INVESTIGATION OF THE SUITABILITY OF CASSAVA FLOUR AND ALGAL STARCH FOR BACTERIAL CELLULOSE PRODUCTION

by

Hüma Kurtoğlu Uzyol

BS. in Env.E., Yildiz Technical University, 2007

MS. in Env.E., Yildiz Technical University, 2009

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APPROVED BY:

Prof. Melek Türker Saçan .....
(Thesis Advisor)

Prof. Dilek Kazan .....

Prof. Ebru Toksoy Öner .....

Assoc. Prof. Burak Demirel .....

Assoc. Prof. İ. Raşit Bilgin .....

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INVESTIGATION OF THE SUITABILITY OF CASSAVA FLOUR AND ALGAL STARCH FOR BACTERIAL CELLULOSE PRODUCTION

Bacterial celluloses have a wide range of applications in many areas such as environmental, medical, food and electronics.

In the present study, the suitability of two carbon sources were tested for BC production. Cassava flour, as a starch-rich material, was tested to be used as a carbon source after the hydrolysis of its starch content to glucose. Since starch is among the valuable components of microalgal biomass, glucose obtained by the hydrolysis of starch from *Chlorella vulgaris* was also tested to be used in BC production. While algae has a lower starch content than cassava flour, several stress conditions were attempted to increase the starch content of *C. vulgaris*.

To the best of our knowledge, the present study is the first report on the use of hydrolysates of cassava flour and algal starch as carbon sources in the production of BC by *Komagataeibacter hansenii*. BC yields on dry weight basis were 1.202±0.005 $g.L^{-1}$, 1.138±0.004 $g.L^{-1}$, and 1.104±0.002 $g.L^{-1}$ from glucose (as control), cassava flour, and algal starch, respectively. The results of the characterization studies have revealed that the morphological and chemical characteristics of the BCs produced from cassava flour and algal starch were similar to the BC produced from glucose.

BAKTERİYEL SELÜLOZ ÜRETİMİ İÇİN KASAVA UNU VE ALG NİŞASTASININ UYGUNLUĞUNUN ARAŞTIRILMASI

Bakteriyel selülozların çevre, tıp, gıda ve elektronik gibi çeşitli alanlarda geniş ölçekte kullanımı söz konusudur.

Bu çalışmada, iki karbon kaynağının bakteriyel selüloz üretimi için uygunluğu test edilmiştir. Nişasta bakımından zengin bir materyal olan kasava unu, nişasta içeriğinin glikoza hidrolizinden sonra bir karbon kaynağı olarak kullanılmak üzere test edilmiştir. Nişastanın mikroalg biyokütlesinin değerli bileşenlerinden biri olmasından dolayı, *Chlorella vulgaris*'ten sağlanan nişastanın hidrolizi ile elde edilen glikoz da bakteriyel selüloz üretiminde kullanılmak üzere test edilmiştir. Algin nişasta içeriği kasava ununa göre oldukça düşük olduğundan, nişasta içeriğini arttırmak amacıyla *C. vulgaris* üzerinde birkaç stres koşulu denenmiştir.

Bildiğimiz kadarıyla bu çalışma, kasava unu ve alg nişastası hidrolizatlarının *Komagataeibacter hansenii* tarafından bakteriyel selüloz üretiminde karbon kaynağı olarak kullanıldığı ilk çalışmadır. Glikoz (kontrol olarak), kasava unu ve alg nişastasından elde edilen bakteriyel selüloz (kuru ağırlık olarak) sırası ile 1.202±0.005 $g.L^{-1}$, 1.138±0.004 $g.L^{-1}$ ve 1.104±0.002 $g.L^{-1}$ şeklindedir. Karakterizasyon çalışmalarının sonuçları, kasava unu ve alg nişastasının kullanımı ile üretilen bakteriyel selülozların, glikozdan elde edilen bakteriyel selüloz ile benzer morfolojik ve kimyasal özelliklere sahip olduğunu göstermiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Units used
BBM	Bold's basal medium	
BC	Bacterial cellulose	
CCAP	Culture Collection of Algae and Protozoa	
CrI^{IR}	Crystallinity index	
ddH_2O	Double-distilled water	
DSM	Deutsche Sammlung von Mikroorganismen	
DW	Dry weight	(g)
f^{IR}	Mass fraction	
FM	Fermentation medium	
FTIR	Fourier transform infrared spectroscopy	
GO	*Gluconobacter oxydans* medium	
HPLC	High performance liquid chromatography	
HS	Hestrin and Schramm medium	
MCC	Microcrystalline cellulose	
OD_{680}	Optical density at 680 nm	
OECD	Organisation for Economic Co-operation and Development	
SEM	Scanning electron microscopy	

1. INTRODUCTION

The use of environmentally friendly materials is increasingly gaining the attention of scientists in parallel with the increasing problems related to the pollution, and growing population all around the world. Biomaterials can be derived either from nature or synthesized in the laboratory, and have become a globally popular research and development area. Improved biotechnologies that give importance to the utilization of agricultural/industrial wastes and food products as feedstocks will become more popular in the near future (Hong et al. 2008, 2011, 2012; Zeng et al. 2011; Vazquez et al. 2013). Of the biomaterials, bacterial cellulose is a widely used polymer in the world (Chawla et al. 2009; Zhong et al. 2013).

Bacterial cellulose is a microorganism based homopolymer of β-(1,4) glucose with the formula ($(C_6H_{10}O_5)_n$), mainly produced by various strains of the genera *Komagataeibacter* (formerly known as *Gluconacetobacter*, *Acetobacter*) (Li et al. 2012; Ma et al. 2012; Huang et al. 2013). The ability of *Acetobacter xylinum* on the cellulose synthesis has been extensively studied (Hestrin et al. 1954; Ha et al. 2012; Sunagawa et al. 2012; Kose et al. 2013; Mamlouk et al. 2013; Mohite et al. 2014). Knowing that the studies on bacterial cellulose production by *Komagataeibacter hansenii* are scarce in the literature, *K. hansenii* was selected as a bacterial cellulose producer in the present study.

Bacterial cellulose is distinguished from plant-based cellulose by its high purity. It does not contain residual hemicellulose or lignin. Moreover, the unique properties of bacterial cellulose including high elasticity, high crystallinity, high mechanical strength, high water-holding capacity, high specific surface area, good biocompatibility and high porosity have made bacterial cellulose a very potentially important material in many application areas (Bielecki et al. 1989; Zhong et al. 2013).

Bacterial cellulose is a fermentation product; and substrates, especially the carbon sources, affect the economical efficiency of microbial fermentation processes. Most of the studies with *Acetobacter* strains used only sugars (glucose, fructose, etc.) as carbon sources to produce bacterial celluloses (Jung et al. 2005; Zhou et al. 2007; Ge et al. 2011).

However, using pure sugars was found to be expensive (Zhong et al. 2013). Therefore, in order to increase the economical efficiency of the fermentation processes, a number of investigations have been conducted to find low or no value materials (e.g. beet molasses (Sunagawa et al. 2012), waste glycerol (Kose et al. 2013), etc.) for bacterial cellulose synthesis.

On the other hand, starch can also be considered as an alternative carbon source since it is a polymer of glucose. It has been reported by several researchers that cassava is a low-cost material as a starch source for bioethanol production processes (Rattanachomsri et al. 2009; Mussatto et al. 2010; Shanavas et al. 2011; Yoonan et al. 2012). Cassava (*Manihot esculenta* Crantz), also known as manioc or yuca in some parts of the world, has been a major food crop and it is a very important source of carbohydrate (Agu et al. 1997). Attempting to find an inexpensive way to obtain glucose, an agricultural product, cassava flour, was used in the present study and the suitability of cassava flour was firstly investigated for the production of bacterial cellulose.

In addition to cassava flour, algal starch was also firstly tested as an alternative material for the production of bacterial cellulose.

Microalgae are unicellular, photosynthetic microorganisms, and are known to have high protein, starch and oil content. Yen et al. (2013) underlined the importance of microalgal biorefinery studies that are related with the use of components (carbohydrates, lipids, proteins) of microalgae. Starch is among the valuable components of microalgal biomass (Doucha et al. 2009; Douskova et al. 2009; Mann et al. 2009). Algae accumulate starch as a carbon and energy source (Ballicora et al. 2004), and there have been several studies carried out to increase starch content of algae under different conditions (sulfur limitation, cycloheximide inhibition, nitrogen starvation, and different light intensities) (Branyikova et al. 2011; Dragone et al. 2011; Fernandes et al. 2012).

Knowing that the freshwater algae, *Chlorella vulgaris,* is a potential candidate for bioethanol production in terms of their carbohydrate content, it was of our interest to test if *Chlorella vulgaris* produced continuously in Ecotoxicology and Chemometrics Laboratory (Institute of Environmental Sciences, Bogazici University) can be a promising carbon

source for the fermentation processes ending with bacterial cellulose. Additionally, to increase the starch content of *Chlorella vulgaris*, different stress parameters such as nutrient starvation (nitrogen, sulfur) and dark cultivation were used.

Several hydrolysis methods have been used for the conversion of starch to glucose (Johnson et al. 2009; Rodrigues et al. 2011; Fernandes et al. 2012; Yoonan et al. 2012). Of these methods, the performances of enzymatic and acid hydrolysis were compared regarding the obtained glucose yields from cassava flour and algal starch.

Many optimization strategies were developed to enhance the yield of bacterial cellulose (Krystynowicz et al. 2002; Khajavi et al. 2011; Zakaria et al. 2012; Huang et al. 2015). Hence, knowing that a variety of parameters including incubation type, incubation period, pH, initial glucose concentration and the addition of ethanol affect the yield of bacterial cellulose, all of these parameters were tested to obtain the highest yield of bacterial cellulose in the present study. After the determination of optimum conditions, the suitability of hydrolysates from cassava flour and algal starch was investigated for the production of bacterial cellulose.

Generally, scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) analysis are used for evaluating the morphological and chemical characteristics of bacterial celluloses (Keshk et al. 2006; Yan et al. 2008; Halib et al. 2012; Gayathry et al. 2014; Neera et al. 2015). Several studies reported that using different carbon sources may led to differences on the structure of bacterial cellulose (Yan et al. 2008; Zhong et al. 2013). Therefore, in the present study, FTIR and SEM analysis were performed in order to investigate the microscopic and molecular structure of the bacterial celluloses produced from cassava flour, algal starch, and glucose.

2. AIM OF THE STUDY

To the best of our knowledge, the present study is the first report on the use of cassava flour and algal starch for the production of bacterial cellulose.

The specific objectives of this study were:

- to determine starch contents of cassava flour and *C. vulgaris*,
- to investigate the effects of stress parameters on *C. vulgaris* in Bold's basal medium in terms of its starch content,
- to compare the performances of enzymatic and acid hydrolysis regarding the obtained glucose yields from cassava flour and algal starch,
- to optimize fermentation conditions (i.e. incubation type, incubation period, pH, initial glucose concentration, and ethanol concentration) of *K. hansenii* for bacterial cellulose production,
- to use the hydrolysates of cassava flour and algal starch for bacterial cellulose production,
- to explore morphological characteristics of the produced bacterial celluloses by SEM,
- to analyze chemical characteristics of the produced bacterial celluloses by FTIR.

3. THEORETICAL BACKGROUND

3.1. Bacterial Cellulose

Polysaccharides play an important role in life sciences because of their superiority in chemical and physical properties (Morgan et al. 2013). They can be mainly categorized in two ways regarding their morphological localization, as intracellular polysaccharides and extracellular polysaccharides. Cellulose is found within the homopolysaccharides group of extracellular polysaccharides and it is a widely used biopolymer in the world (Chawla et al. 2009; Zhong et al. 2013).

Traditionally, celluloses have been produced from plants, however some species of the bacteria can secrete cellulose in the form of fibers. Bacterial cellulose is distinguished from plant-based cellulose by its high purity and it does not contain residual hemicellulose or lignin. Moreover, the unique properties of BC including high elasticity, high crystallinity, high mechanical strength, high water-holding capacity, high specific surface area, high porosity, good biocompatibility and biodegradability have made BC a very potentially important biomaterial (Bielecki et al. 1989; Zhong et al. 2013; Ruka et al. 2014).

Bacterial cellulose is a microorganism based homopolymer of β-(1,4) glucose with the formula ($(C_6H_{10}O_5)_n$), (Ma et al. 2012). Bacteria synthesize cellulose fibrils to generate ribbons (Figure 3.1.) and the biofilm formation occurs during synthesis (Klemm et al. 2011; Huang et al. 2013; Morgan et al. 2013; Shah et al. 2013). In some bacterial species, BC is produced reversely by synthesizing cellulose and building nanofibril bundles instead of nanocellulose formation by fibrillating fibers (Yousefi et al. 2013).

Figure 3.1. Basic structure of bacterial cellulose (Edited from Huang et al. 2013)

The biosynthesis of BC is a biochemical network containing a large number of key enzymes (Figure 3.2.) and the regulation of the enzymes controls the production of cellulose (Ha et al. 2011; Li et al. 2012; Huang et al. 2013). Therefore, the cellulose biosynthetic pathways and related enzymes have been studied extensively (Hestrin et al. 1947; Gromet et al. 1957; Ross et al. 1991; Stauner et al. 2013).

Figure 3.2. Proposed biochemical pathways for the production of BC

(Edited from Ha et al. 2011)

3.1.1. Application areas of bacterial cellulose

Bacterial cellulose has a great biological affinity, compatibility, degradability and with its many unique characteristics, it provides good performance as a biomaterial (Li et al. 2012; Chen et al. 2013b).

Bacterial cellulose is of great research interest in many fields including food and beverage processing, electronics (ear diaphragms, display devices), textile, paper manufacturing, packaging, cosmetics, enzyme immobilization, photocatalysis application, biosensors, dye decolorization, adsorption, membranes for separation, fuel cells, pharmaceutical and medical (drug carrier, artificial blood vessel, antibacterial wound dressing, tissue engineering scaffolds) applications (Indrarti et al. 2012; Huang et al. 2013; Katepetch et al. 2013; Kurniawan et al. 2013; Lin et al. 2013; Mondal, 2013; Mohite et al. 2014; Santos et al. 2015).

Several samples of studies with BC in various application areas are listed below.

Lu M. et al. (2011) prepared a highly magnetic composite adsorbent material with nano-Fe_3O_4 particles for the effective separation of cadmium ions.

Zhu et al. (2011) studied the adsorption of bovine serum albumin (BSA) and Pb^{2+} ions on BC pellicles. The BSA-BC and Pb-BC complexes were treated with sodium hydroxide and sodium citrate solutions, respectively. The recovery ratios were around 90 % for BSA, and around 75 % for Pb. They concluded that BC is a promising material for bioseparation and can be used in sewage treatment systems.

Feng et al. (2012) suggested the use of BC/graphene nanocomposites as electrically conductive materials for electrochemical devices.

Liu et al. (2012) prepared BC–Ag/AgCl nanocomposites and antimicrobial activity of these composites were tested against *Escherichia coli* and *Staphylococcus aureus*.

Katepetch et al. (2013) synthesized BC pellicles with ZnO particles by the absorption of zinc ions to BC and the nano crystalline ZnO particle-incorporated BC sheets were studied in terms of their antibacterial activity. They indicated that BC was a good alternative to be used as an antibacterial wound dressing material.

In the study of Li et al. (2015), BC was produced by *Gluconacetobacter xylinus* (ATCC 53582) at 30°C, for 14 days, in a controlled humidity level of 40 %. Bacterial cellulose-hyaluronan composite films were prepared and the percentage of hyaluronan was found as 0.1. They performed experiments on a full-skin injury model in Wistar rats and reported that BC-hyaluronan composite films were suitable in terms of their ability to enable normal skin breathing as wound dressing materials.

Sun et al. (2010) produced BC by *Acetobacter xylinum* NUST 5.2, then TiO_2 particles were coated to BC by surface hydrolysis method to serve as a supporting photocatalyst for methyl orange (as a model pollutant) degradation under UV irradiation.

3.1.2. Bacterial cellulose producers

Bacterial celluloses are generally produced by gram negative bacteria (Morgan et al. 2013). Especially members of *Komagataeibacter* genera (as known as *Gluconacetobacter*, *Acetobacter*) produce high amounts of cellulose, hence they mostly used for commercial purposes (Iguchi et al. 2000; Ma et al. 2012; Ruka et al. 2014). *Gluconacteobacter* species can be divided into two groups: **nitrogen fixers** (*G. diazotrophicus, G. liquefaciens, G. sacchari, G. azotocaptans, G. johannae*), and **cellulose producers** (*G. xylinus, G. europaeus, G. intermedius, G. oboediens, G. hansenii, G. entanii*) (Dellaglio et al. 2005).

The first production of BC using *Acetobacter xylinum* was reported by Brown in 1886, and the ability of *Acetobacter xylinum* on the cellulose synthesis has been widely studied (Hestrin et al. 1954; Ha et al. 2012; Sunagawa et al. 2012; Kose et al. 2013; Mamlouk et al. 2013; Mohite et al. 2014).

In the present study, knowing that the studies on BC production by *Komagataeibacter hansenii* are scarce in the literature, *K. hansenii* was selected as a BC producer.

3.1.2.1. *Komagataeibacter hansenii* as a BC producer. In honor of Dr. Kazuo Komagata, a famous Japanese microbiologist, this genus was named as *Komagataeibacter*. *Komagataeibacter hansenii* is a non-motile, mesophilic, rod-shaped, aerobic, gram negative bacterium that has a length of 1.0-3.0 µm, and colonies are white-creamy and smooth (Yamada et al. 2012). The scientific classification of *K. hansenii* was given in Table 3.1.

Table 3.1. The scientific classification of *K. hansenii*

Domain	Bacteria
Phylum	Proteobacteria
Class	Alphaproteobacteria
Order	Rhodospirillales
Family	Acetobacteraceae
Genus	*Komagataeibacter*
Species	*Komagataeibacter hansenii*
Full scientific name	*Komagataeibacter hansenii*
Type strain	+

3.1.3. Carbon sources for bacterial cellulose production

Pure sugars such as glucose, maltose etc. are generally used in BC production (Hungund et al. 2010; Castro et al. 2012, Ruka et al. 2014). Castro et al. (2012) isolated the *Gluconacetobacter medellensis* strain, and conducted a study for BC production with different carbon sources such as glucose, sucrose, fructose, mannitol, maltose, cellobiose, xylose and galactose. However, glucose was generally reported as the best carbon source for BC production (Khajavi et al. 2011; Rani et al. 2011; Castro et al. 2012).

Because of economic reasons, researchers have investigated to use agricultural/industrial wastes as carbon source instead of pure sugars especially for large scale of BC production (Sunagawa et al. 2012; Kose et al. 2013).

In the scope of this, the suitability of two different starch-based carbon sources were tested for BC production in the present study. A simplified schematic diagram from starch hydrolysis to bacterial cellulose synthesis is shown in Figure 3.3.

Figure 3.3. A schematic diagram from starch hydrolysis to BC synthesis (Present study)

<u>3.1.3.1. Microalgae as a carbon source.</u> Depending on the increasing global energy consumption, environmental friendly biotechnological studies have gained importance. Microalgae have various advantages such as high photosynthetic rate, less land requirement, effective carbondioxide fixation, high contents of lipid and starch compared to terrestrial plants. These advantages have made microalgae a preferred feedstock for industrial applications (Mcconnell et al. 2012; Suali et al. 2012; Chen et al. 2013a).

Recently, the concept of “algorefinery/biorefinery” has been inspired from the petroleum refinery concept (Safi et al. 2014). It reflects a platform that integrates a process to fractionate the components of a biomass to produce multiple products. The conversion of valuable components in the biomass into refined products for various applications represents additional benefits from the algae (Uzyol and Saçan, 2016).

Microalgae are mainly used for biofuel production. Besides their lipid and carbohydrate contents that could potentially be converted to practical biofuel and some other products (Figure 3.4.), microalgal proteins are also valuable and used as animal feed.

Consequently, all contents of microalgal biomass are worth to be used for various applications, such as pharmaceutical chemicals, nutritional supplements, dyes for food and

cosmetics (Rodjaroen et al. 2007; Varfolomeev et al. 2011; Lammens et al. 2012; Priyadarshani et al. 2012).

Figure 3.4. Algae to biofuel processing (Edited from Sabarsky 2010)

For instance, a saline water microalgae, *Dunaliella salina* contains an ingredient which affects positively the energy metabolism of skin, and triggers cell proliferation. Moreover, collagen synthesis in skin can be stimulated by an extract of *Chlorella vulgaris*. *Chlorococcum* is used for bioethanol production by dark fermentation of starch, and also preferred because of its lipid content for biodiesel production. *Arthrospira* can be used in human nutrition because of its high protein content. The concentrated suspensions and frozen biomass of *Nannochloropsis oculata* can be used as a cultured feed for the rotifer *Brachionus plicatilis* (Spolaore et al. 2006; Busi et al. 2013).

Chlorella vulgaris

Chlorella vulgaris was discovered as the first microalgae by a Dutch researcher, Martinus Willem Beijerinck, in 1890. While the name chlorella means green in Greek, Latin suffix ella specifies its microscopic size (Figure 3.5.). *Chlorella vulgaris* is a unicellular freshwater microalgae and it has been present on earth for 2.5 billion years (Safi et al. 2014).

Figure 3.5. Microscopic view of *Chlorella vulgaris*
(© Culture Collection of Algae and Protozoa – with permission)

Chlorella vulgaris cells are spherical, have a diameter of 2-10 µm, and most of their structural elements are the same with plants. *Chlorella vulgaris* cells have a single chloroplast with two layers enveloping membrane made by phospolipids; while the outer membrane controls the permeability of ions and metabolites, inner layer takes role in protein transport. In addition to this, chloroplast of *C. vulgaris* has green photosynthetic pigments, called as chlorophyl-a and chlorophyl-b (Rai et al. 2013; Safi et al. 2014). *Chlorella vulgaris* belongs to the following scientific classification, given in Table 3.2.

Table 3.2. The scientific classification of *C. vulgaris*

Domain	Eukaryota
Kingdom	Plantae
Division	Chlorophyta
Class	Trebouxiophyceae
Order	*Chlorellales*
Family	*Chlorellaceae*
Genus	*Chlorella*
Species	*Chlorella vulgaris*

Chlorella gained attraction of German scientists as an untraditional food source in early 1900s with regards to its protein content (45.5 %, on dry weight basis). In 1950s, the Carnegie Institution of Washington successfully achieved the growth of this microalgae in large scale for CO_2 reduction. Nowadays, Japan is the world leader in *Chlorella* consumption. In terms of immune-modulating and anti-cancer properties, *Chlorella* is used for medical applications (Safi et al. 2014).

Chlorella is well studied under heterotrophic, mixotrophic and photoautotrophic conditions to investigate and enhance algal biomass production processes (Liu et al. 2010; Lau et al. 2014).

Cell wall polysaccharides and accumulated carbohydrate of *C. vulgaris* can be converted into fermentable sugars, and *C. vulgaris* is considered as a promising feedstock for bioethanol production via microbial fermentation (Chen et al. 2013a).

There are various strategies to increase the lipid and starch contents of microalgae for biofuel production. Some of these strategies are nitrogen, sulfur, and phosphorus limitation, dark cultivation, CO_2 supplement, excessive exposure to light, pH shift, temperature variation and excess iron in medium (Branyikova et al. 2011; Choix et al. 2012; Chen et al. 2013a; Ho et al. 2013; Juneja et al. 2013; Safi et al. 2014).

In the present study, *Chlorella* vulgaris was exposed to nitrogen starvation, sulfur starvation, and dark cultivation in order to enhance its starch content for BC production.

3.1.3.2. Cassava flour as a carbon source. Cassava, *Manihot esculenta Crantz*, is a popular starchy plant cultivated in tropical countries which is one of the most important crops, after rice and maize (Johnson et al. 2009; Rolland et al. 2012; Yuangsaard et al. 2013).

Figure 3.6. A photograph of cassava flour (Present study)

Cassava can grow with minimum chemical requirements (fertilizers etc.) on arid areas, and it is resistant to harsh conditions that makes it a low-cost and sustainable agricultural feedstock (Burns et al. 2012; Rosenthal et al. 2012; Zhang et al. 2016).

Cassava stores starch in the roots, thus cassava roots have become a good source of carbohydrates (Zevallos et al. 2016). While starch is the main component of cassava, cassava peels contain the cyanogenic glycosides, linamarin and lotaustralin which are naturally occurring, but potentially toxic compounds that release hydrogen cyanide (Ki et al. 2013).

Cassava flour is a powdery material (Figure 3.6.) obtained by grinding and sifting the roots of cassava plant. Generally, cassava flour consists of starch (mainly), other polysaccharides, sugar, protein, lipid and some inorganic materials (Charoenkul et al. 2011).

There are several studies that are related with bioethanol production from cassava (Rattanachomsri et al. 2009; Shanavas et al. 2011; Yingling et al. 2011; Yoonan et al. 2012; Zhang et al. 2013). Mussatto et al. (2010) reported that cassava is a low cost material as a starch source for bioethanol production compared to other raw materials (Table 3.3.).

Table 3.3. Raw materials and costs as a carbon source

Groups	Raw Material	Cost (US$/L)
Simple sugar	Sugarcane	0.16-0.22
	Sugar beet	0.60-0.68
	Whey	0.42-0.49
Starch	Corn	0.25-0.40
	Wheat	0.42
	Cassava	0.18

In the light of the characteristics mentioned above, cassava flour was selected as a carbon source for the fermentation of BC in the present study.

3.1.4. Optimization of bacterial cellulose production

Medium selection and optimization of growth conditions are essential to produce high amounts of bacterial cellulose. They may also affect the morphology and/or properties of

BC (Krystynowicz et al. 2002; Chawla et al. 2009; Ruka et al. 2012; Zakaria et al. 2012; Dayal et al. 2013).

Style of fermentation affects the synthesis of BC. Two types of cultivation methods are usually used in BC production, namely static and agitated cultivation. Under static culture conditions, a gelatinous membrane of BC is accumulated on the culture surface whereas under agitated culture conditions, BC is produced as an irregular mass such as granule, pellet and fibrous strand (Hong et al. 2008; Zhu et al. 2011; Ha et al. 2012; Tanskul et al. 2013; Wu et al. 2013; Huang et al. 2015). Krystynowicz et al. (2002) reported that Cel^+ colonies (cellulose producers) are dominant under static cultivation. However, it was also stated that there are Cel^- mutants (cellulose nonproducers) that causes a decline in cellulose synthesis under agitated cultivation (Watanabe et al. 1998; Esa et al. 2014).

Sheykhnazari et al. (2011) reported that hydrogen and C-H bonds increased with the increasing fermentation time for the synthesis of BC. Different incubation periods were tested to attain higher yields of BC (Ge et al. 2011; Khajavi et al. 2011; Ha et al. 2012; Mohite et al. 2013).

pH is another parameter that affects the synthesis of BC (Bielecki et al. 1989; Rani et al. 2011; Ha et al. 2012; Tanskul et al. 2013; Huang et al. 2015). Bielecki et al. (1989) indicated that although the optimum pH value depends on the bacterium strain, it generally ranges from slightly acidic to neutral for BC synthesis.

Several studies reported that initial glucose concentration of fermentation medium affects the BC production. Increasing the initial glucose concentration leads to gluconic acid formation, which causes a drop of the pH, thus inhibits the growth of bacteria and BC production (Masaoka et al. 1993; Son et al. 2001; Rani et al. 2011; Zahan et al. 2014).

Incorporation with some ingredients to BC is also possible to attain a better yield of cellulose (Jung et al. 2005; Zhou et al. 2007; Ha et al. 2011; Lu Z. et al. 2011; Liu et al. 2012; Kose et al. 2013; Ul-Islam et al. 2013). Previous reports stated that the presence of ethanol improves the synthesis of bacterial cellulose (Naritomi et al. 1998; Krystynowicz

et al. 2002; deFaveri et al. 2003; Park et al. 2003; Yunoki et al. 2004; Hutchens et al. 2007; Li et al. 2012).

In the present study, the effects of incubation type and period, pH, initial glucose concentration, and the addition of ethanol as a supplementary material on the production of BC by *K. hansenii* were investigated.

4. MATERIALS AND METHODS

4.1. *Chlorella vulgaris*

Chlorella vulgaris (CCAP 211/11B) was taken from Ecotoxicology and Chemometrics Laboratory, Institute of Environmental Sciences, Bogazici University, Istanbul, Turkey.

4.1.1. Cultivation of *Chlorella vulgaris*

Chlorella vulgaris was cultivated in a modified Bold's basal medium (Table 4.1.). All chemicals were of analytical grade and supplied by either *Sigma* or *Merck*.

Table 4.1. Bold's basal medium

Component	Stock Solution ($g.L^{-1}$ ddH_2O)	Quantity Used (mL)
$NaNO_3$	75.0	10.0
$CaCl_2.2H_2O$	2.5	10.0
$MgSO_4.7H_2O$	7.5	10.0
$K_2HPO_4.3H_2O$	7.5	10.0
KH_2PO_4	17.5	10.0
NaCl	2.5	10.0
Trace elements solution		6.0
Vitamin B1		1.0
Vitamin B2		1.0

Bold's basal medium (enriched with nitrate three times, and the addition of two vitamins) was prepared as described by CCAP, Scottish Marine Institute, UK. Recipes for stock solutions of trace elements, vitamin B1 and B2 were given in Appendix A. The final pH was adjusted to 6.8 by using 0.1 N NaOH and/or 0.1 N HCl, then the media was sterilized by autoclaving at 121°C, for 20 min, under 1.5 psi.

Algal cultivation was performed according to the algal growth inhibition test (No:201) recommended by Organisation for Economic Co-operation and Development for

toxicity determination (OECD, 2006). The control cultures of algal toxicity experiments, that are routinely carried out in Ecotoxicology and Chemometrics Laboratory, were used in BC production. The details of the test conditions were given in Table 4.2.

Table 4.2. Test conditions for *C. vulgaris*

Test type	Static non-renewal, batch test
Initial cell density	$1.5x10^5$ cells.mL^{-1}
Temperature	24.0±0.6°C
Light quality	Cool white fluorescent lighting
Light intensity	60 µmol photons $m^{-2}s^{-1}$
Photoperiod	Continuous illumination
Replicates	3
Growth medium	Bold's basal medium

Cultivation of algae was carried out in a growth chamber (*Digitech*). *Chlorella vulgaris* cells were cultured in 500 mL Erlenmeyer flasks (made of borosilicate glass) containing 100 mL of medium under batch conditions (Figure 4.1.). The flasks were shaken daily by hand, and growth of *C. vulgaris* was monitored by measuring the absorbances at 680 nm with a spectrophotometer (*BIORAD SmartSpec*^{TM} *3000*).

Figure 4.1. *C. vulgaris* culture in Erlenmeyer flasks

<u>4.1.1.1. Stress conditions for algal cultivation.</u> In order to investigate the effect of cultivation conditions on the starch content of algae, *C. vulgaris* was exposed to different stress conditions. The stress parameters used in the present study were nitrogen starvation,

sulfur starvation and dark cultivation. Effects of stress conditions on algal growth were evaluated by OD_{680} measurements. All experiments were carried out in triplicate.

Cultivation conditions of algal culture are given in Table 4.2. Algal culture samples were centrifuged at 4000 g for 10 min. After centrifugation, cells were double-washed with sterile ddH_2O to perform complete removal of medium components. These medium-free algal cells were resuspended in different media which presented various cultivation conditions (given below), and the pH values were kept at 6.8.

- Normal cultivation (BBM, continuous light, at 24.0±0.6°C)
- Dark cultivation (BBM, lights turned off, at 24.0±0.6°C)
- Nitrogen starvation (BBM without nitrogen, continuous light, at 24.0±0.6°C)

  NaOH (0.35 $g.L^{-1}$) was added to the BBM instead of $NaNO_3$ (0.75 $g.L^{-1}$).
- Sulfur starvation (BBM without sulfur, continuous light, at 24.0±0.6°C)

  $MgCl_2$ (0.029 $g.L^{-1}$) was added to the BBM instead of $MgSO_4.7H_2O$ (0.075 $g.L^{-1}$).

4.1.2. Determination of algal cell density and dry cell weight

A calibration curve was generated to estimate dry cell weight ($g.L^{-1}$) of algal culture from optical density. Ten mL culture samples were filtered through pre-weighed 0.45 µm pore size filters (*Millipore*), then dried in an oven at 80°C to a constant weight for dry cell weight determination. Optical density of algal culture was monitored simultaneously, at 680 nm by a spectrophotometer (*BIORAD SmartSpec^{TM} 3000*).

Similarly, a plot of absorbance versus algal cell density ($cells.mL^{-1}$) was prepared. Cell counting was performed by a microscope (*Leica DM 4000B)*, using a hemacytometer (*Neubauer* chamber). First of all, a small aliquot of the cell suspension was transferred by Pasteur pipette into the *Neubauer* chamber (Figure 4.2.). Then, six separate counts (total count number; six) per chamber were recorded for the large centre square (a total of 25 medium squares), with a magnification of 100.

Figure 4.2. The central large square of *Neubauer* chamber

Since the volume of the counted square equals to 10^{-4} mL (1 mm *1 mm * 0.1 mm; length * width * depth), the average number of cells per mL was calculated from the given equation [4.1]:

$$\text{Cell density (cells.mL}^{-1}) = \frac{\text{Total cell count}}{\text{Total Count \# of the large centre square}} \times 10^4$$

Equation [4.1]

4.1.3. Preparation of freeze-dried algal lysates

Algal culture samples were collected at the beginning of stationary phase, and harvested by centrifugation at 4000 g for 10 min. Cells were double-washed with sterile ddH_2O, then dried in an oven at 80°C to a constant weight.

In order to maintain cell disruption before starch determination, ultrasonic pretreatment was applied on the dried algal cells in sterile ddH_2O by a sonicator (*Branson 5500*). Fundamental principle of this pretreatment was the conversion of the sonic energy into mechanical energy. Cell wall was destroyed by the influence of microbubbles and starch packages were released. In this way, the use of carbohydrate components of algae became possible.

For algal cell disintegration, sonication was conducted at 45°C for 15 min, with a constant frequency of 40 kHz and an output power of 2.2 kW in continuous mode, as described by Jeon et al. (2013). Then, algal lysates were freeze-dried (by *Christ Alpha 1-2 LD plus)* for starch analysis.

4.2. Cassava Flour

Cassava flour was kindly provided by Dr. Aziz Akın Denizci and Dr. Güldem Utkan from Council for Scientific and Industrial Research, Ghana, Africa.

Cassava flour was dried in an oven at 105°C to a constant weight before starch analysis and hydrolysis procedures.

4.3. Starch Analysis and Hydrolysis

Starch analysis and hydrolysis procedures were applied to dried cassava flour and freeze-dried algal lysates.

4.3.1. Analysis of total starch

Starch analysis was assayed by the hydrolysis of starch to glucose with amylolytic enzymes (α-amylase and amyloglucosidase) according to the procedure provided by *Megazyme* (Appendix B; Total Starch Assay procedure, © Megazyme International Ireland 2011). Analyses were performed in triplicate. Starch content was calculated as percentage of dry weight by equation [4.2]:

$$\text{Starch (\% DW)} = [\ \Delta A \times (F/W) \times FV \times 0.9\ ] * DF \qquad \text{Equation [4.2]}$$

Where, ΔA is the absorbance read against the reagent blank; F is the conversion factor from absorbance to µg; W is the weight in miligrams of the sample analysed, FV is the final volume, 0.9 is the adjustment ratio from free D-glucose to anhydrous D-glucose, lastly DF is the dilution factor.

4.3.2. Hydrolysis of algal starch and cassava flour

Both enzymatic and acidic methods were used to compare the efficiency of hydrolysis in terms of glucose yields. Triplicate experiments on the hydrolysis were conducted at 10 % (w/v) substrate concentrations.

In the enzymatic method, alpha-amylase (*Liquozyme SC 4X*) and glucoamylase (*Spirizyme Excel*) enzymes were used following the manufacturer's instructions (*Novozymes*, Denmark).

1) First, gelatinization step was done at 70°C, 30 min, 200 rpm, with a pH of 5.5.
2) Secondly, liquefaction step was started (pH 5.5), and α-amylase enzyme (43000 $U.mL^{-1}$) was added, hydrolysate was incubated at 85°C in a water bath for 30 min.
3) In the end, glucoamylase enzyme (20000 $U.mL^{-1}$) was added for saccharification step, at 60°C, 200 rpm, and pH 4, for 3 hours.

The acidic method was applied using H_2SO_4 in two steps. In the first step, 72 % (v/v) H_2SO_4 was used and hydrolysate was kept at 30°C, for 1h. In the second step, hydrolysate was treated with 4 % (v/v) H_2SO_4 at 121°C, for 1 h (Laurens et al. 2012).

After cooling to room temperature, hydrolysates were neutralized by 0.1 N NaOH, and were filtered through 0.22 µm pore size syringe filter (*Techno Plastic Products AG*) prior to their introduction into the high performance liquid chromatography (HPLC) system, then stored at +4°C.

4.3.3. Determination of glucose concentration

Glucose concentrations were determined by HPLC system *Class LC 10* (*Shimadzu*, Japan) using refractive index detector equipped with a MetaCarb 87H column 300x7.8 mm (*Agilent Technologies*, US). 0.01N sulfuric acid was used as mobile phase with a flow rate of 0.4 $mL.min^{-1}$, and column temperature was 35°C. A calibration curve was prepared using glucose as standard.

Glucose yields of starch from algae and cassava flour were given as percentage of dry weight (% DW).

4.4. Production of Bacterial Celluloses

Komagataeibacter hansenii was used for the production of BC. *Komagataeibacter hansenii* (DSM 5602) was purchased from German Collection of Microorganisms and Cell Cultures.

4.4.1. Culture medium and fermentation conditions

In order to maintain *K. hansenii* culture, *Gluconobacter oxydans* (GO) medium was used (Table 4.3.). All chemicals were of analytical grade and supplied by either *Sigma* or *Merck*.

A single colony of *K. hansenii* from GO plate was taken with a sterile loop and inoculated into 5 mL GO medium. This culture tube was incubated at 28°C in an orbital shaker (*B.Braun*) at 180 rpm, for 24 h. Then, 100 µL of the 24-h old culture was inoculated into 5 mL GO medium, and incubated for 48-h at the same conditions (28°C, 180 rpm).

Table 4.3. Basal medium for cultivation of *K. hansenii*

Gluconobacter oxydans medium, pH 6.8	
Chemical	Concentration % (w/v)
Glucose	10.0
Yeast extract	1.0
$CaCO_3$	2.0
Agar(*)	1.5

(*) Added for solid media

Two media were tested for BC production (Table 4.4.). The first medium was a modified nutrient broth with the addition of glucose and NaCl, and it was called as Fermentation medium (FM). The second medium was Hestrin and Schramm medium (HS).

Table 4.4. Media tested for BC yields

Fermentation medium (FM), pH 6		Hestrin and Schramm medium (HS), pH 6	
Chemical	Concentration % (w/v)	Chemical	Concentration % (w/v)
Glucose	1.5	Glucose	2.000
Meat extract	0.3	Yeast extract	0.500
Peptone	0.5	Peptone	0.500
NaCl	0.5	Na_2HPO_4	0.270
		Citric acid	0.115

Two and a half mL of the 48-h old *K. hansenii* culture was used as inoculum for 50 mL of each of FM and HS media. All experiments were conducted in triplicate at a temperature of 28°C, under static incubation conditions for 7 days.

Fermentation of BC was conducted in static and agitated conditions. For static conditions, *K. hansenii* culture in FM medium (pH 6) was incubated in an oven at 28°C for 7 days. For agitated fermentation, *K. hansenii* culture in FM medium (pH 6) was incubated at 28°C in an orbital shaker at 180 rpm, for 7 days. Both conditions were studied in triplicate.

4.4.2. Determination of optimal conditions for BC production

A variety of parameters including incubation type, incubation period, pH, glucose concentration, and ethanol concentration were tested to obtain the highest yield of BC.

All parameters were optimized using one factor at a time approach, and corresponding experiments were carried out in triplicate. Inital pH values were adjusted by using 0.1 N NaOH and/or 0.1 N HCl. The pH measurements were performed by *Hanna Instruments* HI 22091 pH-meter. Ethanol (≤ 100 %) was sterilized by membrane filtration (*Techno Plastic Products AG*, pore size 0.22 µm), and added to the media in aseptic conditions.

Incubation type (static, agitated), incubation period (7-14-21 days), pH values from 4 to 7, initial glucose concentrations from 0.5 to 1.5 % (w/v) and ethanol concentrations in

the range of 0.0-1.0 % (v/v) were investigated to find optimum conditions of BC synthesis by *K. hansenii* (Table 4.5.).

Table 4.5. Parameters tested to optimize BC production (Present study)

Incubation type	Static Agitation
Incubation period (d)	7 14 21
pH	4 5 6 7
Initial glucose concentration % (w/v)	0.5 1.0 1.5
Ethanol concentration % (v/v)	0.0 0.5 1.0

After each optimization step, analysis of glucose by HPLC and pH measurements were performed in the residual culture medium.

4.4.3. Use of hydrolysates in BC production

After the determination of optimal conditions, glucose at the selected medium was replaced by the hydrolysates of algal starch, and cassava flour. As a control, glucose (*Merck*) was used, and BCs were obtained according to the optimized production conditions.

Removal of bacterial cells and medium components from BCs was achieved by the treatment with 0.1 N NaOH, at 80°C, for 20 min. Then, BC pellicles were washed with sterile ddH_2O until neutrality (pH 7), and lyophilized by a freeze dryer (*Christ Alpha 1-2 LD plus*) before characterization studies. Bacterial cellulose yields on dry weight basis were given as $g.L^{-1}$ (g of *BC* / L of *fermentation medium*).

4.5. Characterization of Bacterial Celluloses

The microfibrillated structure of the previously lyophilized BCs was investigated under a scanning electron microscope (SEM) (*JEOL/JSM 6510LV*) by taking micrographs of gold-coated (with *Polaron Emitech SC7640 Sputter Coater*) samples.

Analyses for the structural differences were recorded by a fourier transform infrared (FTIR) spectrometer (*Thermo Scientific Nicolet 6700*), equipped with a *Smart Orbit* high performance diamond attenuated total reflectance accessories, in the transmittance mode with a resolution of 1 cm^{-1} in the range of 4000 – 400 cm^{-1}. Microcrystalline cellulose (MCC) was used as a reference.

5. RESULTS AND DISCUSSION

5.1. Growth of *Chlorella vulgaris*

Growth curves of *Chlorella vulgaris* in Bold's basal medium (control), nitrogen starvation medium, sulfur starvation medium, and BBM under dark cultivation condition are shown in Figure 5.1.

Figure 5.1. Growth of *C. vulgaris* in different cultivation conditions (error bars represent standard deviation of the mean, n=3)

As seen in Figure 5.1., *C. vulgaris* showed no growth under dark cultivation conditions. Besides that, *C. vulgaris* enters the exponential growth on the 5th day for other cultivation conditions. The 10th day was observed to be the beginning of the stationary phase, and the starch content of algae was determined at this phase where the growth rate of biomass started to decrease. The growth curves of *C. vulgaris* in different media were similar, however, cell yields were found different for each media. Sulfur starvation and

nitrogen starvation slowed down the growth rate of *C. vulgaris* compared to control conditions. This result is consistent with the reports of Branyikova et al. (2011) and Dragone et al. (2011). Sulfur starvation resulted in a slight decrease in growth compared to nitrogen starvation.

Several studies supported the selection of stationary phase for the determination of starch content similar to our finding (Rodrigues et al. 2011; Fernandes et al. 2012; Razaghi et al. 2014). For instance, Razaghi et al. (2014) stated that starch accumulation occured at stationary phase for *Porphyridium cruentum* microalgae. Therefore, stationary phase was suggested to measure the starch content of algae.

5.2. Algal Cell Density and Dry Cell Weight

A plot of absorbance versus algal cell density (cells.mL^{-1}) was given in Figure 5.2., and equation [5.1] presents their linear relationship.

Absorbance (OD_{680}) = 7E-08 * Algal cell density (cells.mL^{-1}) Equation [5.1]

Figure 5.2. Relationship between the absorbance and cell density of *C. vulgaris*

Figure 5.3. Relationship between the absorbance and dry cell weight of *C. vulgaris*

In addition to cell density, a linear relationship was obtained between the average values of absorbance and dry cell weight ($g.L^{-1}$), given in Figure 5.3. for three replicates, and resulted in equation [5.2].

Absorbance (OD_{680}) = 4.5345 * Dry cell weight ($g.L^{-1}$) Equation [5.2]

Hu (2014) also measured optical density at 680 nm for *C. vulgaris* sp. cultivated in BBM, and described dry cell weight (DCW) in terms of $g.cell^{-1}$ using equation [5.3] below:

$$\frac{g}{cell} = \frac{dry\ weight\ (\frac{g}{mL})}{cell\ concentration\ (\frac{cell}{mL})}$$

Equation [5.3]

To compare our results with the study of Hu (2014), dry cell weight values corresponding to the 8th day of growth were calculated as $g.cell^{-1}$, and given in Table 5.1.

Table 5.1. Literature comparison of dry cell weight and cell density values

	DCW (g.cell^{-1})	OD_{680} (0.2)	OD_{680} (0.4)	References
C. vulgaris	$1.14x10^{-11}$	$3.8x10^{6}$ cells.mL^{-1}	$7.8x10^{6}$cells.mL^{-1}	Present study
	$1.54x10^{-11}$	$4.0x10^{6}$ cells.mL^{-1}	$8.0x10^{6}$ cells.mL^{-1}	Hu (2014)

In the present study, the algal cell density values were found as $3.8x10^{6}$ cells.mL^{-1} and $7.8x10^{6}$ cells.mL^{-1} for absorbance values of 0.2 and 0.4 (OD_{680}), respectively. Similar results were reported by Hu (2014) as far as the number of cells and dry cell weight were concerned (Table 5.1.).

5.3. Starch Content of *Chlorella vulgaris* and Effect of Stress Conditions

It is known that starch and lipid synthesis of microalgae were triggered by different cultivation conditions (Chen et al. 2013a; Safi et al. 2014). Therefore, in order to grow *C. vulgaris* for producing biomass rich in starch, three different stress conditions, nitrogen starvation, sulfur starvation and dark cultivation were tested in the present study.

Starch contents of algal culture determined under different conditions are given in Table 5.2. The algal starch content determined by the total starch assay procedure was 5.27±0.04 % (DW) under normal cultivation conditions. Similar to our finding, Dragone et al. (2011) reported that *Chlorella vulgaris* sp. had a starch content of 5.43 % (DW) at normal cultivation conditions.

Table 5.2. Starch contents as % (DW) of *C. vulgaris* under different cultivation conditions

	Control	Stress conditions			
Strain	Normal cultivation	Nitrogen starvation	Sulfur starvation	Dark cultivation	References
C. vulgaris (CCAP 211/11B)	5.27±0.04	7.14±0.17	5.00±0.07	1.35±0.04	Present study *
C. vulgaris (CCALA 924)	18.00	35.00	50.00	4.00	Branyikova et al. (2011)
C. vulgaris P12	5.43	41.00[a]	-	-	Dragone et al. (2011)
C. vulgaris P12	2.50	34.00	-	-	Fernandes et al. (2012)

* Values were expressed as mean ± SD (n=3)
a: Nitrogen and iron free medium used

Figure 5.4. *C. vulgaris* exposed to various stress conditions
(a) dark cultivation, (b) nitrogen starvation, and (c) sulfur starvation

Algal culture flasks under different cultivation conditions are shown in Figure 5.4. First of all, dark cultivation experiments were performed under batch conditions. Dark cultivation had a negative impact on the formation of starch for *Chlorella vulgaris* in the present study even though it stimulated the lipid synthesis in another microalgae, *Dunaliella virdis* (Juneja et al. 2013). The relative content of starch decreased to low levels (1.35±0.04 % of DW) under the dark cultivation conditions (Table 5.2.). In the study of Branyikova et al. (2011), starch content of algae was found 18.00 % (DW) at normal cultivation condition, which is greater than our result (5.27±0.04 % of DW). A possible reason of this difference may be the use of light intensities range from 16 to 780 µmol photons $m^{-2}s^{-1}$ in their study, and dark cultivation decreased the starch content to low level, 4.00 % (DW) which corresponds to 78 % decrease in starch content compared to normal cultivation (Table 5.2.). In the present study, starch content was also negatively affected by dark cultivation and a 75 % decrease was observed in starch content compared to control.

Nitrogen is one of the most important element which takes place in all structural and functional proteins in algal cells (Juneja et al. 2013; Blair et al. 2014). Nitrogen starvation was resulted in 35 % increase on the starch content compared to that of the normal cultivation condition in the present study (Table 5.2.). This can be explained by algae metabolism as described by Scott et al. (2010). The main principle of starch accumulation is, if there is not enough nitrogen for protein synthesis required for growth, excess carbon from photosynthesis is directed into storage molecules such as triacylglycerol or starch. In the study of Fernandes et al. (2012), there was an increase in starch content from 2.50 % (DW) to 34.00 % (DW) for control and nitrogen starvation, respectively. This increase may be explained by continuous carbon dioxide (CO_2) feed of *Chlorella vulgaris* sp. in

comparison with the present study. Dragone et al. (2011) also studied the nitrogen starvation for *C. vulgaris* P12. They limited nitrogen and iron in the medium, and these changes resulted in an increase in the starch content from 5.43 to 41.00 % (DW). This high yield of starch can be explained by the effect of nitrogen starvation stress in the presence of iron limitation on the stimulation of starch synthesis.

Sulfur is found in the structure of proteins, lipids and several metabolites, and it plays a major role in electron transfer. Since sulfur storage is limited, cells require a continuous uptake of this element from the environment (Zhang et al. 2004). The effect of sulfur starvation stress can be seen on Table 5.2. In the present study, sulfur starvation made a slight decrease in the starch content of *C. vulgaris* compared to that of the normal cultivation condition. Branyikova et al. (2011) studied the sulfur starvation, and contrary to our result, they found an increase in the starch content from 18 % (DW) to 50 % (DW) for control and sulfur starvation, respectively (Table 5.2.). The use of higher light intensities (range from 16 to 780 µmol photons $m^{-2}s^{-1}$) may be the reason for this increase compared to the present study.

Of the three stress conditions tested in the present study, nitrogen starvation medium had the highest yield of starch. Therefore, algal cells grown in nitrogen deficient medium were used to obtain algae-based glucose for fermentation of BC.

5.4. Starch Content of Cassava Flour

The starch content of cassava flour was found as 80.09±2.19 % (DW) which was consistent with the literature (Moorthy et al. 1996; Charoenkul et al. 2011).

Table 5.3. Starch contents of cassava flour

Starch % (DW)*	References
82.80±3.18	Moorthy et al. (1996)
82.54±3.42	Charoenkul et al. (2011)
80.09±2.19	Present study

* Values were expressed as mean ± SD (n=3)

Starch contents of cassava flours generally varied between 79 and 86 % (DW), concerning the given intervals in Table 5.3.

5.5. Determination of Glucose Concentration

Glucose contents of algae and cassava flour were determined after starch analysis. Figure 5.5. showed the calibration curve for glucose standards at a specific concentration range (0-10 $mg.mL^{-1}$) derived from seven concentration levels. The equation of the fit line is; y = 3E-07x; where, y is glucose concentration ($mg.mL^{-1}$), and x is the peak area ($x10^5$).

Figure 5.5. Calibration curve for glucose standards

In HPLC analysis of glucose, retention time was observed as 12.81 min. The identification of the peak was confirmed with known concentrations of glucose standards injected individually through the HPLC (Figure 5.6.). Overlapped glucose peaks in HPLC indicated high precision in retention time reproducibility, and represented a good response for the peak area.

Analyses of the hydrolysates showed that there were high amounts of glucose and the detected maltose (retention time: 10.76 min) percentages were negligible (data not shown). Moreover, the concentration range of glucose standards was found suitable for the analytes.

Figure 5.6. Overlaid HPLC chromatograms of glucose standards

The glucose was the only product of the hydrolysates confirmed by HPLC, and no interfering peak was observed.

5.6. Evaluation of the Hydrolysis of Cassava Flour and Algal Starch

Glucose yield was 85.60±0.20 % (DW) for the enzymatic hydrolysis of cassava flour in triplicate experiments (Figure 5.7.). The glucose yields as % (DW) obtained from enzymatic hydrolysis with the addition of amylolytic enzymes (α-amylase and amyloglucosidase) were slightly higher than those of sulfuric acid hydrolysis.

Figure 5.7. Glucose yields for enzymatic and acid hydrolysis of cassava flour (error bars represent standard deviation of the mean, n=3)

The glucose yield was 80.50±0.70 % (DW) after the acid hydrolysis of cassava flour in the present study. The variation in glucose yields regarding the applied hydrolysis methods is given in Table 5.4. Johnson et al. (2009) studied the enzymatic hydrolysis of cassava to obtain glucose, and the yield was in a range of 22.00-25.00 % (DW). Yoonan et al. (2012) achieved a glucose yield between 33.94 and 40.24 % (DW) after the acid hydrolysis of cassava. Rattanachomsri et al. (2009) applied enzymatic hydrolysis for cassava, and the yield of glucose was 71.60 % (DW).

Table 5.4. The glucose yields of cassava flour hydrolysis from different studies

Method	Glucose yield % (DW)	References
Enzymatic hydrolysis	22.00-25.00	Johnson et al. (2009)
Enzymatic hydrolysis	71.60	Rattanachomsri et al. (2009)
Acid hydrolysis	33.94-40.24	Yoonan et al. (2012)
Acid hydrolysis	80.50±0.70	Present study *
Enzymatic hydrolysis	85.60±0.20	

* Values were expressed as mean ± SD (n=3)

These variations in the glucose yields could be caused by the use of different enzymes and/or acid concentrations in the hydrolysis methods (Table 5.4.). Our results revealed that both methods were successful concerning the yield based on the percentage dry weight of glucose hydrolyzed from cassava flour, and the efficiency of enzymatic hydrolysis was higher than that of acid hyrolysis.

The starch content of *C. vulgaris* was found as 5.27 and 7.14 % (DW) for the normal cultivation and nitrogen starvation conditions, respectively. Thus, hydrolysis of algal starch were conducted to investigate glucose yields for both of the mentioned cultivation conditions. The yield as the percentage dry weight of glucose for the nitrogen starvation was higher than that of normal cultivation (Figure 5.8.). However, the glucose yield of enzymatic hydrolysis was lower than acid hydrolysis of algal starch for both cultivation conditions.

Figure 5.8. Glucose yields obtained from enzymatic and acid hydrolysis of algal starch for normal cultivation (control) and nitrogen starvation conditions (error bars represent standard deviation of the mean, n=3)

The highest glucose yield, 10.5 % (DW) was obtained from acid hydrolysis of algal starch in nitrogen starvation. The glucose yield of nitrogen starvation from enzymatic hydrolysis was 6.5-6.7 % (DW), whereas the glucose yields of normal cultivation from enzymatic hydrolysis and acid hydrolysis were 4.9-5.5 % (DW) and 8.5–8.9 % (DW), respectively (Figure 5.8.). Rodrigues et al. (2011) performed hydrolysis of algal starch by cellulase, xylanase, and amylase enzyme mixture. In their study, *Chlorella homosphaera*, and *Chlorella zofingiensis* were used as carbon sources, 2.9 % and 5.0 % (DW) were the glucose yields, respectively. Marsalkova et al. (2010) and Ho et al. (2013) also used different enzyme mixtures which were composed of glucanase, xylanase, cellulase and amylase. Their glucose yields were 8.6 % (DW) for *C. vulgaris* FSP-E, and 7.8 % (DW) for *C. vulgaris* sp., respectively. Guo et al. (2013) reported glucose yields as 2.7 % (DW) for *Mychonastes afer* PKUAC 9 (sequential hydrolysis with dilute sulphuric acid and a combination of alpha and glucoamylases) and 5.7 % (DW) for *Scenedesmus abunda*ns PKUAC 12 (sequentially hydrolyzed with dilute sulphuric acid and cellulase mixture).

The results obtained in our study were remarkable when compared with the previously reported data concerning the yields of glucose from algal starch hydrolysis. The reason of high glucose yield in the present study could be the use of ultrasonication for cell disintegration before hydrolysis.

5.7. Production of Bacterial Celluloses

5.7.1. Cultivation of bacteria and selection of BC production medium

K. hansenii colonies grown in GO plates are depicted in Figure 5.9. Appearance of zones of clearing indicated that acid was produced during the growth of *K. hansenii*, and reacted with the calcium carbonate content of the GO medium.

Figure 5.9. Clear zones below *K. hansenii* colonies

Chawla et al. (2009) stated that medium was one of the important parameters affecting the BC production. In the present study, FM and HS media were compared regarding their BC yields throughout the triplicate experiments. A photograph of produced BCs is demonstrated in Figure 5.10.

Figure 5.10. Produced BCs from FM and HS media

The BC yields were 0.620±0.005 $g.L^{-1}$ and 0.422±0.007 $g.L^{-1}$ for FM and HS media, respectively. Thus, FM medium was selected for the BC production by *K. hansenii*.

Ruka et al. (2012) studied the production of BC by *G. xylinus* using different types of media. The tested media were prepared as described by the previous reports of Hestrin et al. (1954), Yamanaka et al. (1989), Toyosaki et al. (1995), Son et al. (2003) and Zhou et al. (2007). Similar to our finding, Ruka et al. (2012) concluded that HS medium had a lower efficiency than the other tested medium regarding the produced BC yields.

5.7.2. Optimal conditions for BC production

5.7.2.1. Selection of incubation type. In the present study, static incubation was more efficient than agitated incubation, and there was an approximately 3.5-fold increase in the BC yield, as $g.L^{-1}$ (Figure 5.11.). Static incubation resulted in a BC yield of 0.612±0.003 $g.L^{-1}$, whereas agitated incubation resulted in 0.176±0.004 $g.L^{-1}$ BC yield.

Figure 5.11. BC yields in static and agitated conditions
(error bars represent standard deviation of the mean, n=3)

Esa et al. (2014) reported that different forms of cellulose were produced under static and agitated cultivation conditions. Tanskul et al. (2013) described that the forms of produced celluloses were three dimensional interconnected reticular pellicle and irregular shape sphere-like cellulose particle under static and agitated conditions, respectively. In the present study, different forms of cellulose were also observed (Figure 5.12.) under static and agitated conditions, however, regarding the BC yields the static incubation was selected as an optimum condition for the fermentation of BC by *K. hansenii*. Static

cultivation was indicated as an economic and practical way to obtain bacterial cellulose by the previous reports of Hong et al. (2008) and Wu et al. (2013). Consequently, Zhu et al. (2011) noted that BC, which was synthesized at static condition, could be widely used in medical, biological and chemical industries regarding its high biodegradability and good biocompatibility characteristics.

Figure 5.12. (a) BC pellicle in static condition, (b) BC pellet in agitated condition

In the present study, the low yield of BC under agitated condition can be attributed to the presence of Cel^- mutants (cellulose nonproducers) that causes a decline in cellulose synthesis as stated by Watanabe et al. (1998) and Esa et al. (2014). In accordance with this, Krystynowicz et al. (2002) reported that Cel^+ colonies (cellulose producers) were dominant under static cultivation. This conclusion was supported by the studies of Ha et al. (2012) and Huang et al. (2015). Ha et al. (2012) reported that static incubation resulted a better yield (8.74 $g.L^{-1}$) compared to agitated incubation (3.92 $g.L^{-1}$) in the production of BC by *G. hansenii* PJK. Similarly, Huang et al. (2015) used *G. xylinum* for the synthesis of cellulose and reported that static incubation resulted in a BC yield of 4.10 $g.L^{-1}$, whereas agitated incubation was resulted in 0.65 $g.L^{-1}$ BC yield.

<u>5.7.2.2. Determination of incubation period.</u> After selection of the incubation type for BC production by *K. hansenii*, bacterial culture was incubated statically for 21 days with 7-day intervals. The obtained BC yields are shown in Figure 5.13.

7-day incubation resulted in 0.617±0.006 $g.L^{-1}$ BC yield. On the 14th day of incubation, BC yield significantly increased to 0.707±0.002 $g.L^{-1}$. However, 21-day incubation had a BC yield of 0.706±0.004 $g.L^{-1}$ which was similar to that of the day 14. Therefore, 14-day incubation of *K. hansenii* was chosen for BC production.

Figure 5.13. BC yields on the 7th, 14th, and 21st day of incubation (error bars represent standard deviation of the mean, n=3)

In literature, there are various incubation periods resulting in different BC yields. Mohite et al. (2013) found that the optimum incubation period was 6 days (5 $g.L^{-1}$) for *G. hansenii* NCIM 2529 whereas Ge et al. (2011) obtained 1.08 $g.L^{-1}$ BC yield by *G. hansenii* J2 after 8 days.

Khajavi et al. (2011) reported that 16-d incubation was the best in terms of BC yield (0.507 $g.L^{-1}$) by *A. xylinum* (ATCC 23768). Ha et al. (2012) studied 5, 10 and 15-d incubation periods for *G. hansenii* PJK, and 15-d resulted in the highest BC yield (8.74 $g.L^{-1}$). Sheykhnazari et al. (2011) stated that after 14 days of incubation there was no more increase in the BC yield by *G. xylinus* (BPR 2004).

These results indicate that incubation period is strain dependent and may be affected by various parameters such as incubation type, supplementary material, pH and type of carbon source.

<u>5.7.2.3. Optimum pH value.</u> After the optimization of incubation type and period, the effect of pH was investigated. Experiments were performed in triplicate in a pH range of 4 to 7. BC yields were 0.478±0.006 $g.L^{-1}$, 0.575±0.007 $g.L^{-1}$, and 0.706±0.005 $g.L^{-1}$ for pHs 4, 5, and 6, respectively.

Figure 5.14. Effect of pH on BC production
(error bars represent standard deviation of the mean, n=3)

As shown in Figure 5.14., BC yield was the highest at pH 6 and increasing the pH of the medium above 6 (i.e., pH=7) resulted in a decrease in the yield of BC. Therefore, pH 6 was selected as an optimum value, regarding the BC yields of the tested pH range.

Tanskul et al. (2013) found that pH 3.5 was the optimum value for the BC synthesis by *Rhodococcus sp. MI 2*. They noted that below pH 3.5 there was no growth observed for the bacteria.

Huang et al. (2015) studied the effect of pH values ranged from 4.0 to 7.0 on BC production by *G. xylinus.* They found that pH 6.5 was optimum, and pH values < 5.5 negatively affected the BC yield.

Ha et al. (2012) investigated the effects of only two pH values which are 3.5 and 5.0 on BC production by *G. hansenii* PJK. The BC yields were 0.68 $g.L^{-1}$ and 2.03 $g.L^{-1}$, respectively. They also studied the performance of another bacteria, namely, *Acetobacter xylinum* (ATCC 23769) for the BC production at the same pH values. For this case, the BC yields were 6.56 $g.L^{-1}$ and 1.87 $g.L^{-1}$, for pHs 3.5, and 5.0, respectively. Rani et al. (2011) studied a pH range of 2.5-9.5 and reported that the optimum pH was 5.5 for the production of BC by *G. hansenii* UAC09. As it can be seen from these results, optimum pH is strongly

dependent on the strain used for BC production, and it generally varies from 5 to 6 for *K. hansenii* strains.

5.7.2.4. Effect of initial glucose concentration. Since glucose is a well-known carbon source for microbial fermentation studies, different initial glucose concentrations in FM medium were tested for BC production. Initial glucose concentrations used were 0.5, 1.0, and 1.5 % (w/v) in FM medium (pH 6), and cultures were incubated at 28°C, under static cultivation, for 14 days.

Figure 5.15. Effect of initial glucose concentration on BC production
(error bars represent standard deviation of the mean, n=3)

The minimum BC yield (0.344±0.004 $g.L^{-1}$) was observed for 0.5 % (w/v) initial glucose concentration, whereas the highest BC yield 0.886±0.006 $g.L^{-1}$ was obtained in the presence of 1.0 % (w/v) initial glucose concentration in FM medium. However, 1.5 % (w/v) initial glucose concentration decreased the BC yield (Figure 5.15.).

This result is consistent with the previous reports of Masaoka et al. (1993), Son et al. (2001) and Rani et al. (2011). Of these studies, Son et al. (2001) and Rani et al. (2011) reported that the initial glucose concentrations up to 4.0 % (w/v) resulted in an enhancement for BC yields obtained from both *Acetobacter sp*. A9 and *G. hansenii* UAC09. However, they also reported that initial glucose concentration above 4.0 % (w/v) decreased the BC yield because of the formation of gluconic acid which was able to inhibit

the growth and hence BC production. A similar study was reported earlier by Masaoka et al. (1993). Their findings indicated that more than 2.0 % (w/v) initial glucose concentration caused a decrease in the production of BC by *A. xylinum* IFO 13693. Zahan et al. (2014) investigated the effect of initial glucose concentration in a range of 0.5-5.0 % (w/v). They found that the 1.0 % (w/v) initial glucose concentration provided the highest yield of BC synthesized by *Acetobacter xylinum*, similar to our finding for *K. hansenii* in the present study.

5.7.2.5. Addition of ethanol. As depicted in Figure 5.16., the addition of 0.5 % (v/v) ethanol to FM medium resulted in an increase in BC yield ($g.L^{-1}$) from 0.890±0.005 to 1.207±0.004. However, 1.0 % (v/v) ethanol supplement decreased the BC yield.

Figure 5.16. Variation in BC yields with the addition of ethanol
(error bars represent standard deviation of the mean, n=3)

Alcohol may act as an electron donor for bacteria to provide extra energy, therefore using ethanol in fermentation medium may enhance cellulose synthesis (deFaveri et al. 2003; Yunoki et al. 2004; Hutchens et al. 2007; Santos et al. 2013). Krystynowicz et al. (2002) reported that the addition of 1.0 % ethanol into stationary cultures increased the efficiency of BC production by *Acetobacter xylinum* E_{25}. Li et al. (2012) also noted that ethanol supplementation stimulated the increased synthesis of BC. However, there are several studies stating that if ethanol addition is above its optimum value which is specific for the used strain, BC production decreases due to the inhibition of growth caused by the

accumulation of acetate (Naritomi et al. 1998; Park et al. 2003; Ha et al. 2011). Park et al. (2003) performed the production of BC by *Gluconacetobacter hansenii* PJK (KCTC 10505BP). In their study, the addition of 1.0 % (v/v) ethanol increased the yield of BC from 1.30 $g.L^{-1}$ to 2.31 $g.L^{-1}$. Son et al. (2003) investigated the effect of ethanol addition on BC synthesis by *Acetobacter sp*. V6. They reported that the BC yield increased from 1.31 $g.L^{-1}$ to 4.16 $g.L^{-1}$ in Hestrin Schramm medium by the addition of 0.6 % (v/v) ethanol. Jung et al. (2005) reported the production of BC by *Gluconacetobacter hansenii* PJK (KCTC 10505BP) in the presence of 1.0 % (v/v) ethanol, and the BC yield was 1.72 $g.L^{-1}$, on dry weight basis.

The results of the optimization studies used to obtain high-yield of BC are given in Table 5.5. After the improvement of BC production conditions, there was approximately a 2-fold increase in the yield of BC, as $g.L^{-1}$.

Table 5.5. BC yields throughout the optimization of the BC production conditions

Parameters	Incubation type	Incubation period (d)	pH	Glucose* concentration % (w/v)	Ethanol concentration % (v/v)	BC yield (g. L^{-1})
Incubation type	**Static**	7	6	1.5	0.0	0.612± 0.003
	Agitation					0.176 ±0.004
Incubation period (d)	Static	7	6	1.5	0.0	0.617 ±0.006
		14				0.707±0.002
		21				0.706 ±0.004
pH	Static	14	4	1.5	0.0	0.478 ±0.006
			5			0.575 ±0.007
			6			0.706 ±0.005
			7			0.514 ±0.003
Glucose* concentration % (w/v)	Static	14	6	0.5	0.0	0.344±0.004
				1.0		0.886±0.006
				1.5		0.709±0.004
Ethanol concentration % (v/v)	Static	14	6	1.0	0.0	0.890 ±0.005
					0.5	1.207±0.004
					1.0	0.985 ±0.006

(*) Glucose provided by analytical grade glucose

Selected parameters are written in bold

5.7.3. Use of hydrolysates in BC production

Hydrolysates of cassava flour by enzymatic hydrolysis, and hydrolysates of algal starch by acid hydrolysis were prepared in order to use for the optimized conditions of BC production.

Finally, the replacement of glucose in FM medium by hydrolysates of algal starch and cassava flour gave promising and comparable results regarding the yields of BC (Table 5.6.).

Table 5.6. Comparison of the produced BC yields

Concentration and source of glucose for BC production	BC yield ($g.\ L^{-1}$)
1.0 % (w/v) glucose provided by analytical grade glucose	1.202±0.005
1.0 % (w/v) glucose provided by hydrolysate of algal starch	1.104±0.002
1.0 % (w/v) glucose provided by hydrolysate of cassava flour	1.138±0.004

Throughout 14 days, *K. hansenii* was incubated statically at 28°C, in the FM medium (pH 6) which consisted of 1.0 % (w/v) glucose, and 0.5 % (v/v) ethanol. BC yields were 1.202±0.005 $g.L^{-1}$, 1.104±0.002 $g.L^{-1}$, and 1.138±0.004 $g.L^{-1}$ for glucose, algal starch, and cassava flour, respectively (Table 5.6.). A photograph of the produced BCs is given in Figure 5.17.

At the end of the optimization study, analysis of glucose by HPLC and pH measurements were performed for the residual culture media. There was no significant difference monitored in the pH values when compared to the initial pH values (data not shown). In addition to this, the initial glucose concentrations decreased to zero at the 14th day of incubation, confirmed by HPLC analysis.

Figure 5.17. BCs produced (a) from glucose, (b) from hydrolysate of algal starch, and (c) from hydrolysate of cassava flour

At the end of 14th day of incubation, produced BCs were prepared for the characterization studies.

5.8. Characterization of Bacterial Celluloses

5.8.1. Morphology of the bacterial celluloses

SEM micrographs for BCs produced from glucose (a-b), from hydrolysate of algal starch (c-d), and from hydrolysate of cassava flour (e-f) are given in Figure 5.18. SEM images were taken at the same magnification (5000x), and scale bar was 5µm.

In Figure 5.18. (a-c-e), fibrillated structures were not visible because of the produced BCs were covered by *K. hansenii* cells. Removal of the bacterial cell debris was achieved by NaOH treatment. Hence, after alkaline wash, three dimensional web-like structures and microfibrils became visible in Figure 5.18. (b-d-f).

Reticulated fibril arrangement with size and density variations can be easily observed by SEM micrographs. BC samples showed highly fibrous network-like structures consisting of ultra fine cellulose microfibrils. As it is indicated in Figure 5.18. (d), majority of fibrils of BC produced from hydrolysate of algal starch were slightly broader than microfibrils produced from glucose, and from hydrolysate of cassava flour which were shown in Figure 5.18. (b) and (f), respectively.

On the other hand, three dimensional web-like structure of BC produced from hydrolysate of cassava flour seemed denser than BC produced with the hydrolysate of algal starch, and glucose. It is known that some morphological differences may occur due to the use of different carbon sources. Zhong et al. (2013) observed thinner fibers for BC by *G. xylinus* CGMCC 2955 from glycerol containing medium, similar to our findings obtained from cassava flour.

Morphological observations were in agreement with the reports of Yan et al. (2008) and Castro et al. (2012). Castro et al. (2012) noted that the microfibrils in the network structure were randomly located, and microfibrillar ribbons were constant sized as a result of organism doubles the microfibril synthesis before division. Furthermore, Yan et al. (2008) reported that incubation type affects the morphology of BC. They indicated that most of the *A. xylinum* strains synthesize the BC as a gelatinous membrane form under static conditions, and statically incubation results in straighter microfibrils.

Overall, these micrographs in Figure 5.18. (b-d-f) indicate that the use of algal starch and cassava flour for BC production by *K. hansenii* does not alter the major microfibril structures in the BC pellicles.

Figure 5.18. SEM micrographs of BCs produced (a-b) from glucose, (c-d) from hydrolysate of algal starch, and (e-f) from hydrolysate of cassava flour (magnification 5000x, scale bar 5μm)

5.8.2. Chemical composition of the bacterial celluloses

FTIR spectroscopy which allows analysis of molecular composition was employed to detect the occurrence of new peaks or any peak shift that could be attributed to structural differences between the obtained BCs.

First of all, a spectra of MCC was taken as a reference which showed peaks at 3328 cm^{-1} for -OH stretching, 2888 cm^{-1} for C-H stretching, and 1640 cm^{-1} for H-O-H bending vibration of water molecules that prove the basic cellulose peaks in the range of 4000 – 400 cm^{-1} (Figure 5.19.).

Figure 5.19. FTIR spectra of microcrystalline cellulose

The FTIR spectra of MCC, and BCs produced from glucose, hydrolysate of algal starch, and hydrolysate of cassava flour was shown in Figure 5.20. (a), (b), (c) and (d), respectively. Halib et al. (2012) described that the pattern of the FTIR spectra varies due to the origin of cellulose. Our results showed that FTIR spectra of the produced BCs had a very similar pattern when they compared to the spectrum of MCC with slight shiftings in wavenumbers.

The stretching peak of -OH was found around 3300 cm^{-1} (Yang et al. 2013; Gayathry et al. 2014), the bands for C-H stretching were found around 2900 cm^{-1} (Lu et al. 2014; Kumbhar et al. 2015), H-O-H bending bands were found around 1640 cm^{-1} (Ashori et al. 2012), and bands for CH_2 bending were found around 1400 cm^{-1} (Zhong et al. 2013; Feng et al. 2015). Functional groups for FTIR spectra of MCC and the produced BCs were listed in Table 5.7.

Figure 5.20. Overlapping FTIR spectra of (a) MCC, and spectra of BCs (b) from glucose, (c) from hydrolysate of algal starch, and (d) from hydrolysate of cassava flour

Table 5.7. Functional groups for FTIR spectra of MCC and the produced BCs

	Peak positions (wavenumber, cm^{-1})			
	-OH	C-H	H-O-H	CH_2
MCC	3328.9	2888.8	1640.1	1427.5
BC from glucose	3346.0	2902.8	1636.2	1426.3
BC from hydrolysate of algal starch	3346.2	2905.2	1640.9	1428.9
BC from hydrolysate of cassava flour	3284.7	2916.5	1632.1	1427.2

These results were in close agreement with the previous reports of Mohite et al. (2013) and Neera et al. (2015). One of the reason for slight shifts of wavenumbers in spectra could be the bond length differences caused by electronegativity changes of neighbour atom. On the other hand, conjugation could be another reason for peak shifting (Kline 1999; Fan et al. 2012). The spectral band around 1730 cm^{-1} in Figure 5.20.(c) represents the C-O stretching vibration which might be caused by the presence of impurities from esters or acids. However, the employment of biorefinery approach can significantly eliminate these impurities.

Moon et al. (2011) noted that cellulose I is known as natural cellulose, and triclinic structure of the cellulose I (I_α) is dominant for bacterial cellulose. Furthermore, absorbance values at 900 cm^{-1} and 1430 cm^{-1} are related to amorphous and crystalline structure; and the absorbance ratio of these peaks (A1430/A900) is evaluated as the crystallinity index (CrI^{IR}) of cellulose I (Oh et al. 2005; Yan et al. 2008; Khajavi et al. 2011). Greater CrI^{IR} value means great absorbance at 1430 cm^{-1} and low absorbance at 900 cm^{-1} so more crystalinne and less amorphous structure.

In addition to this, the mass fraction values of cellulose I_α (f^{IR}) were estimated from equation [5.4] (Keshk et al. 2006), and given with the CrI^{IR} values in Table 5.8.

$$f^{IR} = A750/(A750 + A710) \qquad \text{Equation [5.4]}$$

Table 5.8. Literature comparison of crystallinity index and mass fraction values for BCs

BC producer	**Carbon source**	**Incubation type**	**CrI ^{IR}**	**f ^{IR}**	**References**
			FM medium	**FM medium**	
K. hansenii	glucose	static	4.39	0.59	Present study
	algal starch		4.25	0.58	
	cassava flour		4.13	0.56	
BC producer*	**Carbon source**	**Incubation type**	**CrI ^{IR}**	**f ^{IR}**	
			HS medium	**HS medium**	
10245	glucose	static	3.05	0.44	Keshk et al. (2006)
13693			3.12	0.44	
13772			3.02	0.44	
13773			3.95	0.43	
14815			3.09	0.43	
15237			3.30	0.43	
BC producer**	**Carbon source**	**Incubation type**	**CrI ^{IR}**	**f ^{IR}**	
			HS medium	**HS medium**	
1.1812	glucose	static	5.13	0.56	Yan et al. (2008)
		agitated	2.23	0.37	

* *G. xylinus*
** *A. xylinum*

As shown in Table 5.8., Keshk et al. (2006) studied the BC production from glucose under static conditions by using six species of *G. xylinus*. The crystallinity index and mass fraction values reported in their study were between 3.02 and 3.95, 0.43 and 0.44, respectively for different species of *G. xylinus*. These crystallinity index and mass fraction values were lower than the values obtained in the present study. Yan et al. (2008) studied the production of BC using glucose by *A. xylinum* under static and agitated conditions. They found that both of the crystallinity index and mass fraction values decreased dramatically in agitated cultivation conditions compared to static incubation. In static incubation, crystallinity index of BC was 5.13, which was greater than that of the present study.

These results indicated that crystallinity index and mass fraction values of BCs were strain dependent, and could be changed due to the use of different incubation type.

6. CONCLUSIONS

This study demonstrated for the first time that both enzymatic hydrolysate of cassava flour and acid hydrolysate of algal starch can be used as suitable carbon sources in the production of BC by *Komagataeibacter hansenii.*

Bacterial cellulose yields on dry weight basis were 1.202±0.005 $g.L^{-1}$, 1.138±0.004 $g.L^{-1}$, and 1.104±0.002 $g.L^{-1}$ from glucose (as control), cassava flour, and algal starch, respectively. The produced BCs were characterized by SEM and FTIR. Among FTIR spectra, bacterial celluloses were compared with pure reference, microcrystalline cellulose, and similar chemical characteristics were obtained for both of the produced BCs. In addition to this, SEM results revealed that our bacterial celluloses had highly fibrillated structure, as it was observed in control, which is a glucose-based BC. The starch content of algae used in the present study was low, however, the morphological and chemical characteristics of the BC produced from algal starch were similar to BCs produced from glucose and cassava flour.

As a starch-rich material, the conversion of starch in cassava flour to glucose both by enzymatic and acid hydrolysis gave higher yields of glucose than algae. In addition, it was determined that enzymatic method was more efficient than the acidic method for the hydrolysis of cassava flour. Cassava is a promising, high-yielding multipurpose crop in all tropical and subtropical countries of the world. To the best of our knowledge, the present study is the first report on the use of cassava flour for the production of bacterial cellulose. Based on the knowledge generated in this study, the hydrolysis process of cassava flour may be developed for the industrial scale production of BC.

On the other hand, the success of acid hydrolysis was higher than enzymatic method in the case of *Chlorella vulgaris.* There were several stress parameters attempted to increase the starch contents of *C. vulgaris*, however, nitrogen starvation made about 35 % increase in the starch content compared to that of the normal cultivation condition. The use of *C. vulgaris* as a source of glucose for the production of BC was promising. The continuation of this study can include optimization of the preculturing conditions of algae,

aiming to increase starch yields. Bacterial cellulose is an advantageous biomaterial with its unique properties. There is no information regarding the utilization of algae-based glucose for BC production among the variety of biotechnological applications of microalgae in literature. In this sense, a novel and very important biotechnological application of algae is proposed in the present study. Accordingly, integrating the use of algae-based glucose in BC production with biorefinery concept will lead to have various beneficial aspects. Potential advantages include achieving large scale production at low cost, delivering global economic importance, as well as preserving the environment.

Lastly, to provide better synthesis conditions for *K. hansenii*, a comprehensive optimization study including; incubation type and period, initial glucose concentration, pH, and the use of ethanol as a supplementary material was paved the way for achieving higher BC productivities in future studies.

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APPENDIX A: RECIPES FOR STOCK SOLUTIONS

Trace elements solution

0.75 g Na_2EDTA was dissolved in 1 liter ddH_2O and the listed minerals (given below) were added respectively.

$FeCl_3.6H_2O$	97.0 mg
$MnCl_2.4H_2O$	41.0 mg
$ZnCl_2$	5.0 mg
$CoCl_2.6H_2O$	2.0 mg
$Na_2MoO_4.2H_2O$	4.0 mg

Vitamin B1

0.12 g Thiaminhydrochloride was added to 100 mL ddH_2O. The solution was sterilized by using membrane filter (*Techno Plastic Products AG*, pore size 0.22 µm).

Vitamin B2

0.1 g Cyanocobalamin was added to 100 mL ddH_2O. 1 mL of this solution was taken and final volume made up to 100 mL with ddH_2O. The solution was sterilized by membrane filtration (*Techno Plastic Products AG*, pore size 0.22 µm).

APPENDIX B: TOTAL STARCH ASSAY PROCEDURE

K-TSTA 07/11 - Protocol (c)

(© Megazyme International Ireland 2011)

1. Mill cereal, plant or food product to pass a 0.5 mm screen.

2. Add milled sample (~100 mg, weighed accurately) to a glass tube (16 x 120 mm).

3. Wet with 0.2 mL of aqueous ethanol 80 % (v/v) to aid dispersion, and stir the tube on a vortex mixer.

4. Add a magnetic stirrer bar (5 x 15 mm) and 2 mL of 2 M KOH to each tube and re-suspend the pellets by stirring for approximately 20 min in an ice/water bath over a magnetic stirrer.

5. Add 8 mL of 1.2 M sodium acetate buffer (pH 3.8) to each tube with stirring on the magnetic stirrer. Immediately add 0.1 mL of thermostable α-amylase and 0.1 mL of amyloglucosidase, mix well and place the tubes in a water bath at 50° C.

6. Incubate the tubes for 30 min with intermittent mixing on a vortex mixer.

7. For samples containing > 10 % total starch content; quantitatively transfer the contents of the tube to a 100 mL volumetric flask (using a water wash bottle). Use an external magnet to retain the stirrer bar in the tube while washing the solution from the tube with a water wash bottle. Adjust to 100 mL with distilled water and mix well. Centrifuge an aliquot of the solution at 1800 g for 10 min.

8. For samples containing < 10 % total starch content; directly centrifuge the tubes at 1800 g for 10 min (no dilution). For such samples, the final volume in the tube is approximately 10.4 mL.

9. Transfer duplicate aliquots (0.1 mL) of the diluted solution to the bottom of glass test tubes (16 x 100 mm).

10. Add 3.0 mL of GOPOD Reagent to each tube (including the D-glucose controls and reagent blanks), and incubate the tubes at 50°C for 20 min.

11. D-Glucose controls consist of 0.1 mL of D-glucose standard solution (1 $mg.mL^{-1}$) and 3.0 mL of GOPOD Reagent. Reagent Blank solutions consist of 0.1 mL of water and 3.0 mL of GOPOD Reagent.

12. Read the absorbance for each sample, and the D-glucose control at 510 nm against the reagent blank.