Bachelor's Thesis

Bachelor's Degree in Chemical Engineering

Production of Bacterial Nanocellulose by Fermentation Process

Thesis

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Summary

Bacterial cellulose, a water-insoluble exopolysaccharide produced by some bacteria, has unique structural and mechanical properties and is highly pure as compared to plant cellulose. In contrast to plant cellulose, bacterial cellulose, with several remarkable physical properties, has proven to be a remarkably versatile biomaterial and can be used in a wide variety of applied scientific endeavors. It also can be grown to any desired shape and structure to meet the needs of different applications. It has been used in the food industry for applications such as low-calorie desserts, salads, and fabricated foods. It has also been used in the paper manufacturing industry to enhance paper strength, the electronics industry in acoustic diaphragms for audio speakers, the pharmaceutical industry as filtration membranes, and in the medical field as wound dressing and artificial skin material.

Fermentation is a metabolic process that converts sugar into acids, gases, or alcohol. It occurs in yeast and bacteria, and also in oxygen-starved muscle cells, as in the case of lactic acid fermentation. Fermentation is also used more broadly to refer to the bulk growth of microorganisms on a growth medium, often with the goal of producing a specific chemical product.

In this project, a particular fermentation process is studied, using *Gluconacetobacter xylinum sucrofermentans* (BPR2001) to produce bacterial nanocellulose in static or agitated culture medium, changing the carbon source in different samples and comparing the results to know which the best processes to obtain the wanted product are.

Produced bacterial cellulose has been characterized by IR spectroscopy and scanning electron microscopy. Evolution of fermentation has been monitored by optical density.

Clearly the agitated mode has provided higher yields than the static one, an observation confirmed by other published works on this process. The scientific community's explanation for this fact is that oxygen is more easily transported to bacteria in agitated systems. Static mode fermentations provide better quality microfibers than the agitated mode, as can be seen from SEM photographs.

The most productive fermentations in stirred mode provided approximately 0.5 g of cellulose per liter. In the static mode, the final concentration decreased to 0.2 g/L, in a double time of fermentation (14 days).

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1. Glossary

ALR	Airlift Bioreactor
BC	Bacterial cellulose
BPR 2001	*Gluconacetobacter xylinum sucrofermentans*
CECT	Colección Española de Cultivos Tipo
CMCF	Carboxymethyl cellulose foam
CTA	Cellulose Triacetate
ETSEIB	Escola Tècnica Superior d'Enginyeria Industrial de Barcelona
GRAS	Generally recognized as safe
NCMFC	Noncompartmented microbial fuel cell
SEM	Scanning electron microscope
SFW	Saccharified food wastes
SMC	Smooth Muscle Cells

2. Preface

2.1. Origin of the project

The research group at the *Chemical Engineering Department* from the ETSEIB has carried out an intense research labor in the field of new polymers for more than 20 years, and now it is time to have also a look at new biopolymers like bacterial cellulose, which applications are being investigated in various fields.

2.2. Motivation

The main motivation of the project was to find a method of producing composite sustainable natural systems as an alternative to raw materials derived from oil. Nowadays it is important to find alternative methods to achieve a further step towards a sustainable chemical industry that respects the environment.

Bacterial cellulose is a natural and renewable material, with superior mechanical properties compared to vegetal cellulose, with high porosity and high capacity of water absorption as well as being biodegradable and having biocompatibility (useful for medical applications).

It has the advantage of being much more chemically pure avoiding extraction processes.

Moreover, the industry of composite materials, materials which have superior properties than the conventional ones, also requires more products made with renewable and sustainable processes. This is why it was decided to carry on this project with the purpose of understand the necessary steps and the best conditions to obtain bacterial cellulose.

3. Objectives

3.1. Main Aim

The objective of this work is obtaining bacterial cellulose through bacteria fermentation process and studying BPR2001 efficiency by changing some parameters of the culture.

3.2. Specific aims

In more specific terms, the objectives of this work are the following:

- To carry out the fermentation of *Gluconacetobacter xylinus sucrofermentans* to obtain bacterial cellulose.
- Performing the fermentation process in static culture.
- Performing the fermentation process in agitated culture.
- Performing the fermentation process with several carbon sources.
- Evaluate the yields of each culture.
- Studying the nanocellulose that has been produced.
- Improving the efficiency of the reaction by modifying the culture medium.
- Studying the effect of the agitation in the fermentation.
- To know the relations between the properties of cellulose and the conditions of fermentation.

4. Introduction

4.1. Cellulose

Cellulose is the earth's major biopolymer and is of tremendous economic importance globally. It is the major constituent of cotton (over 94%) and wood (over 50%). Together, cotton and wood are the major resources for all cellulose products such as paper, textiles, construction materials, and cardboard, in addition to cellulose derivatives such as cellophane, rayon, and cellulose acetate. Cellulose from major land and forest plants and cotton is assembled from glucose, which is produced in the living plant cell from photosynthesis. In the oceans, however, most cellulose is produced by unicellular plankton or algae using the same type of carbon dioxide fixation found in photosynthesis of land plants. In fact, it is believed that these organisms, the first in the vast food chain, represent nature's largest resource for cellulose production. Without photosynthetic microbes, all animal life in the oceans would cease to exist. Several animals, fungi, and bacteria can assemble cellulose. However, these organisms are devoid of photosynthetic capacity and usually require glucose or some organic substrate synthesized by a photosynthetic organism to assemble their cellulose (Keshk, 2014).

Cellulose $(C_6H_{10}O_5)_n$, with "n" value of between 10,000 and 15,000 is a natural polymer consisting of a long chain carbohydrate covalently attached. In Figure 1 the molecular structure of cellulose is shown.

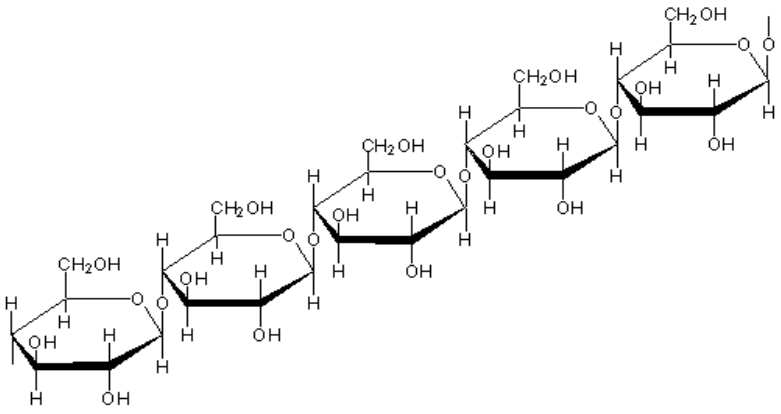

Figure 1.Cellulose internal structure (Stijn Hommes, 2016)

The glucose molecules in the cellulose are linked between carbon atoms 1 and 4 of each of them using links type beta (β-1,4-glycosidic) (Tort-Agell, 2016). This is why cellulose is insoluble in water. In this long chain of glucose hydrogen bonds between the hydroxyl groups are established originating crystalline structures. These structures form so-called micelles, which include from 60 to 70 molecular chains. 20 micelles form a microfibril and 250 microfibrils form a fibril. The final compact fiber has until 1500 fibrils and it is very rigid (Grande-Cruz, 2014) (Apuntes de biologia celular. Carbohidratos.) (Aula virtual de Biología. Polisacáridos.).

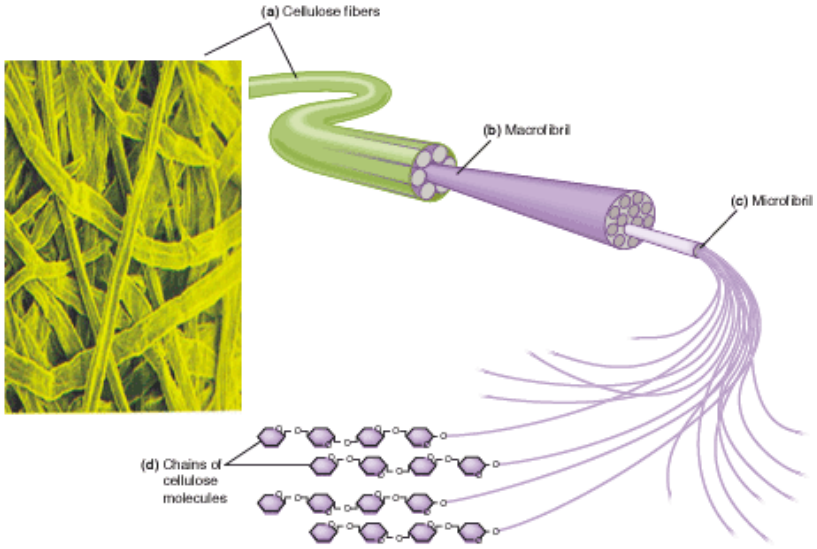

Figure 2. Cellulose fibrils (Tort-Agell, 2016)

Regarding the molecular weight of cellulose, it depends on the main source (wood, cotton, etc.), on the method of isolation and on the method of determining the molecular weight used. What is clear is that the native cellulose has a high degree of polymerization greater than 10000 and an average molecular weight of 2000000 (Kaplan, 1998) (Moon, Martini, Nairn, Simonsen, & and Youngblood, 2011).

4.1.1. Types of cellulose

Polymorphism is the existence of more than one crystalline form, different in physical and chemical properties such as solubility, density, melting point, glass shape or optical or electrical properties. In the case of cellulose there are four different polymorphisms (Quintano-Quirino, 2015).

The cellulose microfibrils mentioned above consist of two different regions. The crystalline region consists of highly tidy molecules while molecules in amorphous or para-crystalline region are not so.

The crystallinity of cellulose varies depending on the type of cellulose. In this way the cellulose has 4 allotropic forms including cellulose I, II, III_1, III_{11}, IV_1, i IV_{11}.

Cellulose I is found in nature and is commonly known as native cellulose. It has two types of crystalline structure; I_α and I_β, which can coexist in different proportions depending on the origin. Cellulose I_α is produced by bacteria and algae in highest proportion while I_β predominates in plants (wood, cotton, etc.). Both differ in crystal packing, molecular shaping and hydrogen bonds. Cellulose I can be converted into other polymorphic forms using different treatments. John Mercer, an English scientist, discovered in 1844 a way to convert cellulose I into cellulose II called mercerization (Quintano-Quirino, 2015) (Moon, Martini, Nairn, Simonsen, & and Youngblood, 2011).

Cellulose II is the most stable structure of technical relevance. It can be obtained from cellulose I either by a treatment with concentrated sodium hydroxide (mercerization process) or by a solubilization followed by precipitation and regeneration, altering the original structure of cellulose I. It is also obtained from *Gluconacetobacter xylinum* bacteria, and algae species Halicystis. The difference between the cellulose I and cellulose II is based on the dimensions of the unit cell and the polarity of the chains.

Cellulose III can be obtained from cellulose III by a reversible reaction using boiling water and hydrochloric acid. The forms of cellulose III_1 and III_{11} are obtained from cellulose I and II, while cellulose IV_1 and IV_{11} may be obtained by heating celluloses III_1 and III_{11} in glycerol medium (Moon, Martini, Nairn, Simonsen, & and Youngblood, 2011) (Quintano-Quirino, 2015).

Figure 3 includes all the reactions known to transform a polymorphic form to another.

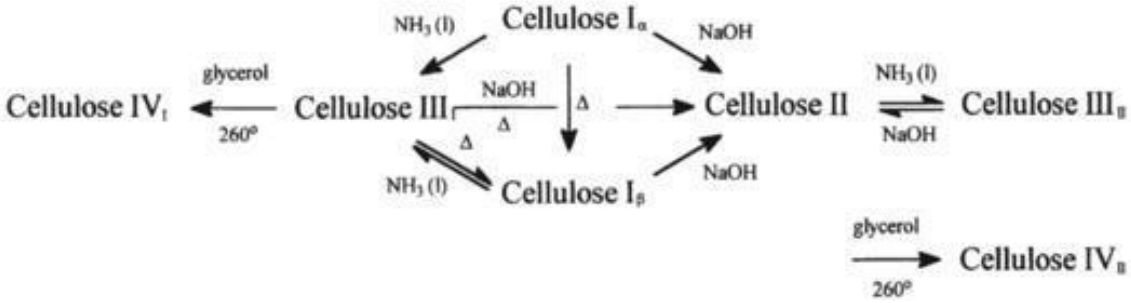

Figure 3. Different transformations of cellulose polymorphisms (Tort-Agell, 2016)

4.2. Bacterial cellulose

As it is said before, one way to reduce the demand from plants is the production of cellulose using microbial systems. Bacterial cellulose was first reported by Adrian Brown, who observed that sometimes a not-expected solid mass was formed at the surface of his fermentation medium. The compound was later identified as cellulose and the author proposed the name *Bacterial xylinum* for the microorganism. The bacterium was given several names including *Acetobacterium xylinum* and *Bacterium xylinodes.* It was later named as *Acetobacter xylinum* and became the official name according to the International Code of Nomenclature of Bacteria (1958). Now the bacterium is called *Gluconacetobacter xylinum*.

Unlike plant cellulose, BC does not require extra processing to remove unwanted impurities and contaminations and therefore it can retain a greater degree of polymerization. BC also demonstrates unique properties, including high degree of crystallinity, water retention value, tensile strength, and moldability. (Cheng, 2010)

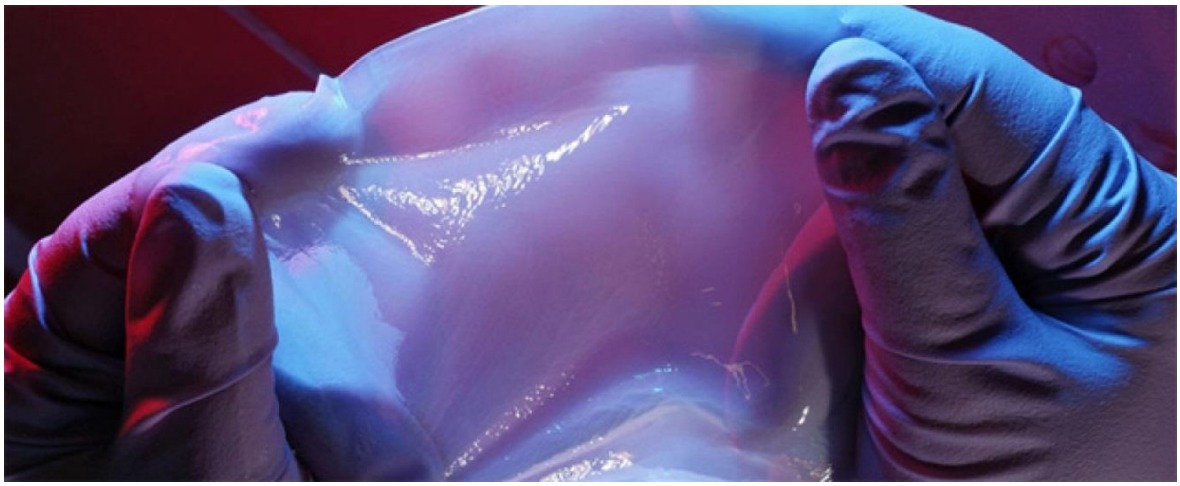

Figure 4. Bacterial nanocellulose

The molecular formula of bacterial cellulose $(C_6H_{10}O_5)_n$ is the same as that of plant cellulose, but their physical and chemical features are different. Bacterial cellulose is preferred over the plant cellulose as it can be obtained in higher purity and exhibits a higher degree of polymerization and crystallinity index. It also has higher tensile strength and water holding capacity than that of plant cellulose, making it more suitable raw material for producing high fidelity acoustic speakers, high quality paper and dessert foods. Fibrils of bacterial cellulose are about 100 times thinner than that of plant cellulose, making it a highly porous material, which allows transfer of antibiotics or other medicines into the wound while at the same time serving as an efficient physical barrier against any external infection. It is therefore used extensively in wound healing. Microbial cellulose exists as a basic structure known as microfibrils, which are composed of glucan chains interlocked by hydrogen bonds so that a crystalline domain is produced. This microfibrillar structure of bacterial cellulose was first described by Mühlethaler in 1949. Electron microscopic observations showed that the cellulose produced by *Acetobacter xylinum* occurs in the form of fibers. The bacteria first secreted a structurally homogeneous slimy substance within which, after a short time, the cellulose fibers were formed. *Acetobacter xylinum* produces two forms of cellulose: cellulose I, the ribbon-like polymer; and cellulose II, the thermodynamically more stable amorphous polymer. Microfibrillar structure of bacterial cellulose is responsible for most of its properties such as high tensile strength, higher degree of polymerization and crystallinity index. Bacterial cellulose is used as a diet food and to produce new materials for high performance speaker diaphragms, medical pads and artificial skin.

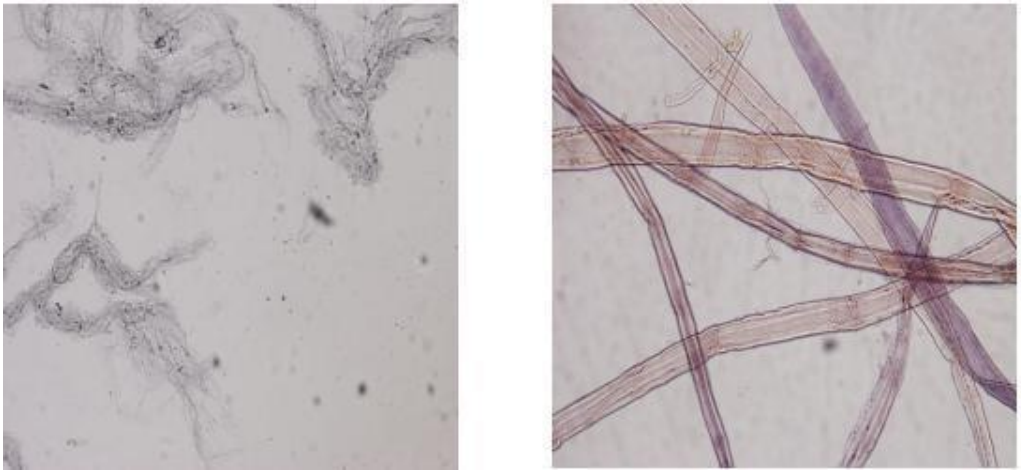

Figure 5: On the left side, BC fibers. On the right side, vegetal cellulose fibers (Tort-Agell, 2016)

Relatively high cost of the production of cellulose may limit its application to high value-added products as well as speciality chemicals. Significant cost reductions are possible with improvements in fermentation efficiency and economics of scale, the lower limit of the cost of microbial cellulose being determined by the price of the raw material substrates. Consequently, Acetobacter cellulose may always be more expensive to produce than conventional sources of cellulose. For this reason, successful commercialization of Acetobacter cellulose will depend on careful selection of applications where its superior performance can justify its higher cost. (Prashan R. Chawla, Ishwar B. Bajaj, Shrikant A. Survase and Rekha S. Shingal, 2009).

Once produced, bacterial cellulose forms a hydrogel (Figure 6). This cellulose hydrogel consists of a 0.9% bacterial cellulose, and a 99.1% water. Of this amount of water, 0.3% is bound water, while 98.8% is free water.

Figure 6. BC hydrogel

Free water can be removed very easily through various forms of dehydration, while the bound water is retained and attached to the cellulose fibers by adsorption and forming capillaries between 0.5 and 1 micrometer. Hydrated gel fibers have dimensions of 50 Nm. The most used dehydration techniques are lyophilization, drying and hot pressing (Laszkiewicz, B. and Cuculo, J. A., 1993).

Because of its crystalline structure, bacterial cellulose has excellent mechanical properties, which make it, as mentioned above, an ideal material for the reinforcement of composite materials. The mechanical properties of the BC are studied mainly from dry BC sheets. The

modulus of elasticity of the sheet can vary between 16 and 18 GPa depending on the drying method (air or hot pressing). The tensile stress reaches 260 MPa and deformation up to 2.1%, although it depends on cleaning method used after removal of the BC culture medium (Laszkiewicz, B. and Cuculo, J. A., 1993).

In the case of the analysis of bacterial cellulose gels without drying, the modulus of elasticity varies between 8 and 12 MPa and tensile stress between 15 and 20 MPa.

Table 1 shows the mechanical properties of bacterial cellulose compared to other polymers:

MATERIAL	ELASTICITY MODULUS [GPA]	TENSILE STRESS [MPA]	ELONGATION [%]
BACTERIAL CELLULOSE	15-35	200-300	1.5-2.1
POLYPROPYLENE (PP)	1-1.5	30-40	100-600
POLYETHYLENE TEREPHTHALATE (PET)	3-4	50-70	50-300
CELLOPHANE	2-3	20-100	14-40

Table 1: Mechanical properties of bacterial cellulose and other organic materials (Moon, Martini, Nairn, Simonsen, & and Youngblood, 2011)

Solubility depends on various factors: structure, molecular weight and origin among others. However, the bacterial cellulose is dissolved using an 8.5% NaOH solution, and the solubility is increased by the addition of 1% urea (Amigo, Salvador, & Sahuguillo, 2009).

The thermal degradation of BC pure and untreated (as hydrogel), begins between 290 and 298ºC. Its melting point is 120,4ºC and its glass transition temperature (Tg) is 13,9ºC. Note that when performing alkaline treatments to gels, a Tg between 41,4ºC and 48,8ºC is obtained and the values of the thermal decomposition temperature increase, reaching 343,2ºC and 370,0ºC (Moon, Martini, Nairn, Simonsen, & and Youngblood, 2011).

It is interesting to compare the properties of bacterial cellulose with vegetal cellulose to justify

the growing interest in this material.

Figure 7 shows the IR spectrum of bacterial cellulose and softwood cellulose. In this spectrum can be observed that the composition of both fibers is very similar. The significant difference for some absorption peaks is derived from the residual lignite plant fibers and the water absorbed by the samples of bacterial cellulose.

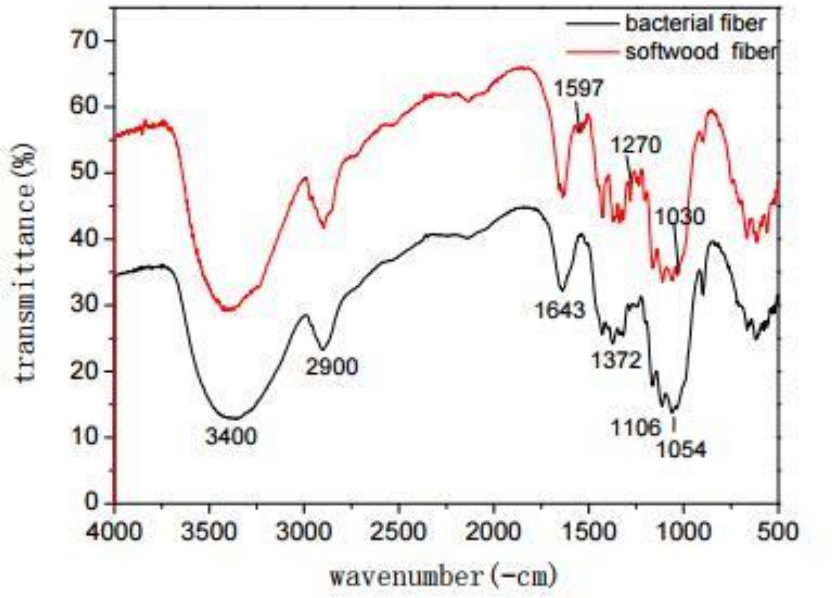

Figure 7: IR spectrum of bacterial cellulose and vegetal cellulose

The index of crystallinity obtained by this spectrum showed that the bacterial cellulose was slightly higher in the softwood one. Therefore, it can be concluded that special properties of bacterial cellulose fibers are due to these macro-reticulated and not the molecular structure.

The following table compares the size of the BC fibers and the vegetal cellulose ones:

	LENGTH (MM)	**WIDTH (MM)**
BACTERIAL FIBERS	0.69	19.87
VEGETAL FIBERS	1.05	31.64

Table 2. Length and width of bacterial fibers vs. vegetal fibers (Tort-Agell, 2016)

Comparing the mechanical properties of the cellulose with bacterial cellulose obtained from cotton fibers, the modulus of elasticity of the bacterial cellulose becomes considerably higher

than the other ones.

4.3. Applications

Bacterial cellulose exhibits several unique properties, such as high tensile strength and water content; several recent publications have focused on its applications or potential applications in various fields. White and Brown (1989) first evaluated the properties of microbial cellulose commercial applications. The important features outlined include no delignification required during processing, high water retention ability as if never dried, and the capacity of being formed into any shape or size, shape retention, and formation from a wide variety of substrates, and properties which can be controlled during synthesis. The applications of BC are summarized in the following categories (Cheng, 2010).

4.3.1. Acoustic transducer diaphragm

Yamanaka and Watanabe (1994) described potential and current applications of BC or composites in the area of acoustic transducers. In the field of acoustics, a diaphragm is a transducer intended to inter-convert mechanical vibrations to sounds, or vice versa. It is commonly constructed of a thin membrane or sheet of various materials, suspended at its edges. The first high value application was acoustic speaker diaphragms. The exceptional shape retention ability of BC, measured as the tensile strength, coupled with the high internal loss of the material make it ideal for speaker diaphragms. The novel diaphragms demonstrated two distinctive properties: high sonic velocity and low dynamic loss, and have been marketed by Sony Corp as loudspeakers and headphones (Iguchi et al., 2000).

4.3.2. Paper manufacturing

Johnson and Neogi (1989) reported that highly branched, reticulated BC pellets produced from agitation culture are suitable for the production of high-quality paper. They also developed composites containing glass fibers, calcium carbonate, and copper powder. Yamanaka and Watanabe (1994) also illustrated that the addition of disintegrated BC to paper pulp make possible to create a paper with higher tensile strength.

Figure 8. Bacterial cellulose paper

4.3.3. Filtration

The specific application of BC as a filtration material was examined by Takai (1994). Several polymers, such as polyethylene glycol, carboxymethyl cellulose, carboxymethyl chitin, and other cellulose-based polymers, were incorporated into the cellulose by simply adding the materials to the starting medium. Some of these polymers showed very high solute rejection which makes them useful for ultrafiltration as well as pervaporation. Another study focused on the evaluation of filtration properties of BC, testing its usefulness as a dialysis membrane (Shibazaki et al., 1994). When compared to a commercial dialysis membrane made of regenerated cellulose, the BC film showed a significantly higher permeation rate and a greater molecular weight cut-off. An additional benefit of the material as compared with the regenerated cellulose was that the added tensile strength allowed the use of a thinner membrane.

4.3.4. Pharmaceutical and medical applications

Microbial cellulose has high tensile strength, high porosity and microfibrillar structure. Chronic wounds such as venous leg ulcers, bedsores, and diabetic ulcers are difficult to heal, and they represent a significant clinical challenge both for the patients and for the healthcare professionals. The treatment of chronic wounds involves the application of various materials (hydrocolloids, hydrogels, biological or synthetic membranes) that provide a moist wound healing environment, which is necessary for optimal healing. According to the modern approaches in the field of wound healing, an ideal wound dressing system must display similarity to artificial skin, both structurally and functionally. The characteristics of modern

wound dressing materials are: nontoxic, non-pyrogenic and biocompatible; ability to provide barrier against infection; ability to control fluid loss; ability to reduce pain during treatment; ability to create and maintain a moist environment in the wound; provide easy and close wound coverage; enable introduction or transfer of medicines into the wound; ability to absorb exudates during inflammatory phase; display high mechanical strength, elasticity and conformability; display an optional shape and surface area, and allow for easy and painless healing of the wound. As microbial cellulose is a highly porous material, it allows the potential transfer of antibiotics or other medicines into the wound, while at the same time serving as an efficient physical barrier against any external infection. It satisfies the requirements of modern wound dressing material. As it is said before, BC has high water holding capacity and the size of fibrils is about 100 times smaller than that of plant cellulose.

Jonas and Farah (1998) worked on efficacy of BC as a temporary skin substitute called “Biofill®”. They discussed some clinical results when applied to burns and other skin injuries. Biofill® showed positive indications of diminished post-surgery discomfort, faster healing, immediate pain relief, reduced infection rate, improved exudates retention, and, most important, reduced treatment time and cost (Prashan R. Chawla, Ishwar B. Bajaj, Shrikant A. Survase and Rekha S. Shingal, 2009).

Meftahi et al. (2009) reported a novel cellulose film coated with cotton gauze which exhibits a 30% higher water absorbency and wicking ability than 25 native cellulose film, and is more suitable for wound dressings. Dissolvable carboxymethyl cellulose foam (CMCF) dressing was reported to be adopted as a substitute of routine nasal packing in functional endoscopic sinus surgery. The results demonstrated that CMCF dressing is associated with lower levels of localized pain and postoperative bleeding and synechia formation.

Figure 9. Cellulose dressing

The applicability of BC pellicle as a substrate for animal cell culture has also been examined. The first detailed report was presented by Watanabe et al. (1993). They cultured eight kinds of cells on the membrane, which was comparable to that achieved in plastic Petri dishes. The ionic charge, roughness of the membrane, and adsorption of collagen are crucial factors promoting cellular adhesion to the membrane surface. Backdahl et al. (2006) examined the interaction between BC and smooth muscle cells (SMC). The BC pellicle holds 99% water and hence leaves room for cell ingrowth and proliferation. The results demonstrated that SMC adhered to and proliferated on the BC film. A 40 μm ingrowth of SMC was observed after 2 weeks of cultivation.

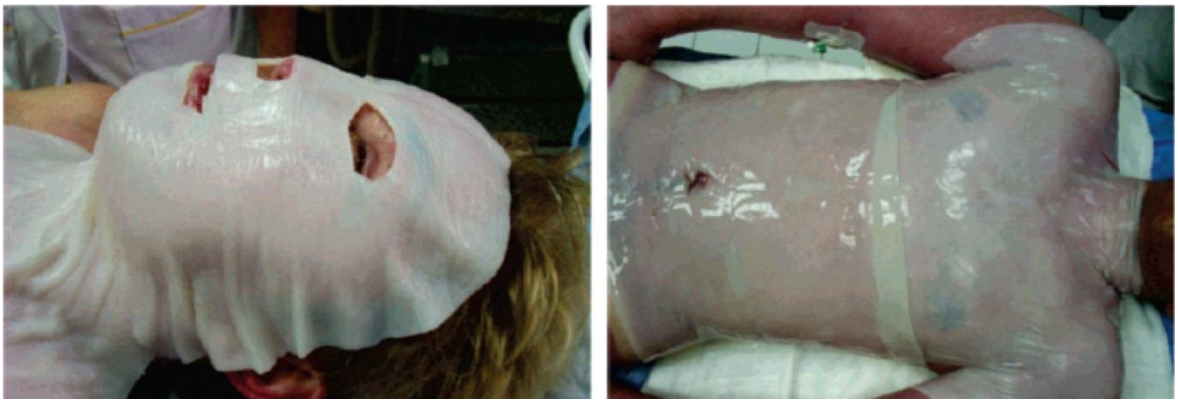

Figure 10. Bacterial cellulose dressing applied on wounded face and torso.

Methyl cellulose is also used in cell culture to study viral replication. A single layer of cells are grown on a flat surface first, and then infected with a virus for a short time (Watanabe, 1982). This assay is reliable and useful for the titration of ecotropic murine leukemia virus. Kohno et al. (1998) employed acetate/methyl cellulose as staining solution for contrast-enhancement in the EM images of human immunodeficiency virus (HIV) from low temperature-embedded samples.

Cai and Kim (2009) reported a cellulose/polyethylene glycol (BC/PEG) composite, which exhibits higher capability of forming fibroblast cell adhesion and proliferation than 26 the pure BC.

In addition to serving as a support for cell growth, modified BC film can be applied as an anti-adhesion and anti-proliferative material. Extremina et al. (2009) reported that a cellulose triacetate (CTA) membranes with the antibiotic imipenem (IPM) entrapped (CTA-IPM) were

developed. The bacterial adhesion tests showed a statistically significant decrease in the adhesion of S. epidermidis to CTA-IPM compared with its adhesion to CTA alone. With this invention, a BC membrane with anti-adhesive and anti-proliferative properties can provide a better simulation of the in vivo clinical situation.

BC became a proposed new biosynthetic vascular graft material since traditional methods may cause intimal hyperplasia, poor blood flow and surface thrombogenicity. In a microsurgical study, the BC implants were attached in an artificial defect of the carotid artery of rats for one year. This long term result showed the incorporation of the BC under formation of neointima and ingrowth of active fibroblasts (Schumann D, Wippermann J, Klemm D, Kramer F, Koth D, Kosmehl H, Wahlers T, Salehi-Gelani S, 2009).

4.3.5. Food applications

Okiyama et al. (1993) suggested several applications for BC in the food industry; such as thickening agents, low-calorie desserts, salads, or fabricated food. A 3% paste of cellulose was added to a chocolate drink in place of xanthan gum. Viscosity comparisons were nearly identical after mixing, but heat treatment caused a severe drop in viscosity for the xanthan gum drink, but the cellulose did not drop its viscosity. Addition of the cellulose to ice cream prevents flow after melting as a result of increase shear stress. Similar results were shown when the cellulose paste was added to 27 tofu, pasty condiments, and boiled fish paste. The authors, therefore, concluded that BC is widely applicable in food industry.

Figure 11. Typical Philippine dessert called "Nata de Coco"

BC has been determined to be ‘‘generally recognized as safe’’ (GRAS) and accepted by the Food and Drug Administration in 1992. It has important applications in a variety of food formulations, especially when low use levels, lack of flavor interactions, foam stabilization, and stability over wide pH range, temperature, and freeze–thaw conditions are required. BC in combination with other agents such like sucrose and CMC improve the dispersion of the product. Potential applications also include low-calorie additive, thickener, stabilizer, texture modifier, pasty condiments, and ice cream additive. Its high crystallinity, high water holding capacity, large surface area, elasticity, mechanical strength, and biocompatibility enable BC to be used as a support for cell immobilization.

Ton and Le (2011a) proved that the immobilized yeasts on BC exhibited much higher metabolic activity and resistance to unfavorable conditions during wine fermentation in comparison with free yeasts. Later, Ton and Le (2011a) studied the suitability of wine yeasts immobilized on BC to perform a repeated batch fermentation in winemaking. The results showed that during 10 consecutive cycles of the repeated batch fermentation, the sugar uptake rate of the immobilized yeast increased from 1.71 g/l/h (cycle 1) to 3.28 g/l/h (cycle 7) and then reduced to 2.75 g/l/h (cycle 10). They concluded that the application of yeast immobilization in winemaking enhanced economic effectiveness of the production-line because of cost reduction in inoculum preparation and simple separation of yeast at the end of the fermentation.

Recently, some studies found that BC composite containing soluble polysaccharide is a useful model for the in vitro fermentation of plant dietary fibers in a nutritional study. Others suggested the use of BC nanocrystals in the fabrication of edible, biodegradable and high-performance gelatin nanocomposite films for food packaging applications (Shin-Ping, et al., 2013).

4.3.6. Other applications

Modification and incorporation with other ingredients to make novel BC composites broaden the spectrum of BC applications.

A graphite film has been prepared by pyrolysis of BC (Yoshino et al., 1990). A highly graphitized film with very high electrical conductivity has been made by pyrolysis at 2900ºC. Shah and Brown (2005) produced an electricity conducting (or semi-conducting) BC sheet by

depositing ions around the micro fibrils to provide conducting pathways and then immobilizing electrochromic dyes within the microstructure. The device has the potential to be extended to various applications, such as e-book tablets, e-newspapers, dynamic wall papers, rewritable maps and learning tools. Yoon et al. (2006) also incorporated multiwalled carbon nanotubes (MWCNTs) into BC pellicles to produce high electricity conducting polymeric membranes.

Another application is as a membrane inside as amperometric glucose sensor. Both in vitro and in vivo testing compared sensors made from BC and wood cellulose. All data showed that membranes made from BC were stable six to seven times longer than those that were made from wood. Although the applications of BC rarely use the pellet type, BC beads (0.5 – 1.5mm) have also been used as a substrate for enzyme immobilization (Wu and Lia, 2008). The immobilized glucoamylase demonstrated its stability against changes in the pH value and temperature. Seo et al. (2009) reported a noncompartmented microbial fuel cell (NCMFC) and adopted semipermeable cellulose acetate film as a cathode material, which can selectively keep protons and hence maintain the redox potential difference between the anode and cathode. The hydrophilicity of cellulose acetate can be modified; Matam et al. (2009) reported that an engineered cutinases can improve the degree of substitution of hydroxyl group onto cellulose acetate fibers. An increase on the hydroxyl groups at the fiber surface is 25% for diacetate and 317% for triacetate after a 24 h treatment, respectively.

The latest trends in BC applications reside in the formation of new nanocomposites that enhance the versatility of this biomaterial. Different type of micro/nano particles can be suspended in the bacteria culture media during the formation of cellulose fibrils. There is also a patent about in situ bioproduction and composition of bacterial cellulose nanocomposites (Laborie and Brown 2008). There are extensive studies on the use of BC in electronic devices. Evans et al. (2005) patented a method for the deposition of metals in BC for the construction of fuel cells and other electronic equipment. Recent studies also disclosed the potential of nanocellulose as substrates for flexible optoelectronic and photonic devices. Legnani et al. (2008) prepared an organic light emitting diode (OLED) device using flexible bacterial cellulose sheet deposited with SiO2. The maximum luminance was measured to be 1200 cd/m^2. Ummartyotin et al. (2012) also reported the successful fabrication of OLED display using a transparent bacterial cellulose nanocomposite film as substrate (Shin-Ping, et al., 2013).

4.4. Bacterial cellulose obtaining methods

The current methods of BC production are static culture, agitating culture, and the airlift reactor. Large scale, semicontinuous, and continuous fermentation will be dominant to meet commercial demand. In all cases, the main objective is to achieve maximum production of BC with optimum form and suitable properties for the application for which it is intended. Through genetic modification or strain selection, several strains can now produce cellulose in an agitated and aerated bioreactor. BC produced by these new strains under agitated and airlift cultures, however, is formed as reticulated cellulose slurry with limited applications (Shin-Ping, et al., 2013).

4.4.1. Static cultures for BC production

Static cultivation is a relatively simple and widely used method of cellulose pellicle production. The medium is placed into shallow trays, inoculated, and cultivated for 5-20 days until the cellulose nearly fills the tray. *G. xylinus* produces a gelatinous BC pellicle, which has a denser surface on the side exposed to air. The BC production is directly related to the area of air/ liquid surface when the depth is less than 4.5 cm (Okiyama et al. 1992b). An external factor, named the wall effect, should be eliminated as it is the strongest limiting factor for BC production. Schramm and Hestrin (1954) explained that the floating property of BC pellicle is due to the entrapped CO_2 bubbles generated from metabolism of bacteria.

Figure 12. Static BC culture

The traditional static culture represents an expensive way of BC production that may hinder

its industrial application since the productivity is low and long cultivation time is required. It was proposed a new static culture system based on a simple fed-batch strategy to increase the BC productivity to a suitable level for commercial applications, using waste from beer fermentation broth (WBFB) as nutrient source. It was found that WBFB is a better medium than the chemically defined medium for BC production in a fed-batch culture. Seven hundred and fifty g of BC sheet was obtained with 2.5 cm thickness after 30 days of cultivation using WBFB, representing a 2–3 times increase in BC production compared to batch cultivation. The scanning electron micrographs of the sheets showed that the fibrils of BC produced in the fed-batch culture are more crowded and thinner than those produced using chemically defined medium (CDM). With the same purpose, Kralisch et al. (2010) developed a novel, efficient bioreactor for a semi-continuous production of planar bacteria-produced nanocelluloses (BNC) named Horizontal Lift Reactor (HoLiR) equipped with fleeces and foils of selectable length and adjustable height.

This process combines the advantages of static cultivation and continuous harvesting under, the commercial product from the HoLiR, is characterized by comparable material properties as BNC produced from traditional static cultures. Meanwhile, significant cost reduction using HoLiR compared with static cultivation in Erlenmeyer flasks was accomplished.

4.4.2. Submerged fermentation

Several attempts have been made to produce BC using the submerged fermentation, which is more convenient for scale-up production. However, there are two main concerns:

1. Insufficient oxygen supply and irregular shape of produced BC
2. Simultaneous accumulation of non-BC-producing mutants in agitation culture.

Moreover, the insolubility of the cellulose product poses obstacles for nutrition transfer resulting in inhomogeneity. *G. xylinus* BPR2001, as ATCC 700178, which was reported by Toyosaki et al. (1995) exhibited 1.8-fold higher BC productivity than the commonly used strain, *G. xylinus* ATCC 23769 cultivated under agitation. Other strains, such as *G. xylinus* BPR 3001A, *G. hansenii* KCTC 10505BP, and *G. xylinus* NUST4.1 were also reported for their applicability in the agitated fermentation. BC pellets instead of pellicles were obtained in agitated cultures. Airlift bioreactors have stood out as solution for reducing shear stress and avoiding shutdowns in BC production. Song et al. (2009) studied the scale-up BC production in a modified airlift-type bubble column bioreactor, which had a low shear stress and high

oxygen transfer rate (kLa), using saccharified food wastes (SFW) as fermentative medium. With the addition of 0.4 % agar and aeration rate of 1.0 vvm, 5.6 g/l BC was produced in a 50 liters spherical type bubble column bioreactor after 3 days of cultivation. In relation with structural properties, Hu and Catchmark (2010a) investigated the formation of spherelike BC particles under shaking conditions. They found that not all the *G. xylinus* strains can produce these spherelike particles. Results showed that the JCM 9730 strain could form spherelike cellulose particles under agitated culture with a rotational speed above 100 rpm. Approximately, spheres with 0.1–1 and 10 mm diameters were produced at a rotational speed of 200 and 150 rpm, when cultured in 100 ml of medium in a 250 and 150 ml Erlenmeyer flask, respectively. Field emission scanning electron microscope (FESEM) analysis revealed that cellulose particles produced at 150 rpm were hollow with a layered outer shell, while, particles produced at 125 rpm were solid, but the central region was not layered.

4.4.3. Bioreactor design

To meet commercial scale production, though scaling up of static culturing is challenging, several modifications were proposed. Yoshino et al. (1996) designed a new culture vessel, which provided an oxygen-permeable silicone membrane surface in the bottom. By doing this, the rate of cellulose production was doubled since BC pellicles can be formed on the liquid–air surface and on the oxygen-permeable silicone membrane. They also found that the rate of cellulose production on the silicone membrane depended strongly on the degree of roughness of the membrane surface. The rate of BC production was about five times higher on a glossy silicone membrane than on an embossed surface. Hornung et al. (2007) developed a novel bioreactor, which involves the generation of an aerosol spray of glucose and its even distribution to the living bacteria on the medium-air interface. The average growth was 2 mm/day or around 9 g cellulose dry mass/day. BC produced in the aerosol bioreactor showed higher mechanical strength than the traditionally produced product from a static beaker or box culture.

4.4.3.1. Airlift bioreactor

Airlift bioreactor (ALR), which requires lower power supply when compared with agitated bioreactor, is another option for BC production. Chao et al. (1997) first reported the implementation of airlift bioreactor for BC production. Air or oxygen-enriched air was supplied

from the bottom and hence drove the circulation of culture medium. Later, different configuration of airlift bioreactor has been applied to enhance BC production. Chao et al. (2000) employed a 50-l internal-loop airlift reactor and obtained 3.8 g/l BC after 67 h fermentation. Cheng et al. (2002) reported a modified airlift reactor (equipped with three wire-mesh draft tubes), which yielded 7.7 g/l BC after 72 h fermentation. The highest BC concentration to date was 10.4 g/l with a 0.22 g/l/h production rate by using air-lift reactors (Shin-Ping, et al., 2013).

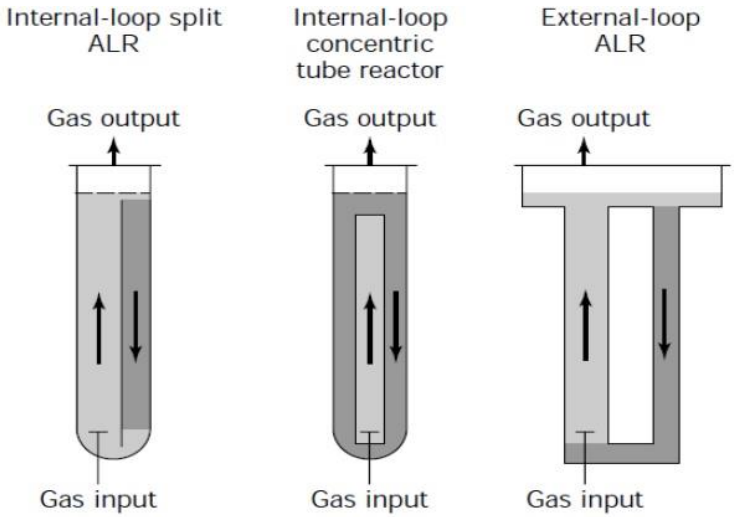

Figure 13. Types of ALRs

4.4.3.2. Rotating disk bioreactor

In agitation or airlift bioreactors, the adhesion of BC to the shaft and the upper part of the vessel causes the homogeneity problem and increases the difficulty of removing the BC product. Another concern is that the produced BC pellets exhibit low mechanical strength which limits its possible applications when compared to BC pellicle. Serafica et al. (2002) first reported the BC production in a rotating disk bioreactor, which consists of a cylindrical inlet for inoculation and several circular disks mounted on a rotating central shaft (Bungay etal.1991).The rotating disk bioreactor was designed that the half area of its disks was submerged in the medium and the other half exposed to the atmosphere, as it is shown in Figure 14 (Shin-Ping, et al., 2013).

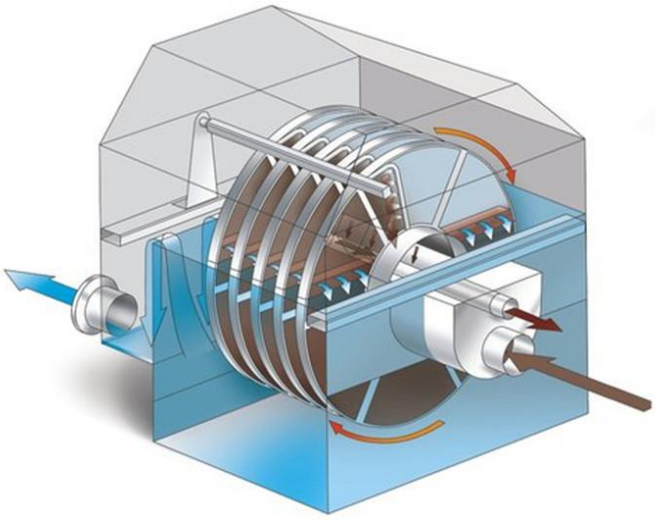

Figure 14.Rotating disk bioreactor

With continuous rotation, the surface of disks is alternately located between the medium and atmosphere. The advantage of this design is that the produced BC will attach on the disks and restore its mechanical strength, in addition to aeration. Later, Krystynowicz et al. (2002) investigated the optimal fermentation condition (medium volume, rotating speed, and number of disks) for BC production. The maximum BC yield was obtained when rotating speed is at 4 rpm with a surface/medium ratio of 0.71. Attempts of adding different solid particles were conducted in order to obtain novel BC composites. Serafica et al. (2002) reported that the incorporation of solid particles into the cellulose matrix was related to the rotating speed and their concentrations. Paper fibers of ordinary cellulose can be incorporated to create composites with enhanced strength and increase the toughness of BC (Mormino and Bungay, 2003). Lin and Cheng (2012) reported a semi-continuous approach by using rotating disk bioreactor containing PCS, a kind of plastic matrix, which can enhance cell adhesion. BC can be produced semi-continuously, and its productivity is approximately 2.58 mg/cm2/day for at least five consecutive runs without the need of reinoculation (Shin-Ping, et al., 2013).

4.4.3.3. Cell immobilization and biofilm reactors

The productivity of BC can be improved by using techniques such as immobilized-cell reactors, cell-recycle reactors, and hollow fiber reactors.

Biofilm reactor, one of the immobilized-cell reactors, is an excellent example of high biomass

density systems, which may reduce the capital costs. Biofilm reactors, moreover, show several advantages over suspended cell reactors, particularly in providing high biomass density, operation convenience, and production yield. Cheng et al. (2009b) successfully enhanced BC production by using a plastic composite support (PCS) biofilm reactor. The results demonstrated that the PCS biofilm reactor yielded BC production (7.05 g/l) that was 2.5-fold greater than the control (2.82 g/l). Moreover, mechanical strength analysis also indicated that the produced BC, similar to pellicle form, improved its tensile strength to a point comparable to that observed in pellicle form, which may broaden its potential applications. However, it is not clear to what extent PCS components are incorporated into the cellulose matrix. Solid nutrient supports present the capability to introduce desired additives into the produced cellulose potentially allowing new engineered materials to be created (Shin-Ping, et al., 2013).

4.4.4. Long-term fermentation

A main limitation of batch fermentation is the time required to restart a new batch. Microorganisms need time to accustom themselves to new fermentation condition. Intense researches have been conducted to develop continuous fermentation processes in order to: minimizing the time for cleaning and sterilizing the bioreactor, omitting the time for seed culture preparation, and shortening the lag time for cell activation and accumulation time of enough biomass. Naritomi et al. (1998) conducted BC fermentation in a continuous manner with ethanol addition. Results demonstrated that, with the presence of 10 g/l of ethanol, 0.95 g/l/h BC production rate and 46 % of BC yield was achieved with a dilution rate of 0.07/h. The BC production was only 0.6 g/l after 36-h fermentation for control in batch fermentation. Ethanol functioned as an energy source for ATP generation, but not as a substrate for BC synthesis. For repeated batch fermentation, Naritomi et al. (2002) studied the broth exchange ratio on BC production. The results indicated that the highest BC production rate (0.43 g/l/h) and BC yield (28 %) were achieved when the broth exchange ratio was 0.9. With a larger broth exchange ratio, the ATP content in cells was low and resulted in a lower BC production rate. Naritomi et al. (1997) patented a process for continuously preparing BC at a production rate of 0.4 g/l/h, by maintaining the concentration of the residual sugar in the culture broth at 20 g/l. More recently, Ruka et al. (2012) proposed a 7-day cultivation period which reached the highest BC productivity. The BC yield reaches a maximum level at approximately 14 days (10 g/l). Lin and Cheng (2012) demonstrated that plastic composite

support (PCS) as solid support features high potential for semi-continuous production of BC. The result shows that BC can be produced semi-continuously, and its productivity reached is approximately 2.58 mg/cm2/day, which can be maintained at least five consecutive runs (Shin-Ping, et al., 2013).

5. Experimental

5.1. Materials and instrumentation

5.1.1. FTIR Bruker Vertex 70 spectrometer

Infrared spectroscopy is a method of analysis that allows obtaining the spectrum of a substance, being it a liquid, a gas or a solid within the IR range. Since each type of bond absorbs radiation at a different wavelength, it is possible to perform the study of the molecular structure of materials. This is precisely one of the most common areas of application.

Figure 15.FTIR Bruker Vertex 70 spectrometer

Throughout the project we have used the FTIR Bruker Vertex 70 spectrometer equipped with an attenuated total reflectance (ATR) device with temperature control to obtain BC spectra.

5.1.2. Refrigerated SIGMA 6K10 Ultracentrifuge

Refrigerated centrifuge SIGMA 6K10 is designed for heavy-duty use in medical laboratories, these SIGMA centrifuges are also well suited for the high requirements in research laboratories, e.g. for investigation of water pollution in environmental research laboratories.

Figure 16.Refrigerated SIGMA 6K10 Ultracentrifuge

This one has a capacity of 2 liters and the centrifugations are carried out at about 8600 rpm, in our case usually for 20 minutes long. Six samples can be centrifuged at the same time.

5.1.3. Multiplace Digital Magnetic stirrer with heating OVAN

Module consisting of 5 or 9 heating units placed in a single platform, with a single electrical connection. Each place works independently. Temperature and magnetic stirring are adjustable, controlled by microprocessor. In our project this device has been used to prepare the culture media, dissolve the components correctly and homogenize the mixture.

Figure 17.Digital magnetic stirrer

5.1.4. Orbital agitator OVAN

Multiplace orbital agitator with 20 places suitable for different sizes of containers. The speed of agitation can be programmed, in our case the agitated fermentations are carried out at 120rpm.

Figure 18.Orbital agitator OVAN

5.1.5. Fisher Mini Centrifuge

For quick spin downs from walls or caps of centrifuge tubes, microfiltration of HPLC samples and cell separations and short-term cold-room use. In our project it has been used for the NaOH cleaning of the BC samples using 2ml Eppendorf tubes. It spins samples to 3000 rpm.

Figure 19.Fisher Mini Centrifuge

5.1.6. Colorimeter Zuzi 4200 A, x = 578nm

A colorimeter is a device used in colorimetry. In scientific fields the word generally refers to the device that measures the absorbance of particular wavelengths of light by a specific solution. This device is most commonly used to determine the concentration of a known solute in a given solution by the application of the Beer-Lambert law, which states that the concentration of a solute is proportional to the absorbance.

In our case, it was used to determine the concentration of bacteria in the growing media to better understand the evolution of the process.

Figure 20.Zuzi 4200 A colorimeter

5.1.7. SELECTA Stoves

- Selecta Stove 80 liters, 48 ºC. For sterile drying of laboratory instrumentation and BC drying.

- Selecta Stove 300 liters, 30ºC. For the correct growth of the bacteria inside the culture medium.

5.2. Culture of bacteria

A nutrient material prepared for the growth of microorganisms in a laboratory is called culture medium. The exact chemical composition in a chemically defined medium is known. The production of bacterial cellulose by *Gluconacetobacter xylinus* depends on the composition of the culture medium, pH, the effect of different sources of carbon and nitrogen, etc.

Once the strain of bacteria is chosen, in this case BPR2001 (*Komagataeibacter sucrofermentans*), it is necessary to introduce it in an appropriate culture medium. The chosen medium was directly proposed by CECT. The composition of this medium is given below:

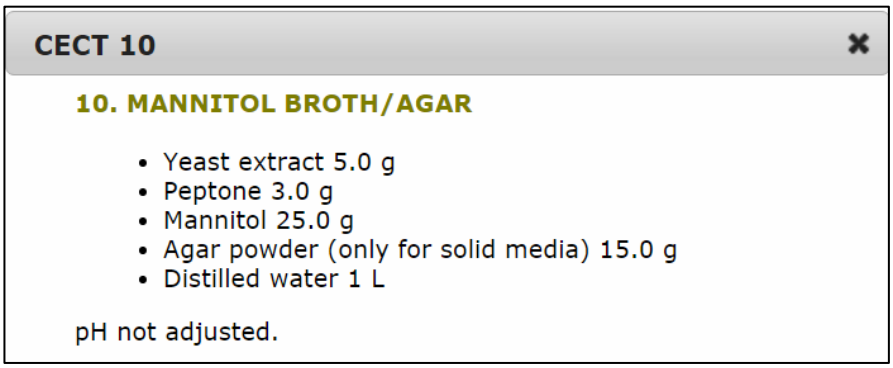

Figure 21.Growth conditions of BPR 2001 by CECT

Instead of producing 1 liter of culture medium, it was decided to produce 300ml: the amounts of each component were reduced to 30% of the initial amounts.

- Yeast Extract 1.5g
- Peptone 0.9g
- Mannitol 7.5g
- Distilled water 300ml

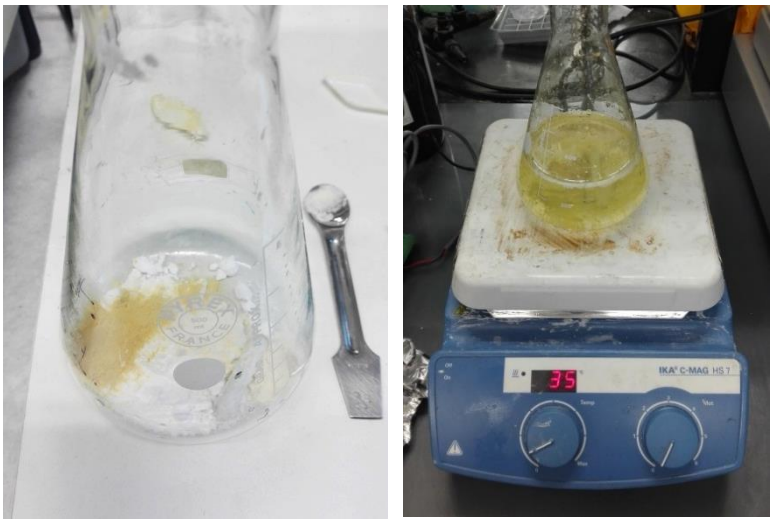

Figure 22.Culture medium preparation

When all the components have been mixed, the mixture is warmed to 35 °C with continuous stirring to dissolve everything properly. The Erlenmeyer flask must be covered to make sure the environmental pollution is as low as possible.

The culture medium is centrifuged and filtered before adding the BPR2001 to eliminate any unwanted bacteria or fungi.

The centrifugation is carried out for a period of 10 minutes using SIGMA 6K10 UltraCentrifuge. To do this, the mixture is introduced in an appropriate container in accordance with this centrifuge. Two more containers with distilled water are used so that the weight entered in the centrifuge is balanced. It is important that the total weight of the mixture + container do not exceed 400g. When the process has been completed, we keep the liquid medium and the solid product is removed.

The liquid obtained is vacuum filtered. This procedure requires the following material:

- Foot
- Clip
- Büchner funnel
- Circular filter paper
- Kitasato
- Rubber Board
- Connection to a vacuum system (suction pump, trompe)

The filtering membranes contain 0.45 micrometers holes and they are chosen because it is wanted to eliminate potential bacteria which size is of about 1 micrometer.

Figure 23. Vacuum filtration

Once the medium is filtered, acquired bacteria can be introduced in it in order to let them reproduce. After introducing it, the Erlenmeyer is covered with a piece of paper to keep it away from other bacteria and it is agitated for 72h at 25ºC.

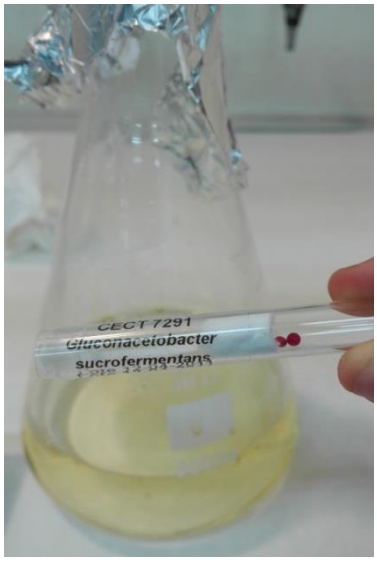

Figure 24. The culture medium is ready to introduce the bacterium

After this time, another centrifugation is carried out for 20 minutes, and the solid part is kept

and introduced in 2ml Eppendorf tubes. These are stored inside the refrigerator until needed.

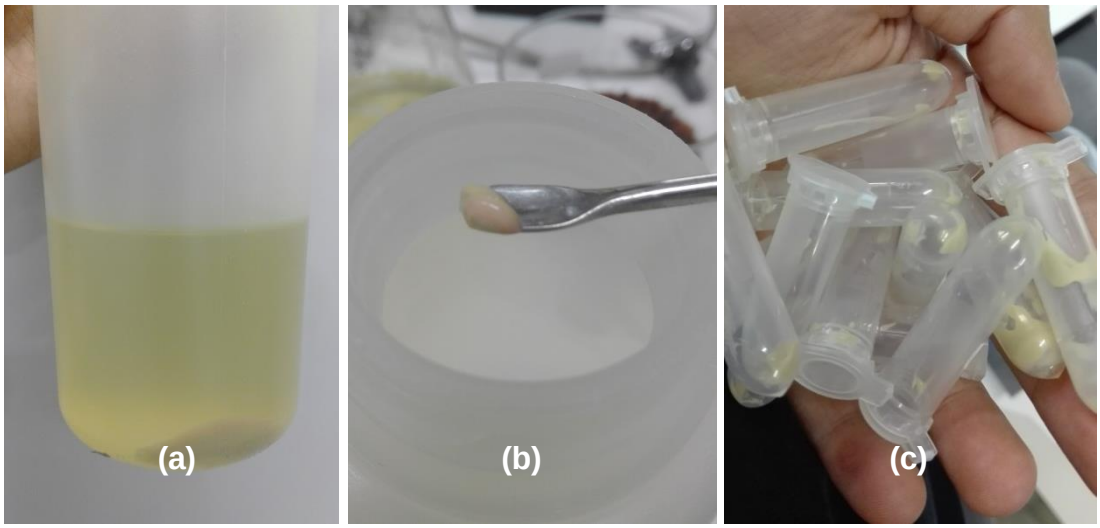

Figure 25. (a) Product of centrifugation process. (b) Gluconacetobacter Xylinum. (c) Bacteria are stored in Eppendorf tubes.

This process is done twice, using the bacteria of one of the Eppendorf tubes instead of buying the bacteria again.

5.3. Preparation of the samples

After having a look at several experimental processes done before, it is decided to be guided by other scientific studies in this field, where a common medium for all samples is used and the carbon source varies in each case to see the effect that it has in the production of BC. It is also decided to make sets of samples for agitated cultivation and other samples for static cultivation.

The common medium has de following composition:

Chemical compound	**Amount (g/l)**
$(NH_4)_2SO_4$	3.3
KH_2PO_4	1
$MgSO_4 \cdot 7H_2O$	0.122

Yeast extract	20
Citric acid	1.5
Acetic acid	0.5

Table 3. Common medium composition

MEDIA PREPARATION

The total number of prepared samples is 30, 15 of them will be agitated and the others will be static cultures. The first step is preparing the common medium; in this case 2.5l are prepared. The corresponding amounts are calculated this way:

$$2{,}5\ l\ media \times 3{,}3\ \frac{g}{l}(NH_4)_2SO_4 = 8{,}25\ g\ (NH_4)_2SO_4$$

The amounts of each compound are shown below:

Chemical compound	**Amount g**
$(NH_4)_2SO_4$	8,25
KH_2PO_4	2.5
$MgSO_4 \cdot 7H_2O$	0.305
Yeast extract	50
Citric acid	3.75
Acetic acid	1.25

Table 4. Total amounts of each compound

In each Erlenmeyer 500 ml of the common medium are introduced and 20 g of the corresponding carbon source are added. Once it is dissolved properly, all the solutions are vacuum filtered and separated in plastic containers (60 ml of solution per container). Then the bacterium is added and the initial pH and optical density of each sample are measured.

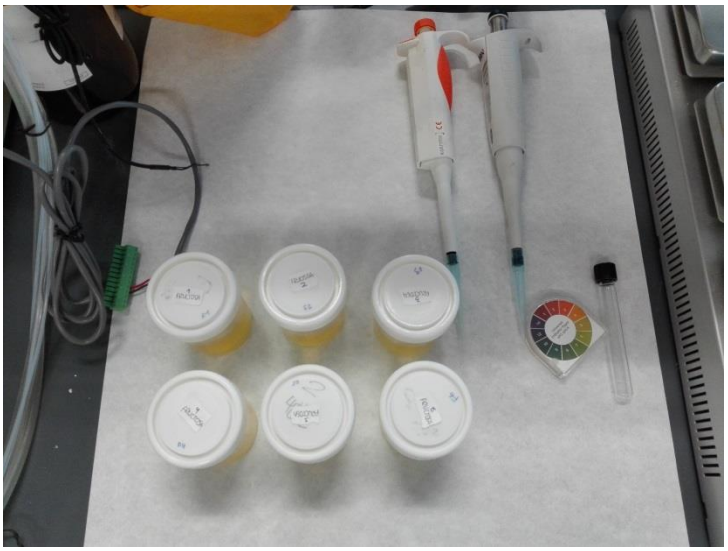

Figure 26. Samples are ready to measure the initial pH

To measure the initial pH, it is used a pH indicator paper. To measure the initial optical density it is used the Zuzy Colorimeter.

All samples are stored in a stove at 28 ºC, agitating three samples of each carbon source and leaving the other three for static culture. The fermentation process begins at this point, and the reaction is followed as the time goes by.

Figure 27. All the samples are ready for beginning the fermentation process

6. Results and discussion

6.1. Initiation of fermentation and preliminary results

When the fermentations have already begun, the progress of the optical density and the pH of the samples are monitored over time. In order not to hamper the quality of static cultures, only one of the three static samples from each carbon source will be used to obtain the necessary data (the sample must be shaken to measure its optical density with the colorimeter and that would change the nanofibers structure). For agitated culture, data will be taken from all samples.

At the end of this section the progression of bacterial growth over time is shown. It is noticed that optical density increases after 50-60h of fermentation time in both kind of cultures.

In terms of pH, it remains constant throughout the experiment at 4.5. The only samples that drastically varied the pH due to the contamination of an external agent were removed at the time of isolating the cellulose: one of shaken mannitol, one of static glucose and one of static lactose.

6.1.1. Agitated cultures fermentation

After 35 hours, BC pellets are formed in the stirred samples of lactose and fructose. In our case, we left the shaken samples fermenting for a period of almost 8 days, and then it starts the washing process and isolated from the BC. This process was carried out by mixing the samples from each set and centrifuging them twice with water, followed by another centrifugation with 0.1M NaOH, another with 1M NaOH, another with water, again with 1M NaOH and to finish and get rid of the NaOH residues the samples were centrifuged twice more with water. All these centrifugations lasted for 20 minutes each.

At this point, the liquid part from the centrifugation is removed and the BC samples are stored in the stove at 48ºC for its proper drying.

Figura 28. Dried BC

Once the BC is dried, the container is weighed with it inside, the BC is stored in another container and the first empty container is again weighed. With the difference of weights we obtain the amount of cellulose produced by each fermentation process (these weights are shown in the "Effects of carbon source" section).

It should be noted that a BC pellet from the fructose and sucrose cultures were stored in the refrigerator in liquid medium for a future study, so the results of the quantities produced by each carbon source are not accurate.

Figure 29. Agitated sucrose BC pellet

6.1.2. Static cultures fermentation

In static cultures, the washing, drying and weighing process of the samples was the same as for the agitated ones.

Figure 30. Dried BC

Even so, some solid floating layers were stored for future study in Petri dishes. For this reason the quantities produced may not be faithful to the actual results.

6.1.3. Optical density figures

6.1.3.1. Static fermentation

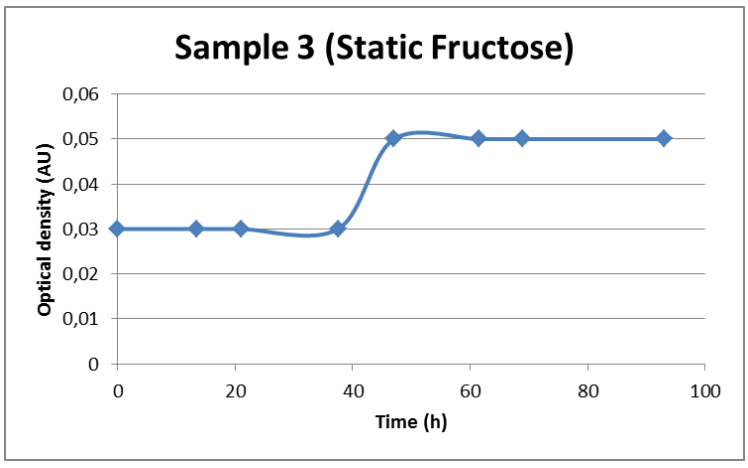

Figure 31. Static fructose optical density progression

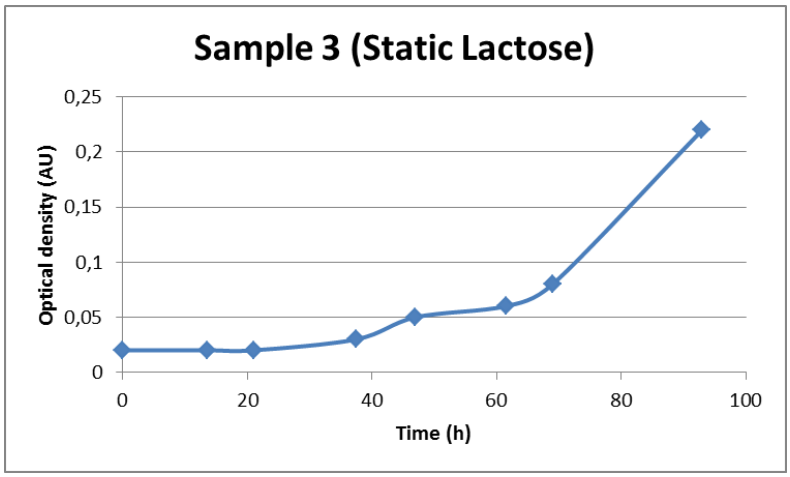

Figure 32. Static lactose optical density progression

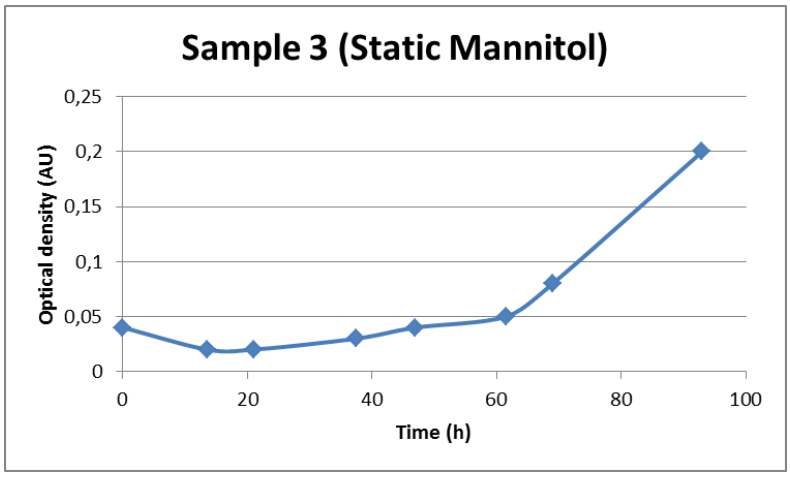

Figure 33. Static mannitol optical density progression

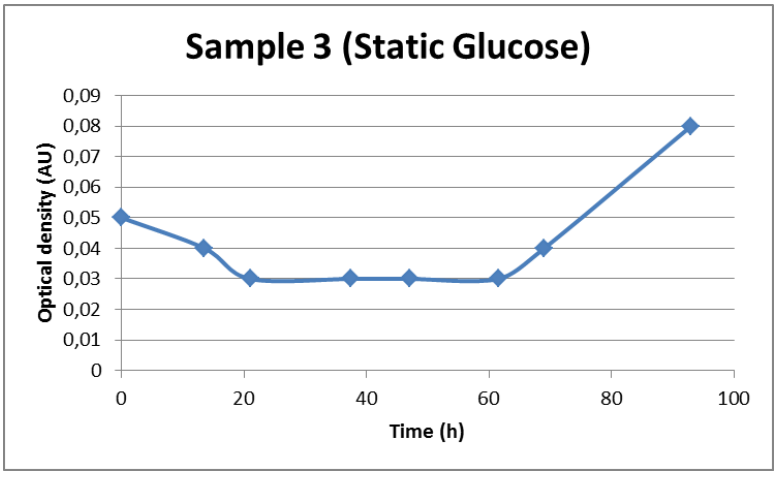

Figure 34. Static glucose optical density progression

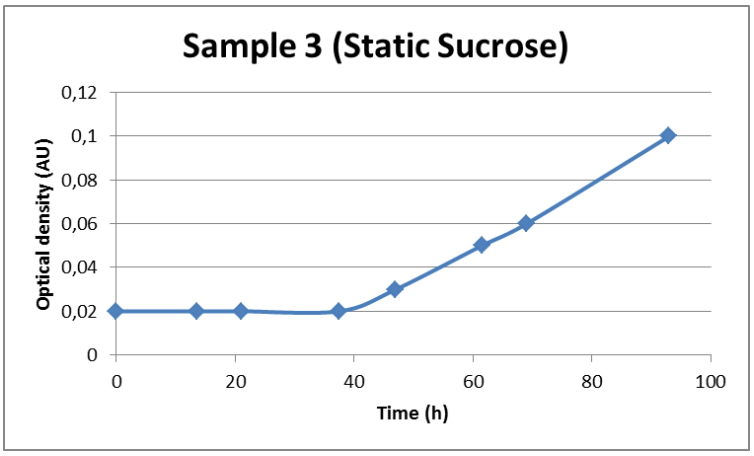

Figure 35. Static sucrose optical density progression

6.1.3.2. Agitated fermentation

FRUCTOSE

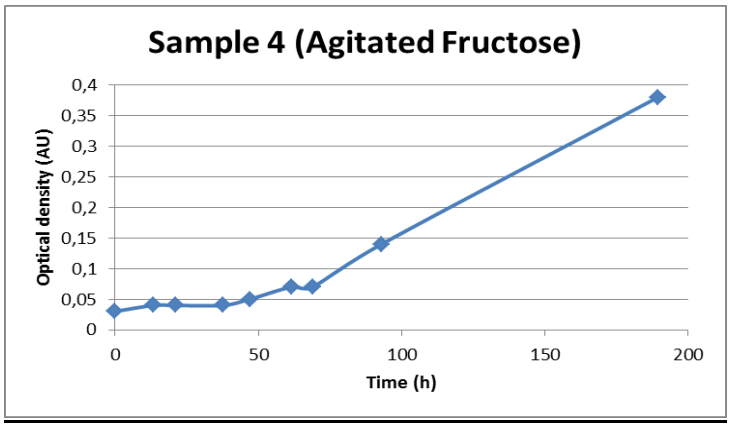

Figure 36. Static fructose optical density progression (sample 4)

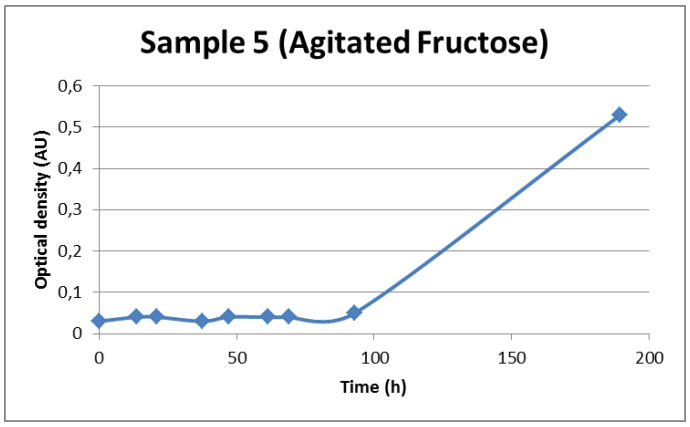

Figure 37. Static fructose optical density progression (sample 5)

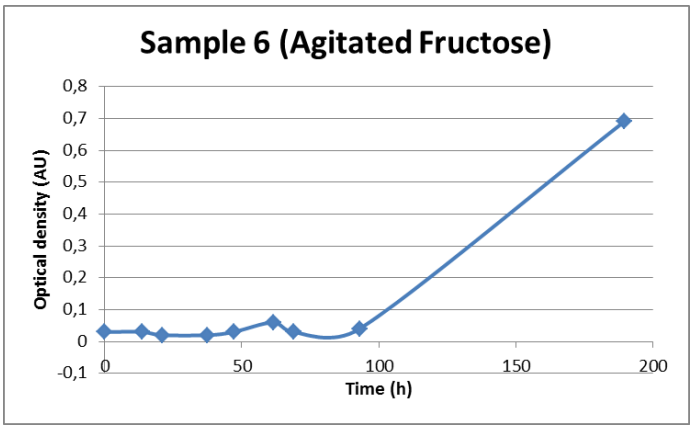

Figure 38. Static fructose optical density progression (sample 6)

LACTOSE

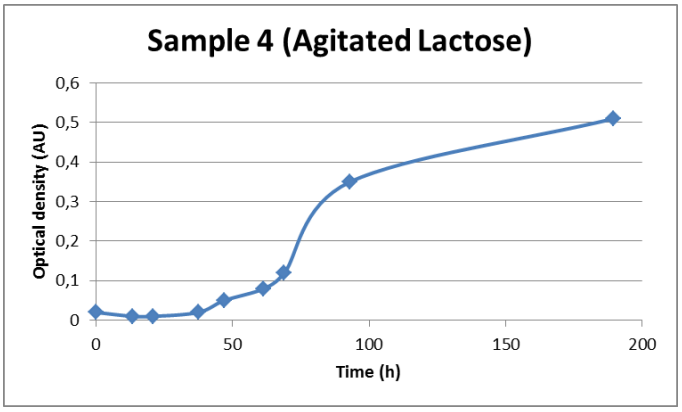

Figure 39. Static lactose optical density progression (sample 4)

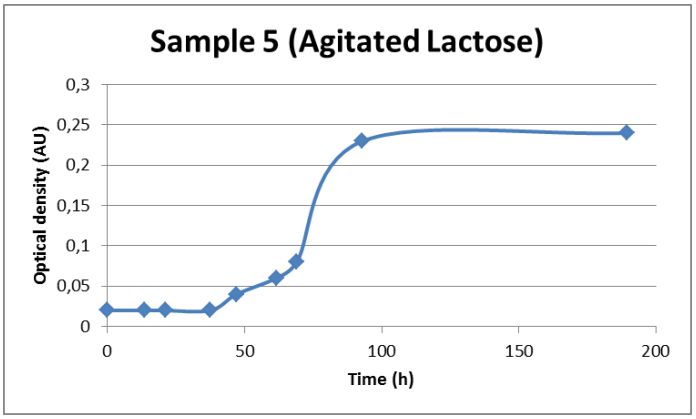

Figure 40. Static lactose optical density progression (sample 5)

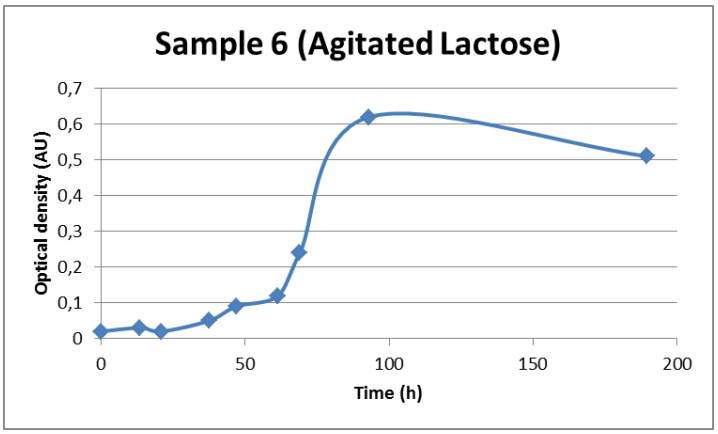

Figure 41. Static lactose optical density progression (sample 6)

MANNITOL

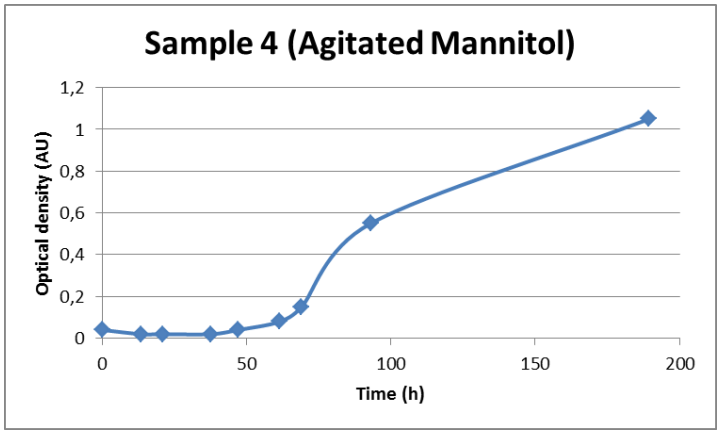

Figure 42. Static mannitol optical density progression (sample 4)

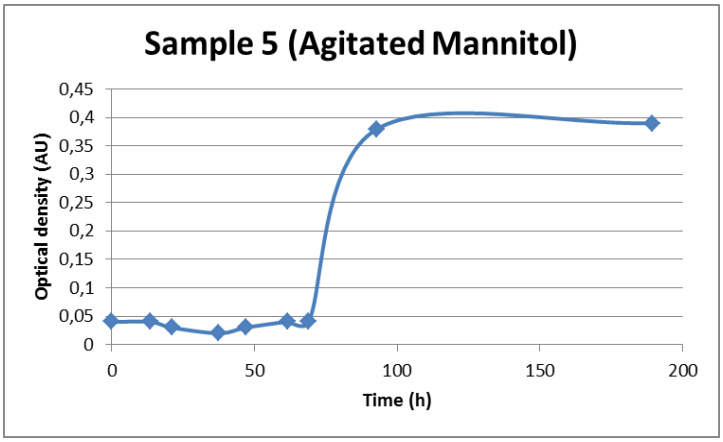

Figure 43. Static mannitol optical density progression (sample 5)

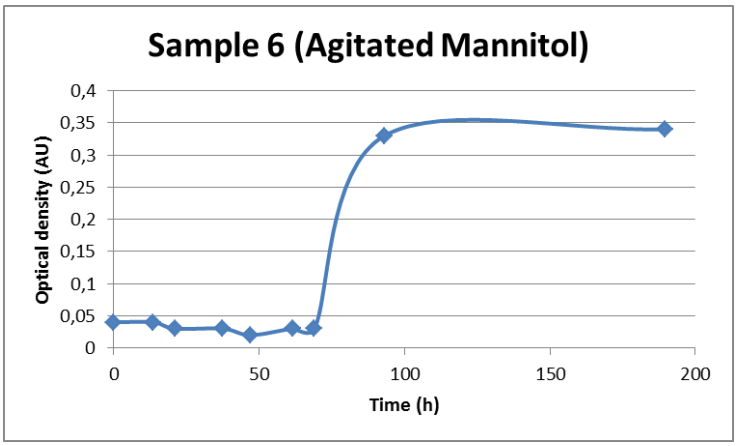

Figure 44. Static mannitol optical density progression (sample 6)

GLUCOSE

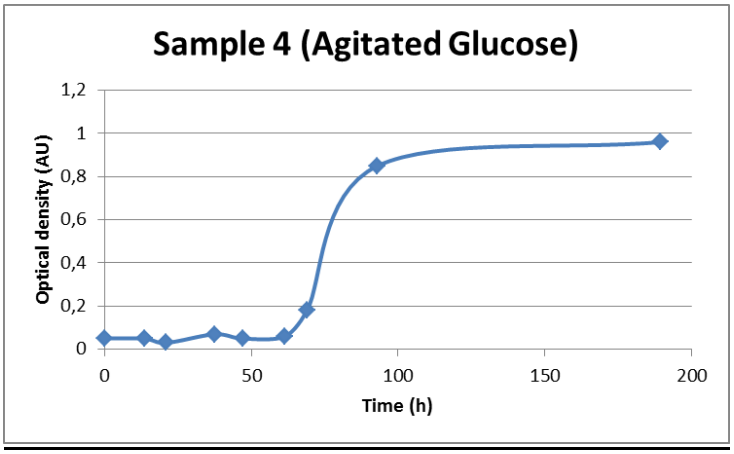

Figure 45. Static glucose optical density progression (sample 4)

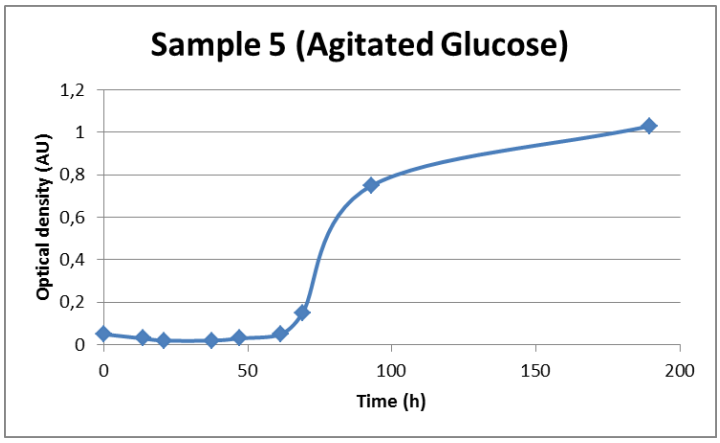

Figure 46. Static glucose optical density progression (sample 5)

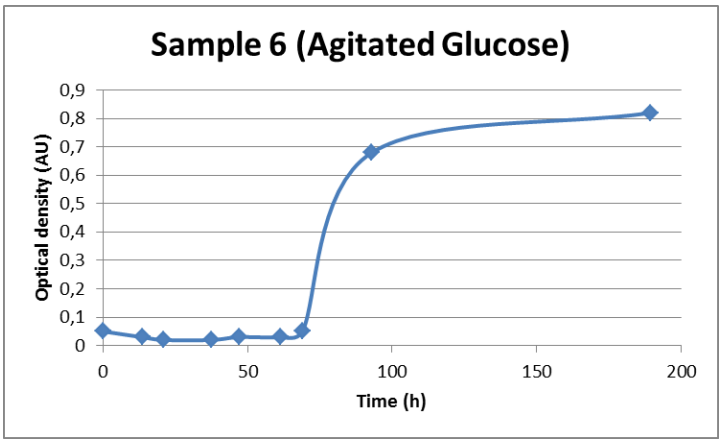

Figure 47. Static glucose optical density progression (sample 6)

SUCROSE

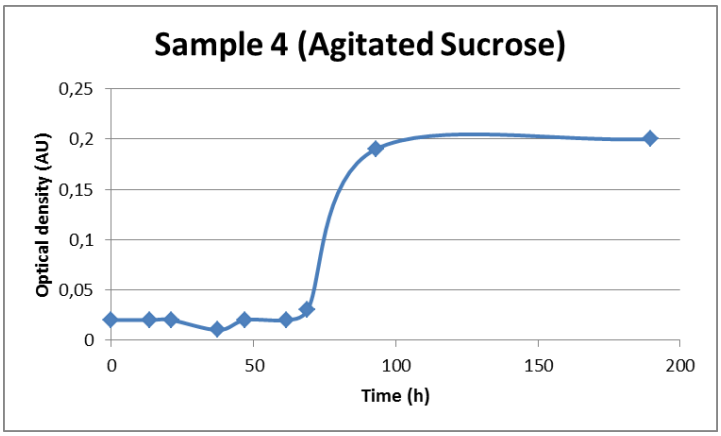

Figure 48. Static sucrose optical density progression (sample 4)

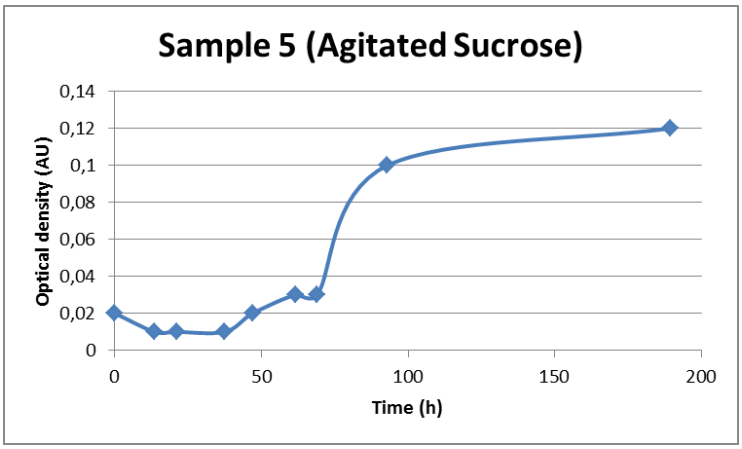

Figure 49. Static sucrose optical density progression (sample 5)

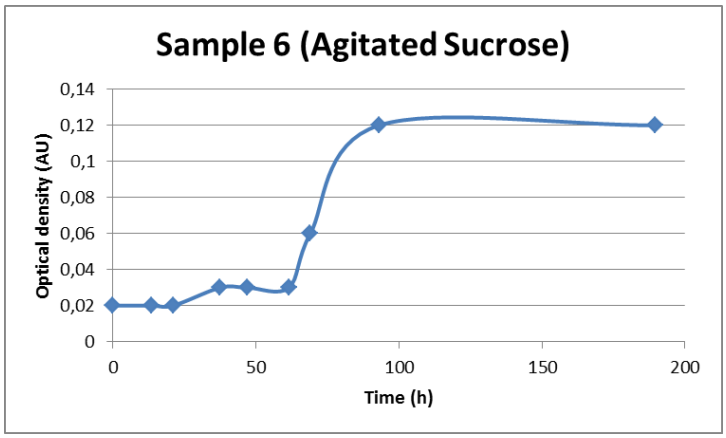

Figure 50. Static sucrose optical density progression (sample 6)

6.1.3.2.1 Average and dispersion of agitated samples

FRUCTOSE

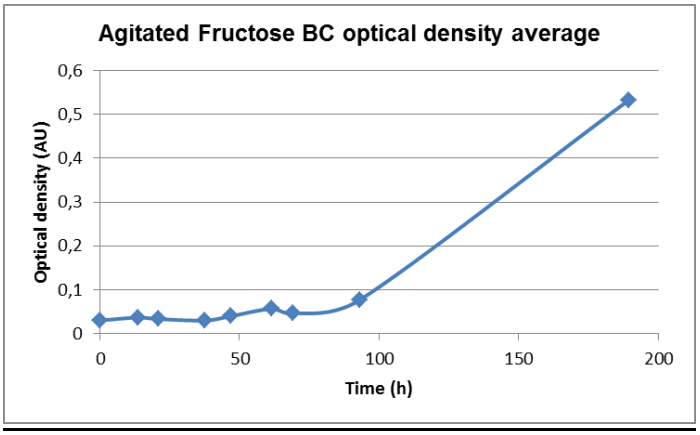

Figure 51. Agitated Fructose BC optical density average progression

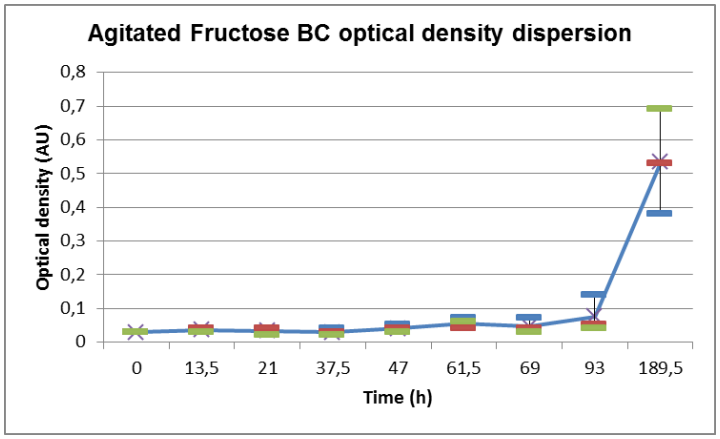

Figure 52. Agitated Fructose BC optical density dispersion

LACTOSE

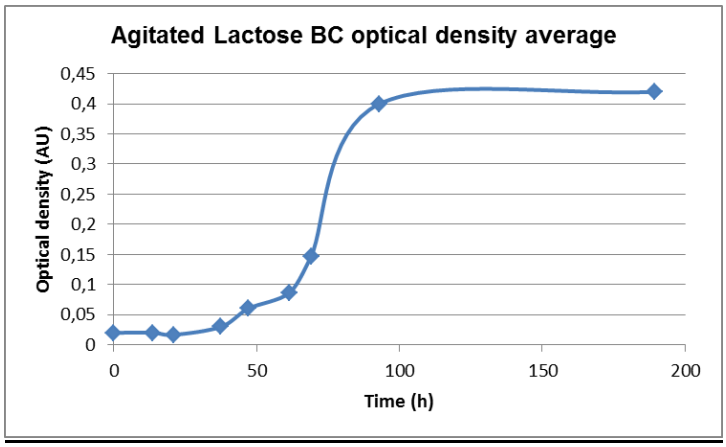

Figure 53. Agitated Lactose BC optical density average progression

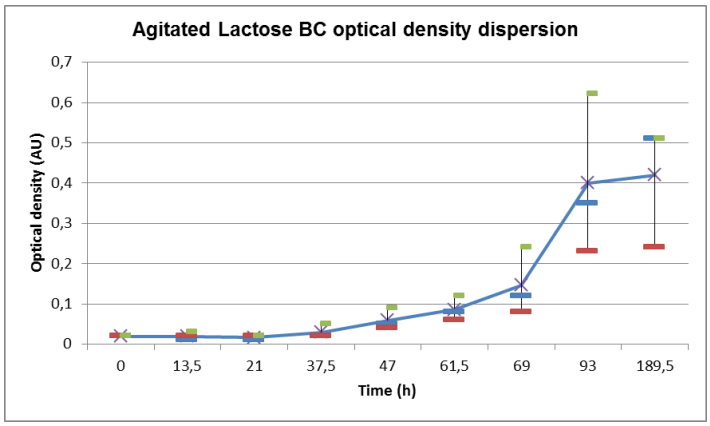

Figure 54. Agitated Lactose BC optical density dispersion

MANNITOL

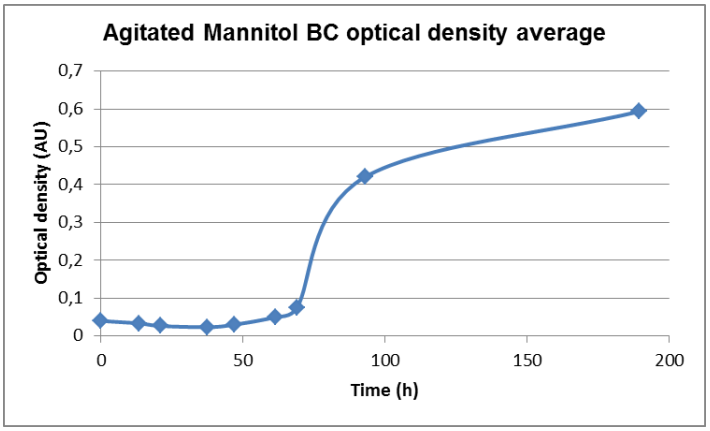

Figure 55. Agitated Mannitol BC optical density average progression

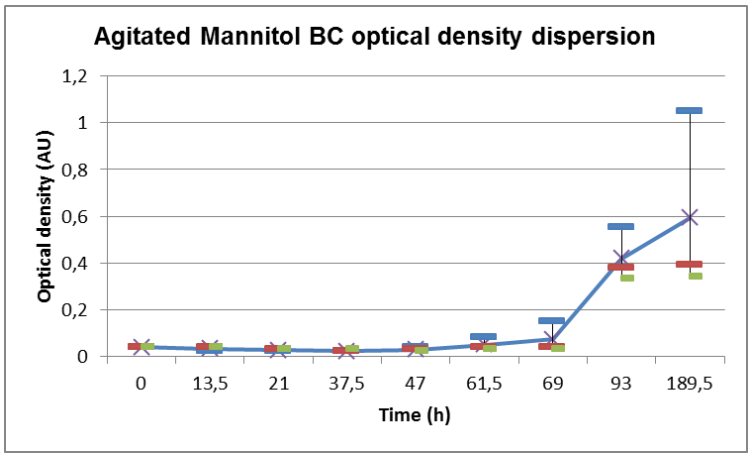

Figure 56. Agitated Mannitol BC optical density dispersion

GLUCOSE

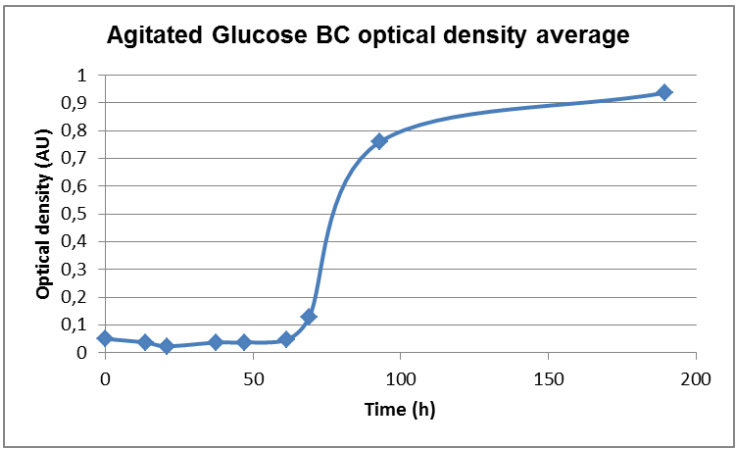

Figure 57. Agitated Glucose BC optical density average progression

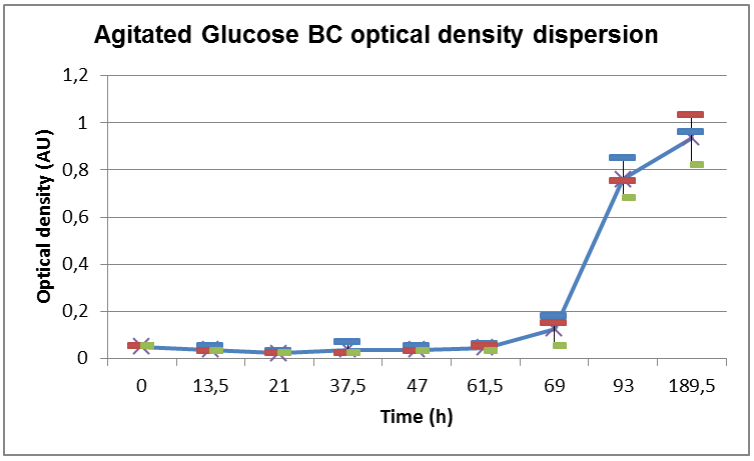

Figure 58. Agitated Glucose BC optical density dispersion

SUCROSE

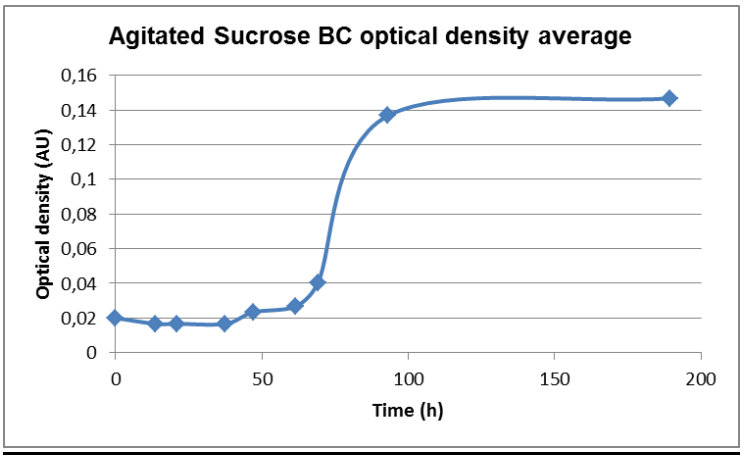

Figure 59. Agitated Sucrose BC optical density average progression

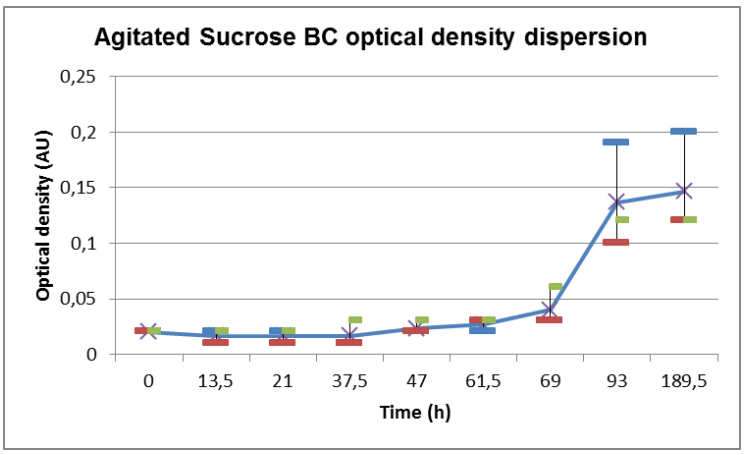

Figure 60. Agitated Sucrose BC optical density dispersion

6.2. Effect of carbon source

Since Schramm and Hestrin published their studies on bacterial cellulose production, the most used culture medium has glucose as carbon source. However, many later studies have looked for new sources to replace it in order to cut costs to make profitable production at industrial level. In addition, several studies have shown that other carbon sources increased production of cellulose, what is because the fact that glucose promotes the production by microorganisms of gluconic acid, and this effect not only decreases the amount of glucose available for polymerization, it also leads to pH levels that affect cell viability (Carreño-

Pineda, Caicedo, & Martínez, 2012).

Table 5 shows the results of a study comparing different carbon sources yield production of cellulose obtained.

CARBON SOURCE	YIELD
GLUCOSE	100
FRUCTOSE	92
GALACTOSE	15
MANNOSE	3
XYLOSE	11
ARABINOSE	14
SORBOSE	11
LACTOSE	16
MALTOSE	7
SUCROSE	33
CELLOBIOSE	7-11
STARCH	18
ETHANOL	4
ETHYLENE GLYCOL	1
DIETHYLENE GLYCOL	1
PROPHYLENE GLYCOL	8
GLYCEROL	93
ARABITOL	620

MANNITOL	380
CITRATE	20
NO CARBON SOURCE	2

Table 5. Comparison of different carbon sources depending on the obtained production yield (Tort-Agell, 2016)

As you can see, arabitol and mannitol are the only two sources that have better cellulose production than glucose.

In the present study, we focused on *G. xylinus* strain BPR 2001, demonstrating its ability to metabolize a range of carbon sources, and highlighting the effect of these substrates on cellulose production.

Efficient cellulose production by this bacterium lies in its ability to synthesize glucose from various carbon substrates, followed by glucose polymerization to cellulose. *G. xylinus* has two main operative amphibolic pathways: the pentose phosphate cycle for the oxidation of carbohydrates and the Krebs cycle for the oxidation of organic acids and related compounds (Ross *et al.* 1991).

The chosen carbon sources to carry out our fermentations were Glucose, Fructose, Sucrose, Mannitol and Lactose. The agitated process was been followed for a 190h period and the static one for two weeks. As it is shown in the table above, the relative amounts of BC produced by all the media compared to the glucose media (considering glucose as 100%) are the following ones:

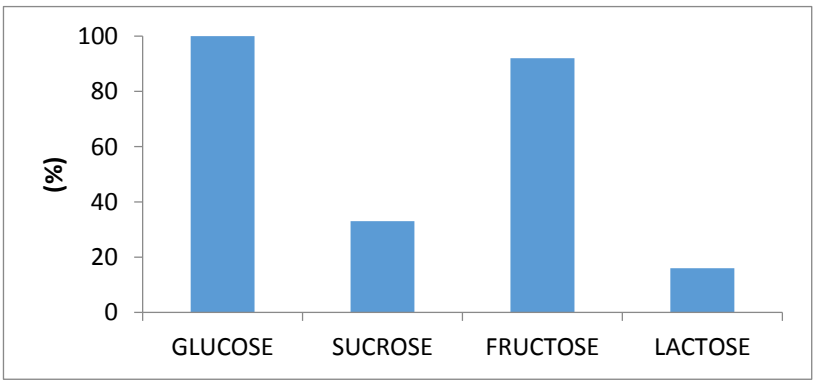

Figure 61.Teoric relative amounts of BC(%) produced by all the media compared to the glucose media

Mannitol samples are not considered as reliable values in this study because at some point they were contaminated with unknown bacteria or fungi and had the worst yield.

Figure 62. Mannitol BC

The amounts of BC produced by each carbon source in this study are shown in the next figures. The first figure shows BC production in agitated culture.

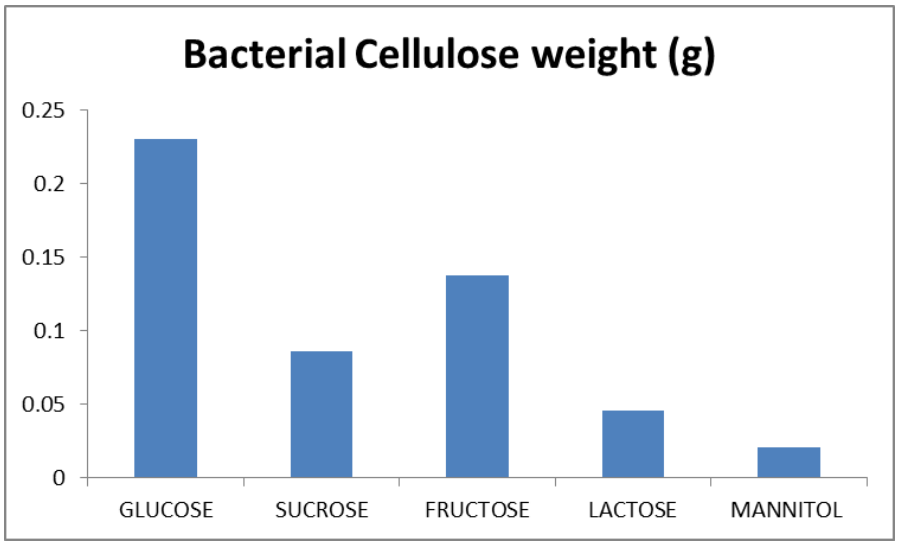

Figure 63. Bacterial cellulose production in agitated culture by each carbon source

As we can see it follows the pattern marked by different studies of agitated cultures. The next figure shows BC production in static culture.

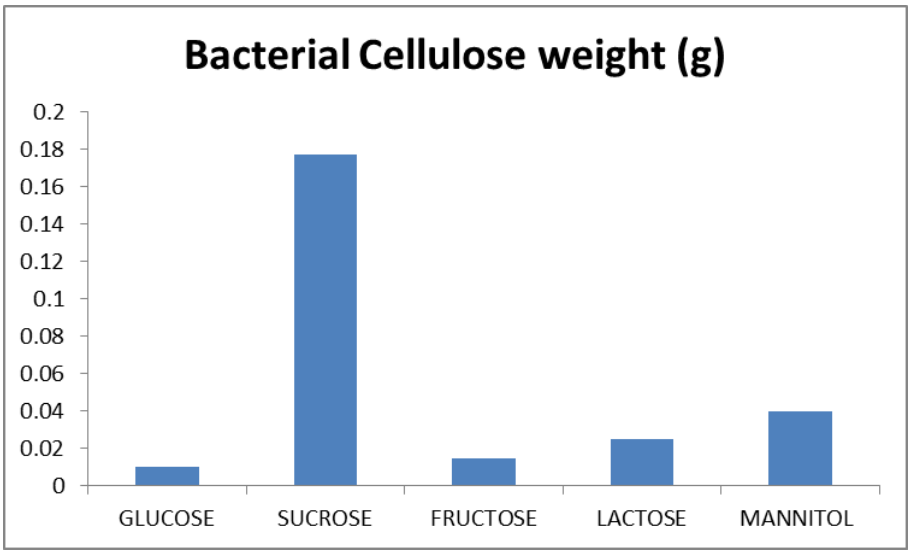

Figure 64. Bacterial cellulose production in static culture by each carbon source

Comparing both methods (Figure 65) we can see that agitated cultures usually have higher yields than the static ones, except in the case of sucrose culture. Despite having looked at other studies and articles, there is no one explaining this fact which comes out of the pattern, so we cannot explain why sucrose has given us much more BC in static culture than in agitated culture. Doing more tests would be necessary to make conclusions or statements about this fact.

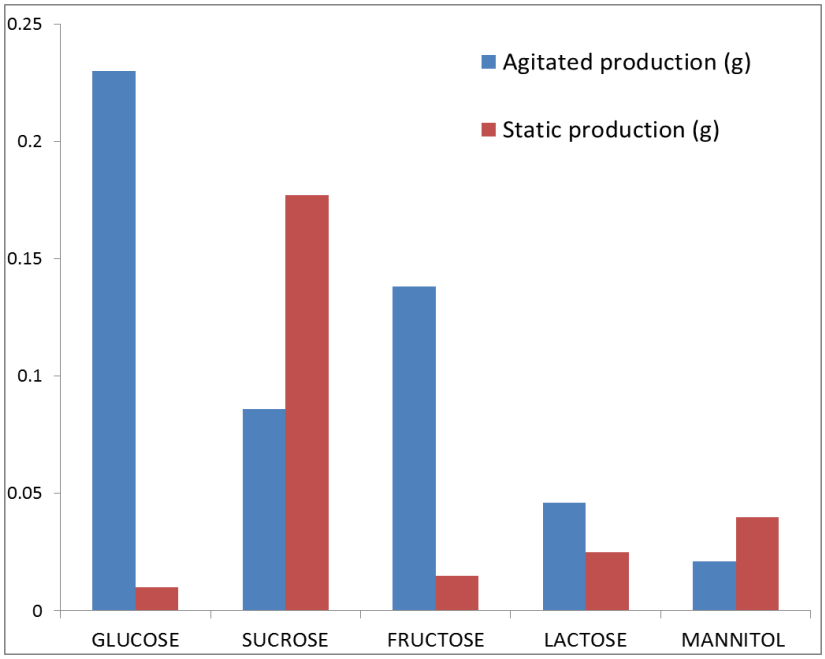

Figure 65. Bacterial cellulose production comparison depending on the agitation

6.3. Agitation effect

Fermentation techniques used to produce bacterial cellulose are static and agitated culture. With each technique cellulose obtained has different morphology (Carreño-Pineda, Caicedo, & Martínez, 2012).

Through the static media, cellulose with a higher Young's modulus, superior tensile strength, higher rate of crystallinity and a higher degree of polymerization is obtained. That is why the main application of cellulose obtained by static culture is in the field of medicine. For less demanding applications of bacterial cellulose, it can be obtained with an agitated fermentation (Grande-Cruz, 2014).

The incubation periods depend on the culture system. Usually, agitated cultures are shaken by 24 to 72 hours while the static cultivation period is between one and two weeks (Carreño-Pineda, Caicedo, & Martínez, 2012).

Therefore, it is interesting to study the effect of cultivation techniques in the production of bacterial cellulose.

In static culture, the cellulose is obtained in gel form, which is about 1% cellulose.

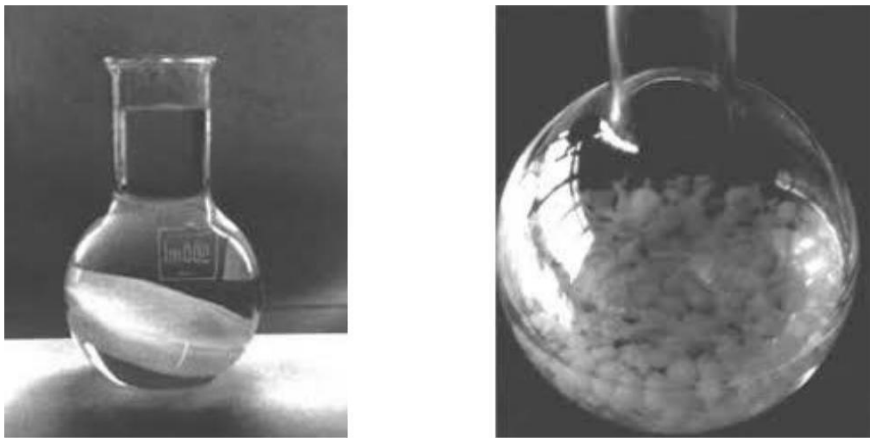

Figure 66. On the left side, static medium cellulose. On the right side, agitated medium cellulose.

Studies show that in the first state, the organism increases its population due to the consumption of dissolved oxygen that is in the culture medium. During this time, the microorganism synthesizes a certain amount of cellulose. At this point, only bacteria that are

near the interface air/culture medium and have oxygen availability can maintain its activity and produce cellulose, which forms overlapping layers. As fermentation time goes by, the thickness of the film increases to generate new layers on the surface, forming a layer structure suspended in culture medium. The main drawback presented by this technique is the high fermentation time and the requirement of a large surface area, which is not suitable in large-scale production.

In fermentations performed in shaken culture it can be obtained pellets or amorphous agglomerations of cellulose fibers, which depend on the type of reactor used and operating conditions. A study in 2013 found that the number of irregular spheres decreased by increasing the volume of the inoculum while the variation in the initial concentration of glucose only had an impact on the diameter of these.

Agitated culture is being investigated with the aim of scaling the process to industrial level, but low productivity reported so far make this scaling more complex. This low productivity is because of the fact that the agitation favors the growth of cells that do not produce the polymer but are substrate consumers. Some authors attribute this phenomenon to mutations that impair the enzymatic machinery responsible for the polymerization of glucose (Carreño-Pineda, Caicedo, & Martínez, 2012).

In this project 15 samples were studied in agitated culture and 15 samples were studied in static culture, both groups with the same temperature and chemical medium components.

In static culture, a layer structure suspended in culture medium was formed. In the case of agitated culture, pellets of cellulose were obtained.

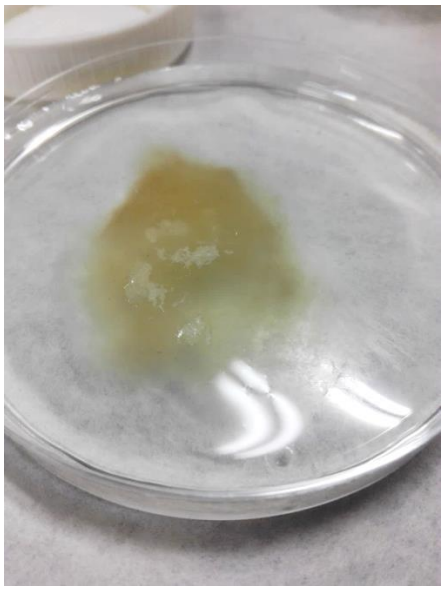

Figure 67. Layer BC structure of static fructose culture

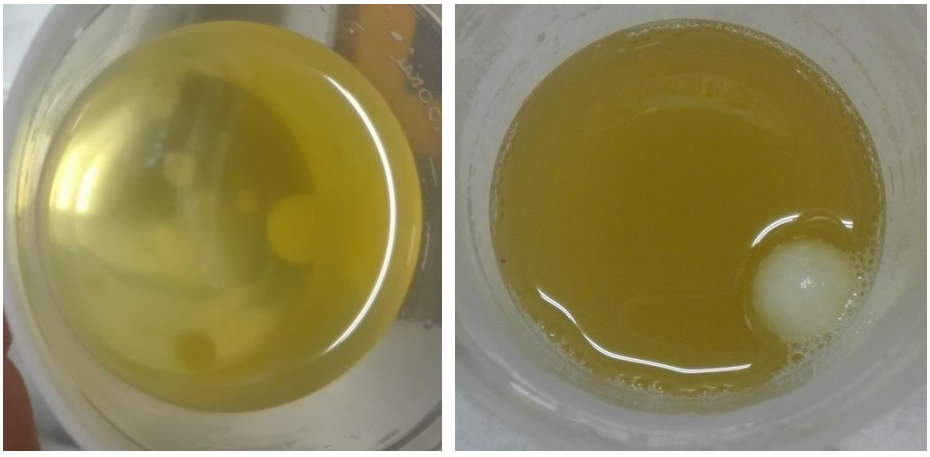

Figure 68. BC pellets of agitated cultures

As it is said before, and as it was expected, agitated cultures produce more BC than static cultures, although the BC quality is better in static cultures.

The next figure shows the structural differences between BC produced in our agitated and static cultures using lactose as a carbon source. These pictures have been taken with an electron microscopy. Both are of BC produced in our project.

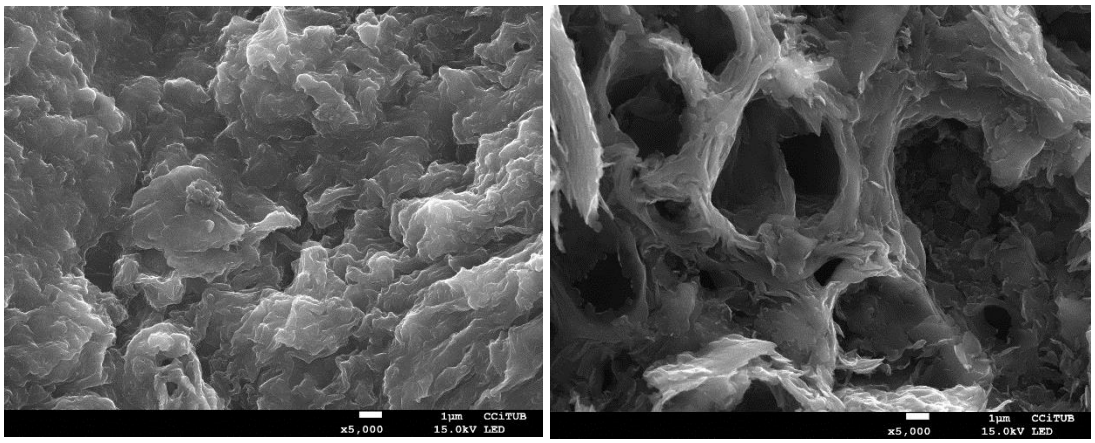

Figure 69. Right side: Agitated Lactose Culture. Left side: Static Lactose Culture

More microfibrils are formed in the static culture while in agitated culture no such complex structures are formed, because when the fibers are agitated they lose quality and less nanofibers are observed. Agitation mechanically modifies the fibers.

It has been observed that the static media tend to be contaminated with other unwanted microorganisms while in the agitated cultures any sample has been contaminated. The following images show a static culture vessel in which fungi have appeared.

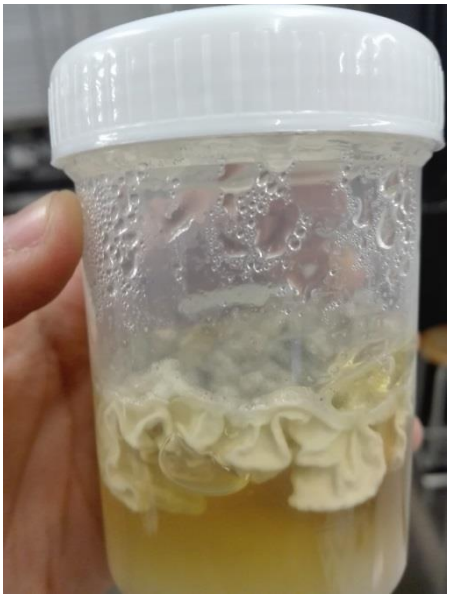

Figure 70. Contaminated sample

Figure 71. Contaminated samples

This is because static cultures are longer and are exposed for longer to contamination. In spite of taking preventive measures to avoid contamination of other microorganisms, the manipulation of each sample cannot prevent the growth of alien species.

6.4. Characterization of the obtained cellulose

6.4.1. Infrared Spectroscopy

In order to evaluate the degree of purity of the obtained bacterial cellulose and the efficiency of the purification method, infrared spectroscopy analyzes were performed. About $1cm^2$ cellulose samples were cut and placed on the spectrometer. The spectra were recorded in the range 600-4000 cm^{-1} with a resolution of 4 cm^{-1} using 16 scans per spectrum in ATR mode.

All recorded spectra showed the same pattern. Standard signals corresponding to typical cellulose are seen, such as OH signals (3300 cm^{-1}) and C-O signals (1100 cm^{-1}). An illustrative spectrum is shown in Figure 72.

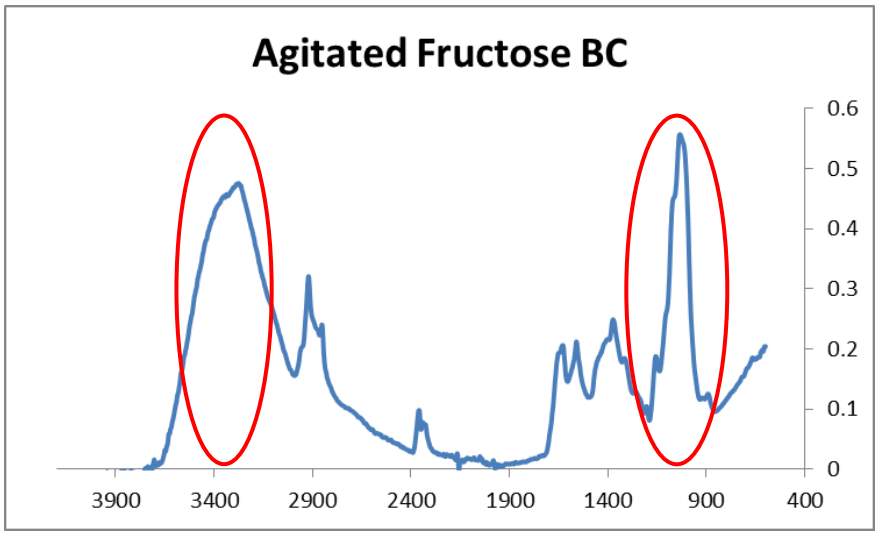

Figure 72. Standard signals corresponding to typical cellulose

In many spectra are also observed signals which do not correspond to cellulose. The peaks at 1650 and 1550 cm^{-1} are not identified with polysaccharides, but can be assigned to functional groups of proteins. In particular, peaks at 1650 cm^{-1} are of the C=O bonds of proteins and the 1550 cm^{-1} signals correspond to the NH link of these compounds.

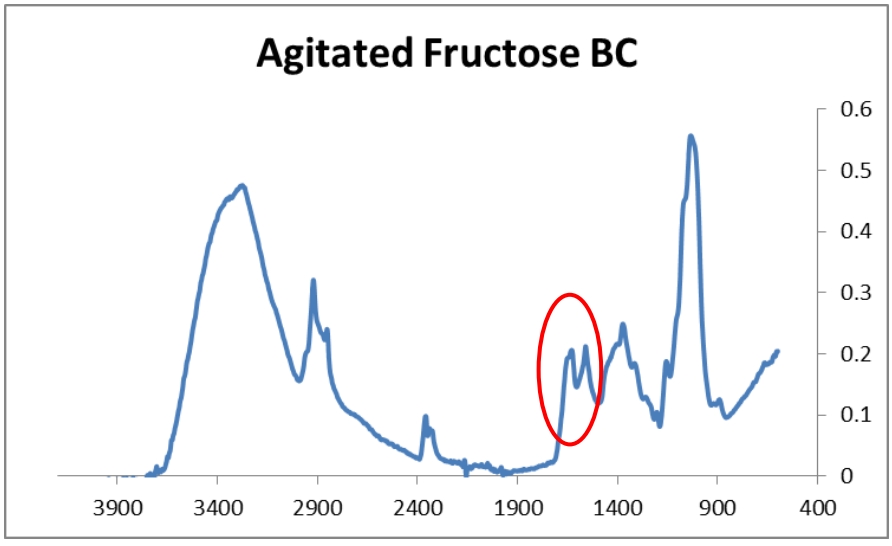

Figure 73. Standard signals corresponding to functional groups of proteins

When the first spectra were obtained, the presence of proteins was noticed. It could happen because the washing method we had chosen from another study probably was not entirely effective, so the process was stopped and the samples were re-washed with 1M NaOH for

longer than the first time. The following figures show the differences between the agitated culture mannitol and fructose BC samples before and after the second NaOH purifying process.

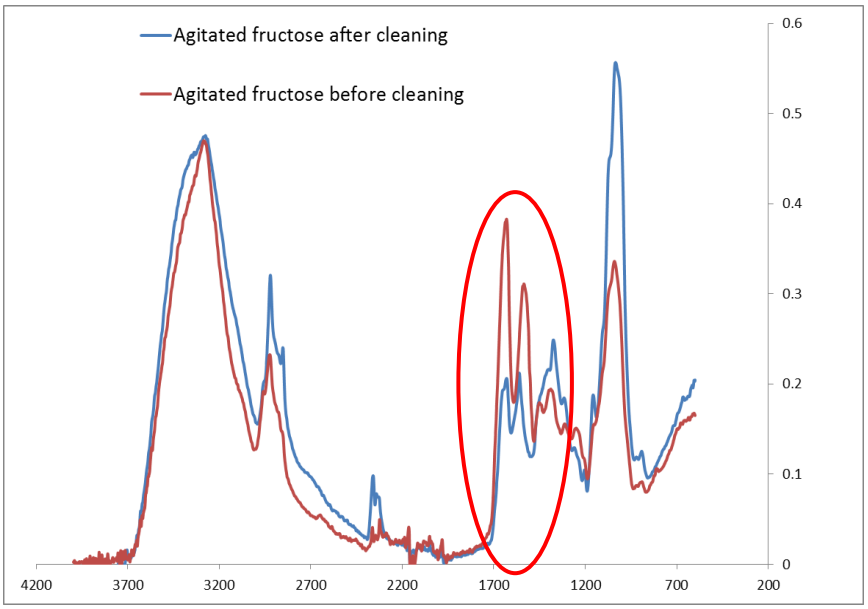

Figure 74. Agitated BC fructose spectrum before and after cleaning

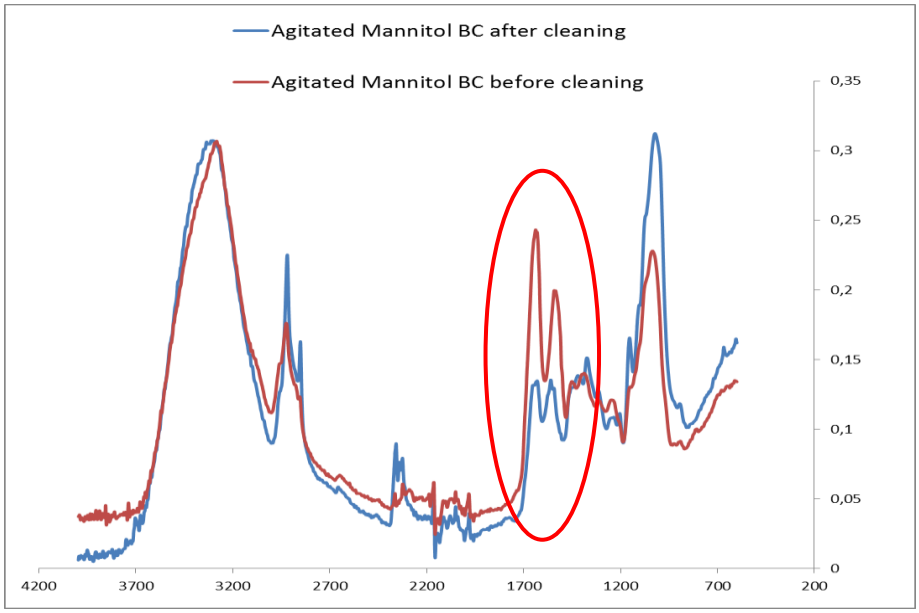

Figure 75. Agitated BC mannitol spectrum before and after cleaning

The protein was not eliminated at all, so maybe NaOH is not the best option for purifying BC.

For later studies it is recommended to do a more intense washing process (more hours and more temperature), because following the recommendations of the studies already done was not enough.

The obtained spectra are showed in the following section.

6.4.1.1. Agitated cultures spectra

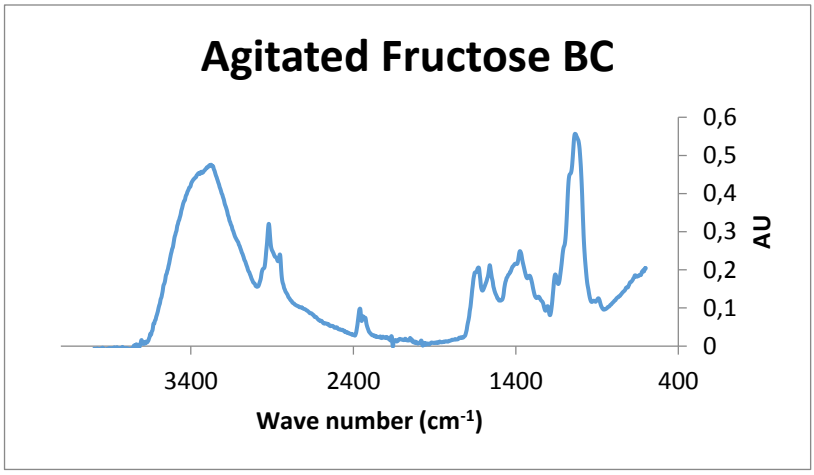

Figure 76. Agitated fructose BC spectrum

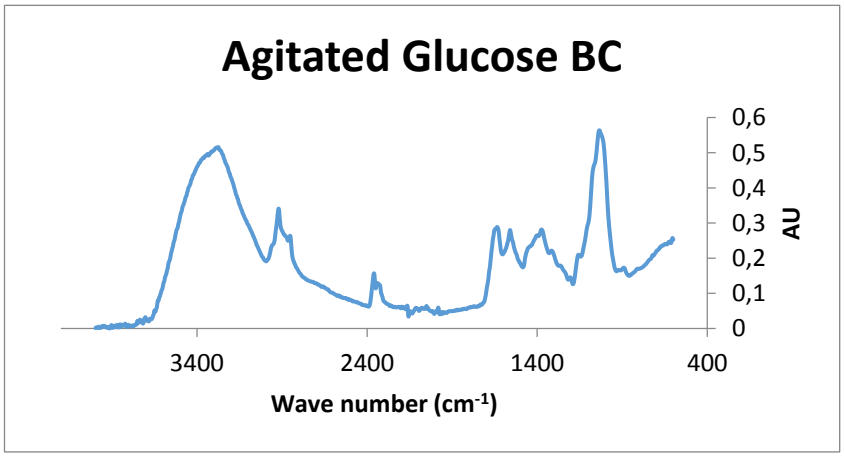

Figure 77. Agitated glucose BC spectrum

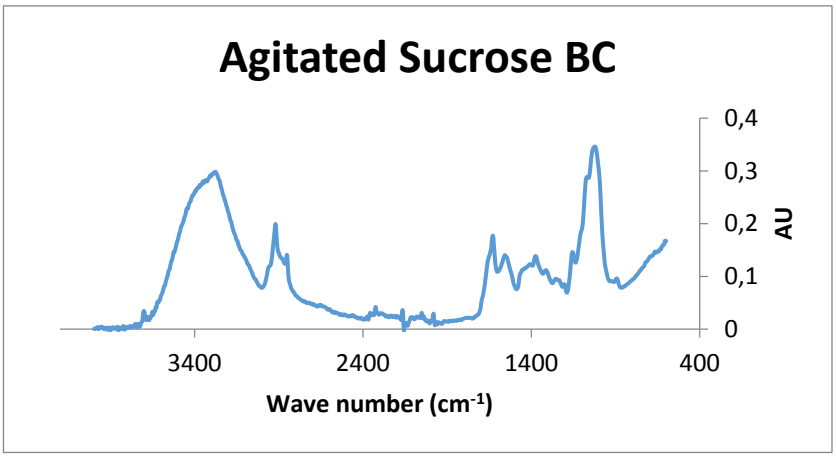

Figure 78. Agitated sucrose BC spectrum

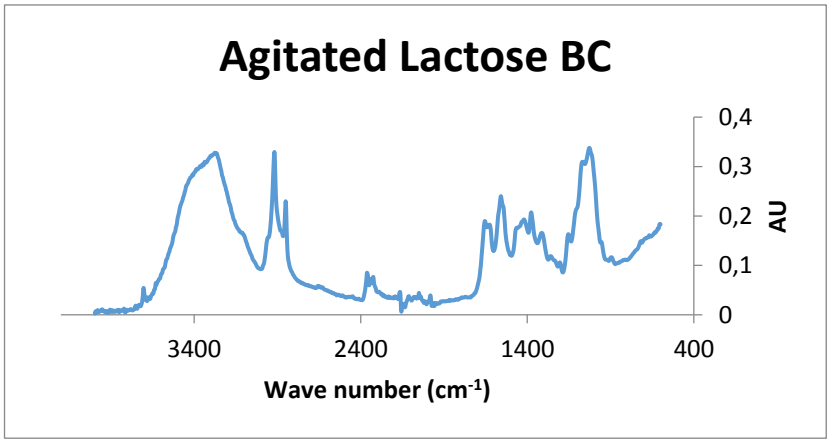

Figure 79. Agitated lactose BC spectrum

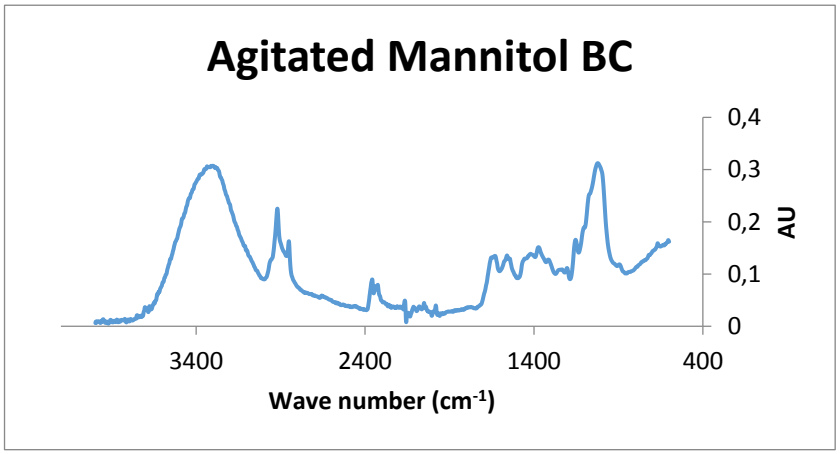

Figure 80. Agitated mannitol BC spectrum

6.4.1.2. Static cultures spectra

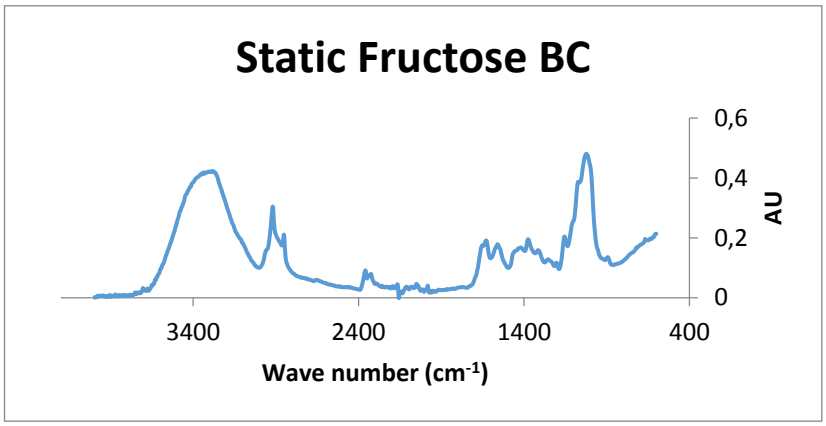

Figure 81. Static fructose BC spectrum

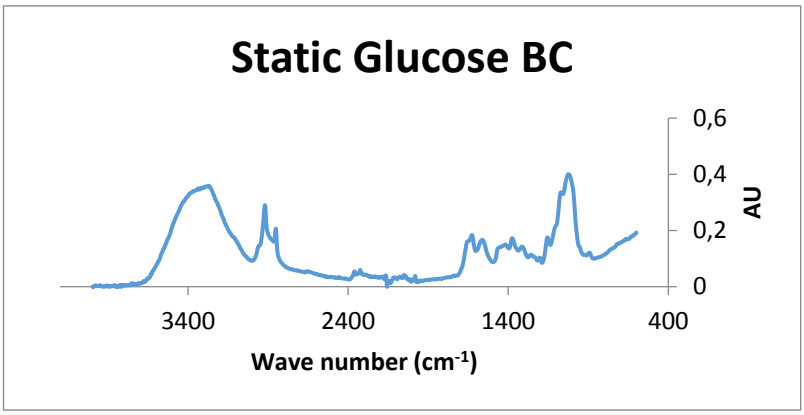

Figure 82. Static glucose BC spectrum

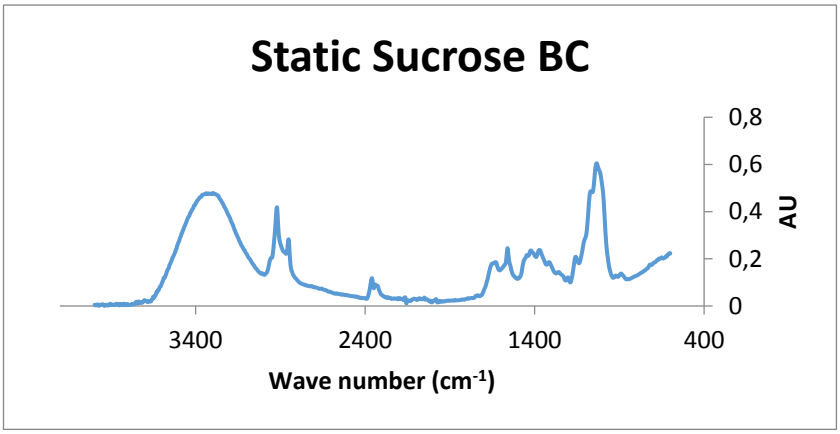

Figure 83. Static sucrose BC spectrum

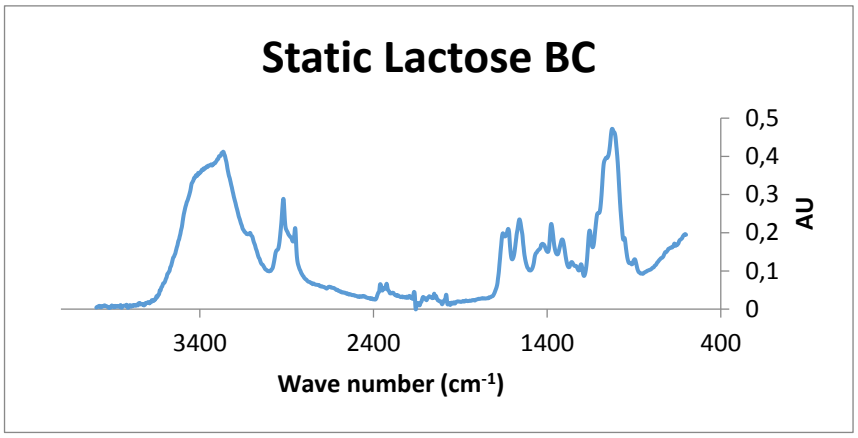

Figure 84. Static lactose BC spectrum

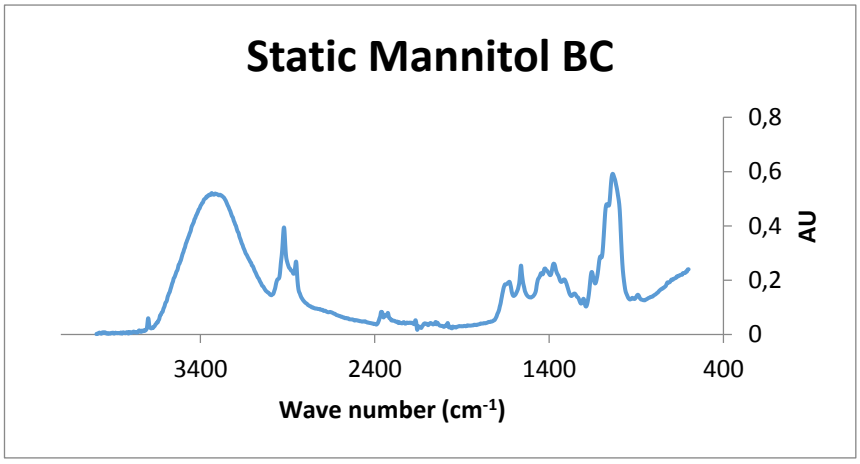

Figure 85. Static mannitol BC spectrum

All the analyzed spectra show, more or less, the same profile. This tells us that chemically the two methods produce the same substance (cellulose). As will be seen later, the most significant differences occur in the morphology of the collected materials.

6.4.2. Electron Microscopy

To characterize the internal structure of the bacterial cellulose the dried samples were sent to CCiT (Scientific and technological centers UB). The analysis was done using the conventional SEM technique (secondary and retro-dispersed electrons).

A scanning electron microscope (SEM) is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that contain information about the

sample's surface topography and composition. The electron beam is generally scanned in a raster scan pattern, and the beam's position is combined with the detected signal to produce an image. Below the photographs taken from our samples are shown.

6.4.2.1. Glucose

Agitated glucose

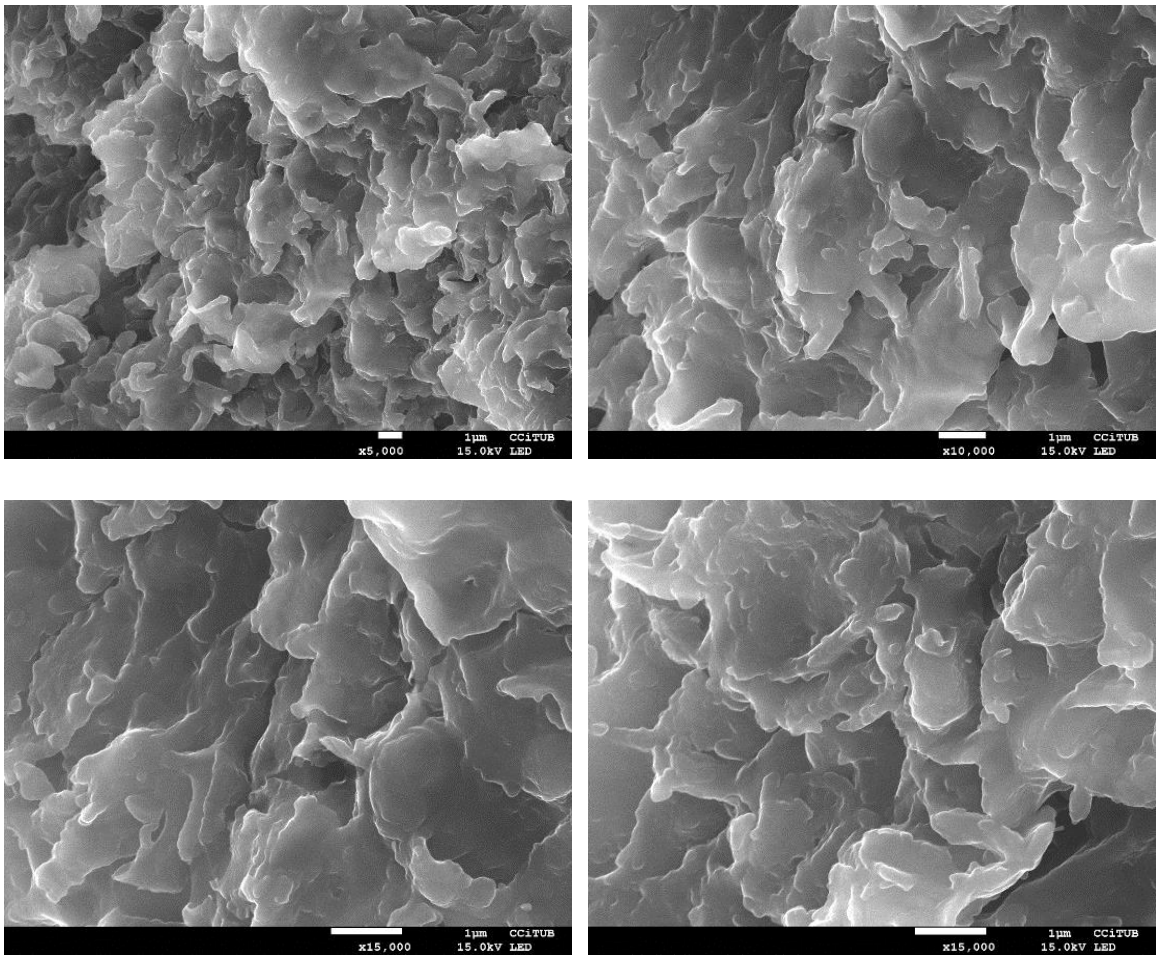

Figure 86. Agitated glucose BC SEM pictures

Static glucose

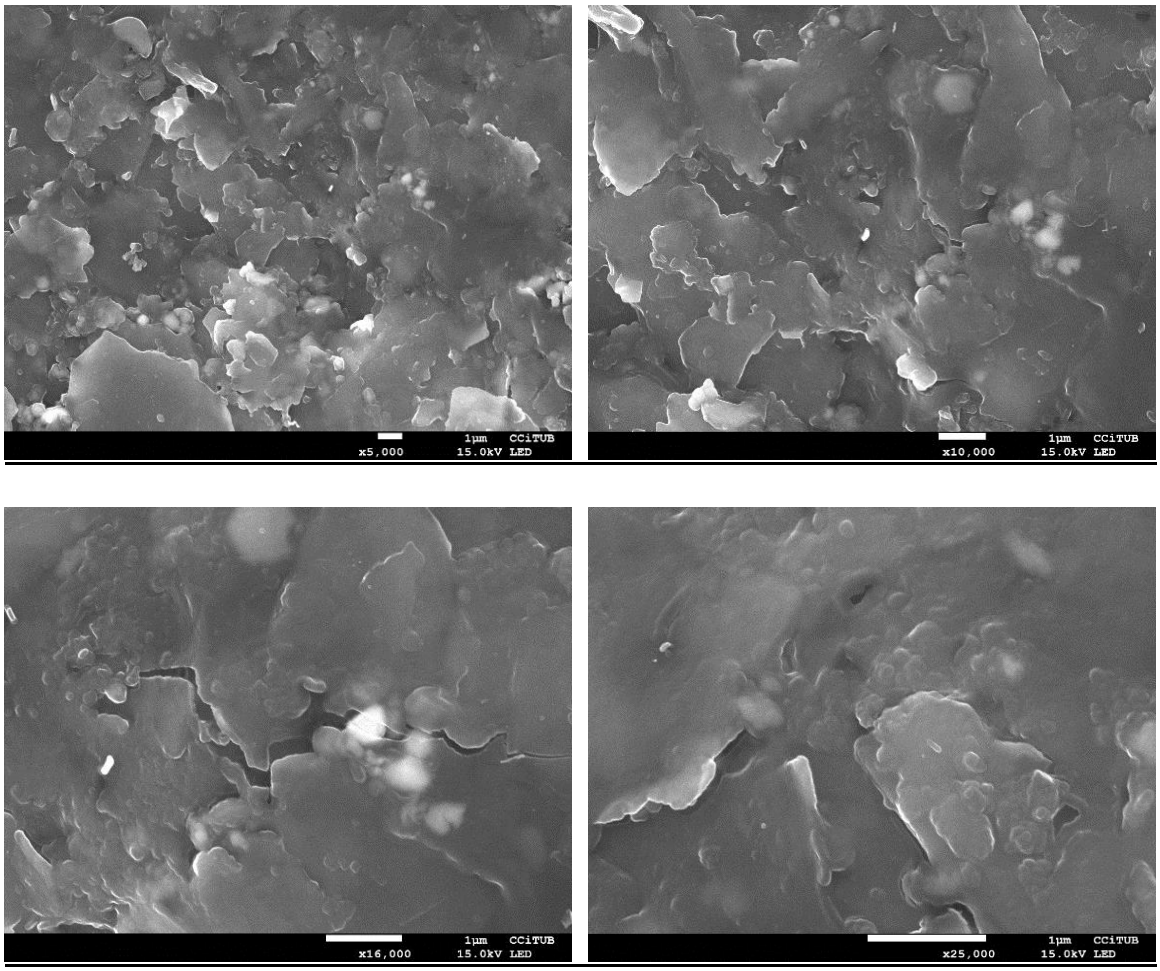

Figure 87. Static glucose BC SEM pictures

6.4.2.2. Fructose

Agitated fructose

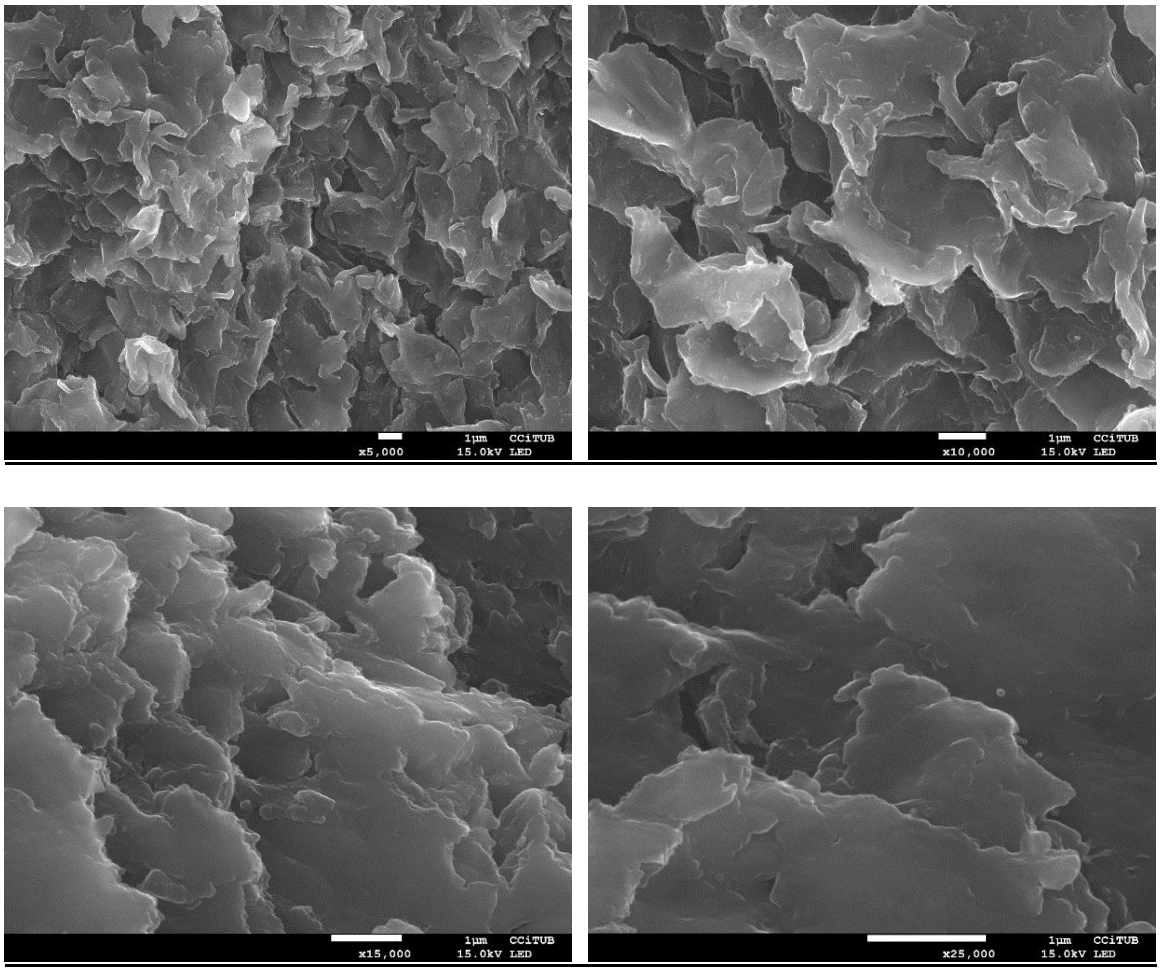

Figure 88. Agitated fructose BC SEM pictures

Static fructose

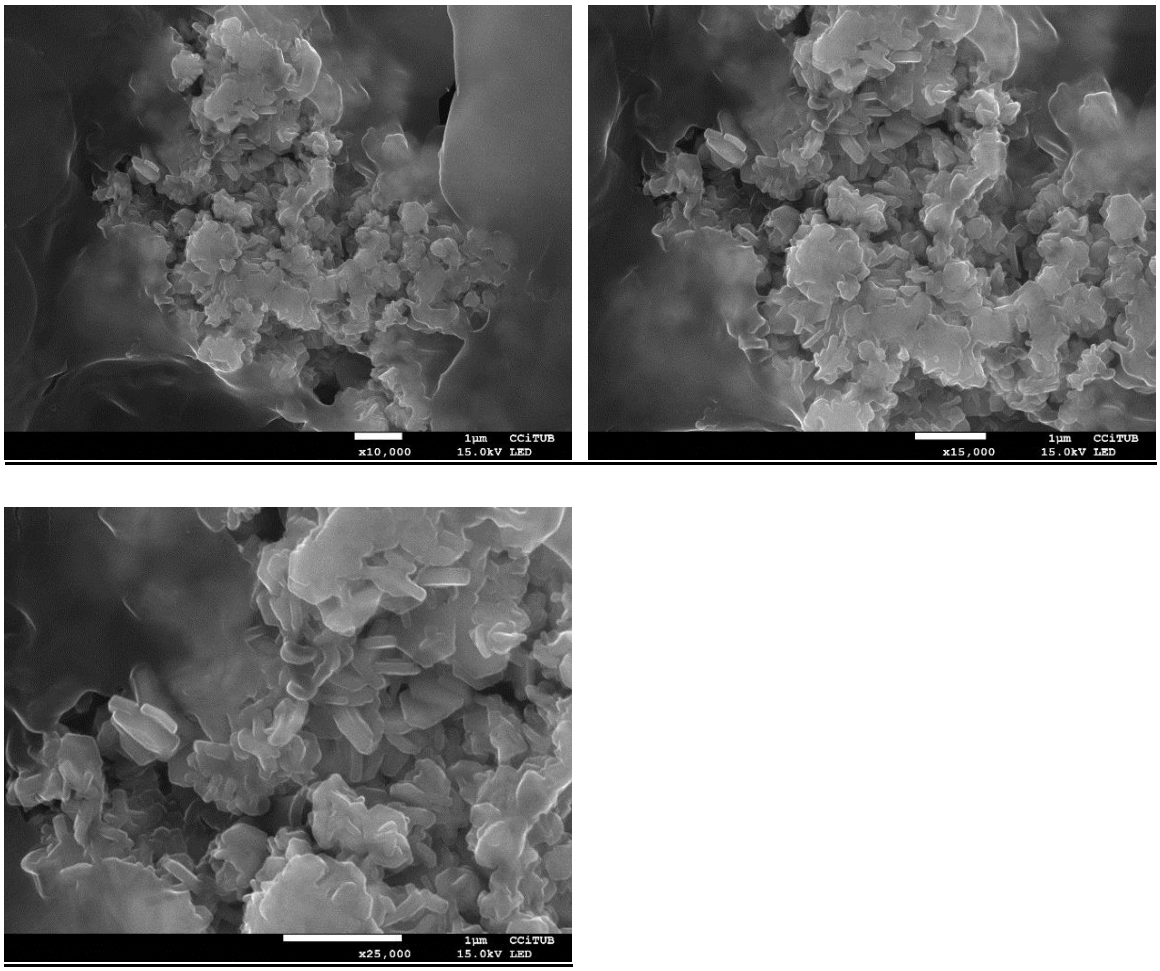

Figure 89. Static fructose BC SEM pictures

6.4.2.3. Sucrose

Agitated sucrose

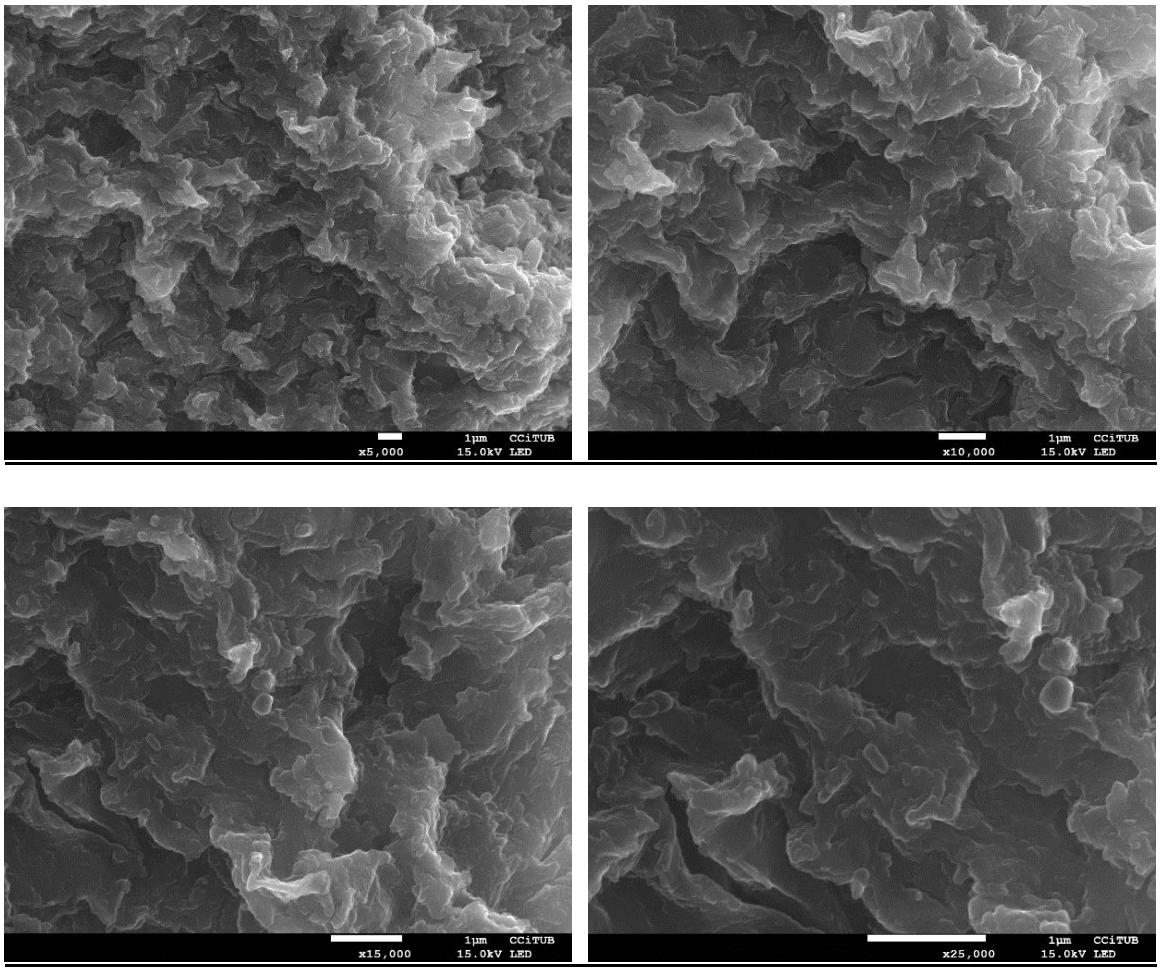

Figure 90. Agitated sucrose BC SEM pictures

Static sucrose

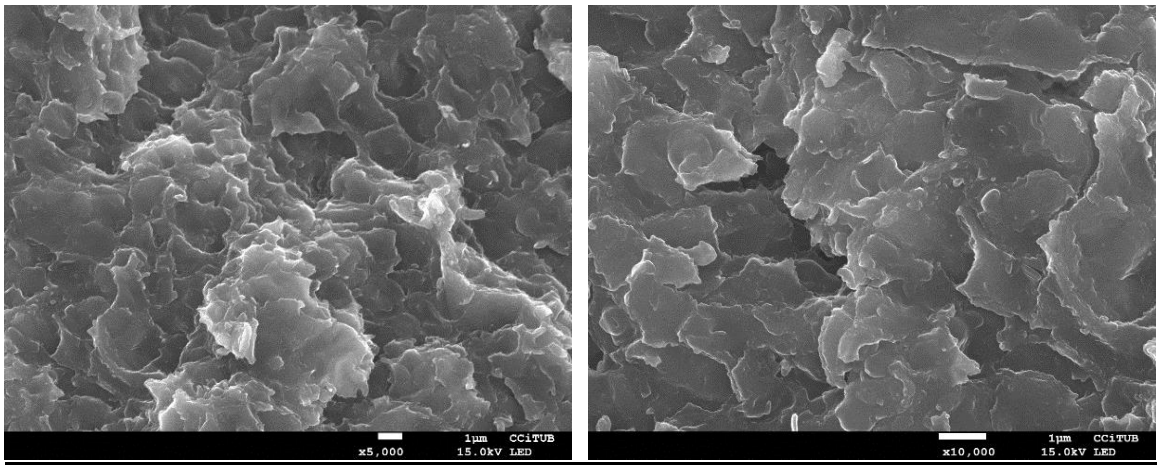

Figure 91. Static sucrose BC SEM pictures

6.4.2.4. Lactose

Agitated lactose

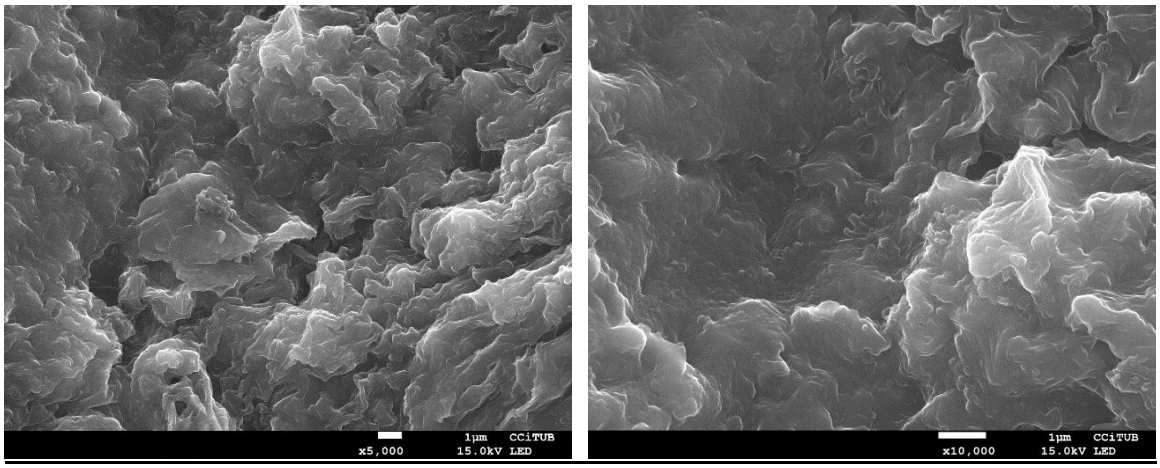

Figure 92. Agitated lactose BC SEM pictures

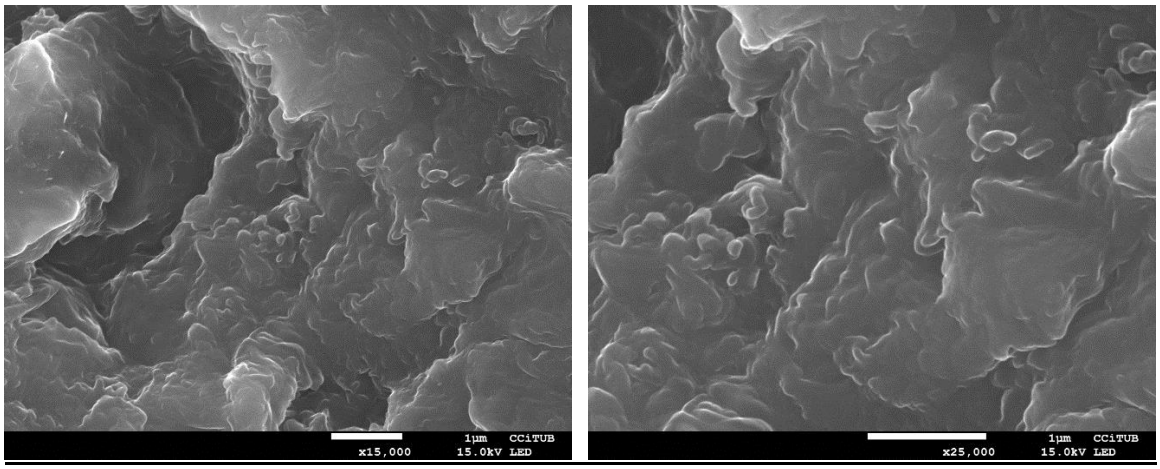

Figure 93. Agitated lactose BC SEM pictures

Static lactose

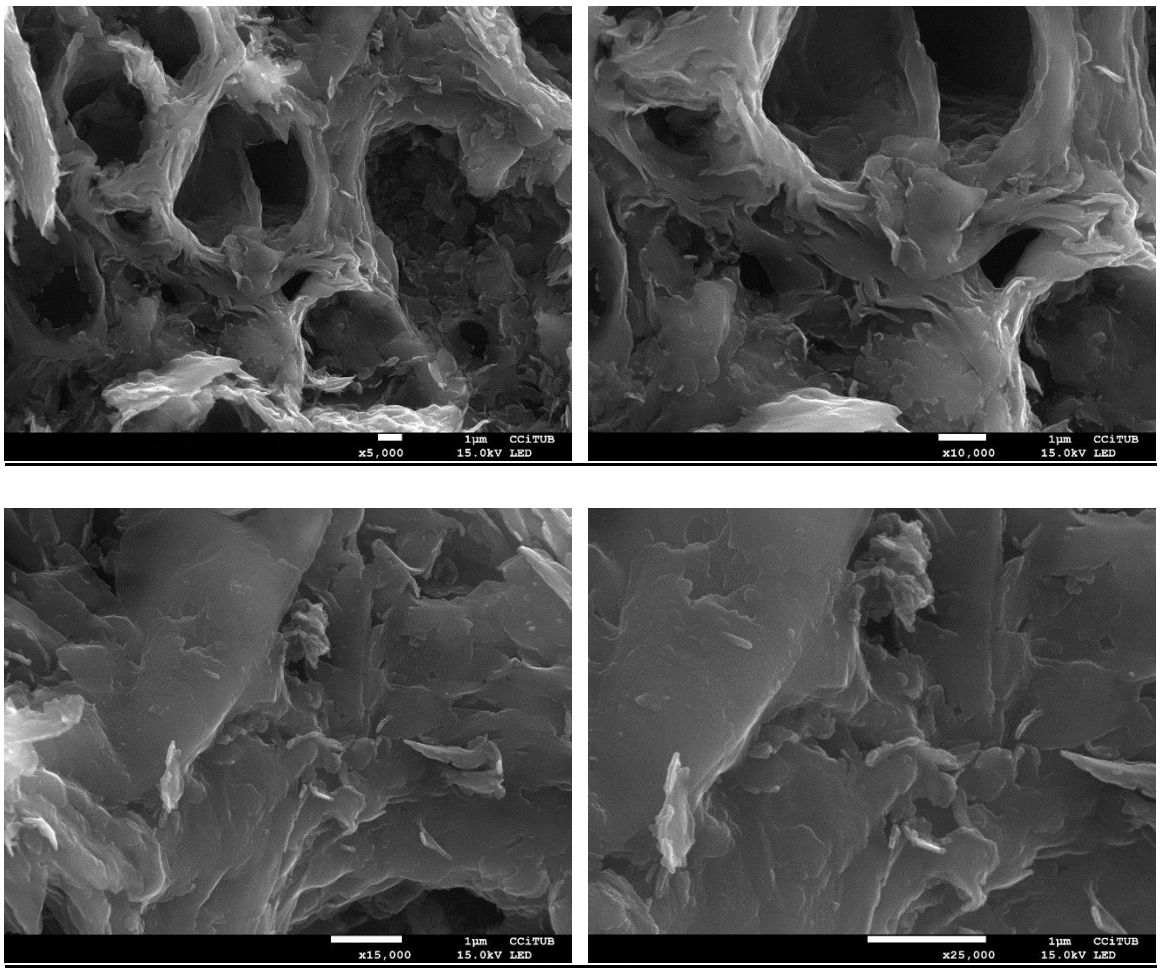

Figure 94. Static lactose BC SEM pictures

6.4.2.5. Mannitol

Agitated mannitol

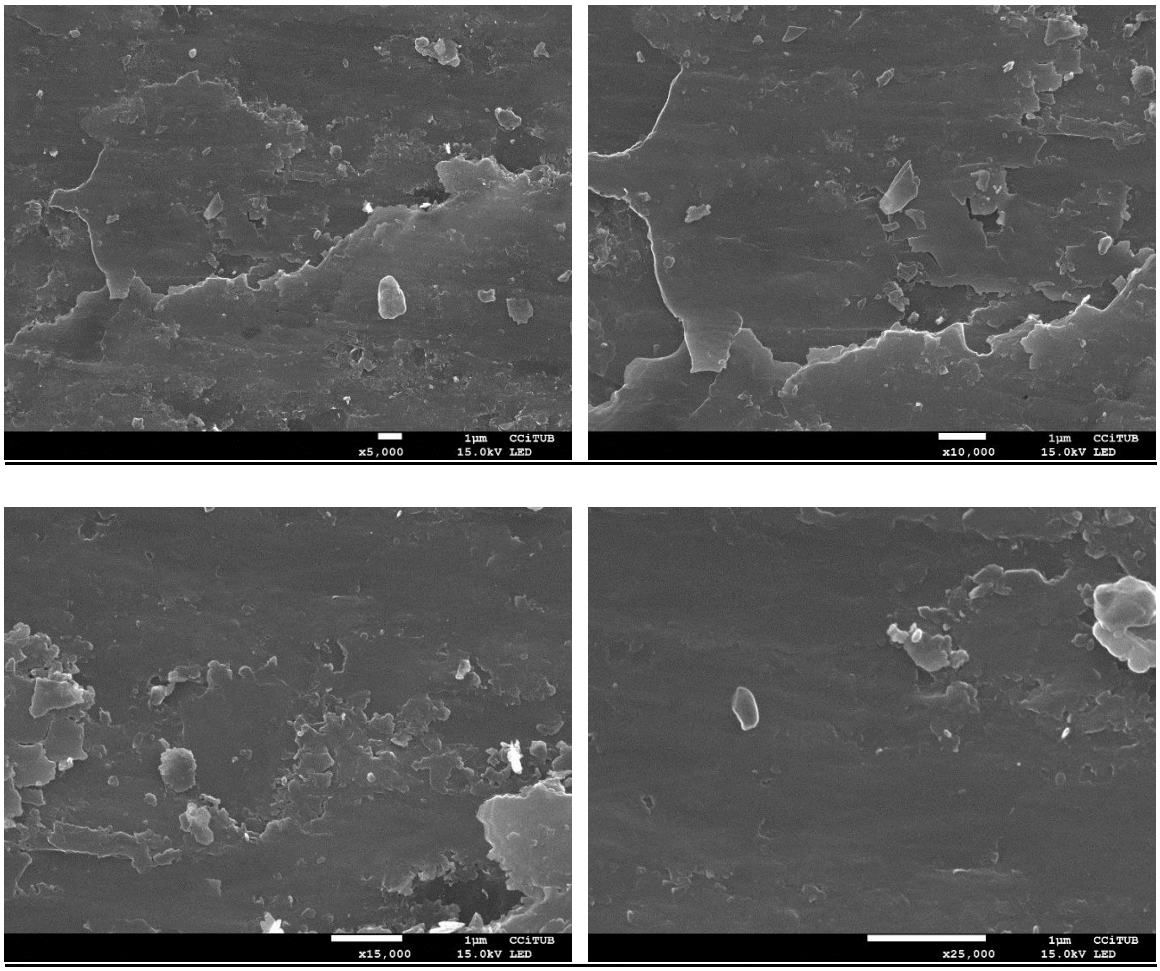

Figure 95. Agitated mannitol BC SEM pictures

Static mannitol

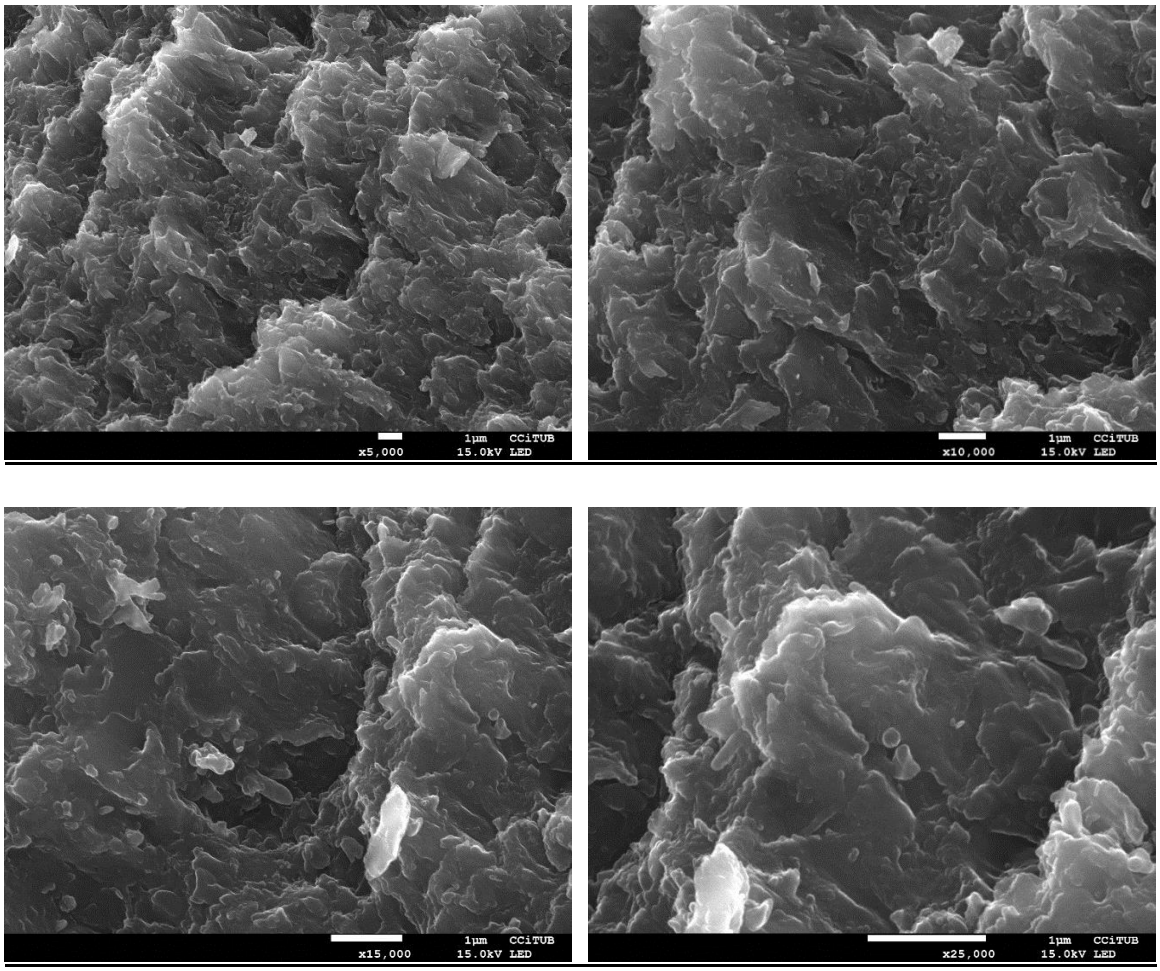

Figure 96. Static mannitol BC SEM pictures

As it can be observed in most cases, samples that are obtained from static processes are sharper than those obtained by agitated processes, indicating that the quality of nanoscopic cellulose is better in static cultures.

This is due to the agitation process that mechanically modifies the cellulose nanofibers that produce a softer, smoother and more agglomerated cellulose.

Effect has been explained by other researchers who have reported the same result (Carreño-Pineda, Caicedo, & Martínez, 2012).

7. Environment evaluation

Environment aspects are considered in the present project, as set in directives of this School to perform Bachelor's thesis and according to the concept of sustainability that should be regulate at any project.

Producing cellulose by this method is a sustainable natural alternative to raw materials derived from oil. It also avoids problems that vegetal cellulose obtaining methods may cause. The environmental impact of paper production is significant, having a number of adverse effects on the environment including deforestation, and air, water and land pollution.

Nowadays it is important to find these alternative methods to achieve a further step towards a sustainable chemical industry that respects the environment.

Bacterial cellulose can cover the demand for new applications which require more cellulose and has excellent mechanical properties that differentiate it from traditional cellulose with the advantage of being much more chemically pure avoiding extraction processes.

Moreover, the industry of composite materials, materials which have superior properties than the conventional ones, also requires more products made with renewable and sustainable processes.

Referring to the experimental part of the project, during all the study, it is taken care to depositing waste (remains of material, pipettes, gloves, etc) in habilitated deposits for this usage.

All residues obtained in the experimental part were non-toxic and biodegradable substances. The only used chemical product that has negative effect on the environment has been NaOH, which has been sent to required disposal plants to apply ideal treatment under appropriate conditions.

To further clarify the environmental impact, all the fermentation processes have resulted in an energy expenditure of 250 kWh (this data corresponds to the processes of shaking (120 kWh), heating (100 kWh) and the expenditure of other equipments in the laboratory (30 kWh)).

According to the current energy MIX, 1 kWh of Catalan electric energy involves an atmospheric emission of 0.302 kg CO_2/kWh. Therefore, carrying out these experiments has involved an emission of 75.5 kg of CO_2 to the atmosphere (which is more or less the equivalent of the amount of CO_2 emitted by a regular vehicle during a 400km displacement).

For vacuum filtration and other laboratory costs the following water cost is calculated:

- Vacuum filtration: it was carried out three times, 2h each time.

$$40\frac{l}{min} \times 60\frac{min}{h} \times 6h = 14400\ l$$

- Cleaning water expenditure $\approx 600\ l$
- TOTAL WATER EXPENDTURE $\approx 15\ m^3$

8. Economic evaluation

It is performed an economic evaluation of the total project cost. It is considered all the costs in terms of material expenses, instrumentation costs and time dedicated of human resources. In material costs, quantity of the product that has been used is considered. The following table shows quantity, material price and total price of expenditure on material:

Product	***Quantity (g)***	***Price per unit (€/g)***	***Total price (€)***
BPR2001	*-*	*-*	*80*
Mannitol	*35*	*0.06*	*2.10*
Glucose	*20*	*0.09*	*1.80*
Sucrose	*20*	*0.09*	*1.80*
Lactose	*20*	*0.05*	*1.00*
Fructose	*20*	*0.04*	*0.80*
Yeast extract	*51.5*	*0.22*	*11.33*
NaOH	*100*	*0.03*	*3.00*
Peptone	*0.9*	*0.19*	*0.17*
$(NH_4)_2SO_4$	*8.25*	*0.05*	*0.41*
KH_2PO_4	*2.5*	*0.05*	*0.13*
Citric Acid	*3.75*	0.04	*0.15*
Acetic Acid	*1.25*	0.03	*0.04*
$MgSO_4 \cdot 7H_2O$	*0.31*	0.05	*0.02*
		Total cost (€)	*102.75*

Table 6. Material costs

Regarding expenses of instrumentation, it is counted the instrumental amortization, the maintenance, required consumed material and power consumption. Moreover, it is counted 250€ approximately in terms of laboratory equipment such as spatulas, syringes, pipettes, globes, ph paper, water, etc.

Tests	***Price per hour(€/h)***	***Total hours***	***Total price (€)***
Centrifuge	*10*	*5.5*	*55*
Stove	*2*	*350*	*700*
Fridge	*2*	*150*	*300*
Agitator	*5*	*190*	*950*
		Total cost (€)	*2005*

Table 7. Power consumption

It is also necessary to consider the economic expenditure associated to the characterization tests carried out in external sites. Their prices are shown below:

Test	***Total price (€)***
Electron Microscopy	*250*
Infrared Spectroscopy	*600*
Total cost (€)	*850*

Table 8. Characterization tests prices

Finally, it is accounted staff costs. In the present project, it is counted one director and one technical researcher as research scholar with their dedication time:

Human resources	***Price per hour (€/h)***	***Dedication (h)***	***Total price (€)***
Technical researcher	*15*	*600*	*9000*
Director	*35*	*120*	*4200*
		Total cost (€)	*13200*

Table 9. Staff costs

Adding all the costs of the above tables and 250 € of laboratory equipment, the total project cost is approximately 16407.75 €.

9. Conclusions

In this project the fermentation of the bacterium *Gluconoacetobacter xylinum sucrofermentans* has been carried out in different conditions and in all the cases variable yields of bacterial cellulose have been obtained. This product has been purified and characterized by IR spectroscopy and by SEM. The most relevant findings are the following.

The bacterium *Gluconoacetobacter xylinum sucrofermentans* has been cultivated with different sugar sources such as glucose, fructose, sucrose, lactose and mannitol. The first two substrates have been the ones that have provided more cellulose.

The bacterium *G xylinum* has been fermented in agitated and static mode. Clearly the agitated mode has provided higher yields than the static one, an observation confirmed by other published works on this process. The scientific community's explanation for this fact is that oxygen is more easily transported to bacteria in agitated systems.

In the agitated mode the formation of pellets or amorphous cellulose clusters, similar to those described in other scientific articles, is observed after 35 h. In the static fermentations the formation of a cellulose layer has been observed after 5 days. Both morphologies are due to the insolubility of cellulose in water.

Static mode fermentations provide better quality microfibers than the agitated mode, as can be seen from SEM photographs. This is because agitation damages microfibers as they form.

The purification of the obtained BC could be improved if the treatment process with aqueous NaOH is carried out during more time and intensity than those described in the references.

The most productive fermentations in stirred mode provided approximately 0.5 g of cellulose per liter. In the static mode, the final concentration decreased to 0.2 g/L, in a double time of fermentation (14 days).

The production profiles in the agitated medium according to the substrate coincide with those described by the experts in this subject.

The best cellulose microfibers were obtained in the static lactose medium. However, it is considered that, with better treatment of the samples, all static fermentations would give this result.

The cost of the project is evaluated at about 16,000 € and has an impact on the planet of about 75 kg of CO2 and a total water waste of 15 m^3.

10. Acknowledgements

In first place, I would like to thank my director Jordi Bou Serra for giving me the chance to perform my bachelor's thesis with him and especially for the patience, support and great help that he have provided to me during these months. I also want to thank professor Xavier Ramis for letting me use the IR spectrometer to characterize BC samples.

At personal level, I would like to thank everyone who has supported me and enjoyed this beautiful stage of my life with me: my family members, my friends and my university partners, thank you all for your support and patience, and especially for believing in me even the times that I have not done it.

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