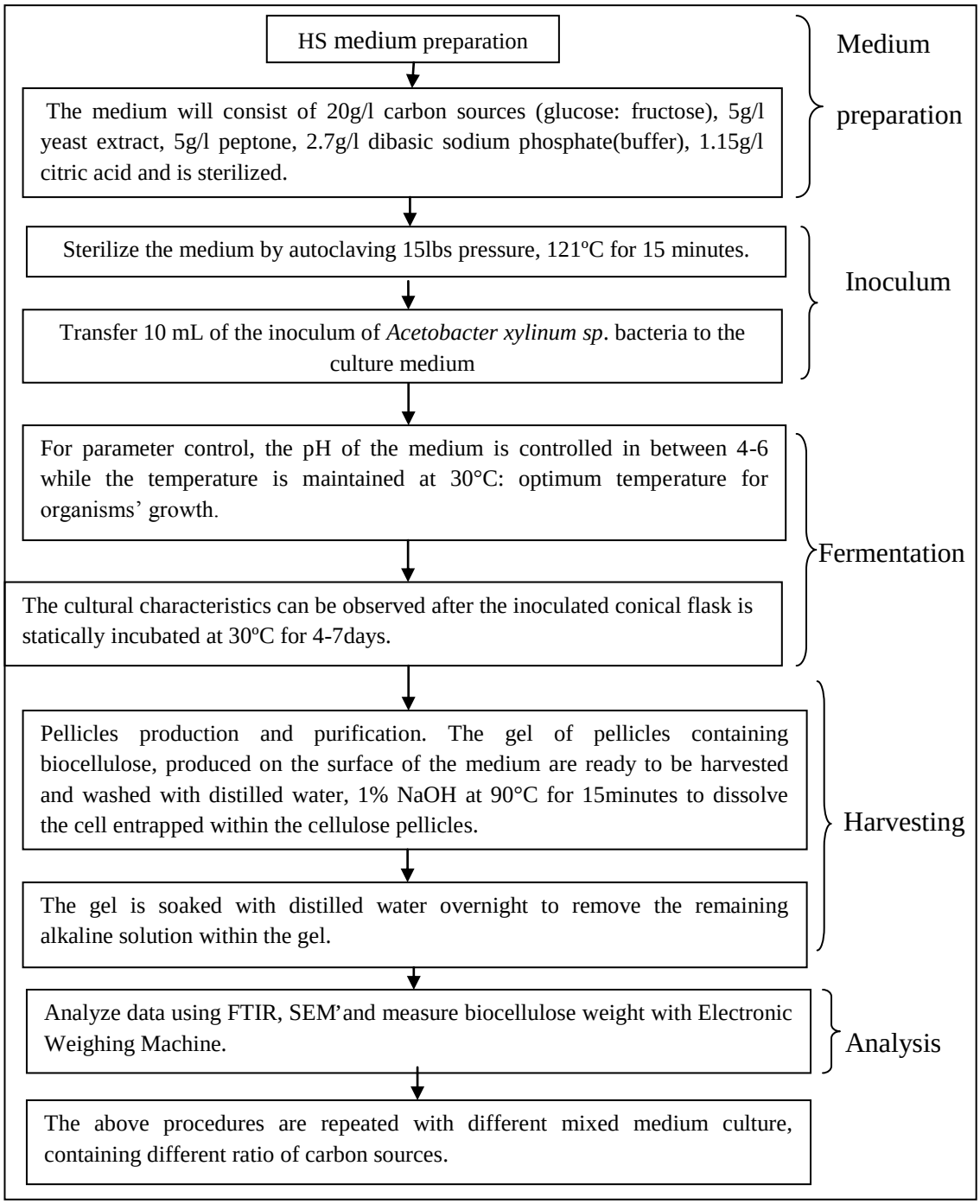

Figure 3.1: Overall methodology flow chart of the biocellulose production

3.4.1 The Optimum Parameters for Biocellulose Production.

The optimum pH for cellulose production was 4.0 to 6.0. Glucose, fructose and glycerol were preferred carbon sources for cellulose production. The yield of cellulose, relative to the glucose consumed, decreased with an increase in initial glucose concentration, and gluconic acid accumulated at a high initial glucose concentration. The decrease in cellulose yield could be due to some glucose being metabolized to gluconic acid (Peter *et al.*, 2000). However, the accumulated gluconic acid did not affect cellulose production. The culture conditions of the bacterium for cellulose production were optimized. The optimum temperature for growth of the bacteria is 30°C, but the organisms can grow well at typical room temperatures (Pourramezan, 2009).

CHAPTER 4

RESULT AND DISCUSSION

4.1 INTRODUCTION

Overall, this study gives result in the most suitable carbon sources ratio of glucose to fructose in the mixed medium culture for biocellulose production enhancement. This identified carbon sources ratio will be important information for the best production of biocellulose by using mixed medium culture. The following discussions in this chapter are the results for the project.

4.2 RELATIONSHIP BETWEEN CARBON SOURCE AND BIOCELLULOSE PRODUCTION

The result in the table 4.1 is the result of biocellulose production in the 11 mixed medium cultures. The result has been plotted into a graph in figure 4.1. Then the result is analyzed and discussed.

Table 4.1: Biocellulose production in mixed culture medium

Composition Ratio, % (Glucose:Fructose)	Cellulose Dried Weight, (g/l)
0:100	0.9
10:90	1.57
20:80	1.475
30:70	1.318
40:60	1.098
50:50	1.00
60:40	0.975
70:30	0.782
80:20	0.722
90:10	0.715
100:0	0.696

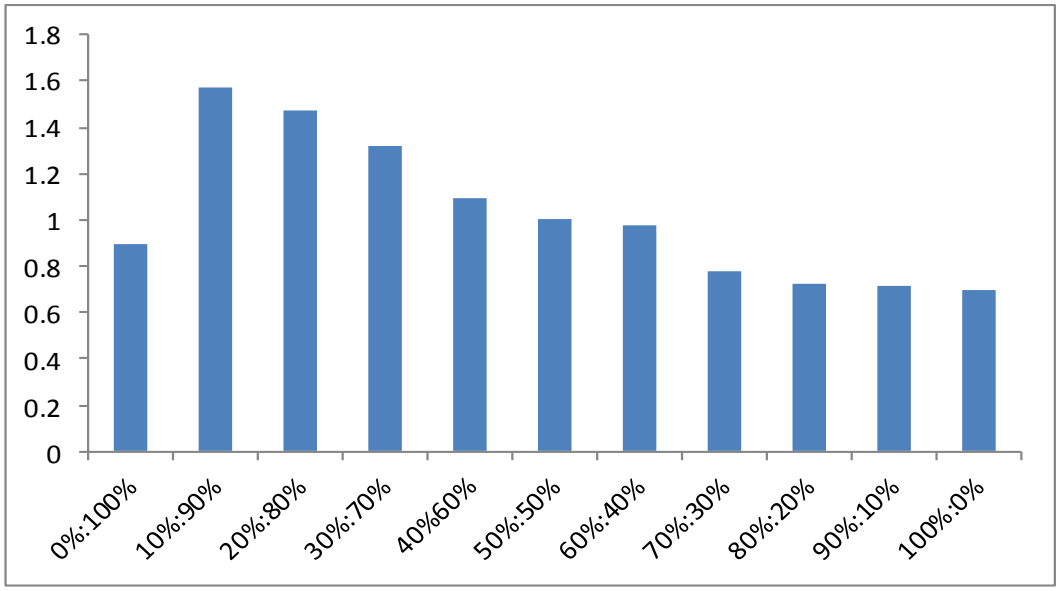

Figure 4.1: Graph of biocellulose dried weight versus composition ratio of carbon sources in mixed culture medium

According to the graph in figure 4.1 above, it shows that the biocellulose production is decreasing when the glucose concentration in the medium culture is increasing. The experiment is brought out with 11 conical flasks (250ml) that contain

Hestrin and Schramm medium, also referred as HS medium. However, each of them comprises of different carbon source composition ratio (Glucose:Fructose) and will lead to different biocellulose production. Two of those conical flasks are prepared with medium culture that contain single carbon source that is glucose alone (100% glucose) and fructose alone (100% fructose) respectively while the other nine conical flasks are prepared mixed medium culture. Each of the nine conical flasks which are prepared with mixed medium culture contains different composition ratio of glucose and fructose concentration which varies from 10% to 90% glucose composition.

From the result, it shows that the conical flask with culture medium that contains the mixture of 10% glucose and 90% fructose lead to the highest biocellulose yield of 1.57g/l. Meanwhile comparing both of the conical flasks which are with different single carbon source each, the conical flask with culture medium that contains fructose alone lead to a higher biocellulose production than the conical flask that contain glucose alone. The biocellulose yield of both conical flasks which contains glucose alone and fructose alone are 0.696 g/l and 0.9 g/l respectively. Anyway, this has proved that the conical flasks with culture medium that contain mixed carbon sources bring about higher biocellulose yield than culture medium that contains only single carbon source. The biocellulose yield in the culture medium that contains mixed carbon sources of glucose and fructose is higher compared to the culture medium that contains only a single carbon source (Young *et al.,* 1997).

However, this statement is only accurate to be used to compare between single carbon source of glucose and mixed carbon sources medium. This is due to the fact that the biocellulose yield in the mixed medium culture is decreasing with the increasing of glucose composition and finally it comes to point that the biocellulose production is less than the biocellulose production from the culture medium that contain single carbon source of fructose alone. This turning point is at the mixed culture medium that contain 70% glucose and above. From the graph and data in the table above, it shows that the production of 70%, 80% and 90% glucose is 0.782 g/l, 0.722 g/l and 0.715g/l respectively while culture medium with single carbon source of fructose lead to the biocellulose productivity of 0.9 g/l. This obviously shows that the productivity from the 3 mixed culture mediums that contain 70%, 80% and 90% glucose respectively are

lower than the medium that only with fructose alone. Anyway, the lowest biocellulose yield belongs to culture medium that contains glucose alone that is 0.696 g/l.

Table 4.2: Relationship of fructose concentration in culture medium with the biocellulose production

Fructose, %	Fructose, g	Biocellulose Dried Weight, g/l
10	0.2	0.715
20	0.4	0.722
30	0.6	0.782
40	0.8	0.975
50	1	1.0
60	1.2	1.098
70	1.4	1.318
80	1.6	1.475
90	1.8	1.57
100	2	0.9

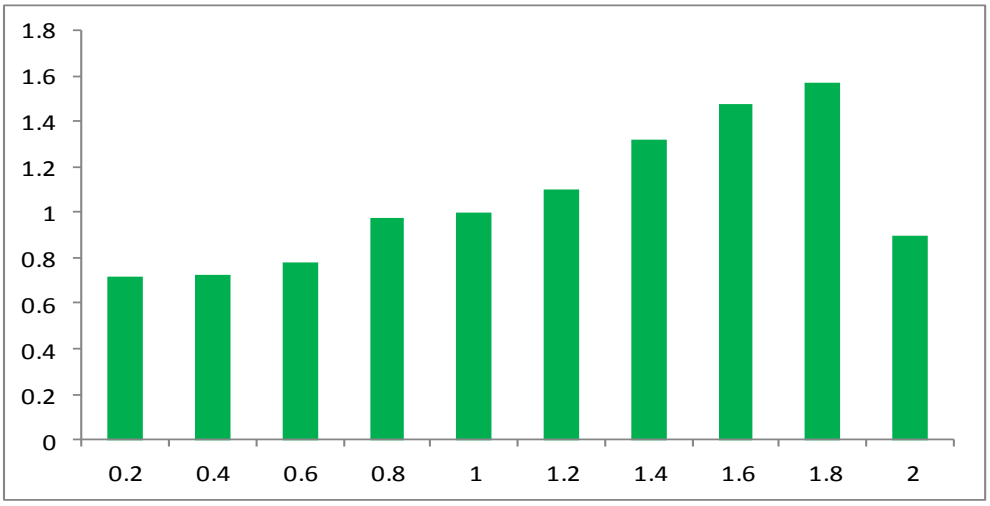

Figure 4.2: Graph of biocellulose dried weight versus fructose weight

The graph above is extracted from the previous result. It gives a clearer image in how the increasing concentration of fructose in the mixed culture medium leads to the increasing of biocellulose yield. Moreover, it also presents that the biocellulose yield is

mainly depends to fructose. Their relationship is proportional to each other. It shows the highest biocellulose yield of 1.57 g/l when the fructose is in the amount of 1.8g, means 90% of the mixed carbon sources with 10% glucose. However, the graph shows a drop to 0.9 g/l although the amounts of fructose supplied in the medium culture is the highest, which is 2g because it indicates the 100% fructose as the carbon source in the culture medium. In other words, it is a single source culture medium.

4.3 RELATIONSHIP BETWEEN PH AND COMPOSITION RATIO OF CARBON SOURCES IN MIXED CULTURE MEDIUM

The result in the table 4.3 below is the result of final pHs in the 11 mixed medium cultures. The result has been plotted into a graph in figure 4.3.

Table 4.3: Composition Ratio of Carbon Sources in Mixed Culture Medium Versus Final pH of The Culture Medium

Composition Ratio, % (Glucose: Fructose)	pH
0:100	5.3
10:90	5.26
20:80	5.14
30:70	4.96
40:60	4.92
50:50	4.87
60:40	4.81
70:30	4.78
80:20	4.68
90:10	4.63
100:0	4.09

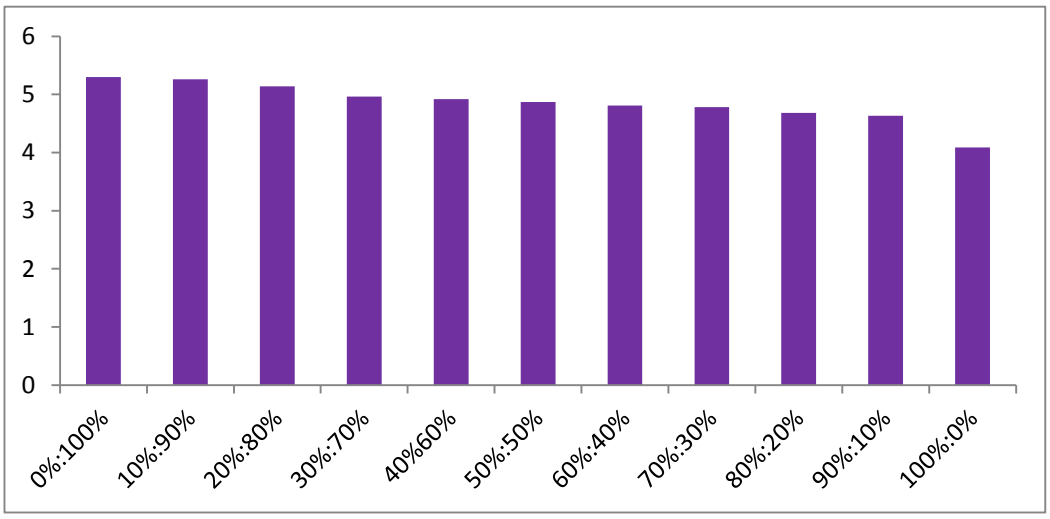

Figure 4.3: Graph of biocellulose production versus pH

Initially, the pH of the 11 culture mediums in each conical flask is all adjusted to 5.5 with acetic acid and pH meter. According to previous researches, the optimum pH for the growth and activity of *Acetobacter xylinum* is in the range of 4 to 7 while pH 6.5 leads to the best biocellulose yield. However, the chance of contamination will increase when the pH approach 7 as many other organism can live under this medium condition (Shazia *et al.*, 2010). Hence, the pH is adjusted to pH 5.5 and so, fewer organisms can adapt to such acidic condition and reduce the chance of contamination. Meanwhile, the culture medium condition cannot be lower than 4 or it will lead to the deactivation of *Acetobacter xylinum* itself.

The graph and data above are the result of the final pH for the project. According to the graph, the pH of the medium culture is decreasing when the glucose concentration is increasing. The medium culture with glucose alone lead to the lowest final pH, that is pH 4.09 while there is only a slightly pH change for the medium culture with fructose alone, that is 5.3.

This is due to the production of glucosidic acid from glucose by *Acetobacter xylinum* that decreases the pH of the culture medium while fructose is not metabolized to be acid. *Acetobacter xylinum* synthesizes glucose to glucosidic acid rather than biocellulose synthesis when the glucose concentration increases in the medium. In other words, higher glucose concentration in culture medium lead to higher production of

glucosidic acid but brings about lower biocellulose production as most of the glucose is already used up to be converted to glucosidic acid. *Acetobacter xylinum* will preferentially synthesize glucosidic acid from glucose when the glucose concentration increases and this is the interesting characteristic of the *Acetobacter xylinum* itself. When glucose coexists with fructose, glucose is preferred to be converted into glucosidic acid while producing cellulose mainly by fructose (Kamide *et al.,* 1990).

In addition to that, the condition of the culture medium is becoming more and more unsuitable for the growth of *Acetobacter xylinum* when the pH is decreasing and approaching to pH 4 because the bacteria will be totally deactivated when the condition of the culture medium is below pH 4. Therefore from graph figure 4.3, it can be observed that the biocellulose production from the medium with fructose alone is higher (0.9 g/l) than the biocellulose production from the medium which is with the glucose alone (0.696 g/l) when comparing cellulose yields of both culture mediums which only contain a single carbon source. However when comparing with the culture mediums that contain mixed carbon sources, the mixed culture medium of 10% glucose and 90% fructose gives highest biocellulose yield although its final pH shows that it has a lower figure than culture medium with fructose alone, that is 5.26. The accumulated glucosidic acid decreases the pH of the culture medium and inhibits biocellulose production (Young *et al*., 1997).

This is because the *Acetobacter xylinum* performs better in mixed culture medium and the low glucose concentration of 10% does not affect its biocellulose production as most of the biocellulose is synthesized from the 90% fructose in the culture medium. Overall, the lowest final pH and the lowest biocellulose yield are the results from the culture medium with glucose alone. In other words, almost all of the glucose is metabolized by the *Acetobacter xylinum* to be glucosidic acid and only a little amount of glucose left for biocellulose synthesize. This is the reason why the increasing amount of the single carbon source of glucose fails to increase biocellulose yield but in fact, deceases the biocellulose yield.

4.4 FTIR TESTING

The following figures of 4.4, 4.5 and 4.6 are the results from the FTIR testing for the 3 biocellulose samples which are produced from mixed medium cultures of 100% glucose, 100% fructose as well as mixture of 90% fructose and 10% glucose respectively.

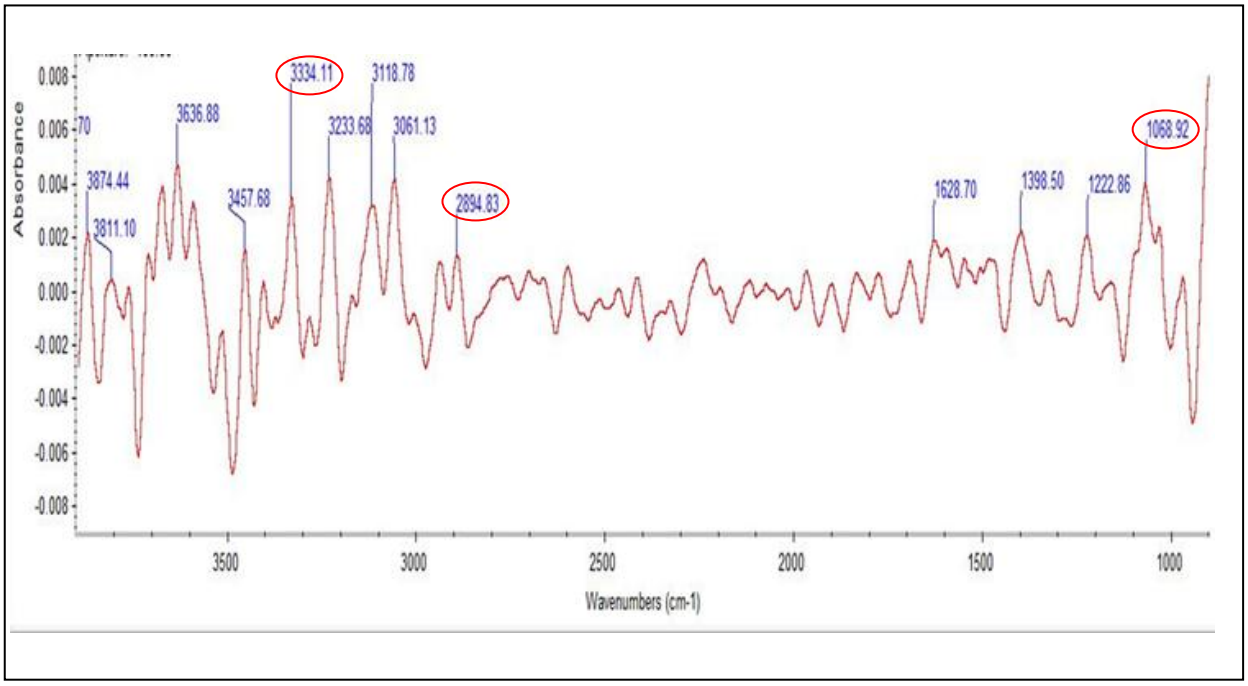

Figure 4.4: FTIR image of biocellulose produced from culture medium with 100% glucose

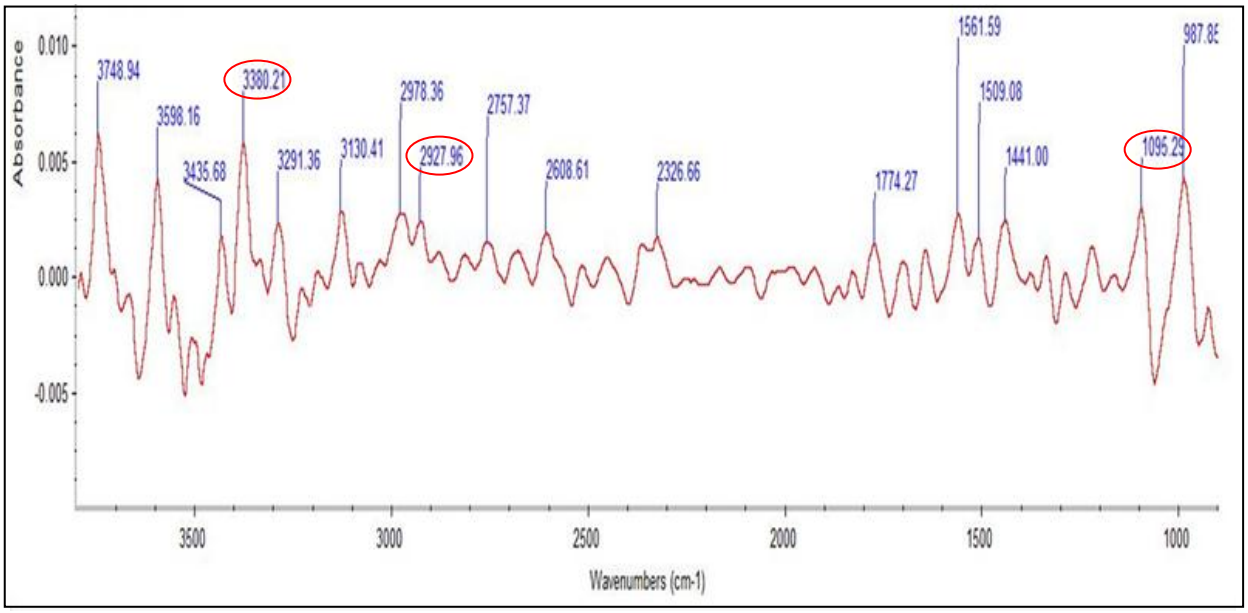

Figure 4.5: FTIR image of biocellulose produced from culture medium with 100% fructose

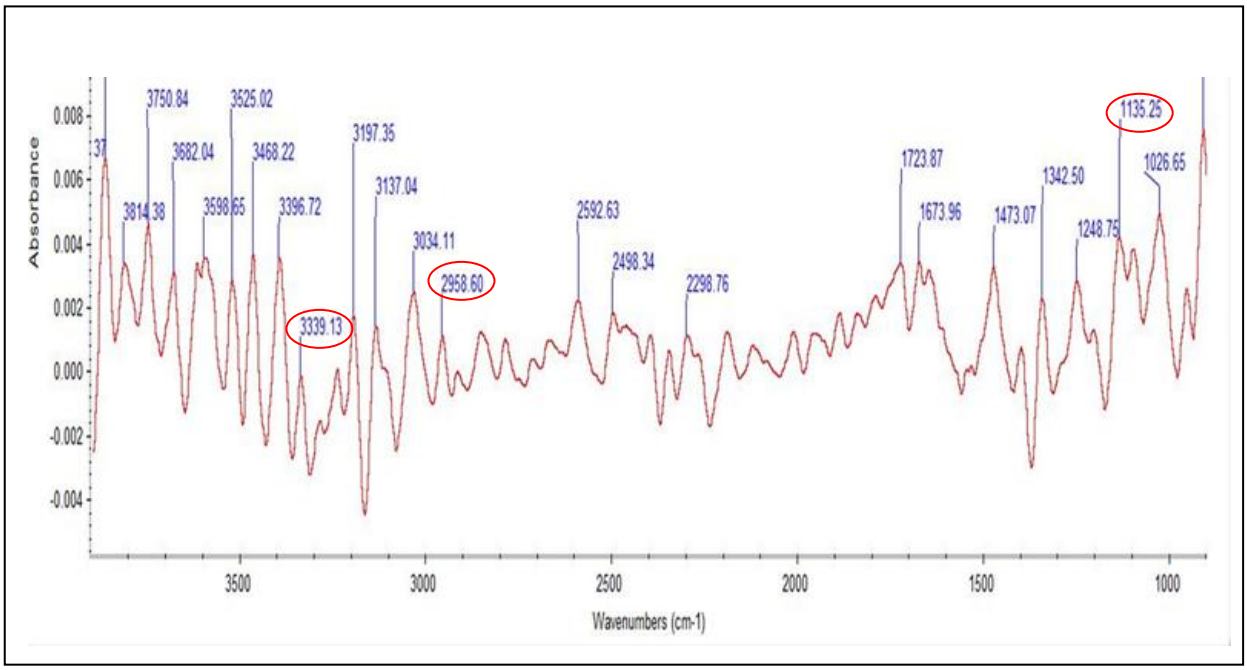

Figure 4.6: FTIR image of biocellulose produced from culture medium with 10% glucose and 90% fructose

There are three biocellulose samples from this project chosen to carry out the FT-IR testing. They are the biocellulose sample from the 100% glucose culture medium, biocellulose sample from 100% fructose culture medium and the biocellulose sample from the mixed culture medium of 10% glucose and 90% fructose. This testing is carried out to ensure that the components produced in those culture mediums are cellulose.

Figure 4.4 shows the FT-IR spectra of the biocellulose from the 100% glucose culture medium. It shows a band at 3334.1/cm which can be associated to the intermolecular and intramolecular hydrogen bonds. Then the spectra also consists of a band at 2894.83/cm that can be related to the C-H stretching and it also has a band at 1068.92/cm which can be associated to the ether C-O-C functionalities. Moreover, it also has a region of 3233.68-3457.68/cm which can be indicated as the intermolecular and intramolecular hydrogen bonds.

Figure 4.5 represents the FT-IR spectra of the biocellulose from the 100% fructose culture medium. It shows a band at 3380.21/cm which can be associated to the intermolecular and intramolecular hydrogen bonds. Then the spectra also consists of a band at 2757.37/cm that can be related to the C-H stretching and it also has a band at 1095.29/cm which can be associated to the ether C-O-C functionalities. Furthermore, it also has a region of 3291.36-3435.68/cm which can be indicated as the intermolecular and intramolecular hydrogen bonds.

Figure 4.6 represents the FT-IR spectra of the biocellulose from the mixed culture medium of 10% glucose and 90% fructose. It shows a band at 3339.13/cm which can be associated to the intermolecular and intramolecular hydrogen bonds. Then the spectra also consists of a band at 2958.6/cm that can be related to the C-H stretching and it also has a band at 1136.25/cm which can be associated to the ether C-O-C functionalities. In addition to that, it also has a region of 3339.13-3468.22/cm which can be indicated as the intermolecular and intramolecular hydrogen bonds.

According to a previous biocellulose research, a band at 3300/cm can be associated to the hydroxyl bonds while the spectra also consists of a band at 2880/cm that can be related to the C-H stretching and it also has a band at 1100/cm which can be

associated to the ether C-O-C functionalities (Saied *et al.*, 2008). Then, the FT-IR spectra region of 3230 -3455/cm can be referred to as the intramolecular and intermolecular hydrogen bonds of the cellulose (Oh *et al.*, 2005). Hence the FT-IR spectra of the three samples consist of bands that similar to the strong bands that appear in a biocellulose. Therefore, it is proved that the component produced form the culture mediums of the project is 100% biocellulose.

4.5 WATER ADSORPTION CAPACITY OF BIOCELLULOSE

The result in the table 4.4 below is the result of water absorption capacity of the samples from the 11 mixed medium cultures. The result has been plotted into a graph in figure 4.7. Water absorption capacity is determined via analysis of the water content within the wet biocellulose.

Table 4.4: Relationship between water content and composition ratio of carbon sources in culture medium

Composition Ratio, % (Glucose:Fructose)	Water Content, %
0%:100%	99
10%:90%	99.13
20%:80%	99.19
30%:70%	99.11
40%60%	99.07
50%:50%	99.05
60%:40%	99.04
70%:30%	99.02
80%:20%	99
90%:10%	99
100%:0%	98.85

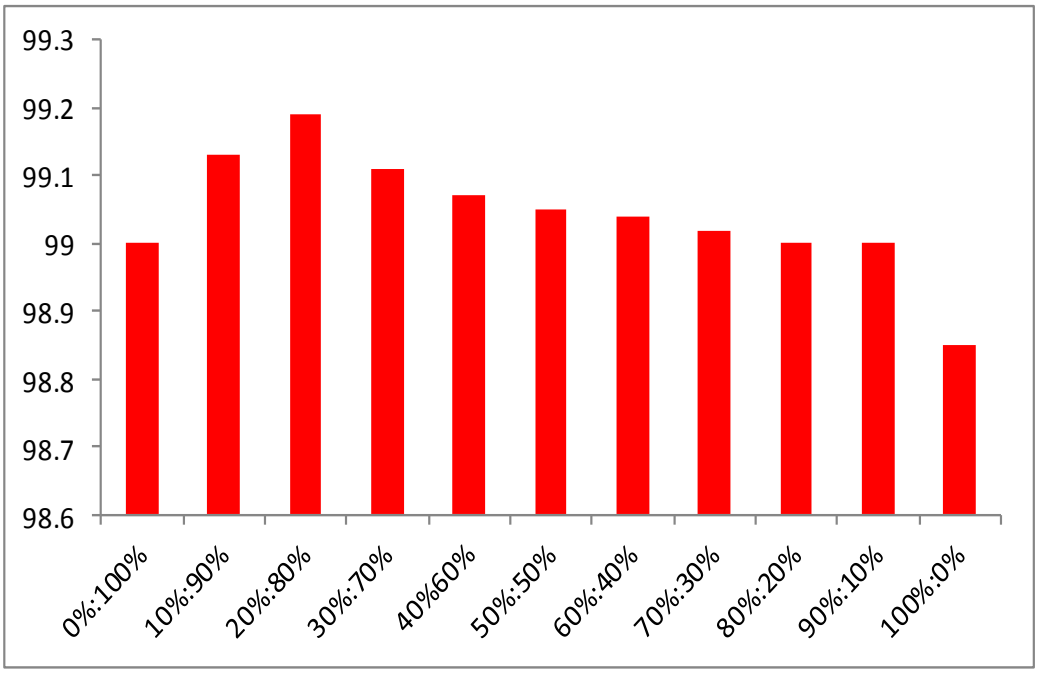

Figure 4.7: Graph of water content versus composition ratio

The data and graph above show the water content for each of the biocelluloses produced from the culture mediums in the project. According to the graph, the highest water content has been determined from the biocellulose that is synthesized in the culture medium that contains the mixed carbon source of 20% glucose and 80% fructose that is with 99.19% water content. The fibrils size of the biocellulose are 100 times smaller than the plant cellulose and this provides the biocellulose with a special nano morphology results that lead to the remarkable water absorbing ability of the biocellulose (Shirai *et al*., 1997). This indicates the mixture of fructose and glucose at a certain ratio can produce biocellulose that possesses strong structures and fine distribution of microbial fibrils which lead to a wider surface area for the biocellulose to hold the big amount of water. The water absorption capacity of biocellulose is analogous to its mechanical strength (Saibuatong *et al.*, 2010).

The biocellulose which is the highest yield from the mixture of 10% glucose and 90% fructose leads to the second largest value of the water absorption capacity that is with 99.13%. It also can be observed that the biocellulose produced from the culture medium that contains fructose alone has a higher water absorption capacity than the biocellulose produced from the culture medium which contains glucose alone. The

biocellulose from the medium with fructose alone has a water content of 99%. Meanwhile, the biocellulose which is produced from the glucose alone has the lowest water content that is 98.85%. The graph shows that the water content is decreasing, meanwhile indicates that the water absorption capacity is decreasing when the glucose concentration is increasing. In other words, the developed biocellulose structure becomes miscellaneous and weak. Hence the ability to hold the water also becomes weaker. The water absorption capacity value is important for the biocellulose to be commercialized as the wound dressing.

4.6 SCANNING ELECTRON MICROSCOPE

The figure 4.8 is the SEM image of the biocellulose surface at magnification of 300X. It is the surface of the enhanced biocellulose which is produced from the mixed medium culture of 10% glucose and 90% fructose. It can be observed that the biocellulose surface is unsmooth and possess non-porous morphology. Pores can not be observed on the surface of the biocellulose. The particles on the surface are the few impurities which are left over after the purification stage.

The figure of 4.9 is the SEM image of the biocellulose cross section at a magnification of 2K X. According to the image, it can be observed that the cross section of the biocellulose is in layered form. It has been reported earlier that the biocellulose is a layer formation (Klemm, 2001).

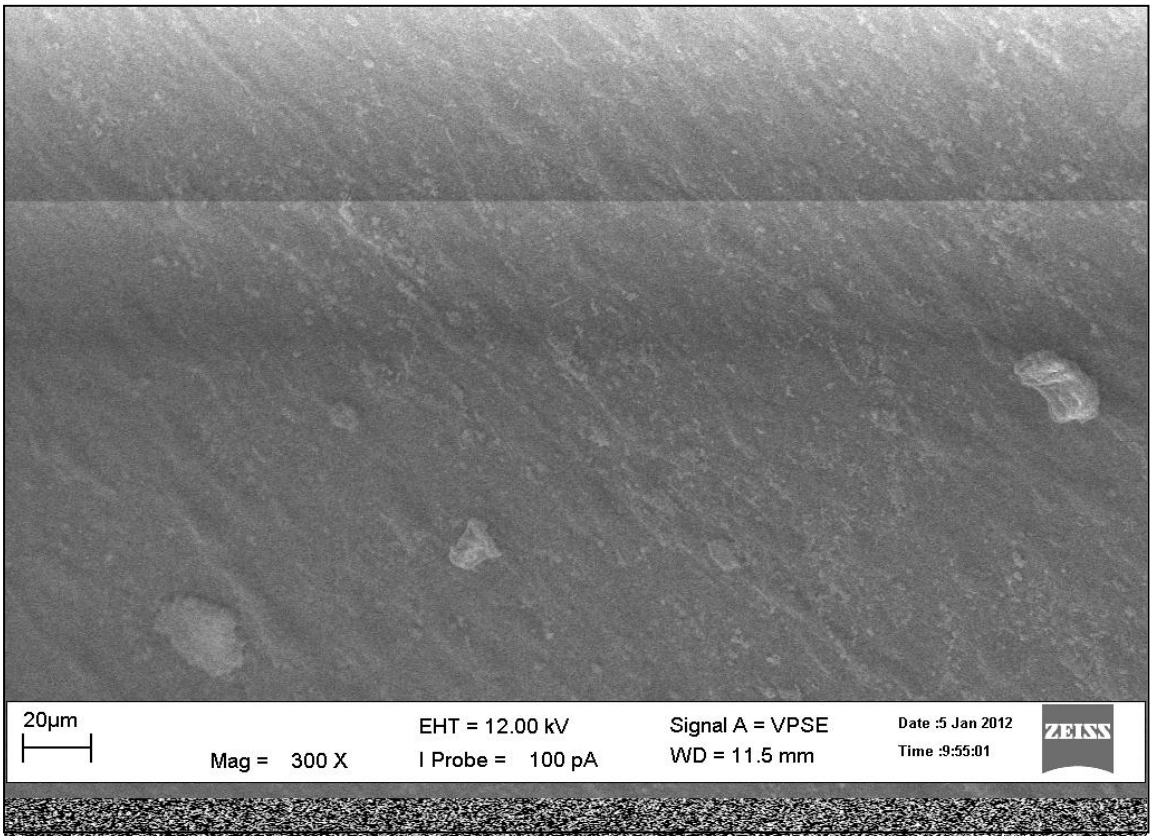

Figure 4.8: SEM image of biocellulose surface

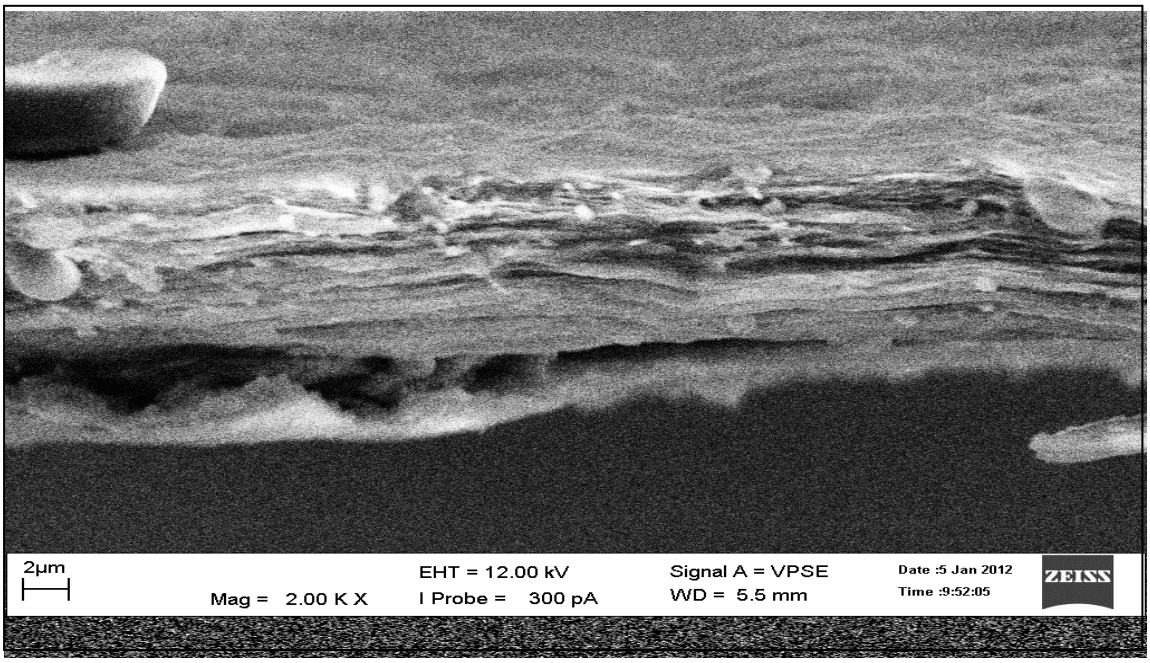

Figure 4.9: SEM image of biocellulose cross section

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 INTRODUCTION

This chapter includes the conclusion of the whole research project of 'Enhancement of Biocellulose Production in Mixed Medium Culture' and the recommendations for the use of future research.

5.2 CONCLUSION

As a conclusion, the application of mixed medium culture for the enhancement of biocellulose production is a success. A suitable composition ratio of the fructose and glucose in the culture medium as the carbon sources for *Acetobacter xylinus* can lead to a high yield of biocellulose. The mixed medium culture that contains 10% glucose and 90% fructose produce the highest biocellulose yield in the project that is with 1.57 g/l. In other words, it is the optimum medium culture for the enhancement of biocellulose production. Increasing the initial concentration of glucose as the carbon source for the *Acetobacter xylinus* in the culture medium fails to increase the biocelulose productivity because glucose is preferred by the bacteria to be converted to glucosidic acid. It is an interesting characteristic of *Acetobacter xylinus* that it can maximize the metabolism of the carbon sources to biocellulose in the mixed culture medium and the cellulose is synthesized by the bacteria mainly by consuming fructose. Therefore, it also means that biocellulose still can be synthesized when the glucose carbon source in a culture medium is limited.

The characterization of the biocellulose produced from the culture mediums in the project has been done by using FT-IR, SEM and water absorption capacity. The FT-IR

spectra has confirmed that the components produced by the *Acetobacter xylinum* in medium cultures are cellulose with the existing bands that represents the strong bonds of hydrogen bonding, carbornyl groups and C-H stretching that represents the cellulose properties. Then, SEM also shows that biocellulose has unsmooth, non-porous and is the formation of layered sheets. Moreover, all of the biocelluloses produced in the project possess high water absorption capacity and this is also one of the main characteristic of biocellulose. The highest water absorption capacity value belongs to the biocellulose produced from the culture medium with carbon source of fructose alone. Hence, the biocellulose synthesized from the fructose alone possess of stronger structures and contain more microbial fibrils that provides it to have a bigger surface area to hold a higher quantity of water within it. The biocellulose produced from the mixture of 10% glucose and 90% fructose lead to a lower water absorption capacity than the biocelluloe synthesized by the fructose alone but it is still acceptable to be commercialized as there is only a slightly difference.

5.3 RECOMMENDATIONS

Further still can be brought out to determine a better method to enhance the biocellulose production. For instance, it has been reported that the biocellulose production can be increased by using agitation method bus has not been applied in the industrial world. In additional to that, further research can be done to determine the suitable organic carbon source such as fruit skins or wastes for biocellulose production to replace carbon sources like glucose and fructose which require higher cost. It is in order to produce biocellulose with lower raw material. Besides, research can be carried out to determine the reason for the special characteristic of *Acetobacter xylinum* that it will prefer to synthesize gluconic acid from the carbon source rather than cellulose when the culture medium contains high glucose concentration.

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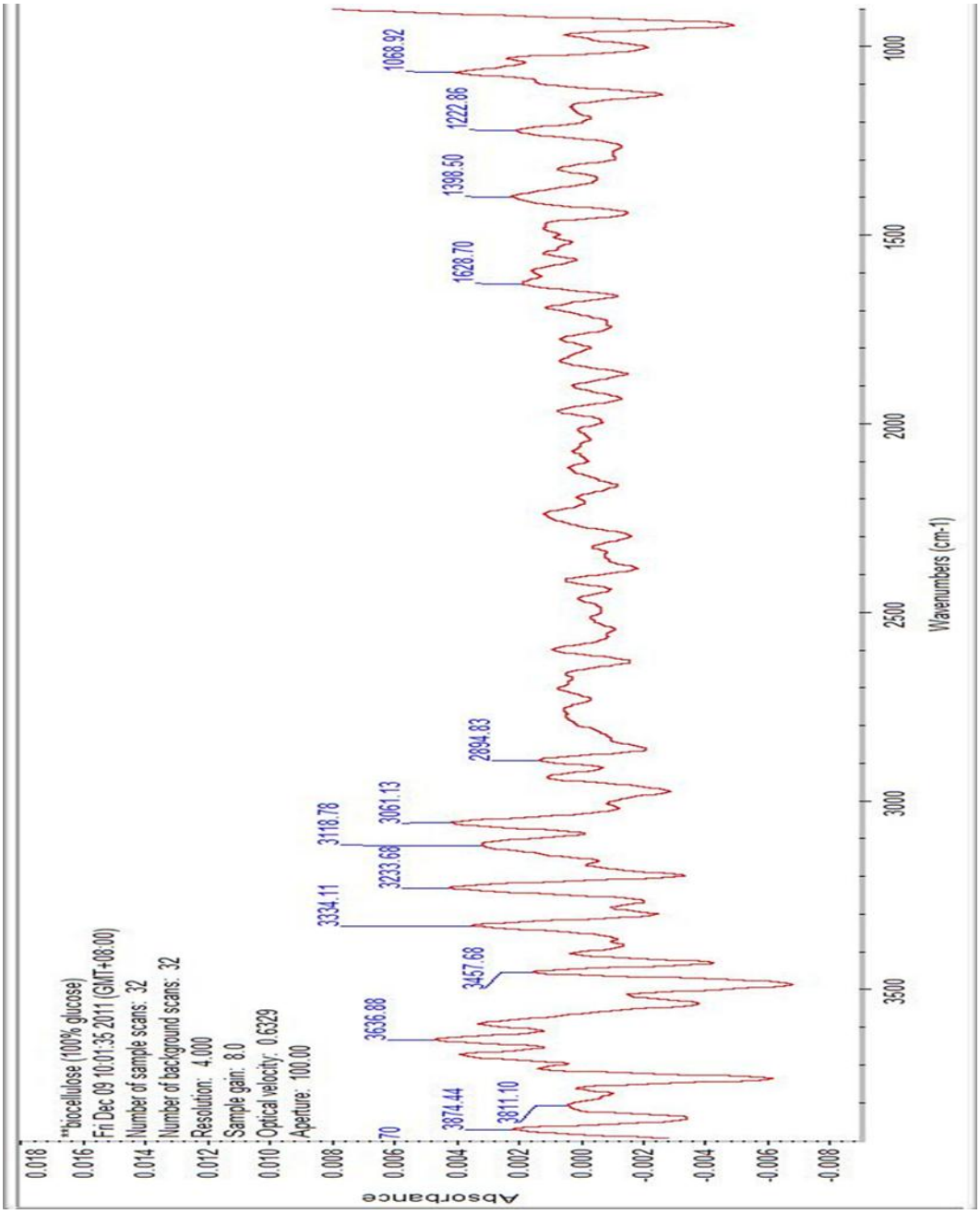
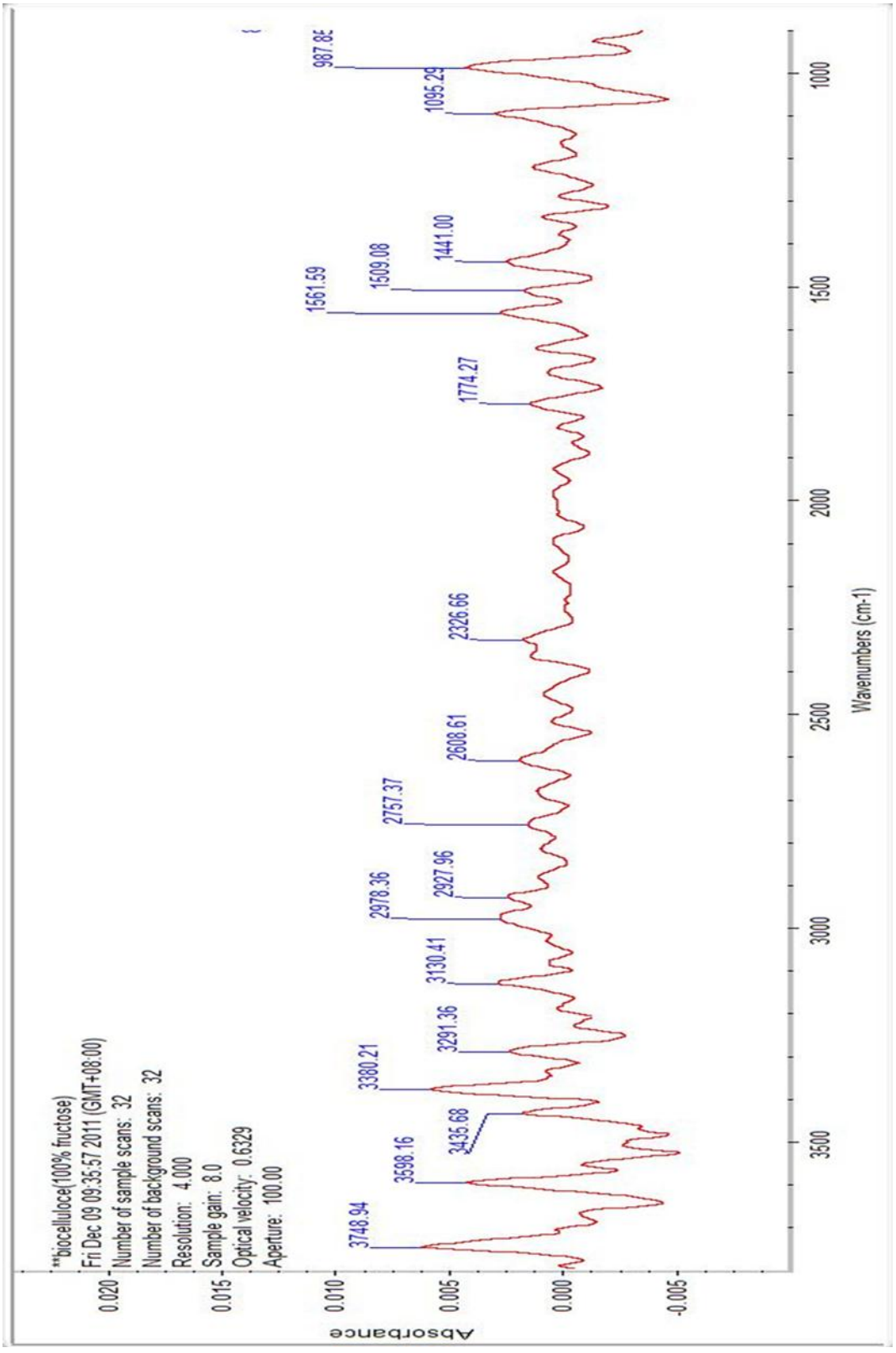
APPENDIX A

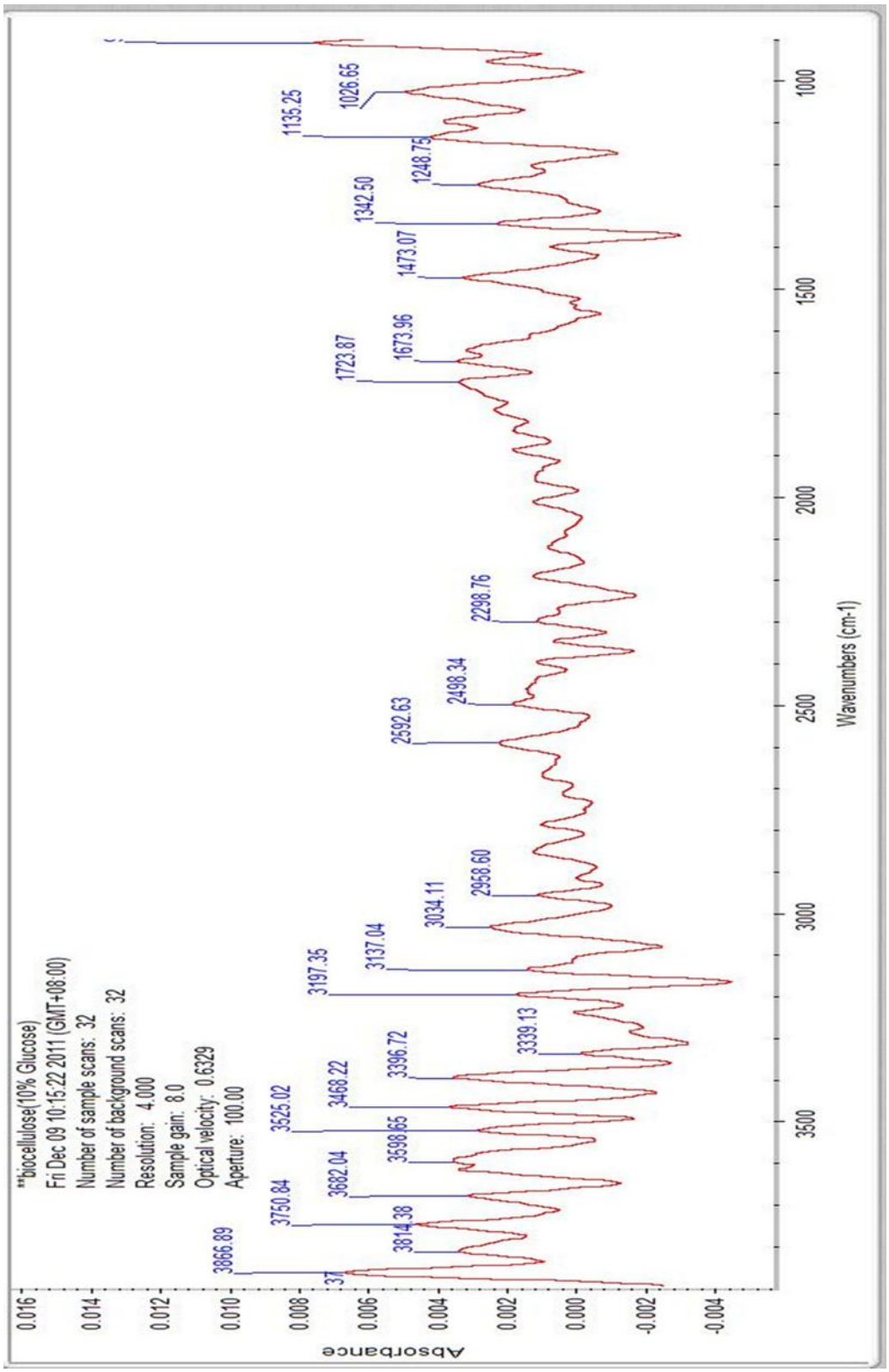
FOURIER TRANSFORM INFRARED SPECTROCOPYMETER



Appendix A1: FTIR spectra of BC from medium culture that contain only glucose



Appendix A2: FTIR spectra of BC from medium culture that contain only fructose



Appendix A3: FTIR spectra of BC from medium culture that contain 10%glucose+90%fructose

APPENDIX B

BIOCELLULOSE PRODUCTION PROCESS

Inoculum Preparation

Prepared Inoculum

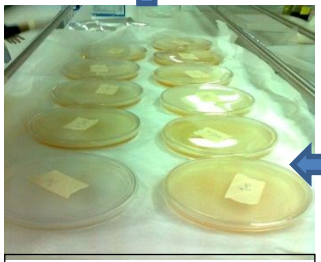
Culture Medium Preparation

Inoculum + Culture Medium

Incubate Samples

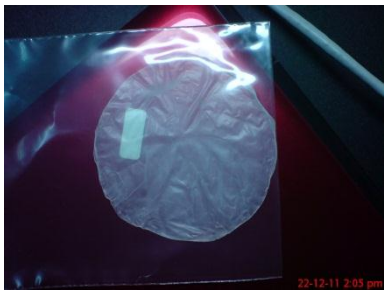
Raw Biocellulose Produced

Biocellulose Treatment in NAOH Solution



Treated Wet Biocellulose

Dry Biocellulose in Oven



DRIED BIOCELLULOSE