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Samara Silva de Souza

BIONANOCELLULOSE: NEW METHODS FOR BIOFILM PRODUCTION AND HIGH-QUALITY BACTERIAL RNA EXTRACTION

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BIONANOCELLULOSE: NEW METHODS FOR BIOFILM PRODUCTION AND HIGH-QUALITY BACTERIAL RNA EXTRACTION

Doctorate thesis for the degree of Doctor in Chemical Engineering presented to the Postgraduate Program in Chemical Engineering at the Federal University of Santa Catarina

Advisor: Prof. Dr. Luismar Marques Porto

Co-advisors: Dr[a]. Fernanda Vieira Berti
Dr[a]. Julia de Vasconcellos Castro

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This thesis was considered adequate for the title of Doctor and was approved in its final form by the Postgraduate Program in Chemical Engineering at the Federal University of Santa Catarina.

Florianopolis, November 22nd of 2017.

____
Prof. Cíntia Soares, Dr.
Coordinator of the Graduate Program in Chemical Engineering

____
Prof. Luismar Marques Porto, Dr.
Advisor, Federal University of Santa Catarina – UFSC

____
Fernanda Vieira Berti, Dra.
Co-Advisor, Federal University of Santa Catarina - UFSC

____
Julia de Vasconcellos Castro, Dra.
Co-Advisor, Federal University of Santa Catarina - UFSC

Examination Board:

____
Prof. Patrícia Poletto, Dra.
Federal University of Santa Catarina - UFSC

____
Daliana Muller, Dra.
Federal University of Santa Catarina – UFSC

____
Prof. Everton Skoronski, Dr.
Santa Catarina State University - UDESC

“It always seems impossible until it is done.”

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It all started on February 28th, 2012 when I decided to commit myself to a Master degree in Chemical Engineering at Federal University of Santa Catarina (UFSC). During the first year, I discovered a completely new world full of ideas. I still remember my first lecture about Genomics and Bioinformatics, the first concepts and subjects that I had never seen before. Despite the long road that a master degree requires, I decided to face the doctorate right after it, and on 15th of April 2014, I was again committed to a post graduate program.

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ABSTRACT

Bionanocellulose, also known as bacterial nanocellulose (BNC), has become an important biomaterial for industrial, technological and biomedical applications. *Komagataeibacter hansenii* ATCC 23769 produces a microstructured BNC biofilm arranged in a 3D network of nanofibrils. Despite its biotechnological relevance, and the fact that the advances of the omics techniques and next-generation technologies have opened a new age in the study of biological systems, this organism has not been investigated at the molecular level and neither the transcriptome assembly nor the genome-scale metabolic model of *K. hansenii* have been elucidated. In this study, we address specifically the bottlenecks of current state of i) transcriptome and ii) metabolic applications in bionanocellulose biofilm production studies. The main challenges associated with those gaps are: i) the difficulty to obtain high-quality RNA from bacteria cells in nanocellulose biofilms due its large contents of polysaccharides and fibers and ii) the need to obtain precise metabolic measures which is a problem when complex culture media is used in the process. First, we developed an optimized extraction method to obtain high-quality RNA from *K. hansenii* cells living/producing BNC biofilms. The method is based on different cell disruption techniques in combination with RNA extraction reagents. For successful isolation of intact RNA, an efficient DNase treatment was performed to remove genomic DNA and guarantee pure RNA samples. The method was evaluated by the quality, quantity and the integrity of RNA samples and it is the first to allow the isolation of highly concentrated and intact RNA from *K. hansenii* cells in BNC biofilms and planktonic states. The developed RNA extraction method that consistently yields high-quality RNA samples can now be used to perform RNA-Sequencing studies allowing gene expression analysis and consequently improving the understanding of BNC synthesis. The second problem we tackle within the current study was the development of a defined minimal culture medium (DMCM) for BNC production. The most common culture medium used to produce BNC is a complex medium that contains yeast extract and peptone thus has undefined chemical composition. The designed DMCM medium is a composed of seven components, disodium phosphate, monopotassium phosphate, sodium chloride, magnesium sulfate, calcium chloride, ammonium chloride as nitrogen source, and glucose as carbon source. Different carbon and nitrogen sources were tested, varying their concentrations and results revealed 25 mM of glucose and 10 mM of NH_4Cl as best

fitted concentrations and carbon and nitrogen sources. Moreover, the developed DMCM culture medium can now be utilized to obtain experimental data to support future *in silico* metabolic investigations for the better understanding of bionanocellulose production by identifying the metabolic capacities and the behavior of the bacterium in terms of growth, nutrient demand and generation of extracellular metabolites. Additionally, the membranes synthesized in DMCM showed a previously unknown transparency, which has not been reported yet without the addition of other substances. The characterization of BNC–Minimal membranes revealed important improvements in some properties such as higher water holding capacity, highly porous surface and better elasticity than the usual membranes. Thus, the defined minimal culture medium proposed here can be exploited to synthesize novel transparent BNC membranes with unique properties of considerable interest to several applications in the biomedical and industrial fields.

Keywords: Bacterial nanocellulose; Bionanocellulose, *Komagataeibacter hansenii*; RNA isolation; RNA-Seq Biofilm; Planktonic; Defined minimal culture medium; Transparent.

RESUMO EXPANDIDO

A bionanocelulose, também conhecida como nanocelulose bacteriana (BNC), tornou-se um importante biomaterial para aplicações industriais, tecnológicas e biomédicas. *Komagataeibacter hansenii* ATCC 23769 produz um biofilme de BNC microestruturado e organizado em nanofibras. Apesar de sua relevância biotecnológica, e o fato de que os avanços das técnicas de ômicas e tecnologias de próxima geração abriram uma nova era no estudo dos sistemas biológicos, este organismo não foi investigado à nível molecular e nem a montagem do transcriptoma nem o modelo metabólico em escala genômica de *K. hansenii* foi esclarecido. Neste estudo, abordamos especificamente os gargalos do estado atual do i) transcriptoma e ii) aplicações metabólicas em estudos de produção de biofilme de nanocelulose. Os principais desafios associados a esses gaps são: i) a dificuldade de obter RNA de alta qualidade de células de bactérias em biofilmes de nanocelulose devido a grande quantidade de polissacarídeos e fibras e ii) a necessidade de obter medidas metabólicas precisas, o que é um problema quando um meio de cultura complexo é usado no processo. Primeiro, desenvolvemos um método de extração otimizado para obter RNA de alta qualidade de células de *K. hansenii* que vivem / produzem biofilmes de BNC. O método é baseado em diferentes técnicas de ruptura celular em combinação com reagentes de extração de RNA. Para o isolamento bem-sucedido de RNA intacto, foi realizado um tratamento de DNase eficiente para remover DNA genômico e garantir amostras de RNA puro. O método foi avaliado pela qualidade, quantidade e integridade das amostras de RNA e é o primeiro a permitir o isolamento de RNA altamente concentrado e intacto de células de *K. hansenii* em biofilmes e em estado planctônico. O método de extração de RNA desenvolvido que produz consistentemente amostras de RNA de alta qualidade agora pode ser usado para sequenciamento permitindo a análise da expressão gênica e consequentemente melhorando a compreensão da síntese de BNC. O segundo problema que abordamos no presente estudo foi o desenvolvimento de um meio de cultura mínimo definido (DMCM) para a produção de BNC. O meio de cultura mais comum usado para produzir BNC é um meio complexo que contém extrato de levedura e peptona, portanto, possui composição química indefinida. O meio DMCM desenvolvido é composto por sete componentes, fosfato dissódico, fosfato monopotássico, cloreto de sódio, sulfato de magnésio, cloreto de cálcio, cloreto de amônio como

fonte de nitrogênio e glicose como fonte de carbono. Diferentes fontes de carbono e nitrogênio foram testadas, variando suas concentrações e os resultados revelaram 25 mM de glicose e 10 mM de NH_4Cl como as melhores concentrações e fontes de carbono e nitrogênio. Além disso, o meio de cultura DMCM desenvolvido agora pode ser utilizado para obter dados experimentais para apoiar futuras pesquisas metabólicas *in silico* para uma melhor compreensão da produção de bionanocelulose, identificando as capacidades metabólicas e o comportamento da bactéria em termos de crescimento, demanda de nutrientes e geração de metabolitos extracelulares. Além disso, as membranas sintetizadas em DMCM mostraram uma transparência previamente desconhecida, que não foi relatada sem a adição de outras substâncias. A caracterização das membranas BNC–Mínimo revelou melhorias importantes em algumas propriedades, como maior capacidade de retenção de água, superfície altamente porosa e melhor elasticidade do que as membranas usuais. Assim, o meio de cultura definido proposto aqui pode ser explorado para sintetizar membranas de bionanocelulose transparentes com propriedades únicas de interesse considerável para várias aplicações nos campos biomédico e industrial. Em resumo, este estudo desenvolveu novos métodos para a extração de RNA bacteriano de alta qualidade e para a produção de biofilmes que permitirão o uso em análises transcriptômicas e metabolômicas para a compreensão dos mecanismos moleculares envolvidos na biossíntese BNC. O método de extração de RNA otimizado foi desenvolvido para produzir amostras de RNA com quantidade, pureza e integridade das células de *K. hansenii* que vivem / produzem biofilmes de BNC. A partir de amostras de RNA de alta qualidade de *K. hansenii* em biofilmes e em estado planctônico, a montagem do transcriptoma poderá ser realizada para identificar genes alvo que têm um alto impacto na síntese de BNC. Em relação ao meio de cultura mínimo definido desenvolvido, esse estudo comprovou a capacidade de *K. hansenii* em sintetizar BNC com limitação de nutrientes. DMCM pode fornecer análises metabólicas precisas e maior reprodutibilidade experimental em comparação com meios complexos.

Palavras Chave: Nanocelulose bacteriana; Bionanocelulose, *Komagataeibacter hansenii*; Isolamento RNA; RNA-Seq; Biofilme; Planctônico; Meio de cultura mínimo definido; Transparente.

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LIST OF ABREVIATIONS

BNC	Bacterial nanocellulose
BNC−Mannitol	Bacterial nanocellulose synthesized in mannitol medium
BNC−Minimal	Bacterial nanocellulose synthesized in defined minimal culture medium
c-di-GMP	cyclic diguanylate monophosphate
C/N	Carbon and nitrogen ratio
DMCM	Defined Minimal Culture Medium
DNA	Deoxynucleic acid
De novo transcriptome	Assembly method of creating a transcriptome without the aid of a reference genome.
HS	Hestrin-Schramm
KEGG	Kyoto Encyclopedia of Genes and Genomes
mRNA	Messenger RNA
M1	RNA sample 1 from BNC biofilms
M2	RNA sample 2 from BNC biofilms
M3	RNA sample 3 from BNC biofilms
M4	RNA sample 4 from BNC biofilms
MA	Pool of the samples M1 and M2 for RNA-Seq
MB	Pool of the samples M3 and M4 for RNA-Seq
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
PE Sequencing	Paired-end Sequencing. Sequence both ends of the same fragment.
PCR	Polymerase Chain Reaction
P1	RNA sample 1 from planktonic
P2	RNA sample 2 from planktonic
P3	RNA sample 3 from planktonic
P4	RNA sample 4 from planktonic
PA	Pool of the samples P1 and P2 for RNA-Seq

PB	Pool of the samples P3 and P4 for RNA-Seq
qPCR	Quantitative real time polymerase chain reaction
Reads	Short (50-200 nucleotide) sequences produced from NGS
RNA	Ribonucleic acid
RNA-Seq	RNA-Sequencing
RPM	Rounds per minute
TBE	Tris-borate buffer
Transcripts	Single-stranded ribonucleic acid (RNA) synthesized by transcription of DNA

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1. WHAT IS THIS THESIS ABOUT?

Bacterial nanocellulose or bionanocellulose (BNC) synthesis has been extensively investigated and the efforts to understand the mechanisms and production of BNC are mainly motivated by the distinguished properties of bacterial nanocellulose-based biomaterials, particularly those that find tissue engineering applications. BNC biofilm production occurs during growth of *Komagataeibacter hansenii* ATCC 23769 in liquid medium under static conditions. The biofilm is formed at the air/liquid interface, from the transition of planktonic cells (free in suspension) to sessile cells (attached to the biofilm). Bacteria present in the biofilms have different characteristics from those growing in suspension, presenting changes in physiology and gene expression (CASTRO; FRANÇA; BRADWELL; et al., 2017; DÖTSCH; ECKWEILER; SCHNIEDERJANS; et al., 2012; DUMITRACHE; KLINGEMAN; NATZKE; et al., 2017; RUMBO-FEAL; GÓMEZ; GAYOSO; et al., 2013). However, the complex of regulatory mechanisms involved in nanocellulose biosynthesis in bacterium such as *K. hansenii*, needs intensive molecular research and a better understanding of the process can be elucidated through advanced omics studies.

With the advances in next-generation sequencing several techniques are routinely being applied in different fields of biology, medicine and engineering. These techniques allow researchers to address important biological questions. It is relevant to highlight that the development of the field of systems biology aims to understand complex biological systems in an integrated network that can make extensive use of omics techniques such as genomics, proteomics, metabolomics, and transcriptomics in combination with computational tools. Through analysis of data gathered from omics techniques we can establish new directions for the elucidation of BNC biosynthesis, its regulation and its physiological roles. In this study, we address specifically the bottlenecks of current state of i) transcriptome and ii) metabolic applications in bionanocellulose biofilm production studies. The main challenges associated with those gaps are: i) the difficulty to obtain high-quality RNA from bacteria cells in nanocellulose biofilms due its large contents of polysaccharides and fibers and ii) the need to obtain precise metabolic measures which is a problem when complex culture media is used in the process.

The transcriptome approach, defined as the complete set of transcripts in a cell and their quantities, in a specific physiological condition (SOREK; COSSART, 2010; SULTAN; SCHULZ; RICHARD; et al., 2008) generates large expression datasets especially suitable for non-model species, such as *K. hansenii.* This approach has a great potential of improvement in our studies, since the transcriptomic comparisons between biofilm and planktonic cultures can provide a comprehensive understanding of gene expression and regulatory mechanisms (CASTRO, JOANA; FRANÇA; BRADWELL; et al., 2017; CREECY; CONWAY, 2015; SOREK; COSSART, 2010; STANTON; EDGER; PUZEY; et al., 2017).

One important detail for the success of transcriptomic analysis is the quantity, purity, and integrity of the RNA. In other words, RNA quality is influenced by the sample's nature and by the method used for RNA extraction. BNC is a polysaccharide and the presence of polysaccharides can interfere during RNA extraction making difficult for bacterial cell lysis and nucleic acid purification. Therefore, an RNA isolation and purification method to obtain a high-quality RNA is a crucial step in gene expression profiling. Some transcriptomes studies have focused on the physiology of bacterial cells in biofilms and planktonic state (CASTRO; FRANÇA; BRADWELL; et al., 2017; DUMITRACHE; KLINGEMAN; NATZKE; et al., 2017). However, the difficulty in obtaining high-quality RNA samples from bacterial cells living/producing nanocellulose biofilms can be a major problem and it has been a barrier to the transcriptome analysis of this organism. We believe that the main reason for the existence of this gap is that, to the best of our knowledge, there is no evidence of an effective method to obtain high-quality RNA of *K. hansenii* in BNC biofilms.

Metabolic studies also have a promising ability to describe cellular phenotypes accurately as they relate to the annotated genome sequence to the physiological functions of the cell. In our previous works, we presented the first core metabolic model developed for a better understanding of *K. hansenii* metabolic pathways, which is a valuable tool for fundamental research, serving as a starting point for metabolic engineering approaches (DE SOUZA; PORTO, 2014; SOUZA, 2014). However, in order to utilize the model with metabolic engineering tools such as flux balance analysis there is a need of data produced with a defined minimal culture medium studies, where "defined minimal" means a controlled amount of carbon and nitrogen with the presence of minimum nutrients.

The most common culture medium used to produce BNC is a complex medium that contains yeast extract and peptone as components which increase the BNC production cost. This complex chemical composition does not allow the establishment of the exact relationship between the amount of carbon and nitrogen present in the medium with the BNC yield. Moreover, the complex medium diminishes reproducibility since it is an undefined composition. To date, many studies have been focused on altering growth conditions to maximize the BNC yield and diminishing the cost of production. However, the majority of the studies supply the bacteria culture medium with complex medium. On the other hand, a defined culture medium has the exact composition and the advantage of being composed of low-cost components, which is desired for BNC production. In this sense, minimal nutritional requirements of an organism summarize its biosynthetic capabilities and contribute to a better control of the performance of microbial cultures under different conditions. A defined medium is essential for designing reproducible biochemical, physiological and genetic studies of microorganisms (CHERVAUX; EHRLICH; MAGUIN, 2000; FAN; ZHANG; ZOU; et al., 2014; SCHNEEBELI; EGLI, 2013). Up until now, a defined minimal medium with strict limitation of nutrients that supports BNC synthesis by *K. hansenii* has not been described in the literature. Thus, the development of a defined minimal culture medium that can support *K. hansenii* growth and BNC synthesis is an important obstacle that needs to be overcome in order to support future metabolic investigations for the better understanding of bionanocellulose production.

There are several contributions to exploit the potential applicability and production of BNC. This research is part of a strategic line of Genomic Engineering Group at the Integrated Technologies Laboratory - InteLab, which aims to contribute to advances in Tissue Engineering, Bioinformatics, Metabolic Engineering, Molecular Biology and Regenerative Medicine. This study is placed in the activity "Systems biology of *Komagataeibacter hansenii*", proposed in the project CELSYS (Genomic Engineering of Cellulose Nanofibers), approved by our group in the announcement MCTI / CTBIOTEC / CNPq N°. 28/2013 - ENGENHARIA DE SISTEMAS BIOLÓGICOS.

1.1 AIMS OF THE STUDY

This thesis aims to provide contributions to a better understanding of bacterial nanocellulose biosynthesis. We try to answer some gaps found in the literature. In particular, we aimed at providing answers to the following questions:

1. How to obtain a high-quality RNA from bacterial cells living/producing BNC biofilms?
2. Will the developed extraction method be suitable to obtain RNA samples from biofilm and planktonic states with enough quantity and quality for transcriptome sequencing?
3. Can the bacteria under minimum nutrient requirements produce bacterial nanocellulose?
4. Which combination of carbon and nitrogen sources in minimal medium allows BNC synthesis?
5. Will the modification of the medium change the BNC properties?

All questions raised are relevant as a guide to understanding bacterial nanocellulose synthesis and were not elucidated in the literature so far. Thus, to achieve the aim of this thesis, the following objectives were proposed:

- To evaluate and compare different techniques and methods for RNA extraction of *K. hansenii* cells in BNC biofilms to guarantee high-quality RNA samples.
- To obtain high concentration and integrity of RNA from *K. hansenii* cells in biofilms and planktonic states suitable for RNA-based analysis.
- To develop a defined minimal culture medium with specific carbon and nitrogen sources that enables *K. hansenii* to synthesize bacterial nanocellulose membranes;
- To characterize the membranes produced under defined minimal culture medium (BNC−Minimal) and compare it with the matrix (BNC−Mannitol) synthesized in complex medium.

1.2 STRUCTURE OF THE THESIS

This thesis is organized as follows:

In Chapter 2 a review regarding each issue investigated is presented. The literature review is focused on highlighting the importance of bacterial nanocellulose (BNC), different aspects of BNC production and its applications. In this chapter, it is also presented the advances of omics sciences and the importance of high-quality nucleic acids extraction for RNA-Sequencing.

Chapter 3 presents an optimized RNA extraction method to obtain high-quality RNA of *Komagataeibacter hansenii* ATCC 23769 living/producing BNC biofilms and planktonic state to perform RNA-Sequencing. Different conditions of cell disruption techniques followed by RNA extraction methods were performed. Four disruption techniques were tested: (I) freeze fracturing (liquid nitrogen), (II) manual crushing, (III) shaking-type bead mills with ceramic beads and (IV) shaking-type bead mills with metal beads. The performance of three RNA extraction methods was tested for: (a) bacterial RNA extraction; (b) plant tissue RNA extraction and (c) phenol-chloroform extraction. With this we aimed at evaluating the performance of those tested conditions to obtain a considerable amount of bacteria RNA from biofilm bacteria producer. The main challenge to obtain high-quality RNA from bacteria cells into the BNC biofilms was the abundance of polysaccharides. BNC contains large quantities of polysaccharides and a high content of fibers that are difficult to break up and remove. More than that, bacterial cells have a thick protective cell wall and the cell disruption technique is important to release intracellular compounds and improve extraction solvent access. For successful isolation of intact RNA, it is also important to remove genomic DNA, which is possible with an efficient DNase treatment. Therefore, in this chapter we presented an optimized RNA extraction method to obtain samples with high quantity, purity, and integrity of *K. hansenii* cells in BNC biofilms and planktonic. The RNA samples with high-quality from both conditions were used for RNA sequencing using Illumina's technology. The only step performed during RNA-Seq analysis was the preprocessing of raw data to perform a quality control of the sequencing and the efficiency of RNA extraction method to obtain samples with high-quality for RNA-Sequencing.

In chapter 4, we evaluate the capability of *K. hansenii* ATCC 23769 bacterium to synthesize BNC under strict limitation of nutrients.

A defined minimal culture medium (DMCM) was developed through the modulation of different carbon and nitrogen concentrations to grow *K. hansenii*. Monosaccharides (glucose and fructose), a disaccharide (saccharose) and sugar alcohols (glycerol and mannitol) were the carbon sources investigated, which were combined with three nitrogen sources separately, ammonium chloride, NH_4Cl; ammonium nitrate, NH_4NO_3; and ammonium sulfate, $(NH_4)_2SO_4$. BNC production yields were calculated based on the dry weight of the produced membranes and the carbon consumption for each condition. BNC membranes synthesized in defined minimal culture medium supplemented with the best combination and concentration of carbon and nitrogen sources, which obtained the highest BNC yield were characterized. Morphological and physicochemical characterizations were performed to compare the BNC−Minimal membranes with those produced in the complex mannitol medium (BNC−Mannitol). In addition, we highlighted all the advantages of a defined minimal culture medium, such as to be composed of low-cost components, which is desired for BNC production, and it has an exact composition that can assists metabolic engineering studies. This study aims to understand the bacterium metabolism so one can use the experimental data to simulate *in silico* metabolism of *K. hansenii* to obtain the optimized parameters to synthesize BNC.

Chapter 5 presents the concluding remarks and the essence of this document, i.e., a critical summary of what was discussed in chapters 3-4 and what to expect in the future. It provides a concise link between all ideas discussed here and aims to provide to the reader the big picture of the investigation carried out and the real meaning of the contributions proposed in this thesis.

Finally, in Chapter 6 all references used in this document are listed. Some evaluation indicators achieved during the doctoral period are listed in the Appendix I.

2 STATE OF ART

2.1 Bacterial Nanocellulose (BNC)

Bacterial nanocellulose (BNC) was first described by Brown in 1886. He identified cellulose as a key component of the gelatinous pellicle formed upon vinegar fermentation by an “acetic ferment”. Studies revealed that this was caused by an acetic acid bacterium producing cellulose, known as *Acetobacter xylinum*, and it proved to be a convenient model organism for studying cellulose biosynthesis (BROWN, A J, 1886). However, it was not until the 20th century that more intensive studies of BNC were conducted. Several decades after the initial discovery of BNC, Browne (1906) studied the cellulose material obtained by fermentation of Louisiana sugar cane juice and confirmed the results by Brown in 1886 (BROWNE, 1906). In 1931, Tarr and Hibbert published the first detailed study of the formation of BNC by conducting a series of experiments to grow *A. xylinum* on complex culture media (TARR; HIBBERT, 1931).

BNC bacteria producers include Gram-negative bacteria species such as *Acetobacter, Azotobacter, Rhizobium, Pseudomonas, Salmonella, Alcaligenes*, and Gram-positive bacteria species such as *Sarcina ventriculi* (SHODA; SUGANO, 2005). The most effective producers of BNC are *A. xylinum, A. hansenii,* and *A. pasteurianus.* Of these, *A. xylinum* is the most studied microorganism for basic and applied studies on BNC due to its ability to produce relatively high levels of polymer from a wide range of carbon and nitrogen sources. Several research groups focus on this specie, consequently most of the BNC discoveries and advances were reported by *Acetobacter xylinum* biosynthesis (KONGRUANG, 2008; MASAOKA; OHE; SAKOTA, 1993).

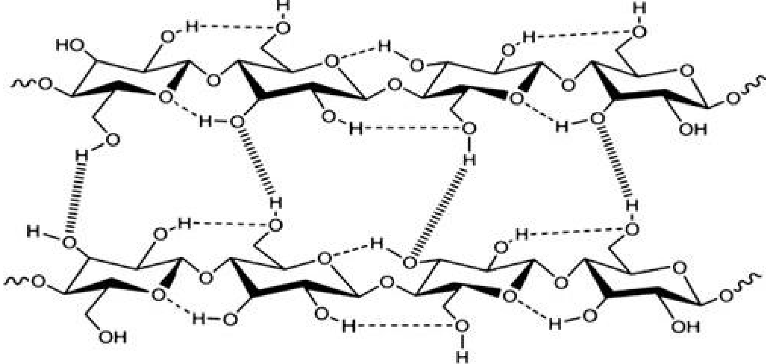
Over the years, the genus of these bacteria has been known under a variety of names, including *Acetobacter* and *Gluconacetobacter*. Five years ago it was renamed once again to *Komagataeibacter* (YAMADA; YUKPHAN; LAN VU; et al., 2012). However, many publications still refer to this organism as *Gluconacetobacter*. To date, 17 species have been accommodated to the genus (YAMADA; YUKPHAN; VU; et al., 2012). The genus *Komagataeibacter* contains several strains of Gram-negative bacteria that are particularly efficient producers of pure nanocellulose, one of which is *Komagataeibacter*

hansenii ATCC 23769 (LISDIYANTI; NAVARRO; UCHIMURA; et al., 2006; PFEFFER; MEHTA; BROWN, 2016; ROSS; MAYER; BENZIMAN, 1991; SAXENA; KUDLICKA; OKUDA; et al., 1994) .

K. hansenii ATCC 23769 produces a microstructured BNC biofilm arranged in a 3D nanofibrils network (JONAS; FARAH, 1998; KLEMM; HEUBLEIN; FINK; et al., 2005; ROSS; MAYER; BENZIMAN, 1991). BNC is distinguished from plant-based cellulose by its high purity, high water holding capacity and good biocompatibility. Also, it does not contain residual hemicellulose or lignin (JOZALA; DE LENCASTRE-NOVAES; LOPES; et al., 2016; MOHITE; PATIL, 2014; TORRES; COMMEAUX; TRONCOSO, 2012). Because of its distinct properties, BNC is particularly well suited for medical, industrial, and commercial applications (BÄCKDAHL et al., 2008; BROWN; WILLISON; RICHARDSON, 1976; IGUCHI; YAMANAKA; BUDHIONO, 2000; WATANABE et al., 1998).

In 1949, the microfibrillar structure of bacterial cellulose was characterized by Muhlethaler (MÜHLETHALER, 1949). Chemically nanocellulose is a linear homopolysaccharide, whose structural unit cellobiose is formed by the union of two glucose molecules linked by glycosidic bonds β-(1→4). These connections provide a linear alignment of the molecule, which allows forming two intramolecular hydrogen bonds within each glucose residue: a link connecting O (6) with the following residue O (2)H and the other connects O (3)H with O (5). The intermolecular hydrogen bond connects the different cellulose chains from the O (3) to O(6)H (Figure 2.1). The glycan chains are composed from 2000 to more than 5000 glucose residues. The total hydrolysis of cellulose produces glucose molecules, while the partial hydrolysis produces cellobiose molecule (HUANG; ZHU; YANG; et al., 2014; RAVEN; EVERT; EICHHORN, 2007; WATANABE et al., 1998).

Figure 2.1 – Representation of nanocellulose, hydrogen bonds inter- and intramolecular. Dashed lines show the intermolecular hydrogen bonds, while the dotted lines show the intramolecular hydrogen bonds.



Source: HUANG et al., 2014.

The hydroxyl groups establishes intramolecular and intermolecular hydrogen bonds which are responsible for the chain rigidity and stability, the formation of nanofibers and to make the nanocellulose insoluble in water and in most organic solvents (KENNEDY; KEEP; CATTY, 1982; WATANABE et al., 1998). The nanocellulose is highly hydrophilic, whereas approximately 99% of its volume consists of a liquid (BÄCKDAHL et al., 2006a; KLEMM et al., 2005).

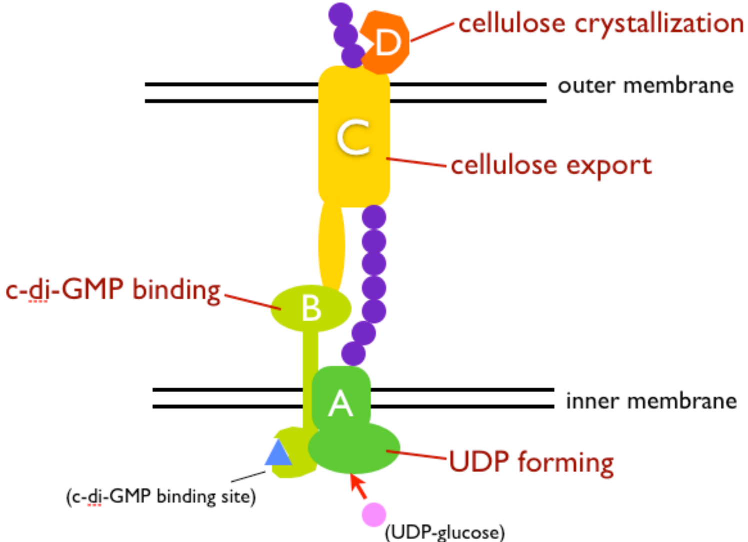
2.1.1 BNC biosynthesis

Bionanocellulose is secreted in the extracellular environment of the bacteria and the chains are grouped in parallel. The organization of nanocellulose fibers is a consequence of its biosynthetic mechanism, since the chains are formed by polymerizing in a particular direction, and then the spontaneous aggregation, while still bound to the cell surface (MORGAN; STRUMILLO; ZIMMER, 2013). BNC biosynthesis involves several regulatory enzymes, while the most important is cellulose synthase, which participates in the transition from UDP-glucose to cellulose. The cellulose synthase enzyme is activated allosterically by monophosphate diguanosine molecule (c-di-GMP), formed from two molecules of guanosine triphosphate (GTP). The

enzyme is activated by that connection, making UDP-glucose into UDP, while β-1,4-glucan chains are formed, which are then used to synthesize cellulose (ROSS; MAYER; BENZIMAN, 1991; STRAP et al., 2011).

The polymerization of glucose chains involves a complex present in the cell membrane, whose genes are organized in an operon (set of genes that are functionally linked to and controlled), as shown in Figure 2.2. The cluster of genes that play the role of producing cellulose is *acs* operon. It contains three major genes, *acsAB*, *acsC* and *acsD*, and the final product is cellulose synthase. The *acsAB* catalyzes the formation of bacterial cellulose; *acsA* is the catalytic subunit which utilizes UDP-glucose to form the basic unit of cellulose; while *acsB* provide the regulatory subunit which has a cyclic di-GMP binding domain. The *acsC* gene is the main composite in the formation of the membrane complex of cellulose synthase and proposed to be involved in the export of the polymer across the bacterial cell wall. The *acsD* gene is involved in the crystallization of the mature cellulose by cleaves the intrastrand ß-1,4 linkages in the cellulose chain and is proposed to have a role in the release of the growing polymer from the cell (IYER et al., 2010; MORGAN; STRUMILLO; ZIMMER, 2013; SAXENA et al., 1994).

Figure 2.2 – Nanocellulose synthase subunits: *operon acs*



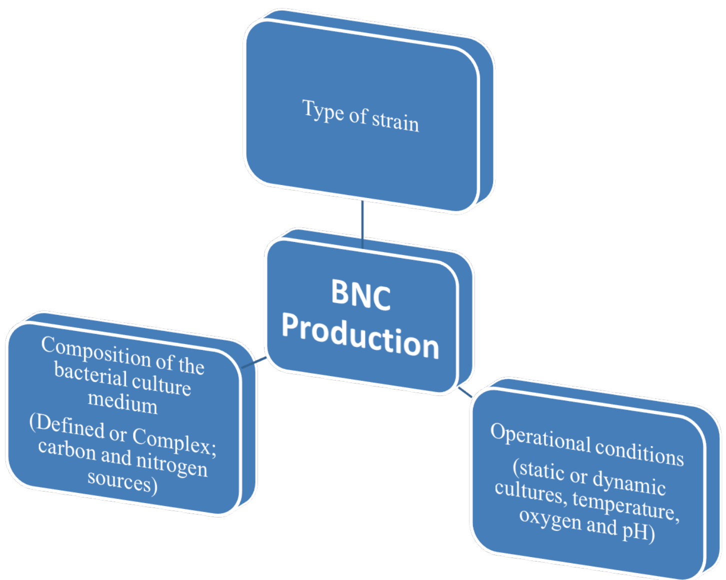
The knowledge gathered so far indicates that c-di-GMP signaling is the most important mechanism causing intensification or decrease in cellulose synthesis but details of exact signaling pathways are unknown (RÖMLING; GALPERIN; GOMELSKY, 2013). Furthermore, molecular biology research of BNC-producing bacterial should reach beyond c-di-GMP signaling. First evidence for potential cAMP-dependent mechanisms importance for BNC production was published by Deng and coworkers (DENG; NAGACHAR; XIAO; et al., 2013), but no other transcription regulation has been documented yet. Despite decades of study, there is much to learn regarding the environmental interactions mediated by BNC and the regulatory pathways that affect gene expression and enzyme activity (FREITAS; ALVES; REIS, 2011).

2.1.2 Culture media and conditions for BNC production

In industrial processes, microbial growth is always in some way controlled by the limited availability of nutrients. The limitation of specific nutrients is frequently used to force microbial cultures into a productive physiological state. Since BNC is an attractive biomaterial for several applications (JOZALA; DE LENCASTRE-NOVAES; LOPES; et al., 2016; KESHK, SHERIF MAS, 2014) an optimized process to produce BNC is desired.

There are several parameters that must be considered in BNC production, such as: type of strain, composition of the bacterial culture medium (defined or complex medium) and operational conditions (static or dynamic cultures, temperature, oxygen and pH) (Figure 2.3). (JOZALA; DE LENCASTRE-NOVAES; LOPES; et al., 2016; KESHK, S.M.A.S.; SAMESHIMA, 2005; RUKA; SIMON; DEAN, 2012; VALEPYN; BEREZINA; PAQUOT, 2012).

Figure 2.3 – Important parameters to consider during BNC production: type of strain; culture media composition and operational conditions.



The most common culture medium used to produce BNC was first described in 1954 by Hestrin and Schramm (HS) (SCHRAMM; HESTRIN, 1954). Hestrin and Schramm is considered a complex medium that contains yeast extract and peptone as components which increases the BNC yield and the production cost (ESA; TASIRIN; RAHMAN, 2014; JOZALA; DE LENCASTRE-NOVAES; LOPES; et al., 2016; VAZQUEZ; FORESTI; CERRUTTI; et al., 2013). Moreover, the complex chemical composition of yeast extract and peptone does not allow to establish the exact relationship between the amount of carbon and nitrogen present in the medium with the BNC yield (JONAS; FARAH, 1998; KESHK, SHERIF MAS, 2014).

For controlled scientific investigations, including growth studies or metabolomic analysis, the use of a complex medium is not ideal. The knowledge of the exact elemental composition of complex media is usually weak, and the media composition can change from batch to batch (JONAS; FARAH, 1998; KESHK, SHERIF MAS, 2014). This may be undertaken by choosing an appropriate combination of carbon and nitrogen sources, which generates a large number of possible combination of nutrients (MIKKELSEN, D.; FLANAGAN; DYKES; et

al., 2009; RUKA; SIMON; DEAN, 2012; VALEPYN; BEREZINA; PAQUOT, 2012). Carbon and nitrogen sources supplied as nutrients to bacteria growth are pivotal to determine BNC yields (RAMANA; TOMAR; SINGH, 2000; SURESH KUMAR; MODY; JHA, 2007). To date, many studies have been focused on altering growth conditions to maximize the BNC yield and diminish the cost of production. However, the majority of those studies supplies the bacteria culture medium with complex chemical components, such as yeast extract and peptone (KESHK, S.M.A.S.; SAMESHIMA, 2005; MIKKELSEN, D.; FLANAGAN; DYKES; et al., 2009; RUKA; SIMON; DEAN, 2012; TABAII; EMTIAZI, 2016; VALEPYN; BEREZINA; PAQUOT, 2012; VAZQUEZ; FORESTI; CERRUTTI; et al., 2013).

A defined medium for BNC production was first reported in dynamic culture by *Acetobacter sp*. More recent studies have optimized this medium, but with the addition of several components (HEO; SON, 2002; MATSUOKA; TSUCHIDA; MATSUSHITA; et al., 1996; SON; KIM; KIM; et al., 2003). Although the medium components and concentrations are known, a defined medium is different from a defined minimal medium. Minimal medium contains only a few necessary nutrients to provide the minimum requirements needed for a particular bacterium or cell.

In recent years, many studies have focused on developing cost-effective culture media for BNC production, such as fruit juices (KUROSUMI; SASAKI; YAMASHITA; et al., 2009), molasses and corn steep liquor (JUNG; LEE; JEONG; et al., 2010), maple syrup (ZENG; SMALL; WAN, 2011), wheat straw (CHEN, LIN; HONG; YANG; et al., 2013), crude glycerol from biodiesel production processes, grape bagasse (VAZQUEZ; FORESTI; CERRUTTI; et al., 2013), residues from the olive oil (GOMES; SILVA; TROVATTI; et al., 2013), waste water of candied jujube (LI; WANG; HUA; et al., 2015), and acetone-butanol-ethanol fermentation wastewater (HUANG, C; YANG; XIONG; et al., 2015). All those studies showed a decrease in BNC production cost through the use of industrial wastes, agricultural residues and by-product streams as culture media. However, those media still contain complex components, such as yeast extract and peptone. In this context, further studies are necessary to investigate the influence of those non-conventional components in the bacteria metabolism to synthesize BNC with distinct properties (CASTRO, CRISTINA; ZULUAGA; PUTAUX; et al., 2011; DAHMAN; JAYASURIYA; KALIS, 2010; KONGRUANG, 2008).

The use of alternative carbon sources can improve BNC production, but it is also necessary to control environmental conditions, such as pH and temperature. Temperature is a crucial factor that affects the growth of microorganisms, thereby influencing BNC production. The fermentation for the synthesis of BNC is usually carried out under static conditions, around 26 °C. The medium becomes turbid, and after a period, a thin biofilm appears on the surface (IGUCHI; YAMANAKA; BUDHIONO, 2000). Importantly, during the process of biofilm formation, nanocellulose pellicle is formed only in areas close to the surface and not throughout the medium. While the system is still maintained, the pellicle remains suspended on the inner wall of the flask by cohesion (IGUCHI; YAMANAKA; BUDHIONO, 2000). In addition to temperature and pH, the dissolved oxygen concentration in the culture medium is an important factor that can affect the production of BNC. In static cultures, the substrate must be transported entirely by diffusion, and because the carbon sources are generally available, the low availability of oxygen can become the limiting factor for cell metabolism and can have a negative effect on BNC production (CHAWLA; BAJAJ; SURVASE; et al., 2009). Ruka and coworkers (2012) discovered that the production of BNC increases as the surface area of static medium and medium volume increase; however, this enhanced yield is also associated with increased cost and production time (RUKA; SIMON; DEAN, 2012).

There are two main methods to produce BNC, a static or dynamic culture. During static condition a thin pellicle (membrane) appears on the surface. The surface that comprises the air/liquid interface has a higher fiber density (top surface) and the opposite surface revealed lower fiber density (bottom surface) (BERTI; RAMBO; DIAS; et al., 2013; KLEMM; SCHUMANN; UDHARDT; et al., 2001). The agitated/dynamic culture, in which nanocellulose is synthesized in a dispersed manner in the culture medium, resulted in irregular pellets, suspended fibers or large three-dimensional biofilms (CZAJA; ROMANOVICZ; BROWN, 2004; KRYSTYNOWICZ; CZAJA; WIKTOROWSKA-JEZIERSKA; et al., 2002; RECOUVREUX; RAMBO; BERTI; et al., 2011). The choice between these two types of production (i.e., static or dynamic culture) depends on the final bionanocellulose application since the morphological, physical, and mechanical properties differ according to the culture method (CHAWLA; BAJAJ; SURVASE; et al., 2009; JEON; YOO; PARK; et al., 2014; KESHK, S.M.A.S.; SAMESHIMA, 2005).

2.1.3 Biofilm and planktonic cells

The formation of nanocellulose biofilm occurs during growth of *K. hansenii* cultivated in the liquid medium, from the transition of planktonic cells (free in suspension) to sessile cells (MORGAN; STRUMILLO; ZIMMER, 2013; RÖMLING, 2002). Studies have shown that the initial interaction of bacteria with a surface is mediated by pili and flagella. In contact with the surface, the bacteria use flagella to move around, forming colonies and starting the production of exopolysaccharides (EPS) (MIKKELSEN et al., 2007; O'TOOLE; KOLTER, 1998). The exopolysaccharides secreted by bacteria play a fundamental role in the structure of the bacterial biofilm. It is believed that biofilm formation is associated, for example, with protection responses against environmental stress factor, such as UV radiation, pH changes and osmotic stress (COSTERTON; STEWART; GREENBERG, 1999; WATNICK; KOLTER, 2000).

Biofilms have been defined as aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of EPS that are adherent to each other and/or a surface (O'TOOLE, GEORGE; KAPLAN; KOLTER, 2000; VERT; DOI; HELLWICH; et al., 2012). Through intercellular interactions, together with the properties of the matrix, the biofilm lifestyle is clearly distinct from that of free-living bacterial cells (planktonic) (KONOPKA, 2009; WATNICK; KOLTER, 2000).

The development of biofilms has been investigated at a molecular level in some bacterial species. Recent genetic and molecular approaches used to study bacterial and fungal biofilms identified genes and important regulatory circuits during the initial cell-surface interactions and during the biofilm maturation (DÖTSCH et al., 2012; MIKKELSEN et al., 2007; O'TOOLE; KAPLAN; KOLTER, 2000). During the state where the bacteria live associated with a biofilm, bacteria express genes that are not observed when they are isolated in planktonic state (COSTERTON; STEWART; GREENBERG, 1999).

Thus, the bacteria living/producing biofilms (it means that bacterial cells live and at the same time produced the biofilm) are greatly different from those growing in suspension, differing in gene expression and physiology. In this context, there is one mechanism to pay attention, which is highly studied and reported in a growing number of bacterial species, called quorum sensing (QS). This mechanism is understood as synchronized changes in genes expression and plays a

crucial role in behavior regulation (including biofilm formation), that must reveal the communication between planktonic and biofilm cells (PATZELT; WANG; BUCHHOLZ; et al., 2013; RUTHERFORD; BASSLER, 2012). Therefore, several efforts have been made to obtain RNA samples with acceptable yield and high-quality from planktonic and biofilms bacteria cells which may allow for the analysis of the functional genomic of those biofilm bacteria producers. The majority of these studies were related to bacteria that produce pathogenic biofilms capable of surviving in the presence of antibiotics and becoming resistant bacteria (CASTRO, JOANA; FRANÇA; BRADWELL; et al., 2017; RESCH; ROSENSTEIN; NERZ; et al., 2005; RUMBO-FEAL; GÓMEZ; GAYOSO; et al., 2013).

2.1.4 BNC applications

The potential of bionanocellulose in several applications is growing because of the advances on BNC production conditions (ESA; TASIRIN; RAHMAN, 2014; JOZALA; DE LENCASTRE-NOVAES; LOPES; et al., 2016). The properties associated with BNC make it an appropriate biomaterial for tissue engineering applications. These applications include: cartilage (SVENSSON; NICKLASSON; HARRAH; et al., 2005), skin substitutes for the recovery of wounds and burns (FU; ZHOU; ZHANG; et al., 2013; GODINHO; BERTI; MÜLLER; et al., 2015; LIN; LIEN; YEH; et al., 2013; METCALFE; FERGUSON, 2007), vascular tubes (HONG; WEI; CHEN, 2015; KLEMM; SCHUMANN; UDHARDT; et al., 2001), and drug delivery (MORITZ; WIEGAND; WESARG; et al., 2014).

Different approaches are used in order to improve BNC properties, including the addition of another component, such as hydroxyapatite to promote osteoblast (TAZI; ZHANG; MESSADDEQ; et al., 2012), polyurethane to improve light emitting diode (UMMARTYOTIN; JUNTARO; SAIN; et al., 2012), polypyrrole for conducting nanocomposites (MULLER; RAMBO; PORTO; et al., 2013), collagen for bone regeneration (SASKA; TEIXEIRA; TAMBASCO DE OLIVEIRA; et al., 2012; ZHIJIANG; GUANG, 2011), among others. The versatility of components that can be added to BNC makes this biomaterial an important source to the development of new products with different applications (IGUCHI; YAMANAKA; BUDHIONO, 2000; JOZALA; DE LENCASTRE-NOVAES; LOPES; et al., 2016; MOHITE; PATIL, 2014).

Grande and coworkers (2009) investigated porous biofilms by adding nanoparticles of hydroxyapatite and carboxymethylcellulose in the culture medium to produce BNC. The resulting material presented fibers with diameters up to 50% lower than the unmodified BNC and pores with dimensions up to 47% greater. All those changes were attributed to the chemical bond between the compounds that provided stabilization of the nanocomposite (GRANDE; TORRES; GOMEZ; et al., 2009).

Pertile and coworkers (2010) studied BNC membranes submitting them to nitrogen plasma in order to modify the surface of the BNC. These membranes chemically incorporated nitrogen groups and increased the porosity. On those modified membranes, fibroblasts, neuroblasts and endothelial cells were cultured and they also showed enhance cell affinity (PERTILE; ANDRADE; ALVES JR.; et al., 2010).

Zimmermann and coworkers (2011) investigated BNC membranes and tubes with hydroxyapatite for applications in bone tissue regeneration. The scaffolds revealed an increase in adherence and confluence of osteoprogenitor cells on the surface of the bone, showing the potential of these scaffolds for this application (ZIMMERMANN; LEBLANC; SHEETS; et al., 2011).

Trovatti and coworkers (2012) developed nanocomposites based on two polysaccharides, pullulan and BNC. The biomaterial was obtained by a green procedure of casting water-based suspensions of pullulan and BNC. The nanocomposites showed considerable improvement in thermal stability and mechanical properties (TROVATTI; FERNANDES; RUBATAT; et al., 2012).

Stumpf and coworkers (2013) investigated the addition of glucose or dextrin into a BNC fermentation mannitol-based medium (BNC-Gl and BNC-De, respectively) under static culture conditions. Both enriched media decreased the BET surface area, water holding capacity, and rehydration rate. Micrographs of the membranes showed effects on fiber density and porosity on both sides of the BNC membranes (STUMPF; PÉRTILE; RAMBO; et al., 2013).

Lin and coworkers (2013) developed BNC-chitosan (BNC-Ch) composites with applicability in wound healing. BNC-Ch composites presented a more compact structure with a smaller pore diameter, an increase in tensile modulus and decrease in the water absorption capacity. Histological examinations revealed that wounds treated with BNC–Ch epithelialized and regenerated faster than those treated with BNC or with the commercially available product Tegaderm™ (LIN; LIEN; YEH; et al., 2013).

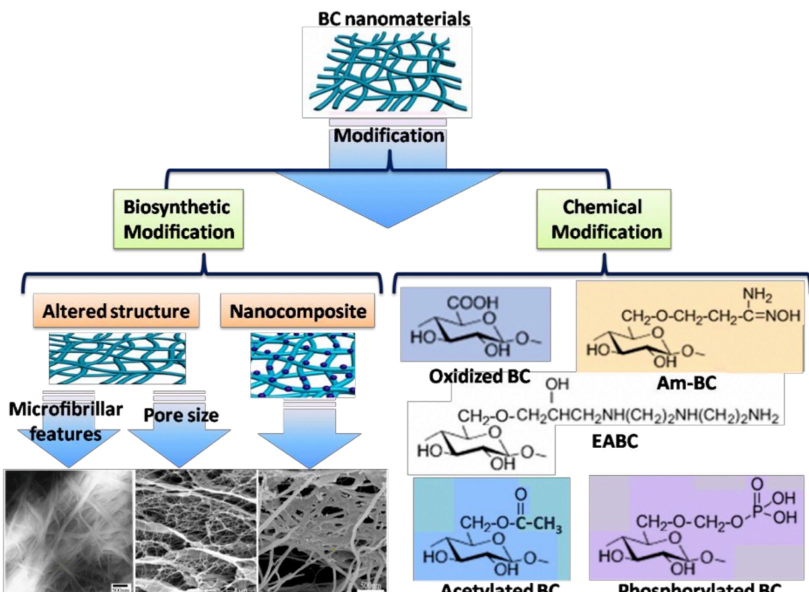
Godinho and coworkers (2014) investigated BNC-Aloe composites by incorporating portions of *Aloe vera* in the growth medium of *Komagataeibacter hansenii*. Chemical interactions, morphology, crystallinity and mechanical properties were affected by supplementation of *Aloe vera*. The results indicated that this biomaterial could be used for several biomedical applications, such as a scaffold for skin substitution and regeneration (GODINHO; BERTI; MÜLLER; et al., 2015).

Ávila and coworkers (2015) evaluated a bilayer scaffold composed of a dense layer of BNC and alginate for use as auricular cartilage. The bilayer BNC scaffolds offered a good mechanical stability and maintained a structural integrity while providing a porous architecture that supported cell growth (ÁVILA; FELDMANN; PLEUMEEKERS; et al., 2015).

Keskin and coworkers (2017) developed a nanocomposite produced by incorporating keratin (isolated from human hair) to the BNC to enhance dermal fibroblast cells attachment. In vitro cell culture experiments performed with human skin keratinocytes and fibroblast cells indicated the potential of novel BC/keratin nanocomposites to be used in skin tissue engineering (KESKIN; SENDEMIR URKMEZ; HAMES, 2017).

All those studies proved that several compounds have been successfully added to BNC by in situ modifications and post modified by methods of impregnation, loading or coating. A variety of surface functionalization through biosynthetic or chemical modification can improve the functionality of BNC and expand its potential application fields (Figure 2.4). Various approaches have been studied regarding the preparation of functional BNC-based nanocomposites by incorporating different substrates including small molecules, inorganic nanoparticles, and polymers on the surfaces of BNC (BRANDES; DE SOUZA; VARGAS; et al., 2016; GENG; YANG; ZHU; et al., 2011; IFUKU; TSUJI; MORIMOTO; et al., 2009; LEE; BLAKER; BISMARCK, 2009; OSHIMA; KONDO; OHTO; et al., 2008; YAMANAKA; SUGIYAMA, 2000).

Figure 2.4 – Schematic illustration of the generalized synthetic routes to modified BNC nanomaterials



Source: (HU, WEILI; CHEN; YANG; et al., 2014)

The functionalization or modification of BNC has been mainly achieved by chemical or mechanical modifications of the nanocellulose matrix or via changing culturing conditions. For a better understanding of the fundamental processes involved in BNC biosynthesis and the regulation of these processes is important the use of omics sciences (FONDI; LIÒ, 2015; SANG; LEE; TAE, 2005). The studies and interest of BNC biosynthesis has increased in the last fifteen years due to the improvement of next-generation sequencing (NGS) technologies, the publication of genome sequences of numerous BNC producers and the increased availability of genetic tools (RÖMLING; GALPERIN, 2015).

2.2 Omics sciences

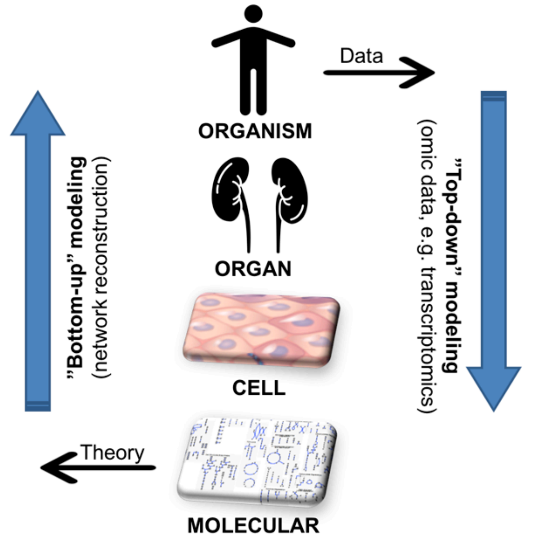
In the past two decades, our ability to study cellular and molecular systems has been transformed by the continuous development of omics techniques (CHEN, LUONAN, 2014; GOMEZ-CABRERO; ABUGESSAISA; MAIER; et al., 2014). The future advancements in understanding biological principles will progressively depend on the development of these techniques that will provide high-resolution data. Omics techniques and sequencing analysis have become key tools in the development of systems biology (CHEN, LUONAN, 2014; FONDI; LIÒ, 2015).

Systems biology approach has been successfully used to identify the components of biological systems and their interactions in order to explain complex cellular processes by using different computational resources (KITANO, 2001; KLIPP et al., 2005; PALSSON, 2006). The goal is to understand complex biological systems by integrating the information from the system components and their relationships from many data sources such as genome, transcriptome, proteome or metabolome (IDEKER; GALITSKI; HOOD, 2001; KIRSCHNER, 2005; KITANO, 2002; STELLING, 2004). In this context, the integration of knowledge, skills and technology from different research disciplines are necessary. For example, the use of mathematics to understand the data collected, computer science to simulate and explain the observations, engineering to develop technologies to measure the system, and physics to develop theories to explain how the system works (CHEN, LUONAN, 2014; IDEKER; GALITSKI; HOOD, 2001; PERCO; RAPBERGER; SIEHS; et al., 2006).

Approaches to modeling biological systems can generally be divided into two ways, often described as top-down and bottom-up (Figure 2.5). These approaches are central in order to assemble information from all levels of biological pathways that coordinate physiological processes (SHAHZAD; LOOR, 2012; BRUGGEMAN; WESTERHOFF, 2015). A bottom-up approach creates a very detailed model of a relatively small subset of biological processes. It encompasses draft reconstruction, manual curation, network reconstruction through mathematical methods, and validation of these models through literature analysis (i.e., bibliomics). Whereas top-down approach collects huge amounts of data and apply statistics to learn and model the system. It encompasses analyses using omics data (e.g.,

transcriptomics) generated through DNA microarrays, RNA-Seq or other high-throughput techniques using appropriate statistical and bioinformatics methodologies.

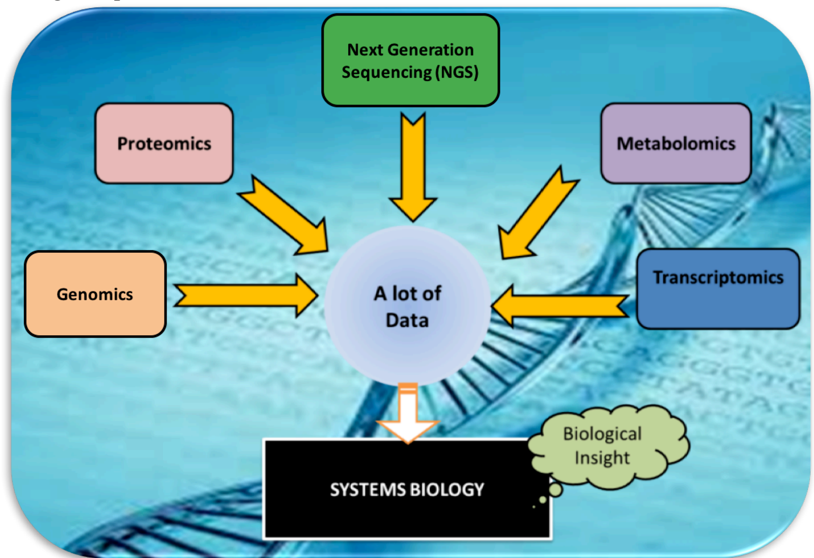
Figure 2.5 – Top-down and bottom-up approaches to modelling biological systems viewed in the context of biological hierarchies.



Source: Adapted from (EDWARDS; THIELE, 2013).

Currently, molecular studies require interaction between genomic, cell analysis, and bioinformatics data, which has gradually introduced an essential role in interpreted and generated results from omics techniques. The development of bioinformatics started with the sequencing analysis and it has provided advances in omics sciences, especially the transcriptome data, which allowed the interrelationship between the functional genome and the encoded information (KYUNG; LUN, 2014; PERCO et al., 2006). There are different types of omics data and bioinformatics resources relevant and related to each omic technique (GOMEZ-CABRERO; ABUGESSAISA; MAIER; et al., 2014; SCHNEIDER; ORCHARD, 2011). The integrative analysis of those omics data sets are the best way to make comprehensive observations of living organisms and to achieve a better understanding of specific biological insights (Figure 2.6).

Figure 2.6 – Genomics, transcriptomics, metabolomics and proteomics with NGS technologies to generate high-throughput omics data to answer underlying biological questions.



These omics sciences and their high throughput technologies have provided a great opportunity to observe all the variables of the system, such as genes, proteins and metabolites, simultaneously. High throughput sequencing of genomes (DNA-Seq) and transcriptomes (RNA-Seq) has made it possible to study the genetic and functional information of any organism (BOJA; KINSINGER; RODRIGUEZ; et al., 2014; GOMEZ-CABRERO; ABUGESSAISA; MAIER; et al., 2014; KITANO, 2001, 2002). In this context, it is strongly demanded in bioinformatics field of study to develop effective computational methods and techniques to deal with all these high-throughput data (PERCO et al., 2006; LIKIĆ et al., 2010; SCHNEIDER; ORCHARD, 2011).

2.2.1 Extraction of nucleic acids

The first crucial step in omics techniques analysis is the extraction of nucleic acids (DNA or RNA) with high quality. Nucleic acids can be extracted from several materials for subsequent processes,

for example polymerase chain reaction (PCR) technique. This technique is used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence (GARIBYAN; AVASHIA, 2013).

The process of extraction and purification of nucleic acids used to be complicated, time-consuming, labor-intensive, and limited in terms of overall throughput. To date, different methods have been developed to extract nucleic acids (CHOMCZYNSKI; SACCHI, 2006; GAMBINO; PERRONE; GRIBAUDO, 2008; RUMP; ASAMOAH; GONZALEZ-ESCALONA, 2010; TAN; YIAP, 2009). For the success of NGS analysis, nucleic acids extracting protocols should result in high purity, yield and reproducibility. Also, the accuracy, speed, and reliability of the method should be maximal, while the contamination should be minimized (TAN; YIAP, 2009). Depending on the kind/nature of the sample, a commercial extraction kit cannot be sufficient to extract the quantity of DNA or RNA needed for NGS analysis, which will demand time and resources to perform an extraction method (PSIFIDI; DOVAS; BRAMIS; et al., 2015; RUMP; ASAMOAH; GONZALEZ-ESCALONA, 2010). In this context, the extraction methods might provide very convenient solutions to extract high quantity and quality of DNA/RNA for NGS applications.

2.2.1.1 RNA extraction in biofilms

The success of any RNA-based analysis, such as RNA-Sequencing (RNA-Seq) or Reverse transcription polymerase chain reaction (RT-PCR), depends on the yield, purity, and integrity of the RNA (BUSTIN; BENES; NOLAN; et al., 2005; NOLAN; HANDS; BUSTIN, 2006). However, different RNA extraction methods can yield RNA samples with variable levels of quality (RUMP; ASAMOAH; GONZALEZ-ESCALONA, 2010; WANG, LIMIN; STEGEMANN, 2010). Currently, there are several methods for RNA extraction available, however, there are only a few published studies comparing RNA extraction from biofilm samples (CURY, J A; SEILS; KOO, 2008; DÖTSCH; ECKWEILER; SCHNIEDERJANS; et al., 2012; PEREZ-OSORIO; FRANKLIN, 2008). Biofilm makes difficult to perform RNA extraction procedure mainly due to the presence of the extracellular

matrix, which is estimated to comprise about 90% of the total biofilm biomass (FLEMMING; WINGENDER, 2010).

BNC contains large quantities of polysaccharides and a high content of fibers that are difficult to break up and remove (DEINEMA; ZEVENHUIZEN, 1971). Polysaccharides have been denominated as an interfering substance (inhibitory substances) for RNA extraction which makes bacterial cell lysis and nucleic acid purification difficult, turning the process of extraction of pure RNA samples into a challenge (BROWN, ROBERT B; AUDET, 2008; JAHN; CHARKOWSKI; WILLIS, 2008; YU; TANG; ZHANG; et al., 2012). Some studies have evaluated RNA isolation strategies in order to eliminate the presence of polysaccharides from the biofilm, as an alternative to minimize variations in the sample purity (ATSHAN; SHAMSUDIN; LUNG; et al., 2012; CURY, JAIME A; KOO, 2007). Based on chemical similarities between polysaccharide-based biofilms and plant tissues some studies have evaluated the efficiency of a plant RNA extraction kit to overcome the challenge of acquiring RNA samples from biofilms with good quality (WANG, CHUNMING; HAO; ZHANG; et al., 2008; WANG, LIMIN; STEGEMANN, 2010). Several companies have developed methods to optimize the RNA extraction but they are often costly. However, in general, a single kit or method do not result in satisfactory quality and quantity of RNA samples (ATSHAN; SHAMSUDIN; LUNG; et al., 2012; JAHN; CHARKOWSKI; WILLIS, 2008; JUNTTILA; LIM; RUDD, 2009; YU; TANG; ZHANG; et al., 2012). In other words, RNA samples quality is directly related to the sample's nature or origin and by the method used to extract them.

2.2.2 Next-Generation Sequencing (NGS) Technologies

DNA sequencing has come a long way since the days of two-dimensional chromatography in the 1970s. With the advent of the Sanger chain termination method in 1977, scientists gained the ability to sequence DNA in a reliable, reproducible manner (SANGER; NICKLEN; COULSON, 1977). A decade later, Applied Biosystems introduced the first automated capillary electrophoresis (CE), based on sequencing instruments, the AB370 in 1987 and the AB3730xl in 1998. These instruments were used for sequencing large part of the Human Genome Project data (GOODWIN; MCPHERSON; MCCOMBIE, 2016; VAN DIJK; AUGER; JASZCZYSZYN; et al., 2014; ZHAO; GRANT, 2011).

The new sequencing technologies, called next-generation sequencing (NGS) technologies, are based on high throughput sequencing, or high-performance sequencing, which resulted in an surge of base sequencing platforms in contrast to the Sanger method. In the NGS millions of fragments are sequenced at the same time, in parallel (Massively Parallel Sequencing - MPS), generating short reads that can be single-end, or can be sequenced from both ends (paired-end reads). The paired-end approach facilitates the detection of genetic rearrangements, structural variations and alternative splice junctions (MACLEAN; JONES; STUDHOLME, 2009; METZKER, 2010).

Currently, these high-throughput sequencing platforms (Figure 2.7) are divided into 1st generation (Sanger sequencing), 2nd generation (454-Life Science, Genome Analyzer - Solexa, SOLiD - Life Technologies, HiSeq and Mi-Seq – Illumina and Ion Torrent - Life Technologies) (VAN DIJK; AUGER; JASZCZYSZYN; et al., 2014), and 3rd generation (Helicos, PacBio RS and Nanopore) (FENG; ZHANG; YING; et al., 2015; GLENN, 2011; QUAIL; SMITH; COUPLAND; et al., 2012).

In 2004, the 454 was introduced by Roche. Roche 454 pyrosequencing by synthesis (SBS) was the first commercially successful second-generation sequencing system developed by 454 Life Sciences in 2004, and it was acquired by Roche in 2007. The sequencing is performed by detecting the nucleotide incorporated by a DNA polymerase. Pyrosequencing relies on light detection based on a chain reaction when pyrophosphate is released (MARDIS, 2008).

In 2006, the Genome Analyzer (GA) was introduced by Solexa which uses the concept of “sequencing by synthesis” providing highly accurate sequencing with a low error rate, even within repetitive sequence regions. The sequencing reaction is conducted simultaneously on a very large number of different template molecules spread out on a solid surface. Illumina purchased Solexa and entered the NGS business in 2007 (RIHTMAN; MEADEN; CLOKIE; et al., 2016).

In 2007, the SOLiD (Sequencing by Oligo Ligation and Detection) technology was marketed by Life Technologies and released in 2008 by Applied Biosystems Instruments (ABI). It is based on 2-nucleotide sequencing by ligation (SBL). This procedure involves sequential annealing of probes to the template and their subsequent ligation. The major disadvantages are the short read lengths (50–75 bp) and the very long run times of 7 to 14 days (MARDIS, 2008).

In 2010, Illumina introduced the HiSeq 1000 and HiSeq 2000 systems (QUAIL; SMITH; COUPLAND; et al., 2012). The technology

of sequencing by synthesis uses removable fluorescently labeled chain-terminating nucleotides that is able to produce a larger output at lower reagent cost. In 2011, Illumina released a benchtop sequencer called the MiSeq, which, although small in size, has an output of 0.3 to 15 Gb and fast turnover rates suitable for targeted sequencing. It can provide sequencing results in 1 to 2 days at much reduced cost. Illumina's new method of synthetic long reads using TruSeq technology apparently improves *de novo* assembly. Today, it is the most successful sequencing system with more than seventy percent dominance of the market.

In 2010, Ion Torrent Systems Inc. introduced the Ion semiconductor sequencing (Ion Proton) based on the detection of hydrogen ions that are released during the polymerization of DNA. Life Technologies (now ThermoFisher Scientific) expanded their NGS portfolio with the acquisition of Ion Torrent and Applied Biosystems (BUERMANS; DEN DUNNEN, 2014; GOLAN; MEDVEDEV, 2013).

Third-generation single-molecule sequencing (SMS) technologies have emerged to reduce the price of sequencing and to simplify the preparatory procedures and sequencing methods. In 2010, Helicos Genetic Analyzer System was introduced by Helicos technology, called. The principle of single molecule fluorescent sequencing is to identify the exact sequence of a piece of DNA (MOROZOVA; HIRST; MARRA, 2009).

In 2011, Pacific Biosciences developed a sequencing system named the PacBio RS using a single molecule real time sequencing (SMRT) method. This system can produce and read lengths of multiple thousands of base pairs, though with a high rate of errors. These errors are corrected using optimized assembly strategies (RHOADS; AU, 2015).

In 2015, the MinION device was developed by the company Oxford Nanopore Technologies (MIKHEYEV; TIN, 2014). This NGS sequencing uses nanopores and the conductivity of ion currents in the pore changes when the strand of nucleic acid passes through it. Changes in electric current indicate which base is present. It is 60 to 85 % accurate, compared with 99.9 % in conventional technologies (JAIN, M; FIDDES; MIGA; et al., 2015; JAIN, MITEN; OLSEN; PATEN; et al., 2016; WARD; KIM, 2015).

Figure 2.7 – First, second and third generation sequencing technologies. The commonly NGS machines and companies.

1st generation:

- Sanger sequencing – Life Technologies

life technologies

2nd generation:

- 454-Life Sciences – Roche

Roche
454 Life Sciences

- Genome Analyser – Solexa

illumina. Solexa

- SOLiD – Life Technologies

Applied Biosystems
part of life technologies

- HiSeq and Mi-Seq – Illumina

HiSeq 2000™ NextSeq 500™ MiSeq™
illumina

- Ion Torrent – Life Technologies

ion torrent by life technologies
ThermoFisher SCIENTIFIC
life technologies

3rd generation:

- Helicos – Helicos Bioscience

Helicos BioSciences Corporation

- PacBio RS – Pacific Bioscience

PACIFIC BIOSCIENCES

- MinION – Oxford Nanopore

Oxford NANOPORE Technologies

These recent technologies allow to sequence DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing, and they have revolutionized the study of genomics and molecular biology (CROUCHER; THOMSON, 2010). While the exact methodology for each system differs, in general, the next generation sequencing workflow involves DNA/RNA preparation, library or template preparation, sequencing and analysis (QUAIL; SMITH; COUPLAND; et al., 2012; VAN DIJK; AUGER; JASZCZYSZYN; et al., 2014; VAN VLIET, 2010).

The development of new technologies and tools to improve the extraction of genetic information from large sets of biological data has allowed the implementation of more effective experiments. All these NGS technologies exhibit advantages and disadvantages, with key differences between the quality of the data and the applications it will support. Many databases and open-source computer packages were developed to store and analyze the data generated by these NGS technologies and to facilitate scientific research. The visualization, analysis, and interpretation of data involve specific knowledge of computational techniques that demand that the researcher learn how to use all necessary tools (LIKIĆ et al., 2010; PERCO et al., 2006; SCHNEIDER; ORCHARD, 2011).

2.2.2.1 RNA-Sequencing and transcriptome analysis

The nucleotide sequence of the yeast alanine was the first RNA molecule reported by Holley (HOLLEY; APGAR; EVERETT; et al., 1965). Since then, several techniques have been used to study the types and levels of RNA in different organisms. The sequencing of expressed RNA using NGS technologies is known as RNA-Seq or Whole Transcriptome Shotgun Sequencing (WANG, ZHONG; GERSTEIN; SNYDER, 2009). The transcriptome can be defined as the complete set of transcripts in a cell, and their quantities, in a specific physiological condition. Those transcripts includes RNA coding (mRNA) and non-coding (rRNA, tRNA, structural RNA, regulatory RNA and other RNAs) (VAN VLIET, 2010; WANG, ZHONG; GERSTEIN; SNYDER, 2009).

The advances in sequencing and bioinformatics have enabled analysis with unprecedented levels of resolution and depth, allowing for comprehensive profiles of RNA species (MCCLURE;

BALASUBRAMANIAN; SUN; et al., 2013; WANG, ZHONG; GERSTEIN; SNYDER, 2009). Northern blot techniques, which required a large amount of RNA, were used in early studies on candidate gene-based RNA expression analysis (ALWINE; KEMP; STARK, 1977). To overcome the limitations of the Northern blot technique and facilitated more efficient gene expression analysis, the technique of reverse transcription quantitative PCR (RT-PCR) was created. This technique is sensitive for mRNA detection and quantitation (BECKER-ANDRÉ; HAHLBROCK, 1989; BUSTIN; BENES; NOLAN; et al., 2005).

The microarray technology (SCHENA; SHALON; DAVIS; et al., 1995) based on the hybridization intensity of the transcripts enabled researchers to characterize the expression levels of thousands of transcripts in different cell types and compare the levels of expression during various physiological conditions. The ability to study the expression of thousands of genes in a cost effective manner has made microarray technology a popular method for transcriptome analysis (HINTON; HAUTEFORT; ERIKSSON; et al., 2004). However, this technology also has major drawbacks such as the high background noise due to cross-hybridization of probes and the inability to detect novel transcripts and their relative quantification (HINTON; HAUTEFORT; ERIKSSON; et al., 2004; SCHENA; SHALON; DAVIS; et al., 1995).

To overcome the low throughput approach of these technologies, researchers investigated high-throughput methods that could provide an overview of the whole transcriptome. This led to the discovery of tag-based sequencing approaches - ESTs (Expressed Sequence Tag), such as serial analysis of gene expression (SAGE) (VELCULESCU; ZHANG; VOGELSTEIN; et al., 1995) and massively parallel signature Sequencing (MPSS) (REINARTZ; BRUYNS; LIN; et al., 2002). The method of SAGE provided an absolute quantification of the transcripts and more precise comparisons of results obtained from different samples. SAGE traditionally uses Sanger sequencing for quantification of tags and is more expensive than microarrays. However, poor mapping of the transcripts to the reference genome, and the inability to detect isoforms, have limited the application of traditional tag-based sequencing technologies in transcriptome analysis (VELCULESCU, 1999; VELCULESCU; ZHANG; VOGELSTEIN; et al., 1995). The development of Massively Parallel Signature sequencing (REINARTZ; BRUYNS; LIN; et al., 2002) and Solexa technology (HU, ZHENFEI; CHENG; WANG, 2015) led to the development of RNA-Sequencing (RNA-Seq) and made a revolutionary impact on

transcriptome analysis in the last few years. RNA-Sequencing has been developed to overcome many of the shortcomings of hybridization-based methods and preexisting sequencing-based approaches (WANG, ZHONG; GERSTEIN; SNYDER, 2009). RNA-Seq is a powerful method for mapping and quantifying transcriptomes developed to analyze gene expression on a global scale. According to the NGS platform, RNA-Seq consists of large-scale sequencing of transcribed regions generating short fragments (reads) with variable length (30-400 bp). The RNA is converted into cDNA and after sequencing, millions of reads need to be analyzed using extensive bioinformatics tools (CAPOBIANCO, 2014; CHU; COREY, 2012; HU; CHENG; WANG, 2015; WANG; GERSTEIN; SNYDER, 2009b). In this context, RNA-Seq became significantly more efficient allowing increased replication and increased power in downstream analysis. The study of the extensive complexity of transcriptomes by RNA-Seq technology has provided a better understanding of the complexity of regulatory mechanisms, functional annotation and differential gene expression, in different organisms (CHU; COREY, 2012; MORTAZAVI; WILLIAMS; MCCUE; et al., 2008).

There are studies that evaluated how gene expression differs in biofilm and planktonic cells through the use of RNA-Sequencing. They provide a quantitative and qualitative analysis of whole transcriptome (RUMBO-FEAL et al., 2013). Dötsch and coworkers (2017 showed distinct patterns of gene expression in biofilms and planktonic conditions by *Pseudomonas aeruginosa*. Many genes of bacterial cells adhered to the biofilm were involved in the adaptation of microaerophilic growth conditions (low oxygen concentration) and production of extracellular matrix (DÖTSCH; ECKWEILER; SCHNIEDERJANS; et al., 2012; SPIERS; BOHANNON; GEHRIG; et al., 2003; WHITELEY; BANGERA; BUMGARNER; et al., 2001). Dumitrache and coworkers (2017) developed a novel bioreactor designed to generate separate sessile and planktonic cell populations of *Clostridium (Ruminiclostridium) thermocellum* for omics studies. This study demonstrated that sessile cells and planktonic cells produces widespread gene expression changes for critical functions of this organism. They provided with this study that physiological insights for those two cells populations were relevant for reverse engineering of industrially relevant phenotypes (DUMITRACHE; KLINGEMAN; NATZKE; et al., 2017).

3 OPTIMIZED METHOD FOR HIGH-QUALITY RNA FROM *Komagataeibacter hansenii* IN BIOFILMS AND PLANKTONIC STATES

3.1 INTRODUCTION

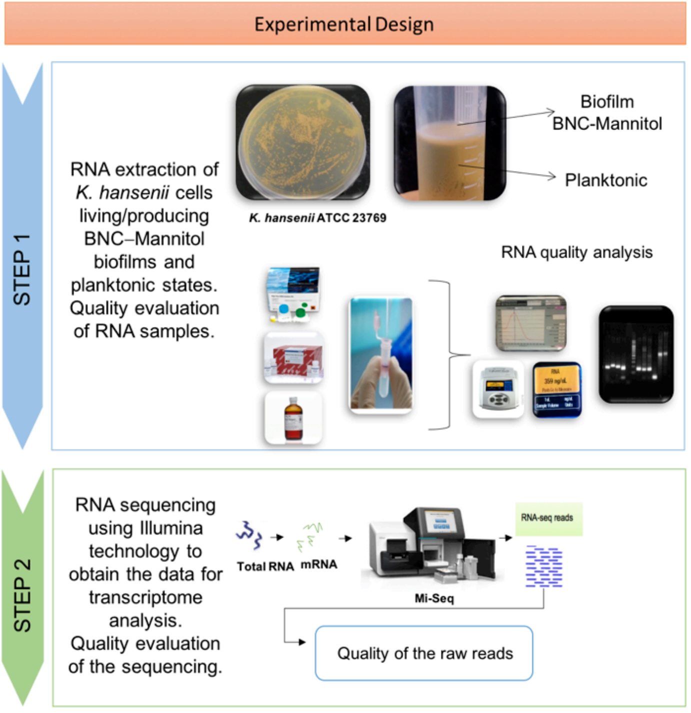
Through the development of NGS technologies, genetic information of *Komagataeibacter hansenii* was gradually revealed in recent years (IYER; GEIB; CATCHMARK; et al., 2010; PFEFFER; MEHTA; BROWN, 2016). However, to the best of our knowledge there are not studies reporting transcriptome profiles of the *Komagataeibacter* bacteria. The difficulty in obtaining RNA samples from *K. hansenii* cells living/producing biofilms can be a major problem and has been a barrier to the transcriptome analysis of this organism. Therefore, it would be desirable to analyze the transcriptome data of this bacterium, which will facilitate the evaluation of its potential use as bionanocellulose producer. To gain a better understanding of the genes and proteins involved in BNC biosynthesis, we chose two conditions to identify the differences between cells living/producing biofilms (sessile cells) and planktonic (free-living cells) of *K. hansenii* ATCC 23769 and thereby identify differences in their gene expression patterns.

De novo transcriptome assembly is an important step towards transcriptome analysis to obtain genome information, such as novel gene discovery and gene expression profile (CAPOBIANCO, 2014; CLARKE; YANG; MARSH; et al., 2013; GRABHERR; HAAS; YASSOUR; et al., 2011; WICKRAMASINGHE; CÁNOVAS; RINCÓN; et al., 2016). However, the crucial step for RNA-Seq analysis is the extraction of high-quality RNA. In this study, we evaluated different conditions to obtain a considerable amount of bacterial RNA from *K. hansenii*. The conditions were based on different cell disruption techniques combined with RNA extraction methods. The main challenge to obtain high-quality RNA samples from bacteria cells living/producing BNC biofilms is due to the fact that BNC contains large quantities of polysaccharides and a high content of fibers that are difficult to break up and remove. For successful isolation of intact RNA samples, it was important to remove genomic DNA with an efficient DNase treatment. In this chapter, we present an optimized RNA extraction method, developed during this work, to obtain high quantity, purity and integrity of bacterial RNA living/producing BNC biofilms and planktonic states for RNA sequencing and transcriptome analysis.

3.2 MATERIALS AND METHODS

Chapter 3 comprises two main steps, as described in Figure 3.1. The first step was the evaluation of different techniques and RNA methods to obtain a considerable amount of bacterial RNA from *Komagataeibacter hansenii* cells living/producing BNC biofilms and planktonic culture. The tested conditions were evaluated by the quality and quantity of RNA samples. In the second step, only RNA samples obtained by the optimized RNA extraction method were sent for sequencing using the platform MiSeq-Illumina. The quality analysis of raw reads was performed to evaluate RNA sequencing. Since the transcriptome assembly and analysis is a group collaboration still under progress, they are not considered as part of this thesis.

Figure 3.1 – The two steps experimental design for the development of RNA extraction method and sequencing of RNA samples to obtain raw reads.



3.2.1 BNC−Mannitol biofilms synthesis

Komagataeibacter hansenii ATCC 23769 obtained from "Coleção de Cultura Tropical (CCT)", Fundação André Tosello (Campinas, SP) was used to produce BNC biofilms. Mannitol medium (ATCC® 23769) was prepared as follows: 25.0 $g{\cdot}L^{-1}$ mannitol, 5.0 $g{\cdot}L^{-1}$ yeast extract and 3.0 $g{\cdot}L^{-1}$ bactopeptone. The medium was adjusted to pH 6.5 with 0.1 M NaOH solution before sterilization by autoclaving (121 °C for 20 min).

K. hansenii was inoculated on mannitol agar plates and incubated at 26 °C for seven days. Bacteria colonies were randomly selected and suspended to the desired starting optical density (OD = 1) at 660 nm in Mannitol medium using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Subsequently, 2.5% v/v of bacteria inoculum was added in Mannitol medium and transferred to 24-well plates (2 mL/well). *K. hansenii* was incubated for fifteen days under static conditions at 26 °C. Bacterial cells living/producing BNC biofilms and living planktonic were submitted for RNA extraction.

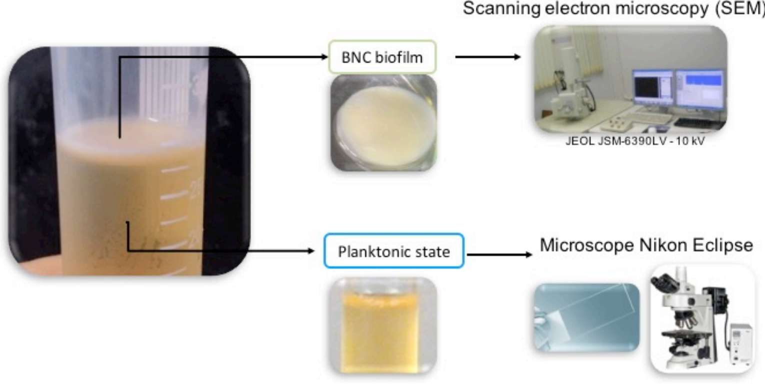
3.2.2 Visualization of *K. hansenii* cells living/producing biofilm and planktonic

In order to achieve an efficient RNA extraction, it was necessary to guarantee enough amount of bacterial cells living/producing BNC biofilms and planktonic culture after fifteen days of incubation. The major difficulty to visualize bacterial cells under the microscope is due to the fact they are small and the cells float in and out of focus.

To visualize bacteria cells living in planktonic state they were placed on a cover slip with a small amount of planktonic suspension and then get a microscopy image with a 40× magnification using a Nikon Eclipse microscope (Nikon) (Figure 3.2).

Scanning electron microscopy (SEM) was performed to visualized *K. hansenii* cells in BNC biofilm fibers, since visualization of bacteria cells living/producing BNC biofilm is hindered by the background from the biofilm under an optical microscope. In this case, BNC biofilms were characterized using a JEOL JSM–6390LV microscope operated at 10 kV(Figure 3.2). Prior to the analysis, the samples were dried by critical point drying (CPD), cut into small pieces and coated with a thin layer of sputtered gold.

Figure 3.2 – Techniques to visualize bacterial cells in BNC biofilms (scanning electron microscopy) and in planktonic state (optical microscope).



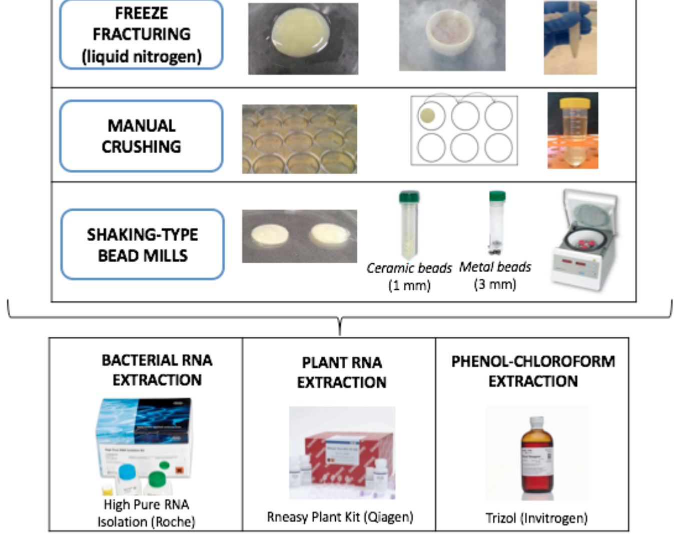
3.2.3 Extraction of total RNA of *K. hansenii* cells from biofilms

Different cell disruption techniques combined with RNA extraction methods were investigated to obtain high-quality RNA of *K. hansenii* cells present in the BNC biofilms. First, we evaluated physical cell disruption techniques to access RNA from bacteria cells within nanocellulose biofilms aiming to eliminate the presence of polysaccharides from RNA samples. We chose physical cell disruption techniques because chemical techniques could interfere in the RNA expression. All disruption techniques were performed following the manufacture's RNA extraction methods as shown in Table 3.1 and Figure 3.3. Three independent RNA extractions were performed for each condition. RNA samples were analyzed in terms of concentration and purity. Only the best RNA samples were used in the following steps.

Table 3.1 – Different conditions of cell disruption techniques followed by RNA extraction methods were performed. Four disruption techniques were tested: (I) freeze fracturing (liquid nitrogen), (II) manual crushing, (III) shaking-type bead mills with ceramic beads and, (IV) shaking-type bead mills with metal beads. The performance of three RNA extraction methods was tested: (a) bacterial RNA extraction; (b) plant tissue RNA extraction and, (c) phenol-chloroform extraction.

Test Condition	Cell disruption techniques		RNA extraction methods
I	Freeze fracturing	(a)	Bacterial RNA
		(b)	Plant RNA
		(c)	Phenol-chloroform
II	Manual crushing	(a)	Bacterial RNA
		(b)	Plant RNA
		(c)	Phenol-chloroform
III	Shaking-type bead mills with ceramic beads	(a)	Bacterial RNA
		(b)	Plant RNA
		(c)	Phenol-chloroform
IV	Shaking-type bead mills with metal beads	(a)	Bacterial RNA
		(b)	Plant RNA
		(c)	Phenol-chloroform

Figure 3.3 – Cell disruption techniques combined with RNA extraction methods. For each disruption technique three RNA extraction methods were tested.



3.2.3.1 Freeze fracturing (liquid nitrogen)

Physical disruption technique was performed using a traditional freeze fracturing (I) with liquid nitrogen grinding in a mortar and pestle. BNC biofilm samples (two BNC biofilms for every assay) were powdered by grinding biofilms and adding liquid nitrogen into the mortar to prevent biofilms thawing. Once the biofilms were ground to a fine powder, the macerate was transferred to 1.5 mL Eppendorf tubes and followed by RNA extraction.

3.2.3.2 Manual crushing

One alternative technique tested was cell disruption by manual crushing (II). In this case, BNC biofilms were crushed and the resulting liquid was collected. This technique eliminates the biofilms before RNA extraction. BNC biofilms (24-well plate size) were crushed and successively washed with PBS (Phosphate Buffered Saline) to obtain the remaining liquid with high-density of bacterial suspension. The remained liquid was precipitated by centrifugation at 8,000× g for 10 min. The supernatant was discarded and the cell pellet was used for subsequent RNA extraction.

3.2.3.3 Shaking-type bead mills

Another disruption technique used was based on shaking-type bead mills (III and IV) performed using MagNa Lyser instrument (Roche Applied Science, Germany). This technique allows processing more samples at the same time. Two kinds of beads were tested: ceramic beads with 1.4 mm diameter (III) supplied by the manufacturer (Roche Applied Science, Germany), and metal beads with 3 mm diameter (IV) (modified procedure) to evaluate their suitability for biofilm disruption. During this technique, it was necessary to use the lysis reagents of each extraction method.

BNC biofilm samples (two BNC biofilms for every assay) were placed into the MagNA Lyser tubes filled with beads (ceramic or metal) and resuspended in 500 µL of the lysis reagents. We tested the following lysis reagents: (a) Bacterial RNA extraction: Lysis buffer, kit High Pure RNA Isolation (Roche Applied Science, Germany); (b) Plant RNA extraction: Buffer RLT, kit RNeasy Plant Mini kit (Qiagen, Germany) and, (c) Phenol-chloroform extraction: Trizol (Invitrogen, USA). The tubes containing BNC biofilm samples were homogenized for 60

seconds at 7500 rpm, and centrifuged at 12,000× g for 10 minutes to precipitate cell debris. The lysate was used for RNA extraction.

3.2.4 RNA extraction methods

Three independent RNA extractions were performed for each tested cell disruption technique as shown in Table 3.1. The following RNA extraction methods were tested: (a) Bacterial RNA extraction, (b) Plant RNA extraction and, the (c) Phenol-chloroform extraction.

Bacterial RNA extraction method was performed using High Pure RNA Isolation Kit (Roche Life Science, Germany, #11828665001), and followed the protocol according to the manufacturer.

Plant RNA extraction method was performed using RNeasy Plant Mini Kit (Qiagen, Germany, #74904). An important step of this method was the use of QIAshredder column that consists of a unique biopolymer-shredding system in a microcentrifuge spin-column format. We followed the manufacturer's protocol for RNA extraction.

Phenol-chloroform extraction method was performed using the reagent Trizol (Invitrogen, USA). The protocol followed as described. The samples were incubated with 500 µL of Trizol reagent for 5 minutes at room temperature. For each disruption technique, different samples were generated: (I) powder from liquid nitrogen, (II) pellet after manual crushing and centrifugation, (III) biofilms that were placed in tubes with ceramic beads, and (IV) biofilms that were placed in tubes with metal beads. After homogenization, samples were incubated for 5 minutes followed by supplementation with 100 µL of chloroform and, then, vigorous mixing for 15 seconds and incubation for 3 minutes at room temperature was performed. Samples were centrifuge at 12,000× g for 15 min at 4 °C, and the upper layer was transferred to a new tube. An aliquot of 250 µL isopropanol was added and the resulting mixture incubated for two hours at -80 °C. When samples were removed from freezer samples were kept for 10 min at room temperature, followed by centrifugation at 12,000× g for 10 min at 4 °C to obtain an RNA pellet. The pellet was washed with 500 µL of ethanol 75%, followed by brief vortex and centrifuged at 7,500× g for 5 minutes at 4 °C. The RNA pellet was then partially air-dried (i.e., not letting the pellet dry completely) to help its further manipulation. RNase-free water

(Invitrogen, USA) was added and the final volume depends on the size of the formed pellet.

Major modification in the conventional phenol-chloroform extraction method was concomitant use of shaking bead mills technique. Initially, after transferred biofilms to the tubes that contained beads, Trizol was added, thus tubes were placed on a rotator to maximize biofilm exposure to the action of this reagent. Subsequently, the mixture was centrifuged before addition of chloroform to prevent carryover of undigested biofilms following the subsequent extraction steps and, thus enhancing RNA purity. Also, in the step to add isopropanol, the samples were kept at -80 °C for two hours, this increases the amount of RNA captured during precipitation with isopropanol.

3.2.5 Extraction of total RNA of *K. hansenii* cells in planktonic

Only the condition of cell disruption technique combined with RNA extraction method which provided RNA samples with high concentration was used to extract RNA from planktonic cells. Thus, after we removed the biofilms from the plate wells, the remaining liquid was mixed and centrifuged at 12,000× g for 10 min at 4 °C, and the pellets were submitted to the defined RNA extraction method, without the necessity of using the disruption technique.

3.2.6 DNase treatment and polymerase chain reaction (PCR)

RNA samples were analyzed in terms of concentration and purity. The condition of cell disruption technique combined with RNA extraction method which provided RNA samples with better quality and concentration were submitted for DNase treatment.

To evaluate any genomic DNA contamination in the RNA samples a PCR amplification using the specific primer *acsAB* (cellulose synthase) was performed. *acsAB* primer was selected based on the *acsAB* sequence of *K. hansenii* deposited in GenBank at NCBI (AB091060). The primer sets were designed using Primer3 (UNTERGASSER; NIJVEEN; RAO; et al., 2007) to be 20−27 base-pairs in length, have a GC content of 40−55%, a melting temperature (Tm) of 55−65 °C, and to produce an amplicon of 90−300 base-pairs. Sequences were checked to be specific in silico using the Primer-Blast program to compare primer sequences to *K. hansenii* genome sequence. RNA samples were used to check the *acsAB* gene using gene-forward

(5-CGACCTATAACGAAGAACTGAGC-3) and reverse (5 TAGTTAAGATTACCGGCCTTTGC-3), with 199 bp amplicon.

Specific PCR reaction was performed within 10 µL of a solution containing 1 µL AccuPrime Pfx Reaction Mix (Invitrogen, USA), 0.1 µL Accu Prime Pfx DNA Polymerase (Invitrogen), 0.4 µL of each primer, 1 µL of template (bacterial DNA as positive control and RNA samples) and the final volume were completed with ultrapure water DEPC (Invitrogen, USA). A thermocycler (Model 2720, Thermo Fisher Scientific, USA) was used for PCR reactions following the protocol described. The PCR program consisted of initial denaturation at 94 ºC for 3 minutes, followed by 30 cycles of 94 ºC for 30 seconds, annealing at 60 ºC for 1 minute, extension at 72 ºC for 30 seconds and a final extension at 72 ºC for 10 minutes.

The addition of a positive control was considered to check the ideal condition of PCR reactions to amplify the target sequence. For that, DNA was extracted from *K. hansenii* using the PureLink Genomic DNA Kit (Thermo Fisher Scientific, USA) and the protocol was followed according to the manufacturer.

RNA samples were followed to DNase treatment with RNase-free DNase Set (Qiagen, Germany, # 79254), following the manufacturer's protocol with a few modifications. First, RNA samples were diluted in order to guarantee the DNase treatment. After optimization, we standardized RNA samples concentration in a range of 100 ng to 300 ng from the total RNA which reacted with 20 µL DNase solution; considering also that for 300 ng to 400 ng total RNA/30 µL DNase solution. Following the proportions, if 20 µL of DNase was used, 80 µL of Buffer RDD is necessary, and 30 µL of DNase/120 µL of Buffer RDD. The RNA sample solution was completed to the final volume of 800 µL with RNase-free water and samples incubated at 25 ºC for 20 min. After the DNase treatment, the samples contained residual DNase I. For this reason, a supplementary purification step was included to eliminate DNase I and other residual components. Sodium acetate (3 M, pH 5.2) was used in a quantity equal to 1/10 of the RNA sample solution. Thus, isopropanol in 1:1 (v/v) proportion was added in the mixture of RNA solution with sodium acetate. The tubes were gently inverted 10 times and incubated at room temperature for 10 minutes. Samples were spinned for 10 min, 12,000× g at 4 ºC. After that, the supernatant was discarded and cell pellet was diluted with 500 µL of 75% ethanol. Samples were spinned for 5min, 12,000× g at 4 ºC.

Supernatant was discarded. The tubes containing the cell pellet were inverted on a clean paper to dry and resuspended with RNase-free water.

RNA samples before and after DNase treatment proceeded with PCR reaction to evaluate the presence/absence of gDNA contamination. The PCR products were analyzed by agarose gel electrophoresis, by loading 5 µL of each amplified product with 2× Blue Juice Loading buffer (Thermo Fisher Scientific, USA) into 1.5% agarose gels stained with SYBR Safe gel stain (Thermo Fisher Scientific, USA), and run for 30 min at 120 V.

3.2.7 RNA Quality assessment (concentration, purity and integrity)

The concentration of RNA samples was measured with fluorescence-based QuantiFluor Dye RNA System (Promega, USA) using Quantus Fluorometer (Promega, USA). RNA samples purity was assessed by the ratios of $A_{260/280}$ and $A_{260/230}$ in a SpectraDrop 384 spectrophotometer (Molecular Devices, USA), with $A_{260/280}$ ratios appreciably < 1.8 and $A_{260/230}$ ratios appreciably < 2 (generally indicating contaminated RNA samples). RNA samples integrity was checked by loading 2 µL of the total RNA mixed with 2× Blue Juice Loading buffer (Thermo Fisher Scientific, USA) and applied to a gel into 1.5% agarose gel (Invitrogen, USA) containing TBE buffer and SYBR Safe Gel Stain (Thermo Fisher Scientific, USA), and electrophoresed at 120 V for 30 min.

3.2.8 Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) method with Tukey test. P-values < 0.05 were considered significant. The results were expressed as mean ± standard error. All experiments were run in triplicate.

3.2.9 Preparation of RNA samples for sequencing

Four RNA samples were obtained from biofilms and planktonic and denominated as M (M1, M2, M3 and M4) for RNA samples extracted from biofilms and as P (P1, P2, P3 and P4) for RNA samples from planktonic. To obtain a high concentration of RNA, it was

performed a pool of two samples of each condition. For example, samples M1 and M2 were mixed and renamed as MA. The same procedure was performed for the other samples. Then, two biological replicates (MA and MB) and (PA and PB) were sent for sequencing.

3.2.10 RNA-Sequencing

The cDNA libraries preparation and high-throughput sequencing were performed by Neoprospecta Microbiome Technologies (Florianópolis, SC, Brazil, https://neoprospecta.com/).

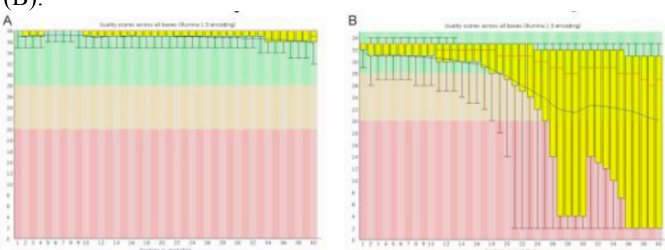
The libraries were sequenced using the MiSeq platform from Illumina, through pair-end methodology. During the libraries preparation, it was utilized the TruSeq Stranded mRNA Library Prep Kit® (Illumina, San Diego, California, USA), which allows the sequencing of both strands of cDNA: forward and reverse. In summary, it is possible to properly know from which sense is each read. A150-cycle format in 2 × 75 bp (paired-end) was used.

Four samples were analyzed, MA and MB for libraries generated by “biofilm” samples and PA and PB for libraries generated by “planktonic” samples. Each sample generated two libraries, R1 and R2, forward and reverse, respectively.

The only step performed during RNA-Seq analysis was the preprocessing of raw data to evaluate the quality control of the sequencing and if the RNA extraction method was efficient in obtain samples with high-quality for RNA-Sequencing.

We tested the quality of sequenced libraries using the FastQC tool (version 0.11.5), developed by Babraham Institute (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). This tool allows the evaluation of raw sequences, in order to guarantee the sequence quality. For these analyses, we used the Graphical User Interface (GUI) environment and default commands. It reports multiple QC metrics, which are reported with a traffic light warning system, normal (green), abnormal (orange) or bad (red), making it relatively easy to interpret results. This step is important, because reads that presented bad qualities (scores lower than 28) are discard and only the remain reads follows the next steps. Figure 3.4 shows the quality of a good data and from a bad data.

Figure 3.4 – FastQC reports from sequences with good quality (A) and bad quality (B).



Source: (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

The next steps for the transcriptome assembly are still under progress. Since the whole transcriptome assembly and gene expression analysis is a group collaboration, they are not considered as part of this thesis.

3.3 RESULTS AND DISCUSSION

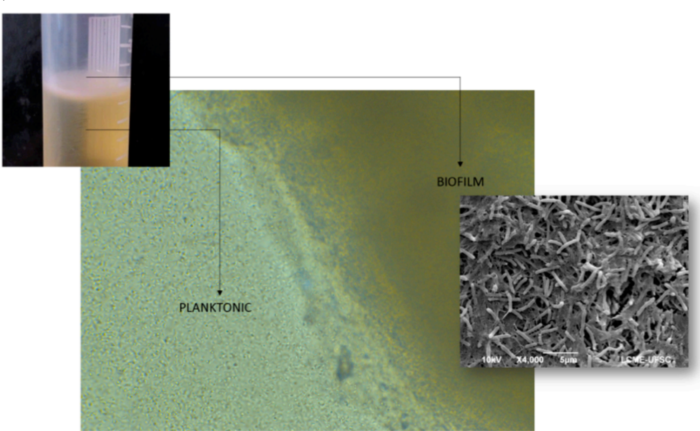
3.3.1 Visualization of bacterial cells living/producing biofilms and planktonic state

Planktonic cells were visualized by optical microscopy and images showed high cell concentration after fifteen days of incubation (Fig. 3.5A).

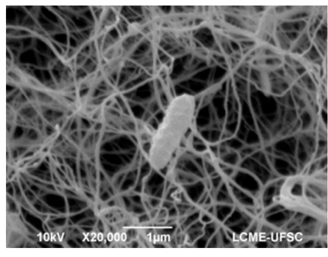
BNC biofilm background makes it very difficult to visualize bacterial cells within the biofilm (Fig. 3.5A). The SEM micrographs (Fig. 3.5A) showed a high number of bacteria cells attached to the nanocellulose fibers. Micrograph of the bacterium *K. hansenii* attached to the fibers of BNC is shown in Figure 3.5B.

Figure 3.5 – (A) Visualization of *K. hansenii* cells in planktonic (microscopy) and in BNC biofilm (SEM micrograph). (B) Micrograph of the bacterium *K. hansenii* in the fibers of BNC at 20,000× magnification.

(A)



(B)



3.3.2 Total RNA extraction of bacterial cells living/producing BNC biofilms

The four tested conditions (I, II, III and IV) based on different cell disruption techniques followed by three RNA extraction methods (a, b and c) resulted in RNA samples analyzed in terms of quantity (RNA concentrations) and quality (RNA purity) as shown in Table 3.2. RNA concentration ranges and the mean of $A_{260/280}$ and $A_{260/230}$ ratios of RNA samples were presented based on three independent RNA extractions performed for each condition tested.

Results revealed that different conditions of cell disruption techniques followed by RNA extraction methods influenced the quality of RNA samples extracted from *K. hansenii* cells living in BNC biofilms. Even using the same condition, RNA samples presented a wide variation in concentration (Table 3.2). In fact, there is no accurate quantification of bacteria cells living in BNC biofilms, resulting in differences in the amount of bacterial cells in biofilm samples and consequently obtaining a distinct range of RNA samples concentrations.

Table 3.2 – RNA sample concentration ranges and purity obtained for all conditions tested of cell disruption techniques followed by RNA extraction methods. Four disruption techniques were tested: (I) freeze fracturing (liquid nitrogen), (II) manual crushing, (III) shaking-type bead mills with ceramic beads and, (IV) shaking-type bead mills with metal beads. The performance of three RNA extraction methods was tested: (a) bacterial RNA extraction; (b) plant tissue RNA extraction and, (c) phenol-chloroform extraction. Three independent RNA extractions (n = 3) were performed for each condition. Absorbance ratios were presented as means ± standard error.

Cell Disruption Techniques	RNA Extraction Methods	Test Condition	Total RNA concentration range (ng/µL)	RNA purity $A_{260/280}$ ratio $A_{260/230}$ ratio
Freeze fracturing (liquid nitrogen)	Bacterial RNA	Ia	6.75–48.69	1.69 ± 0.20 0.96 ± 0.39
	Plant RNA	Ib	179.97–348.23	1.78 ± 0.63 0.72 ± 0.32
	Phenol-chloroform	Ic	28.32–112.05	1.81 ± 0.22 0.96 ± 0.48
Manual crushing	Bacterial RNA	IIa	1.17–25.30	1.22 ± 0.27 1.35 ± 0.11
	Plant RNA	IIb	1.80–16.02	0.96 ± 0.14 1.50 ± 0.13
	Phenol-chloroform	IIc	1.66–11.16	0.94 ± 0.20 1.13 ± 0.03
Shaking-type bead mills (ceramic beads)	Bacterial RNA	IIIa	13.75–96.59	1.32 ± 0.29 1.68 ± 0.81
	Plant RNA	IIIb	35.20–101.53	1.55 ± 0.36 2.08 ± 0.39
	Phenol-chloroform	IIIc	56.47–696.95	2.12 ± 0.39 1.12 ± 0.10
Shaking-type bead mills (metal beads)	Bacterial RNA	IVa	27.18–115.04	2.01 ± 0.10 1.20 ± 0.27
	Plant RNA	IVb	59.46–359.08	2.22 ± 0.26 1.34 ± 0.15
	Phenol-chloroform	IVc	688.33–1501.11	2.67 ± 0.05 1.11 ± 0.09

By comparison, the RNA extraction method (a) was not applicable to any cell disruption technique. Conditions (Ia), (IIa), (IIIa) and (IVa) showed the lowest range of RNA concentrations, 6.75−48.69; 1.17−25.3; 13.75−96.59; and 27.18−15.04 ng/μL, respectively, compared to the conditions using methods (b) and (c). Also, RNA samples obtained from the method (a) presented low $A_{260/230}$ ratio, revealing polysaccharide contamination. Even if this method was specific to extract RNA from bacterial cells, RNA samples were obtained with low concentrations. The hypothesis was that the presence of BNC biofilms interferes in the bacteria extraction resulted in low performance of the lysis buffer.

Results showed that the conditions (IIa), (IIb) and (IIc) were inefficient, giving RNA samples with the lowest RNA concentration range among the tested disruption techniques, 1.17−25.3; 1.8−16.02; and 1.66−11.16 ng/μL, respectively. Those RNA samples presented an average $A_{260/280}$ ratio of 1.04 ± 0.27, which is an indicative of contamination by residual phenol, guanidine or other reagent used in the extraction process. Although manual crushing technique eliminated the biofilms before RNA extraction, this technique did not allow a complete removal of bacteria cells from BNC biofilms. The weak lysis of bacterial cells prevented a satisfactory execution of RNA extraction methods. Several studies reported that microorganisms in biofilms live in a self-produced matrix of hydrated extracellular polymeric substances (EPS), which provide mechanical stability of biofilms, cells adhere to the polymeric surfaces and form a three-dimensional polymer network that interconnects and transiently immobilizes biofilm cells (FLEMMING; WINGENDER, 2010; FLEMMING; WINGENDER; SZEWZYK; et al., 2016).

On the other hand, conditions (Ia), (Ib) and (Ic) presented statistical differences ($p < 0.05$) among them and revealed an increase in RNA samples concentrations compared to the conditions (IIa), (IIb) and (IIc). Condition (Ib) showed high RNA concentrations ranged from 179.97 to 348.23 ng/μL (Table 3.2). Meanwhile, conditions (Ia) and (Ic) resulted in low RNA concentrations, probably because the conditions tested were not adequate for cell lysis to liberate RNA from bacterial cells.

The conditions tested by disruption techniques (III) and (IV) presented several advantages from the other techniques tested. The advantages included reduction of procedure time, extraction of multiple samples at the same time and decreasing of contamination by handling.

There was no need of liquid nitrogen, which is often a hazardous and problematic agent for a large number of samples.

An increase of RNA concentration using conditions (IIIa) and (IVa) compared to (Ia) and (IIa) was observed. Even that, method (a) showed lower RNA samples concentration than the other extraction methods tested. Conditions (IIIb) and (IVb) revealed RNA samples with high-quality and concentration ranged from 35.20 to 101.53 ng/µL and from 59.46 to 359.08 ng/µL, respectively. RNA extraction method (b) uses a silica membrane column to purify RNA, resulting in better quality RNA samples. However, the existence of polysaccharide contamination has been shown to decrease the efficiency of spin-columns significantly (GAMBINO; PERRONE; GRIBAUDO, 2008). Thus, one cannot measure the total amount of bacterial cells living in BNC biofilms and because of that, the column was overloaded with the biofilm samples. On the other hand, conditions (IIIc) and (IVc) showed an increase in the quality of the RNA samples compared to bacterial (IIIa and IVa) and plant (IIIb and IVb) RNA methods. Phenol-chloroform extraction (IIIc and IVc) was more efficient for disrupting the high density of bacterial cells strongly entangled in the BNC biofilms.

In terms of the kind of beads, ceramic and metal, they were completely different in terms of RNA concentrations. Conditions using metal beads, (IVa), (IVb) and (IVc), showed significant differences ($p < 0.05$) and exhibited RNA samples with high-quality compared to the conditions using ceramic beads, (IIIa), (IIIb) and (IIIc). The usual protocol for shaking bead mills, using MagNA Lyzer instrument, is performed with ceramic beads (III). The protocol was modified using metal beads (IV) that are much harder and with a higher diameter that enabled a better bacteria cell disruption within BNC biofilms than ceramic beads and provided a better method performance. For RNA samples provided by condition (IIIc) the concentration ranged from 56.4 to 696.95 ng/µL, while RNA samples from condition (IVc) the concentration ranged from 688.33 to 1,501.11 ng/µL. In terms of RNA purity, the average of $A_{260/280}$ and $A_{260/230}$ ratios using condition (IIIc) was 2.12 ± 0.39 and 1.12 ± 0.10, respectively. For RNA samples provided by condition (IVc) the ratios were 2.67 ± 0.05 and 1.11 ± 0.09, respectively. Phenol-chloroform extraction (c) contributed to obtain a high concentration of RNA, but it is known that this method does not guarantee DNA-free samples and this can contribute negatively to the

absorbance ratios. In this case, DNase treatment is recommended for more accurate quantitation.

Comparison of all tested conditions based in the quality and quantity of bacterial RNA from *K. hansenii* cells living/producing BNC biofilms revealed that condition (IVc) provided RNA samples with high concentration. In terms of cell disruption technique, metal beads for shaking bead mills (IV) provided significantly disruption of bacterial cells from BNC biofilms and improved extraction solvent access. Significant differences ($p < 0.05$) were found for RNA samples concentrations obtained from condition (IVc) compared to conditions (IVa) and (IVb).

3.3.3 DNase treatment and quality analysis

Four RNA samples from BNC biofilms provided by condition (IVc) were denominated as M1, M2, M3 and M4. RNA samples from planktonic followed the same extraction method used for BNC biofilms, without the addition of the disruption technique since bacterial cells were living free in suspension. Four RNA samples from planktonic state were denominated as P1, P2, P3 and P4. The samples followed the same method for the accuracy of RNA sequencing. Thus, eight RNA samples were submitted to DNase treatment. RNA concentration and absorbance ratios from those samples before and after DNase treatment are shown in Table 3.3. RNA is extremely susceptible to degradation due to the ubiquitous presence of RNases in the environment. A careful handling during RNA purification was necessary but also selecting the proper quantification. RNA concentrations were measured using fluorescent dye-based method, which quantitates up to 600 ng/µL. For this reason, RNA samples from biofilms, which showed concentrations ranged from 1,320 to 2,188 ng/µL, were diluted (1:4 in RNase-free water) before measurement.

Table 3.3 – Concentration and absorbance ratios of RNA samples from planktonic (P) and biofilms (M), before and after DNase treatment.

	Before DNase treatment			**After DNase treatment**		
Sample	**$A_{260/280}$ ratio**	**$A_{260/230}$ ratio**	**Conc. (ng/µL)**	**$A_{260/280}$ ratio**	**$A_{260/230}$ ratio**	**Conc. (ng/µL)**
P1	1.95	0.19	180	1.93	1.41	57.0
P2	1.96	0.32	126	2.02	1.70	50.8
P3	2.03	0.21	232	2.03	1.69	120
P4	2.13	0.41	370	2.02	1.61	164
M1	2.71	1.01	415 (1:4)	1.96	1.57	284 (1:4)
M2	2.67	1.01	547 (1:4)	2.10	1.73	200 (1:4)
M3	2.62	0.79	330 (1:4)	1.79	1.27	124 (1:4)
M4	2.67	1.03	330 (1:4)	2.09	1.68	159 (1:4)

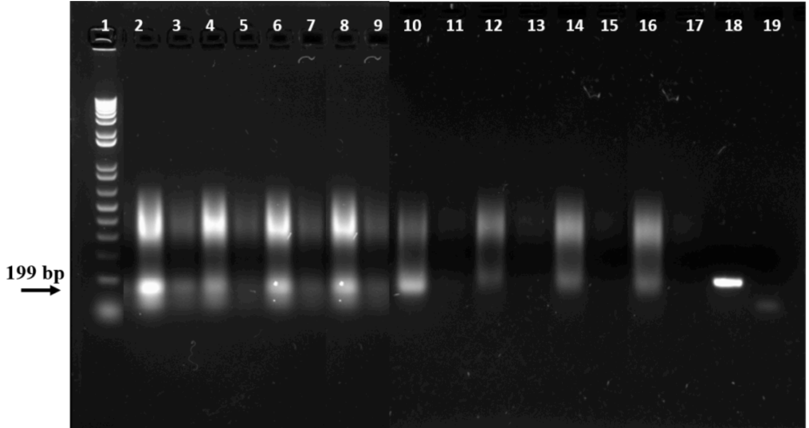
Moreover, for an effective DNase treatment, RNA samples extracted from biofilms needed to be diluted, thus all samples were treated with 30 µL of DNase at around the same concentrations, to guarantee total DNA removal. RNA samples from planktonic state before DNase treatment presented average $A_{260/230}$ ratios of 0.28 ± 0.10, while after treatment the ratio increased to 1.60 ± 0.13. RNA samples from biofilms before DNase treatment presented average $A_{260/280}$ and $A_{260/230}$ ratios of 2.67 ± 0.04 and 0.96 ± 0.11, respectively. After DNase treatment, they presented improvement in the purity, with average $A_{260/280}$ and $A_{260/230}$ ratios of 1.98 ± 0.14 and 1.56 ± 0.20, respectively. After treatment, all RNA concentration decrease, but RNA samples with better purity (Table 3.3) were obtained. This can be explained by the fact that ideal absorbance measurements quantify the concentration of the respective nucleic acid. After treatment, any DNA residual was removed from the samples and RNA concentration measurement was more accurate.

RNA samples before and after treatment were used as templates for PCR reaction aiming to analyze the presence/absence of residual genomic DNA by agarose gel electrophoresis (Figure 3.6). The positive

control was bacterial genomic DNA and *acsAB* was the housekeeping gene.

Figure 3.6 indicates genomic DNA contamination of RNA samples before DNase treatment. The presence of PCR fragments amplified in this reaction was visualized in lanes 2, 4, 6, 8, 10, 12, 14 and 16. RNA samples from biofilms were more contaminated than planktonic samples, as verified on the band intensities. Lane 18 refers to the positive control (bacterial DNA) with amplification product for the gene *acsAB* (amplicon 199 bp). After DNase treatment (lanes 3, 5, 7, 9, 11, 13, 15 and 17) there is a decrease in signal intensity of the PCR products, thereby confirming the efficiency of the treatment.

Figure 3.6 – Agarose gel electrophoresis of PCR amplification. The position of 199 bp band is pointed with an arrow. Lane 1: molecular marker (1 Kb ladder); Lane 2: M1 before treatment; Lane 3: M1 after treatment; Lane 4: M2 before treatment; Lane 5: M2 after treatment; Lane 6: M3 before treatment; Lane 7: M3 after treatment; Lane 8: M4 before treatment; Lane 9: M4 after treatment; Lane 10: P1 before treatment; Lane 11: P1 after treatment; Lane 12: P2 before treatment; Lane 13: P2 after treatment; Lane 14: P3 before treatment; Lane 15: P3 after treatment; Lane 16: P4 before treatment; Lane 17: P4 after treatment; Lane 18: positive control; Lane 19: negative control.



For RNA-Seq analysis two biological replicates of each condition were prepared. Sample PA (pool of samples P1 and P2) and PB (pool of samples P3 and P4) are from planktonic condition; samples MA (pool of samples M1 and M2) and MB (pool of samples M3 and M4 extractions) are from biofilms. Table 3.4 shows the purity, concentration and yield of

the pool samples. The total RNA yield was obtained from the concentrations (ng/µL) and the total volume of RNA samples (22 µL).

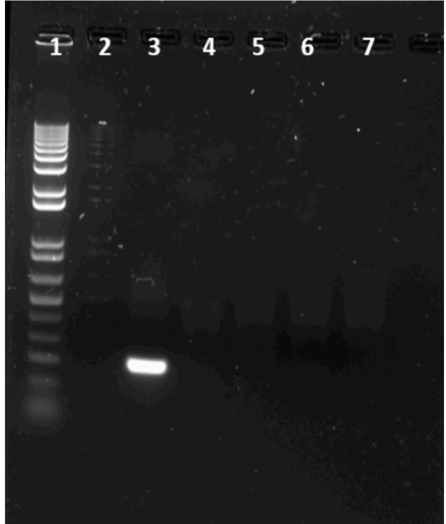
Table 3.4 – Purity, concentration and yield of RNA samples (Pools of RNA samples from planktonic and biofilm extractions).

Samples	$A_{260/280}$ ratio	$A_{260/230}$ ratio	Conc. (ng/µL)	Yield (µg)
PA (P1 + P2)	1.92	1.55	104	2.29
PB (P3 + P4)	2.03	1.66	139	3.06
MA (M1 + M2)	2.01	1.46	275	6.05
MB (M3 + M4)	2.01	1.46	188	4.14

Results showed that RNA-pool samples presented good quality. It was recommended for RNA sequencing an amount of 1 to 5 µg in a total volume of 50 to 100 µL. Samples PA, PB, MA and MB presented total RNA yield above 2 µg. Thus, RNase-free water was added in all samples to the final volume of 70 µL, before been sent for sequencing. As a last guarantee of the quality of final samples (RNA-pool samples), a PCR amplification was performed to verify the absence of gDNA contamination.

As can be seen from Figure 3.7, lane 3 refers to the positive control (bacterial DNA) with amplification product for the gene *acsAB* (amplicon 199 bp). RNA samples PA, PB, MA and MB, used as template did not present PCR amplified fragments (lanes 4, 5, 6 and 7), indicating no contamination with genomic DNA. In fact, the RNA extraction method was able to produce RNA samples with high purity and yield from planktonic and biofilm states.

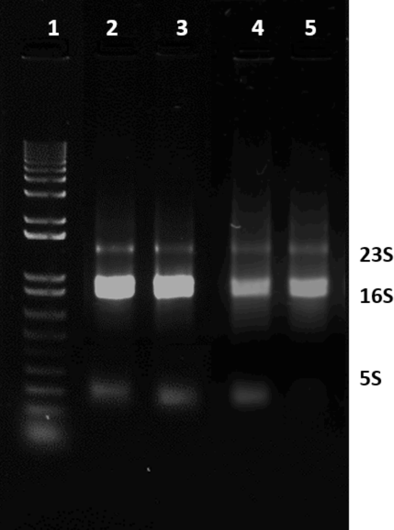
Figure 3.7 – Agarose gel electrophoresis of PCR amplification. Lane 1: molecular marker (ladder); Lane 2: negative control; Lane 3: positive control; Lane 4: PA sample; Lane 5: PB sample; Lane 6: MA sample; Lane 7: MB sample.



To further verify the efficacy of the RNA extraction method, an aliquot of each sample was run on agarose gel electrophoresis to check RNA integrity before RNA sequencing (Figure 3.8).

RNA samples exhibited sharp and distinct 23S and 16S ribosomal RNA bands. The less intense band at the bottom corresponds to 5S RNA. All RNA samples presented high-quality and were intact since the samples did not show smeared bands, demonstrating the integrity of the isolated RNA. Typically, three major bands reflect the typical pattern of the 23S, 16S and 5S bacterial rRNA gene molecules.

Figure 3.8 – RNA integrity of purified samples by 1.5% agarose gel electrophoresis. Lane 1: ladder; lane 2: PA; lane 3: PB; lane 4: MA; lane 5: MB.

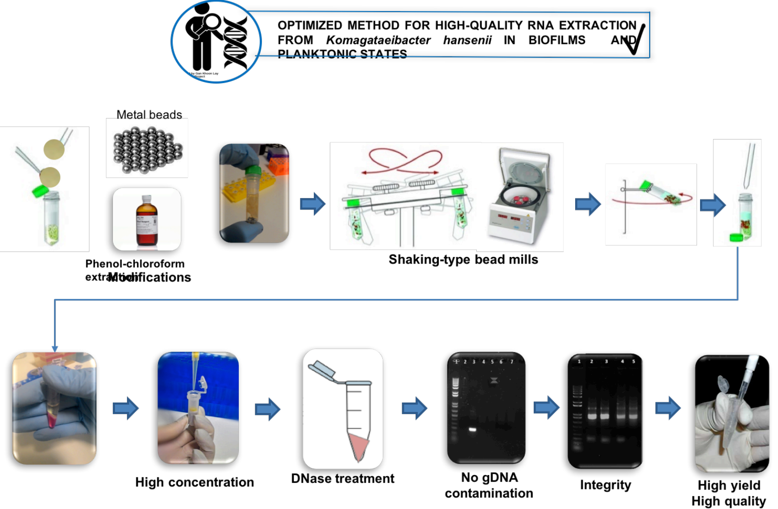


To the best of our knowledge, the method outlined here is the first to allow the isolation of highly pure and intact RNA from *K. hansenii* cells in BNC biofilms. Obtaining high-quality nucleic acids is the primary and most critical step in molecular biology studies, particularly when using difficult material such as bacterial nanocellulose biofilms. The method reported in this study is rapid, relatively non-toxic and applicable for extracting large quantities of high purity RNA from bacterial cells in BNC biofilms. The critical steps of the present method included cell lysis to destroy the cellular structure (cell walls and nanocellulose fibers), separation of desired nucleic acids from cell debris and contaminants and purification of RNA. Due to the dense nature of BNC biofilms, in this method a phenol-chloroform in conjunction with homogenization and mechanical grinding was effectively to lyse the cells. The mechanical method employed metal beads (3 mm) which in the extraction buffer (phenol) disrupted the samples through high level agitation by shaking. Its advantages over other methods (e.g. grinding tissue with liquid nitrogen using a mortar or manual crushing), are in the ability to process many samples at a time with no concerns of cross-contamination. Also, this method provided high yields of total RNA from small quantities of starting material (two

biofilms produced in 24 well-plate). RNA extraction methods using bacterial or Rneasy kits have failed to provide satisfactory yield and purity of RNA from bacterial cells in BNC biofilms.

Condition (IVc) was found to be suitable for RNA extraction of bacterial cells from BNC biofilms. This condition involved modification of steps in the conventional phenol-chloroform protocol compatible with shaking with metal beads technique (Fig. 3.9).

Figure 3.9 – Steps during RNA extraction method. With all modifications and treatments, the method was adequate to obtain RNA samples without contamination, pure and with high yield.



RNA extraction method was also able to extract RNA from bacterial cells living in planktonic state. Furthermore, the designed primer was efficient and generated a single amplicon of the correct size after PCR amplification to detect gDNA contamination. The modifications of DNase treatment were critical for DNA removal, resulting in RNA samples with no genomic DNA. After DNase treatment, the extracted RNA was further purified using 3M sodium acetate and isopropanol, which eliminated the contaminants. Using the described method, high yields of total RNA were extracted, as confirmed by spectrophotometric and electrophoretic analyses. The values of the ratios $A_{260/280}$ and $A_{260/230}$ indicated that RNA samples were

pure and effectively separated from protein, polysaccharides and other metabolites. In conclusion, RNA extracted were of excellent quality and applicable for downstream applications.

3.3.4 RNA sequencing

3.3.4.1 Generation of raw reads and data processing

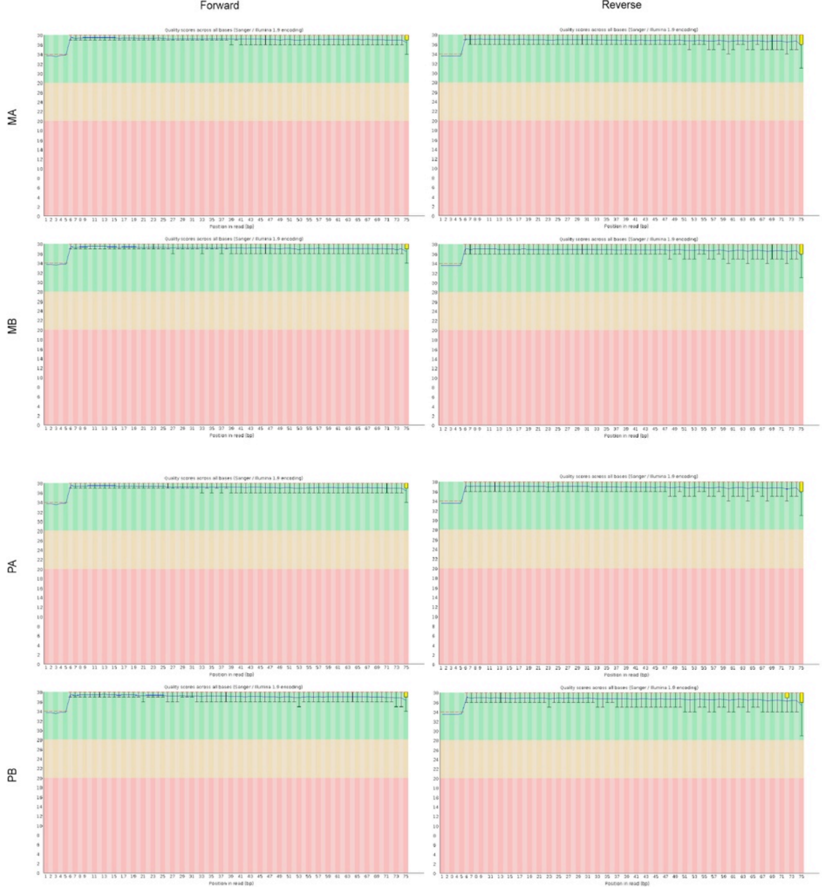
To obtain an overview of the transcriptome of *K. hansenii*, the cDNA libraries were generated from total RNA of cells from biofilm and planktonic states. A total number of 21,974,446 paired-end reads (2×75 bp) were obtained from the four samples on the Illumina MiSeq platform. Four cDNA libraries were prepared generated by RNA-Seq and the number of reads sequenced in each library are shown in Table 3.5.

Table 3.5 – Raw reads of the four cDNA libraries generated by RNA-Seq

Sample	Abbreviation	Total of raw reads
Biofilm A	MA	5,534,577
Biofilm B	MB	4,541,970
Planktonic A	PA	5,259,303
Planktonic B	PB	6,638,596

The only RNA Sequencing analysis presented was the quality control of raw reads. The data processing is the first step in any pipeline for transcriptome analysis. To that aim, we assessed read quality using the FastQC software. All reported reads showed high quality, demonstrated by FastQC analysis. Figure 3.10 presents in the "x" axis the positions of the read bases and in the "y" axis the phred quality scores. Reads on the green section means a good quality (phred > 28); on orange: section a reasonable quality (20 < phred <28); and on red section, a poor quality (phred < 20). A Phred quality score is a measure of the quality of the identification of the nucleobases generated by automated sequencing.

Figure 3.10 – Quality scores across all bases generated by FastQC for forward sequences (R1) and reverse sequences (R2) of the samples MA, MB, PA and PB.

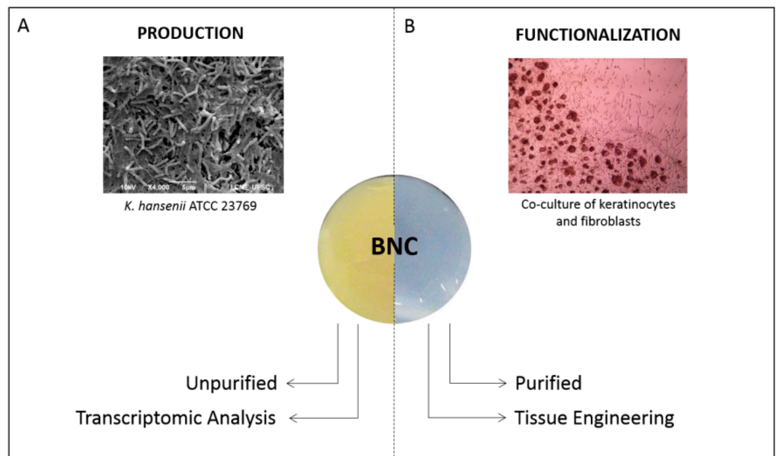


As shown in Figure 3.10, the quality score for all samples were above 28, which means a high-quality of the data sequencing and the efficiency of the developed RNA extraction method to provide samples recommended for RNA-Sequencing.

Although the RNA extraction method developed in this study was performed to extract RNA from bacterial cells living/producing BNC biofilms for RNA-based analysis, we further suggest that the method may have wider applicability for RNA extraction of eukaryotic

cells seeded on BNC biofilms. BNC is a biomaterial with great interest for tissue engineering and the cells interacting with BNC may exhibit distinct patterns of gene expression (BOTTARO; LIEBMANN-VINSON; HEIDARAN, 2002). The importance of obtaining eukaryotic cells' RNA from BNC biofilms is to evaluate global patterns of gene expression to facilitate the formulation of improved biomaterials for tissue engineering applications (Figure 3.11).

Figure 3.11 – *K. hansenii* for BNC production and co-culture of eukaryotic cells for BNC functionalization. (A) Micrograph of *K. hansenii* cells attached to nanocellulose fibers. To obtain RNA from bacterial cells the extraction must be performed with unpurified BNC. (B) Microscopy image of co-culture of keratinocytes and fibroblasts seeded on BNC biofilm, after purification. Co-culture of keratinocytes image gently provided by Pittella, C. Q. P. (Results not published).



BNC has been used as scaffold for 3D *in vitro* tests because presents characteristics that provide an optimal environment for cell culture (KLEMM; KRAMER; MORITZ; et al., 2011; SASKA; TEIXEIRA; TAMBASCO DE OLIVEIRA; et al., 2012). For instance, BNC has a distinctive nanofibrillar structure that may be a good mimicry to the extracellular matrix, as in a wound healing environment. Wound healing is primarily controlled by the proliferation and migration of keratinocytes and fibroblasts as well as complex interactions between these two types of cell. The interactions between the eukaryotic cells living in BNC biofilms can be used to establish new strategies of BNC functionalization (FRASER; TING; MALLON; et al., 2008;

KINGKAEW; JATUPAIBOON; SANCHAVANAKIT; et al., 2010; VARANI; PERONE; SPAHLINGER; et al., 2007; WANG, ZHENXIANG; WANG; FARHANGFAR; et al., 2012). Thus, the RNA extraction method proposed in this study may be an efficient method to extract RNA from eukaryotic cells seeded in BNC biofilms.

In this chapter, we have developed a method for the isolation of high-quality RNA from *K. hansenii* cells living/producing BNC biofilms and planktonic states. Results revealed that condition (IVc) was the most effective resulting in RNA samples with high concentration. The selection of shaking with metal beads as a disruption technique was essential to guarantee a better efficiency of the phenol-chloroform RNA extraction. This condition involved modification of steps in the conventional phenol-chloroform protocol, compatible with the disruption technique. Additionally, DNase treatment allowed the isolation of RNA without genomic DNA contamination. Moreover, the appropriate extraction system for bacterial cells from BNC biofilms provided RNA samples with high yield and integrity, suitable to obtain accurate and reproducible results for RNA-based analysis.

4 A DEFINED MINIMAL CULTURE MEDIUM FOR BACTERIAL NANOCELLULOSE BIOSYNTHESIS

4.1 INTRODUCTION

The advances in the development of biopolymers have demonstrated their importance and potential for a variety of applications such as wound healing (METCALFE; FERGUSON, 2007; SMITH; MOXON; MORRIS, 2016), body implants (NIAOUNAKIS, 2015b), electronic devices (LEGNANI; VILANI; CALIL; et al., 2008; NIAOUNAKIS, 2015a) and also cosmetics (NIAOUNAKIS, 2015c; SIONKOWSKA; LEWANDOWSKA; PLANECKA; et al., 2014).

The versatility of components that can be added to BNC makes this biomaterial essential to the development of new products with different applications (IGUCHI; YAMANAKA; BUDHIONO, 2000; JOZALA; DE LENCASTRE-NOVAES; LOPES; et al., 2016; ROSS; MAYER; BENZIMAN, 1991). Besides the addition of components to modify properties of BNC, researchers have focused in the increasing of BNC production under static and dynamic cultures (CHAWLA; BAJAJ; SURVASE; et al., 2009; CZAJA; ROMANOVICZ; BROWN, 2004; TORRES; COMMEAUX; TRONCOSO, 2012; VANDAMME; DE BAETS; VANBAELEN; et al., 1998) and also altering carbon and nitrogen sources (KESHK, S.M.A.S.; SAMESHIMA, 2005; MIKKELSEN, D.; FLANAGAN; DYKES; et al., 2009; RAMANA; TOMAR; SINGH, 2000; TYAGI; SURESH, 2016). Since BNC is an attractive biomaterial for several applications (JOZALA; DE LENCASTRE-NOVAES; LOPES; et al., 2016; KESHK, SHERIF MAS, 2014) an optimized process to produce BNC is desired. This may be undertaken by choosing an appropriate combination of carbon and nitrogen source in the bacterium culture medium, which generates a large number of combinations related to the amount of bacterium nutrients in the culture medium, mainly limitation/excess of nitrogen and carbon sources (MIKKELSEN, D.; FLANAGAN; DYKES; et al., 2009; RUKA; SIMON; DEAN, 2012; VALEPYN; BEREZINA; PAQUOT, 2012).

Thus, one important aspect in BNC production is to develop a defined culture medium. Here, “defined” means to control the total amount of carbon and nitrogen sources allowing us to understand their influence in the BNC synthesis (JOZALA; DE LENCASTRE-

NOVAES; LOPES; et al., 2016; VAZQUEZ; FORESTI; CERRUTTI; et al., 2013). Carbon and nitrogen sources supplied as nutrients to bacterium growth are pivotal to determine BNC yields (RAMANA; TOMAR; SINGH, 2000; SURESH KUMAR; MODY; JHA, 2007).

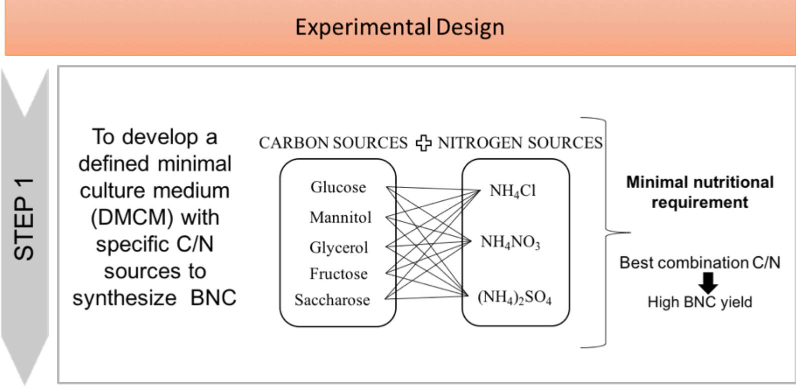
The physiological properties that enable *K. hansenii* to synthesize BNC are not well understood. Transcriptional profiling and genetic analysis can also be used to characterize metabolic pathways and transport systems that are differentially expressed during growth in different media. The comparative transcriptome analysis during *K. hansenii* growth in complex medium and a defined minimal medium can provide important baseline data on the genetic coordination of metabolism in this organism.

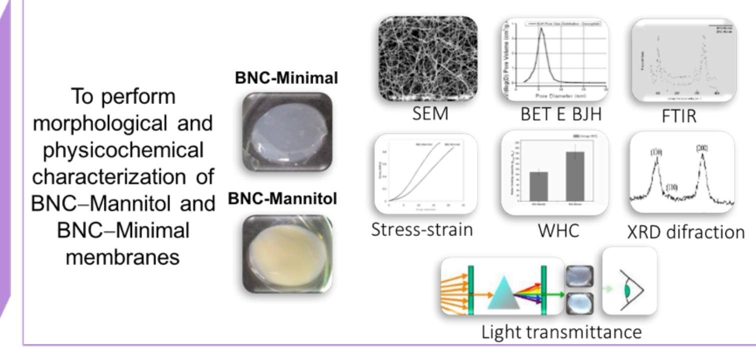
In this chapter, we evaluate the capability of *K. hansenii* ATCC 23769 bacterium to synthesize nanocellulose (BNC) in a variety of controllable chemical conditions through the modulation of carbon and nitrogen concentrations and sources. Different carbon and nitrogen sources were tested, varying their concentrations, as components of a defined minimal culture medium (DMCM) to grow *K. hansenii*. Monosaccharides (glucose and fructose), a disaccharide (saccharose) and sugar alcohols (glycerol and mannitol) were the carbon sources investigated, which were combined with three nitrogen sources separately, ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3) and ammonium sulfate ($(NH_4)_2SO_4$). We aim to understand the bacterium metabolism so one can use experimental data to simulate *in silico* metabolism of *K. hansenii* to obtain optimized parameters to synthesize BNC. The morphological and physicochemical characterization of BNC−Minimal membranes were compared to the usual matrix (BNC−Mannitol) to evaluate how DMCM will influence the properties of BNC−Minimal membranes.

4.2 MATERIALS AND METHODS

Chapter 4 comprises two main steps, as shown in Figure 4.1. The first step was the development of a defined minimal culture medium (DMCM) in order to evaluate the capability of *Komagataeibacter hansenii* to synthesize BNC in a variety of controllable chemical conditions through the modulation of carbon and nitrogen concentrations and sources. In the second step, only the BNC membranes produced in DMCM supplemented with the best combination and concentration of C/N sources were characterized. Morphological and physicochemical characterization were performed to compare the BNC–Minimal membranes with those produced in the complex mannitol medium (BNC–Mannitol).

Figure 4.1 – The two steps experimental design for the development of defined minimal medium and characterization of the BNC membranes.



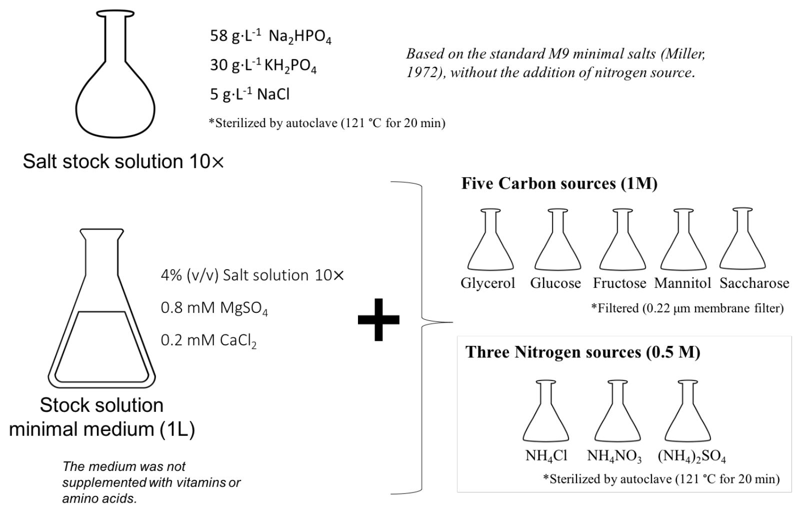


4.2.1 Defined minimal culture medium (DMCM)

A defined minimal medium was prepared as follows: 4% (v/v) of salt solution 10× containing (58.0 $g \cdot L^{-1}$ Na_2HPO_4, 30.0 $g \cdot L^{-1}$ KH_2PO_4 and 5.0 $g \cdot L^{-1}$ NaCl). The composition of the salt solution was based on the standard M9 minimal salts (MILLER, 1972), without the addition of nitrogen source. Solutions of 1 M $MgSO_4$ and 0.5 M $CaCl_2$ were prepared separately and autoclaved at 121 °C for 20 minutes. A stock solution of minimal medium was prepared adding 0.2 mM of $CaCl_2$ and 0.8 mM of $MgSO_4$ to the final volume. The medium was not supplemented with vitamins or amino acids. The medium pH was adjusted to 6.5. Different carbon and nitrogen sources were tested, varying their concentrations as components of DMCM. Each of the following carbon sources (1 M): glucose, saccharose, glycerol, fructose and mannitol were tested in combination with different nitrogen sources (0.5 M): NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$. The carbon sources were prepared and filtered using a sterile 0.22 µm membrane filter and the nitrogen sources were sterilized by autoclave (121 °C for 20 min) (Figure 4.2).

The choice of the carbon sources concentrations to be tested in the defined minimal culture medium was based on the carbon source concentration used in the complex medium. Mannitol medium (ATCC® 23769), one of the complex media used to cultivate *K. hansenii*, generally contains 25.0 $g \cdot L^{-1}$ of carbon source (concentration of 137 mM). From this carbon concentration, a range of carbon sources was selected to be tested in different concentrations. Once the nitrogen source concentrations were undetermined in commonly complex media, we tested a relative carbon and nitrogen sources concentrations (C/N) at ratio of 2.5. In our previous studies with the core metabolic model, we tested the relative ratio (SOUZA, 2014; SOUZA; CASTRO; PORTO, 2017).

Figure 4.2 – Development of a defined minimal culture medium (DMCM).



The molar concentrations of both carbon and nitrogen sources indicated by the four tested conditions (i, ii, iii and iv), as shown in Table 4.1. The carbon sources were also expressed on the C-molar basis concentration.

Table 4.1 – Four tested conditions in defined minimal culture medium; five carbon sources were tested (glycerol, glucose, fructose, mannitol, and saccharose) and balanced as C-molar concentration. NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$ were the nitrogen sources combined separately with each carbon source in the tested conditions.

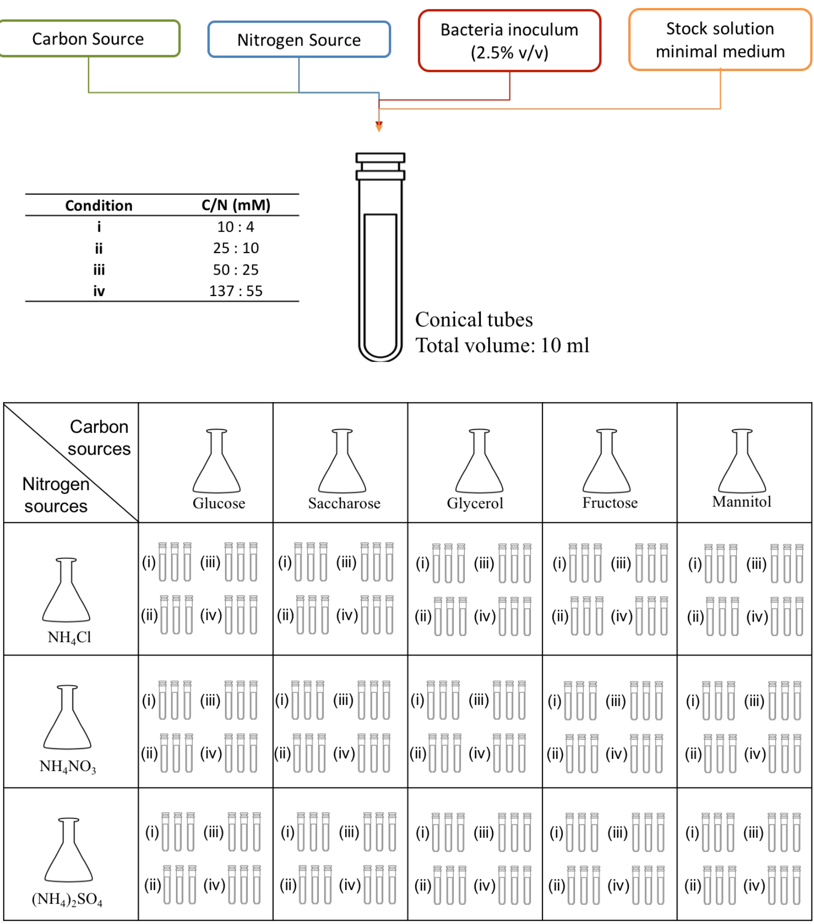
Test Condition	Carbon source concentration (mM)	Carbon source concentration (C-mol)			Nitrogen source concentration (mM)
		Glycerol	Glucose Fructose Mannitol	Saccharose	NH_4Cl or NH_4NO_3 or $(NH_4)_2SO_4$
i	10	0.030	0.060	0.120	4
ii	25	0.075	0.150	0.300	10
iii	50	0.150	0.300	0.600	20
iv	137	0.411	0.822	1.644	55

4.2.2 BNC−Minimal membranes synthesis

Bacteria colonies were randomly selected from the agar plates and suspended to the desired starting optical density (OD = 1) at 660 nm in DMCM using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

K. hansenii was incubated in conical tubes containing a total volume of 10 ml (working volume for the experiments), with 2.5% v/v of bacteria inoculum, each of carbon and nitrogen sources based on the concentrations of the conditions i to iv, and completed the volume with the stock solution of minimal medium. Thus, the experiment consisted of the five carbon sources combined separately with each of the three nitrogen sources in the four distinct concentration conditions (i, ii, iii, iv). For each condition three tubes were prepared and one of them was not added the inoculum corresponding to the control. In this way, it was possible to evaluate the influence of the different sources of C and N and their concentrations in DMCM (Figure 4.3).

Figure 4.3 – Determination of carbon and nitrogen sources concentration in DMCM during the experiment with all combinations in the four tested conditions.

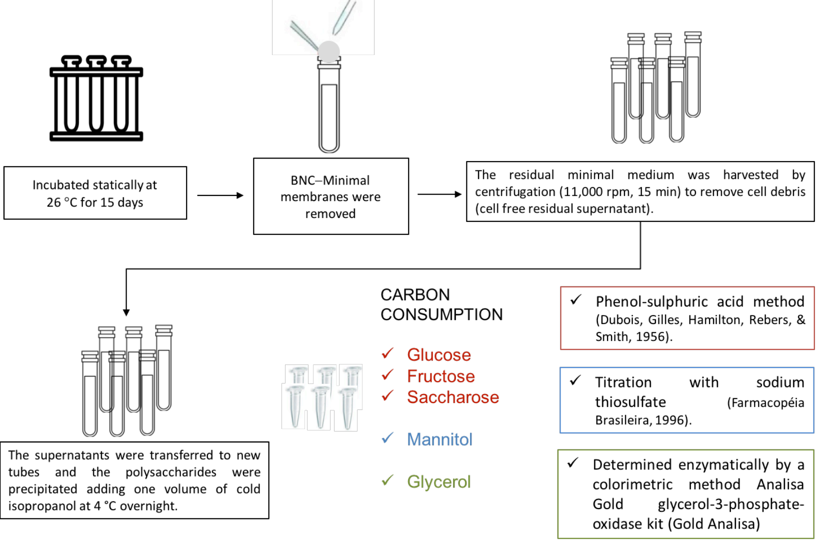


After fifteen days of incubation, BNC–Minimal membranes produced under static conditions at 26 °C were carefully removed, purified with 0.1 M NaOH solution at 50 °C for 24 h and finally rinsed with distilled water to pH 6.5.

4.2.2.1 Quantification of carbon consumption in the residual defined minimal medium

The residual minimal medium from the conical tubes were used to determine the quantification of carbon consumption (Figure 4.4). The residual minimal medium was harvested by centrifugation (11,000 rpm, 15 min) to remove cell debris (cell free residual supernatant). The supernatants were transferred to new tubes and the polysaccharides presented in the supernatant were precipitated adding one volume of cold isopropanol at 4 °C overnight (AZEREDO; OLIVEIRA, 1996; VALEPYN; BEREZINA; PAQUOT, 2012). Then, the upper phase of the residual medium was used to quantify the carbon consumption. Glucose, fructose, and saccharose were measured by the phenol-sulphuric acid method, a colorimetric method to determine total carbohydrates in a sample (DUBOIS; GILLES; HAMILTON; et al., 1956). Mannitol was measured by titration with sodium thiosulfate ("Farmacopéia Brasileira", 1996). Glycerol was determined enzymatically by a colorimetric method using Analisa Gold glycerol-3-phosphate-oxidase kit (Gold Analisa - #MS 80022230062). The calibration curves from standards for all carbon sources were generated to evaluate the concentrations.

Figure 4.4 – Steps during the quantification of carbon consumption in the residual minimal medium.



4.2.2.2 BNC–Minimal membranes dry weight and yield

BNC–Minimal membranes were frozen at -80 °C for 24 h and freeze-dried for 24 hours to determine their dry weight. BNC production yields were calculated based on the dry weight of BNC membranes and the carbon consumption to identify which combination synthesizes more BNC based on the consumed carbon.

The yield of the biosynthesis process was calculated by the expression below:

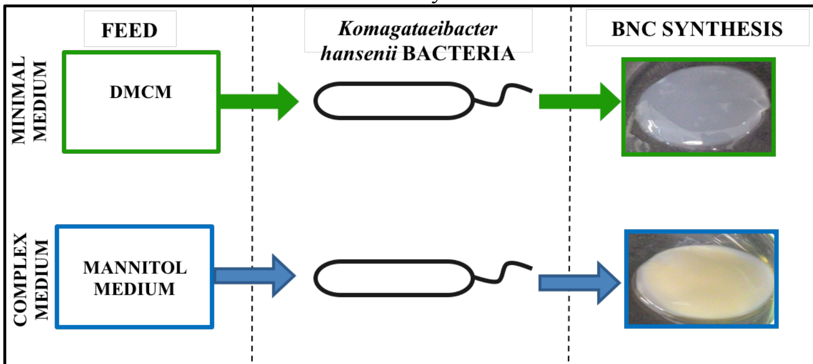
$$BNC_{yield} = \frac{gDW_{BNC}}{C_0\text{-}C_f}$$

where, g DW_{BNC} is the dry weight of bacterial nanocellulose (g), C_0 is the initial concentration (C-mol·L^{-1}) and C_f is the carbon source final concentration (C-mol·L^{-1}).

4.2.3 BNC–Mannitol and BNC–Minimal membranes synthesis

The membranes synthesized in the defined minimal culture medium (BNC–Minimal) with the highest yield were characterized aiming to analyze their morphological and physicochemical properties compared to the membranes synthesized in the complex mannitol medium (BNC–Mannitol) (Figure 4.5).

Figure 4.5 – Two culture media for *K. hansenii* growth: defined minimal culture medium and mannitol medium to synthesize bionanocellulose.



Mannitol medium (ATCC® 23769) was prepared as follows: 25.0 $g \cdot L^{-1}$ mannitol, 5.0 $g \cdot L^{-1}$ yeast extract and 3.0 $g \cdot L^{-1}$ bactopeptone. The medium was adjusted to pH 6.5 with 0.1 M NaOH solution before sterilization by autoclaving (121 °C for 20 min).

K. hansenii bacteria were inoculated on mannitol agar plates that were incubated at 26 °C for seven days. Bacteria colonies were randomly selected and suspended to the desired starting optical density (OD = 1) at 660 nm in Mannitol-based and DMCM supplemented with the best combination of C and N sources. Subsequently, 2.5% v/v of bacteria inoculum was added in the specific medium and transferred to 24 wells plates (2 mL/well) and 96 wells plates (0.2 mL/well) for BNC membrane production. *K. hansenii* bacteria were incubated for fifteen days under static conditions at 26 °C to produce BNC−Mannitol and BNC−Minimal membranes. The membranes were carefully removed from culture plates, purified with 0.1 M NaOH solution at 50 °C for 24 h and finally rinsed with distilled water to pH 6.5.

4.2.4 Statistical analysis

Statistical analysis of differences among BNC yields were performed in R (version 3.2.3) through independent *Student* "t-test" function using 95% confidence interval. *P*-values < 0.05 were considered significant. All experiments were performed in duplicate. Results were expressed as mean ± standard error using "ggplot2" package to plot results in graphical formats.

4.2.5 Characterization of BNC membranes

4.2.5.1 Microstructural characterization of BNC membranes

Scanning electron microscopy (SEM) was performed to analyze the microstructure of BNC–Mannitol and BNC–Minimal membranes surfaces. BNC membranes were characterized using a JEOL JSM–6390LV microscope operated at 10 kV. Prior to the analysis, the samples were dried by critical point drying (CPD), cut into small pieces and coated with a thin layer of sputtered gold.

4.2.5.2. Surface area and pore size analysis

BNC–Mannitol and BNC–Minimal membranes nitrogen adsorption–desorption at 77 K was conducted with a liquid nitrogen trap according to the principle of static volumetric method by a Quantachrome NovaWin version 10.01. The surface area (SA) was calculated by Brunauer–Emmett–Teller (BET) model and pore volume (PV) and distribution pore diameter (PD) were calculated by Barrett–Joyner–Halenda (BJH) model.

4.2.5.3 Water holding capacity (WHC)

The water holding capacity (WHC) was determined by the immersion of BNC in distilled water followed by the weighting of BNC membranes. BNC membranes were frozen at -80 °C for 24 h and lyophilized to determine their dry weight. An average of five samples was considered. The WHC was calculated by the following formula (SHEZAD; KHAN; KHAN; et al., 2010):

$$WHC = \frac{\text{Mass of water removed during drying (g)}}{\text{Dry weight of BNC membranes (g)}}$$

4.2.5.4 X–ray diffraction (XRD)

XRD diffraction spectra of freeze–dried samples were collected on a Philips diffractometer (model X'Pert) with CuKα radiation (λ = 0.154 nm). Measurements were made over a 2θ interval of 5° to 40° with 1°/min steps. The crystallinity index (CrI) was calculated from the reflected intensity data using Segal method (SEGAL; CREELY; MARTIN; et al., 1959) according to the equation below:

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100$$

In Segal equation, CrI expresses the relative degree of crystallinity, I_{002} is the maximum peak intensity (in arbitrary units) of the 002 lattice diffraction and I_{am} is the intensity of diffraction in the same units at $2\theta = 18°$.

4.2.5.5 Mechanical properties measurement

BNC–Mannitol and BNC–Minimal membranes were submitted to tensile tests on a Stable Micro Systems Texture Analyser (Model– TA– HDplus) using a 500 N load cell at room temperature and humidity conditions, operating at a deformation rate of 1 mm/s. To perform tensile test, samples with dimension of 92 × 14 × 0.5 (mm) were used. Young´s modulus (E), tensile strength (σ) and elongation at break (ε_b) were determined from the stress/strain curve. Three specimens were analyzed for each sample group.

4.2.5.6 Light transmittance

The transparency of BNC–Mannitol and BNC–Minimal membranes were examined by light transmittance within the visible range (300–700 nm) using a SpectraMax 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The light transmittance was evaluated as function of the wavelength of the BNC membranes. If the light is transmitted without absorption, it means a 100% transmittance or absorbance equals to zero which corresponds to a totally transparent material.

4.2.5.7 Chemical characterization of BNC membranes

The freeze–dried samples were analyzed by Fourier transformed infrared spectroscopy (FTIR) performed in an Agilent Technology Instrument (model Cary 600), with a resolution of 4 cm^{-1}, in the range of 4000–600 cm^{-1}, using attenuated total reflectance.

4.2.5.8 Statistical analysis

Statistical analysis was performed by the one–way analysis of variance (ANOVA) method with Tukey test. $P < 0.05$ was considered significant. Results were expressed as mean ± standard error. All experiments were run in triplicate at two separated times.

4.3 RESULTS AND DISCUSSION

4.3.1 Effects of carbon and nitrogen sources on BNC-Minimal production yield

The four tested conditions (i, ii, iii and iv) were compared based on the dry weight of BNC membranes and the quantification of each carbon consumption, to determine which combination produced the highest BNC production yield (Fig. 4.6). Figure 4.6 shows the BNC production yields related to the C-mol concentration of the carbon sources used in combination with the nitrogen source tested, NH_4Cl (Fig. 4.6 A), NH_4NO_3 (Fig. 4.6 B) and $(NH_4)_2SO_4$ (Fig. 4.6 C).

Our results revealed that when the highest concentration of carbon and nitrogen sources were tested, conditions (iii) and (iv), a decrease of BNC production yields were observed (Fig. 4.7). These findings indicated that BNC production under strict limitation of nutrients but with high concentrations of carbon and nitrogen sources were not favorable to obtain high yield of BNC.

Similar results were performed by Masaoka et al. (1993) when they investigated the production of BNC by *A. xylinum* IFO 13693 under different concentrations of glucose. Their results showed that BNC yield was higher at initial glucose concentration than with high concentration of glucose (MASAOKA; OHE; SAKOTA, 1993). Son et al. (2003) investigated the BNC production by *Acetobacter* sp. V6 and the yield of BNC production was enhanced when the amounts of carbon source used were up to 1.5% (v/v), but a decrease in BNC production was observed when they used more than 2% glucose (v/v) (SON; KIM; KIM; et al., 2003).

High concentrations of carbon source tend to drastically reduce the pH of the bacterial culture medium, which consequently introduces unfavorable culture conditions for bacteria culture interfering directly in the production of nanocellulose. These conditions showed the pH values around 4 to 4.5. Those alterations in the pH of the bacteria culture medium occur mainly due to the increase in the amount of secondary metabolites produced as product of bacteria metabolism, such as gluconic acid (HWANG; HWANG; et al., 1999; ISHIHARA; MATSUNAGA; HAYASHI; et al., 2002; LIU; ZHONG; ZHANG; et al., 2016).

Figure 4.6 – Bacterial nanocellulose (BNC) production yields (g DW_{BNC}·C-mol^{-1}·L) in a defined minimal culture medium (DMCM) as a function of different initial carbon concentration (C_o, in C-mol·L^{-1}), while keeping constant the carbon/nitrogen ratio at 2.5 (See Table 4.1). Nitrogen sources were varied: (A) ammonium chloride, NH_4Cl; (B) ammonium nitrate, NH_4NO_3; (C) ammonium sulfate, $(NH_4)_2SO_4$.

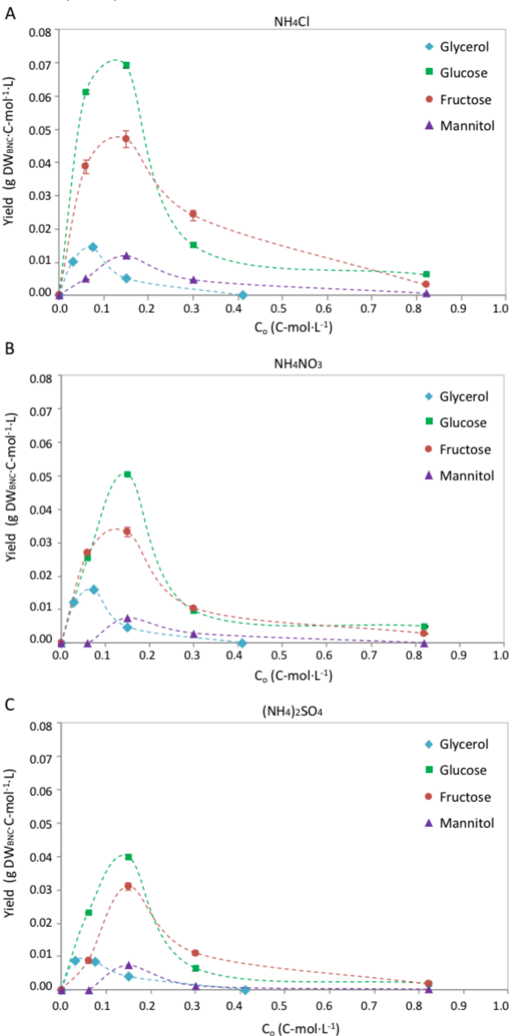
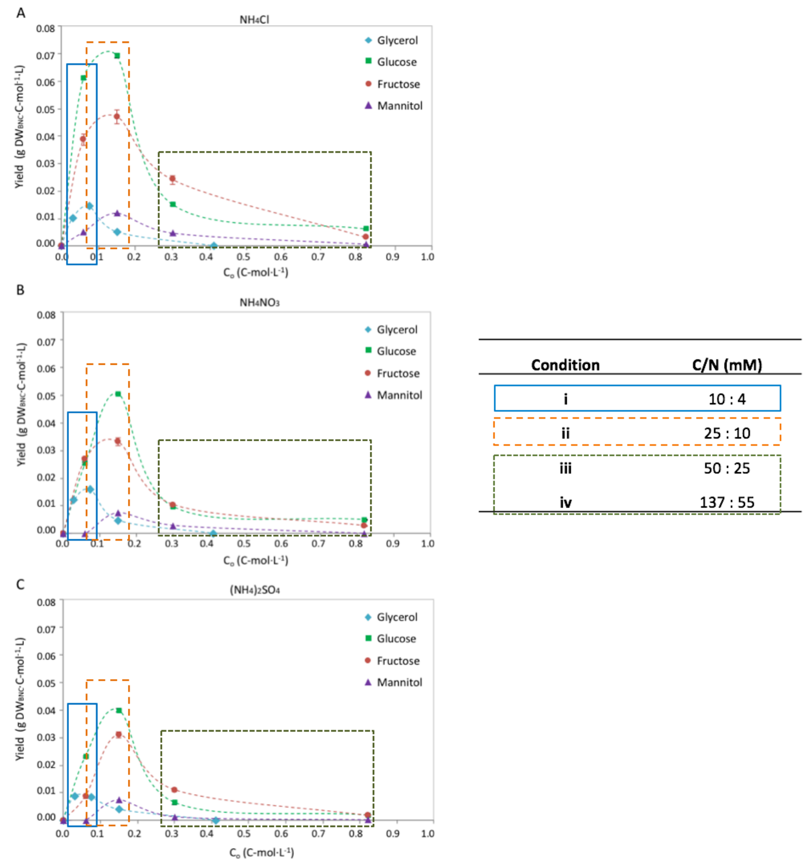


Figure 4.7 – Bacterial nanocellulose production yields with the highlights for each condition tested.



Our results revealed that the concentrations of carbon and nitrogen sources were extremely important to produce high yields of BNC using a defined culture minimal medium. Condition (ii) revealed high BNC production yields with all combinations of carbon and nitrogen sources. On the other hand, with a reduction of the concentration of carbon and nitrogen in DMCM, referred as condition (i) the BNC production yields decreased for all tested combinations compared to condition (ii) (Figure 4.7).

Defined minimal culture medium supplemented with glucose as carbon source in combination with each nitrogen source presented higher BNC production yield than the other carbon sources tested.

Glycerol and mannitol were classified as weak substrates compared to glucose considering the amount of nanocellulose synthesized. Defined minimal medium using fructose allowed the synthesis of BNC, but the BNC production yield was lower than glucose as carbon source. *K. hansenii* bacteria did not produce BNC in all tested conditions that involved saccharose as carbon source. Metabolically, saccharose needs to be hydrolyzed into glucose and fructose in the bacteria periplasm (ROSS; MAYER; BENZIMAN, 1991; VELASCO-BEDRÁN; LÓPEZ-ISUNZA, 2007) to be consumed as carbon source. Mikkelsen et al. (2009) reported that relatively low concentrations of BNC were produced by *K. xylinus* ATCC 53524 when saccharose was used as the sole carbon source (MIKKELSEN, D.; FLANAGAN; DYKES; et al., 2009). These findings corroborate our results although their investigation were performed in HS medium by *K. xylinus* ATCC 53524 and not with *K. hansenii* ATCC 23769 in defined culture minimal medium. Contrary to saccharose, glucose is easily transported through the cell membrane and incorporated into the nanocellulose biosynthetic pathway (OIKAWA; OHTORI; AMEYAMA, 1995; ROSS; MAYER; BENZIMAN, 1991). Mannitol is known to be converted to fructose, and then metabolized to produce BNC. (OIKAWA; OHTORI; AMEYAMA, 1995; ROSS; MAYER; BENZIMAN, 1991). Glycerol, a three-carbon sugar, on the other hand, is introduced into metabolic pathways at the triose phosphate level. The oxidation of triose phosphate is a primary reaction in *K. hansenii* ATCC 23769 for the channeling of sugar carbon from the pentose phosphate pathway into the tricarboxylic acid cycle (TCA cycle). Table 4.2 shows the yield of BNC production by *K. hansenii* cultured in a defined minimal culture medium using glycerol, glucose, fructose and mannitol combined with three nitrogen sources separately in the four tested conditions.

Table 4.2 – BNC production yields using glycerol, glucose, mannitol and fructose for varying nitrogen sources and concentrations, expressed as mean ± standard error.

			Yield (g DW_{BNC}·C-mol^{-1}·L)		
Test condition	**C: N sources concentration (mM)**	**Carbon source**	**NH_4Cl**	**NH_4NO_3**	**$(NH_4)_2SO_4$**
i	10 : 4	Glycerol	0.0101 ± 0.0001	0.0121 ± 0.0002	0.0087 ± 0.0002
		Glucose	0.0612 ± 0.0007	0.0254 ± 0.0004	0.0231 ± 0.0004
		Fructose	0.0387± 0.0021	0.0269 ± 0.0004	0.0086 ± 0.0006
		Mannitol	0.0050 ± 0.0001	0.0000 ± 0.0000	0.0000 ± 0.0000
ii	25 : 10	Glycerol	0.0154 ± 0.0004	0.0158 ± 0.0001	0.0085± 0.0001
		Glucose	0.0717 ± 0.0043	0.0506 ± 0.0001	0.0399 ± 0.0006
		Fructose	0.0470 ± 0.0025	0.0332 ± 0.0015	0.0311 ± 0.0011
		Mannitol	0.0119 ± 0.0001	0.0075 ± 0.0001	0.0075 ± 0.0001
iii	50 : 20	Glycerol	0.0051 ± 0.0001	0.0048± 0.0005	0.0040 ± 0.0001
		Glucose	0.0151 ± 0.0005	0.0097 ± 0.0004	0.0064 ± 0.0007
		Fructose	0.0241 ± 0.0014	0.0105± 0.0002	0.0111 ± 0.0006
		Mannitol	0.0046 ± 0.0001	0.0029 ± 0.0001	0.0013 ± 0.0001
iv	137 : 55	Glycerol	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
		Glucose	0.0061 ± 0.0002	0.0050 ± 0.0001	0.0019 ± 0.0001
		Fructose	0.0030 ± 0.0001	0.0028 ± 0.0002	0.0017 ± 0.0002
		Mannitol	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000

Our results revealed that the concentrations of carbon and nitrogen sources were extremely important to produce high yields of BNC using a defined culture minimal medium. Condition (ii) presented statistical differences ($p < 0.05$) when BNC production yields where compared to all combinations of carbon sources tested and each nitrogen source separately (Table 4.2). When ammonium chloride (NH_4Cl) was combined to the five carbon sources tested different yields of BNC production were obtained. By changing the nitrogen source to ammonium nitrate (NH_4NO_3) and ammonium sulfate ($(NH_4)_2SO_4$) the BNC production yields decreases.

DMCM containing 25 mM of glucose combined with 10 mM NH_4Cl increased BNC production yield 1.5 and 1.8 times compared to NH_4NO_3 and $((NH_4)_2SO_4)$, respectively. Our results showed that 25 mM of glucose combined with 10 mM of NH_4Cl in the defined culture minimal medium was identified as the best combination of C and N sources based on the highest yield of BNC production observed (0.0717 ± 0.0043 g DW_{BNC}·C-mol^{-1}·L). Fructose, glycerol and mannitol combined with NH_4Cl in condition (ii) decreased the BNC production yields 1.5, 4.5 and 6 times compared to glucose, respectively. Several studies reported the effects of carbon sources on BNC production (KESHK, S.M.A.S.; SAMESHIMA, 2005; MIKKELSEN, D.; FLANAGAN; DYKES; et al., 2009; RUKA; SIMON; DEAN, 2012), but only a few studies evaluated the combination of carbon and nitrogen sources for BNC production (RAMANA; TOMAR; SINGH, 2000; YIM; SONG; KIM, 2016). Yeast extract and peptone, which are the basic components of the complex medium developed by Hestrin and Schramm are the preferred nitrogen sources for bacteria culture media (JOZALA; DE LENCASTRE-NOVAES; LOPES; et al., 2016; KRALISCH; HESSLER, 2012). However, the use of complex components in a culture medium does not allow us to determine the influence of carbon and nitrogen sources in the bacteria metabolism.

We elaborated a defined culture minimal medium seeking for more bacteria data information, conditions to obtain high yields of BNC and also to understand the metabolic pathway of BNC producer bacteria in terms of carbon consumption. The rate of carbon consumption in condition (ii) with NH_4Cl as nitrogen source combined with each carbon source is shown in Table 4.3.

Table 4.3 – Carbon consumption and BNC production yields using glycerol, glucose, mannitol and fructose in condition (ii) with NH_4Cl as nitrogen source.

Test condition	C:N sources concentration (mM)	Carbon source	Yield (g DW_{BNC}·C-mol^{-1}·L)	Carbon consumption (%)
ii	25 : 10	Glycerol	0.0154 ± 0.0004	66.5
		Glucose	0.0717 ± 0.0043	89.5
		Mannitol	0.0119 ± 0.0001	58.2
		Fructose	0.0470 ± 0.0025	42.1
		Saccharose	0.0000 ± 0.0000	83.2

The BNC production yields was based on the dry weight of BNC membranes and the carbon consumption. Our results showed a high concentration of consumed glucose (89.5%) in condition (ii) compared to the other carbon sources. Saccharose was consumed almost completely, but metabolically was not sufficient to produce BNC in the DMCM. In other words, 25 mM of glucose and 10 mM of NH_4Cl were appropriate concentration of nutrients with respect to efficient C and N metabolism in order to synthesize BNC. The combination of glucose with NH_4Cl and the particular concentrations were extremely important to obtain the highest BNC production yield using the developed defined culture minimal medium.

To date, many studies have tested several carbon and nitrogen sources aiming to increase BNC yields and decrease production costs. However, in general, they still use complex components, such as yeast extract and peptone as nitrogen sources; and also modifying HS medium through the modulation of carbon source, such as mannitol, glycerol, fructose, sucrose or galactose (MASAOKA; OHE; SAKOTA, 1993b; MIKKELSEN, D.; FLANAGAN; DYKES; et al., 2009; ROSS; MAYER; BENZIMAN, 1991a; SUWANPOSRI; YUKPHAN; YAMADA; et al., 2013). Kurosumi and coworkers (2009) investigated the use of fruit juices as carbon source in HS medium by *Acetobacter xylinum* NBRC 13693. They revealed an increase of BNC yield (KUROSUMI; SASAKI; YAMASHITA; et al., 2009) but the unknown culture media composition is not an interesting strategy to understand bacterium metabolism. In the same context, Gomes and coworkers (2013) investigated the use of residues from the olive oil production industry as nutrient and for the production of BNC by *Gluconacetobacter sacchari* in HS medium (GOMES; SILVA; TROVATTI; et al., 2013). All those studies found that non-conventional

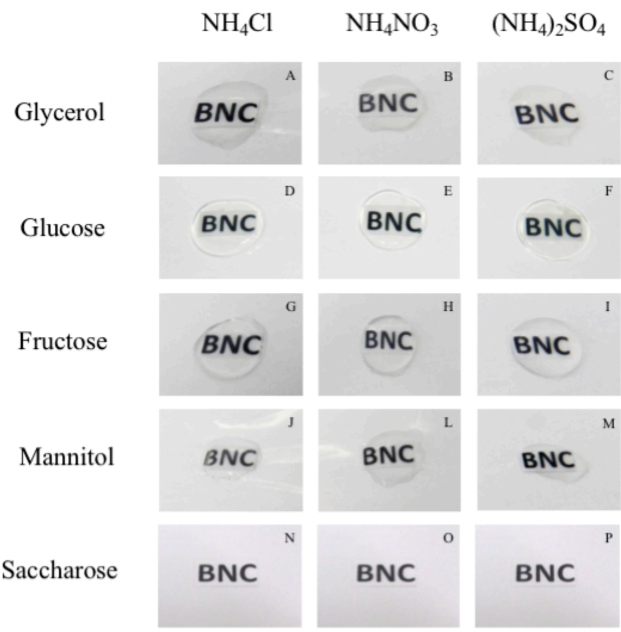
components (industrial and agroindustrial residues) have great potential for BNC production. However, further studies are necessary to investigate the influence of those components in the bacteria metabolism to obtain BNC with distinct properties (CASTRO, CRISTINA; ZULUAGA; PUTAUX; et al., 2011; DAHMAN; JAYASURIYA; KALIS, 2010; KONGRUANG, 2008). Although these studies showed a decrease of the total cost of BNC production, the exact composition of the medium cannot be determined, which makes it difficult to maintain reproducibility. Also, in terms of BNC production, there are several parameters that must be considered, such as: type of strain (passage, species and amount), composition of the bacterial culture medium (minimal or complex medium) and operational conditions (static or dynamic cultures, temperature, oxygen and pH) (JOZALA; DE LENCASTRE-NOVAES; LOPES; et al., 2016; KESHK, S.M.A.S.; SAMESHIMA, 2005; RUKA; SIMON; DEAN, 2012; VALEPYN; BEREZINA; PAQUOT, 2012).

There are no evidences in the literature that supports the relationship between BNC-producing bacteria and different types and amount of carbon and nitrogen sources supplemented in defined culture minimal medium. In this work, we found that 25 mM of glucose combined with and 10 mM of NH_4Cl is an adequate combination and concentration of carbon and nitrogen sources.

4.3.2 BNC−Minimal membranes synthesis

BNC membranes synthesized in condition (ii) presented the highest BNC production yields considering the four conditions tested. Figure 4.8 shows the membranes produced in that condition (25 mM of C and 10 mM of N source) considering all combinations of carbon and nitrogen sources tested.

Figure 4.8 – BNC membranes synthesized in the defined minimal culture medium with all combinations of carbon (glycerol, glucose, fructose, mannitol and saccharose) and nitrogen (NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$) sources tested in condition (ii) (25 mM of carbon source and 10 mM of nitrogen source).



BNC membranes were biosynthesized in all tested conditions, using 25 mM of carbon and 10 mM of nitrogen sources, except when saccharose was used as carbon source (Fig. 4.8 N-P). *K. hansenii* bacteria produced BNC membranes (Fig. 4.8 D-F) with regular shape and higher weight when glucose was used as carbon source. *K. hansenii* bacteria were able to synthesize thin and irregular nanocellulose membranes when glycerol (Fig. 4.8 A-C) and/or fructose (Fig. 4.8 G-I) were used as carbon source. BNC membranes synthesized by *K. hansenii* from the medium supplemented with mannitol were not well-formed (Fig. 4.8 J-M). The highest yield of BNC production was obtained using NH_4Cl as nitrogen source with all combinations of carbon sources. Although some of these carbon sources, such as glycerol and fructose are reported as excellent sources for BNC

production using complex medium (MIKKELSEN, D.; FLANAGAN; DYKES; et al., 2009; RAMANA; TOMAR; SINGH, 2000; RUKA; SIMON; DEAN, 2012), our results showed that the defined minimal medium supplemented with those carbon sources were not efficient for BNC production.

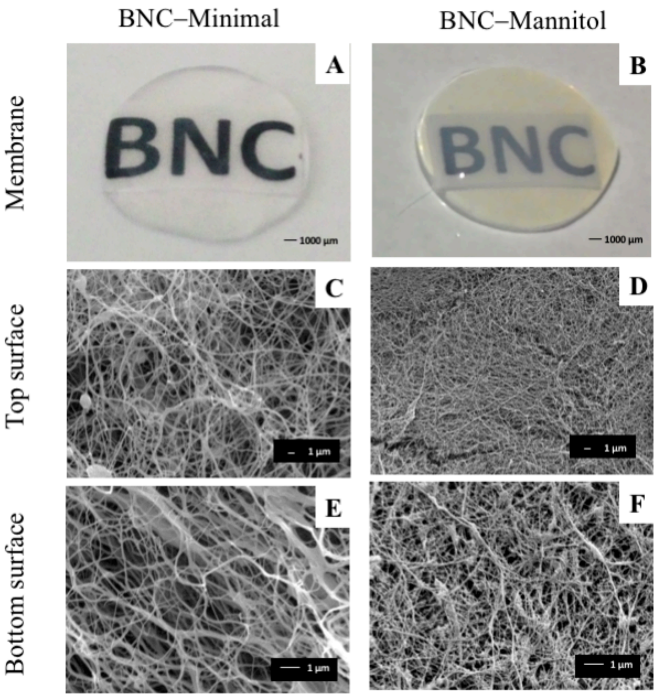
Indeed, we demonstrated that there exists a simple growth requirement for *K. hansenii* to synthesize BNC: a medium composed of seven commercial components, disodium phosphate, monopotassium phosphate, sodium chloride, magnesium sulfate, calcium chloride, ammonium chloride as nitrogen source, and glucose as carbon source. Trace elements may be found on those commercial components.

4.3.3 BNC membranes characterization

BNC–Minimal membranes produced in the defined minimal medium supplemented with 25 mM of glucose and 10 mM of NH_4Cl were characterized and compared with BNC–Mannitol membranes.

BNC–Mannitol and BNC–Minimal membranes were successfully synthesized and characterized considering distinct composition of bacterial culture media. Visual observation (Fig. 4.9 A-B) revealed a higher transparency of BNC membranes produced by Minimal culture medium when compared to those membranes produced by Mannitol culture medium. BNC membranes were microstructurally analyzed by SEM (Fig. 4.9 C–F). BNC membranes microstructures were characterized by a 3D nanofibers network differently organized over the membranes structure. The top surface of BNC–Mannitol membranes were characterized by a dense network of nanofibers (Fig. 4.9 D) and the bottom surface (Fig. 4.9 F) showed a lower density (porous surface), in accordance with previous results published by Berti et al. (BERTI; RAMBO; DIAS; et al., 2013).

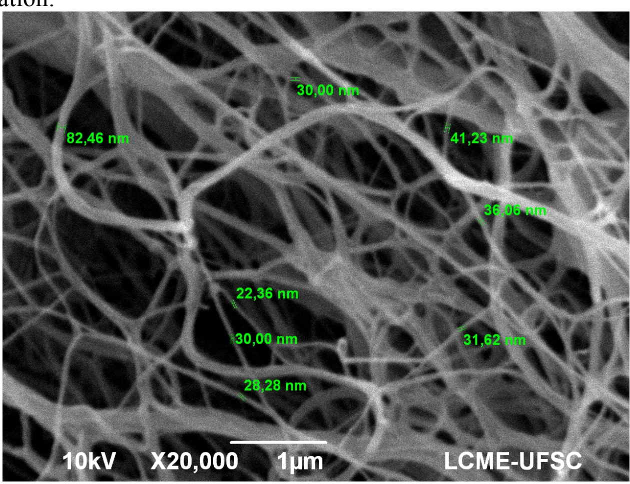
Figure 4.9 – BNC–Minimal (A) and BNC–Mannitol (B) membranes produced in 24 wells plates. SEM micrographs of BNC–Mannitol and BNC–Minimal membranes at 10,000× magnification: (C) Top surface of BNC–Minimal, (D) Top surface of BNC–Mannitol, (E) Bottom surface of BNC–Minimal, and (F) Bottom surface of BNC–Mannitol membranes



Curiously, BNC–Minimal membranes exhibit porous surface on both sides (Fig. 4.9 C-E) of the membranes analyzed. Particularities observed in the nanofibers arrangement over the 3D microstructure were the main characteristic observed when micrographs of BNC–Minimal membrane were compared (Fig. 4.9 C) to BNC–Mannitol membranes (Fig. 4.9 D). The distinct BNC nanofibers arrangement may explain specific transparency of BNC–Minimal membranes produced by *K. hansenii*, since nanofibers organization has been indicated as a major structural factor to define optical properties in biomaterials (CHEN, CHUCHU; LI; DENG; et al., 2012; KOTAKI; LIU; HE, 2006; REDMOND, 2014). In tissue engineering and regenerative medicine applications, biofilms have been highlighted because they hold a highly

porous microstructure which provides an interconnected matrix that allows cell ingrowth (ANNABI; NICHOL; ZHONG; et al., 2010; LOH; CHOONG, 2013). Figure 4.10 shows the arrangement and diameters of the nanofibers in the bottom surface of BNC–Minimal membranes.

Figure 4.10 – Bottom surface of BNC–Minimal membrane at 20,000× magnification.



To the best of our knowledge BNC–Minimal membranes have not been produced, characterized and reported, i.e., never considering this particular bacterial culture medium composition, BNC nanofibers arrangement and visual observation of transparent BNC membranes. Those BNC–Minimal membranes physical characteristics motivated us to explore further their physicochemical properties aiming not only tissue engineering applications but also to obtain more information about those produced transparent membranes.

As expected, BET and BJH results showed that the BNC–Minimal membranes possessed higher pore volume (PV) and surface area (SA) comparable to BNC–Mannitol membranes (Table 4.4). BNC–Mannitol and BNC–Minimal showed a similar pore diameter distribution, ranging from 3.2–31.3 nm to BNC–Mannitol and 3.2–30 nm to BNC–Minimal, respectively. In comparison to BNC–Mannitol, the pore volume of BNC–Minimal increased approximately 44% and 48% on surface area.

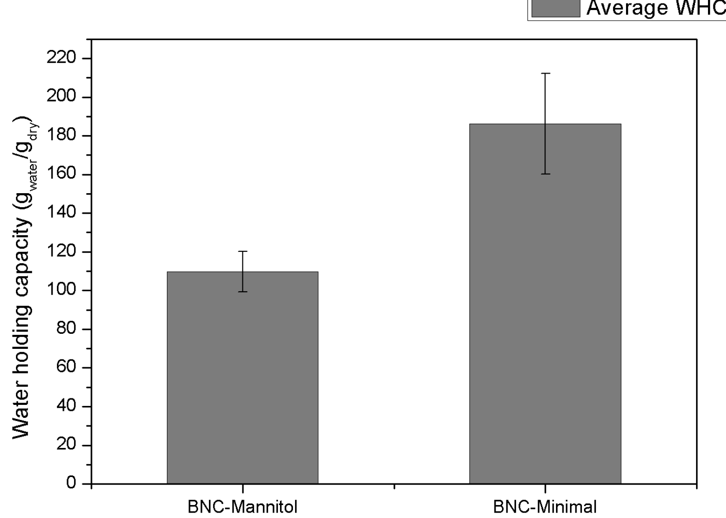
Table 4.4 – Surface area (SA) and pore volume (PV) of BNC–Mannitol and BNC–Minimal membranes

Sample	PV ($cm^3 \cdot g^{-1}$)	SA ($m^2 \cdot g^{-1}$)
BNC–Mannitol	0.293	108.1
BNC–Minimal	0.422	160.6

Important parameters, such as pore size, pore volume, pore distribution, and surface area should be considered in the design of tissue engineering scaffold constructions and also for other applications.

Different water holding capacity was also detected when BNC membranes were compared, BNC–Mannitol versus BNC–Minimal membranes. The WHC for BNC–Mannitol membranes were 111 times their dry weight and the BNC–Minimal membranes absorbed 187 times their dry weight with water (Fig. 4.11).

Figure 4.11 – Water holding capacity (WHC) of BNC–Mannitol and BNC–Minimal membranes.



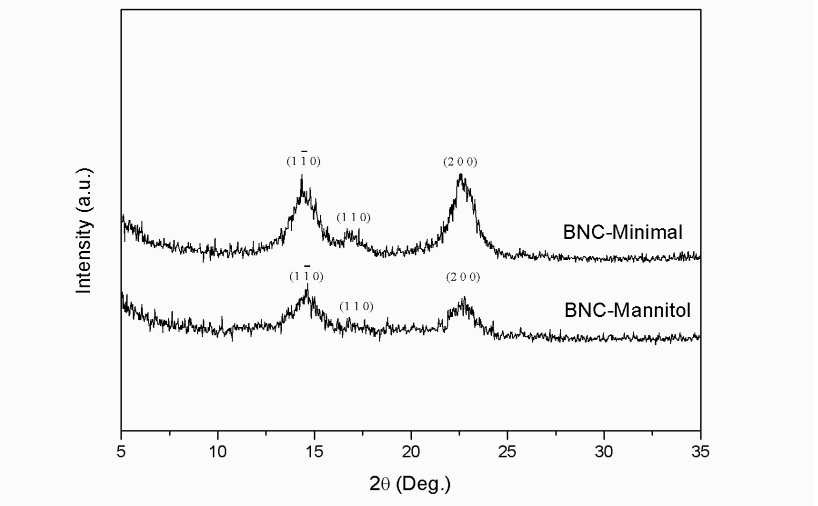
Differences on WHC of BNC membranes can be attributed to their distinct porosity and surface areas. The water molecules are trapped physically on the surface and inside the BNC (WATANABE et al., 1998). BNC–Minimal membranes hold more empty spaces among the BNC nanofibers arrangement, then more water could penetrate and be adsorbed onto those membranes. The results obtained in the present

study showed that WHC of BNC membranes are highly dependent on the surface fiber density of the membranes. WHC has been considered an important property because it is directly involved in the biomedical applications of BNC as a dressing material, for example (KAEWNOPPARAT; SANSERNLUK; FAROONGSARNG, 2008).

4.3.4 Physicochemical properties

Analyzing XRD patterns of the BNC–Mannitol and BNC–Minimal membranes (Fig. 4.12), we observed the presence of three main peaks centered at 14.6°, 16.7° and 22.5° which are attributed to the typical profile of a cellulose I polymorph, as known by the contributions of two distinct crystalline phase organizations, known as I_{α} and I_{β}. (CZAJA; ROMANOVICZ; BROWN, 2004; NIEDUSZYNSKI; PRESTON, 1970).

Figure 4.12 – X–ray diffractograms of BNC–Mannitol and BNC–Minimal membranes. The diffraction peaks for cellulose are labeled.



X-ray diffractograms of BNC–Mannitol and BNC–Minimal membranes revealed that there were no changes in peak intensities located at the crystallographic plane reflections of the analyzed samples which indicate that cellulose I was not transformed into cellulose II polymorph, Figure 4.12. However, BNC–Minimal hold 87% of

crystallinity degree (CrI) while BNC–Mannitol membranes hold 61%, respectively.

In order to investigate the mechanical properties of BNC–Mannitol and BNC–Minimal membranes, samples were submitted to tensile strength analysis. The stress–strain curves were shown in Figure 4.13 and the tensile strength, Young´s modulus and elongation at break of the membranes were shown in Table 4.5.

Figure 4.13 – Stress × strain curves of the BNC–Mannitol and BNC–Minimal membranes. Data representative of one of the triplicates of this analysis.

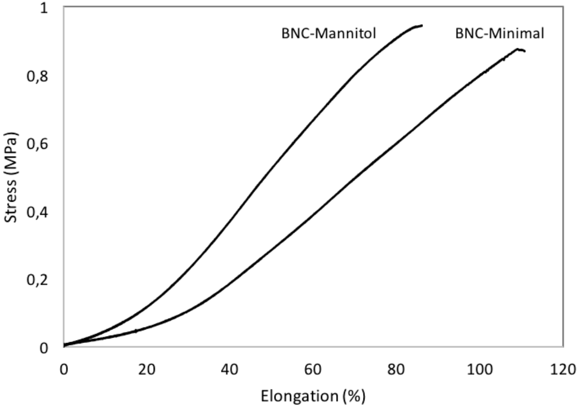


Table 4.5 – Mechanical properties of BNC–Mannitol and BNC–Minimal membranes.

Sample	Tensile strength (MPa)	Young's modulus (kPa)	Elongation (%)
BNC–Mannitol	0.94 ± 0.13	74.37 ± 3.30	80.63 ± 7.58
BNC–Minimal	0.85 ± 0.07	50.11 ± 2.02	111.47 ± 14.50

The tensile strength and elongation tests provided an evaluation of the strength and elasticity of the membranes. The mechanical properties can be correlated to the morphological features of the membranes. It was expected that BNC–Minimal membrane exhibits the lowest tensile strength and Young´s modulus when compared to BNC–Mannitol membranes, due to their surface fiber density. Tensile strength of the BNC–Mannitol and BNC–Minimal were 0.94 ± 0.13

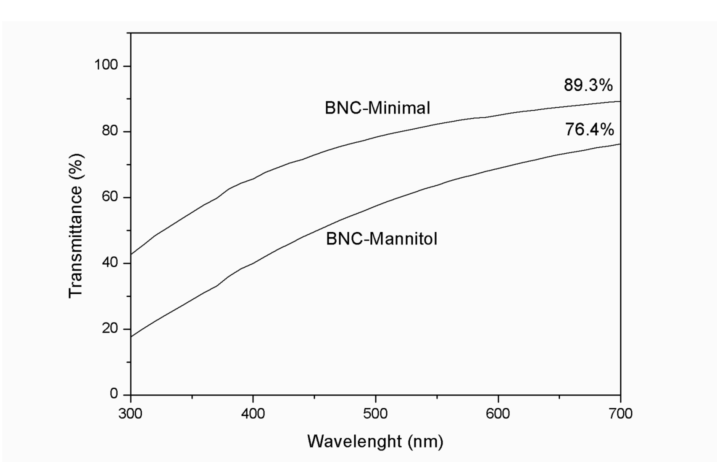
MPa and 0.85 ± 0.07 MPa respectively. As the mechanical properties are closely linked to the microstructure and crystallinity of the material, BNC−Minimal membranes presented unique extensibility and elastic properties, expressed by increasing elongation before breaking, followed by decreasing the Young's modulus and tensile strength of the samples.

BNC−Minimal membranes showed significantly greater elongation (111%, $p < 0.05$) before breaking compared to BNC−Mannitol. The greater elasticity of the BNC−Minimal membranes can be useful in different applications, such as skin and blood vessels substitutes that follow movements and need adequate flexibility. Elasticity of the membrane is important to give flexibility to the cross-linked chains, and to facilitate the movement (ZHU; MARCHANT, 2011). Thus, a compromise between mechanical strength and flexibility is necessary for the appropriate use of the membranes as tissue engineering scaffolds (AHMED, 2015; ZHU; MARCHANT, 2011).

4.3.5 Optically transparent BNC–Minimal membranes

To quantify light transmittance property and transparency, the transmission spectrum of BNC membranes was measured (Fig. 4.14). Light transmittance measurement for BNC–Minimal membranes was 82.3% and for BNC-Mannitol membranes was 63.8%, at 550 nm wavelength. For comparison, a cornea scaffold should present a percentage of light transmission around 80% (at 550 nm) (Beems & Van Best, 1990). BNC–Mannitol membranes achieved 76.4% light transmittance while BNC–Minimal achieved 89.3% considering visible wavelengths (400–700 nm).

Figure 4.14 – Percentage of light transmittance measurements under distinct wavelengths for BNC–Mannitol and BNC–Minimal membranes.



The transmittance of both BNC membranes declined with the decrease of the wavelength, which demonstrated their good absorbing ability for UV lights. The absorbance of UV light is crucial to obtain artificial cornea scaffolds to prevent cornea damage caused by incidence of UV directly to the internal eye tissue (BEEMS; VAN BEST, 1990; WANG, JIEHUA; GAO; ZHANG; et al., 2010).

Besides the light transmittance, the membranes thickness are also critical parameters for those applications. BNC–Mannitol membranes can maintain high transparency during their early growth, because with fewer bacteria incubation time the fibers were distributed unevenly and the membranes formed are still very thin and consequently transparent. If the bacteria incubation time needed had been several days, the light transmittance of those BNC–Mannitol membranes displayed a decrease and subsequently reached a plateau (LUO; ZHANG; XIONG; et al., 2014). However, BNC–Minimal membranes can maintain high transparency even with the increase of their thickness.

Additionally, high porous BNC–Minimal membranes associated with their high transparency property could avoid the background normally present in the investigations of 3D hydrogels-cells microscope analyses for tissue engineering and regenerative medicine (TERM) applications.

Overall, results of the characterization revealed a BNC membrane with distinct properties and optical transparency. FTIR

spectra did not detect any difference between BNC–Mannitol and BNC–Minimal membranes. This evidences that BNC–Minimal membranes synthesized in the Minimal medium are chemically pure and without the addition of another substance. Here, we developed optically transparent BNC membranes using a defined low-cost minimal medium, thereby increasing a wide new range of applications.

Surprisingly, our results revealed that we discovery a cheap and easy pathway to produce a high transparent nanocellulose membranes using the defined minimal culture medium (DMCM). Those transparent nanocellulose membranes have been highlighted for tissue engineering applications, because their microstructure provides an organized matrix that allows cell growth (KHODA; KOC, 2013; ZHU; MARCHANT, 2011). More than that, the lack of optical transparency has limited their widespread use in cell culture which was here surpassed. Cells can be easily visualized using a standard inverted confocal microscope, which is normally difficult to achieve with conventional translucent or opaque membranes (TAM; FISHER; BAKER; et al., 2016; UMMARTYOTIN, S.; JUNTARO, J.; SAIN; MANUSPIYA, 2012). Also, BNC−Minimal membranes can be a better platform for wound healing processes that can be easily monitored without removing the dressing (Figure 4.15) (NOGI; YANO, 2008a; SAITO; SAKURAI; SAKAKIBARA; et al., 2003).

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Figure 4.15 – BNC–Mannitol and BNC–Minimal membranes after purification. BNC–Minimal membranes can maintain high transparency although the time of bacteria incubation takes several days.

Moreover, the transparent nanocellulose membranes are extremely interesting for electronic and optical applications which is confirmed by the number of patents applied in this context (Table 4.6).

Optically transparent biomaterials are essential for several technological applications and the challenge to develop those biomaterials has increased interesting innovative processes to produce them (CHEN, CHUCHU; LI; DENG; et al., 2012; FERNANDES; OLIVEIRA; FREIRE; et al., 2009; OKAHISA; YOSHIDA; MIYAGUCHI; et al., 2009a; RETEGI; ALGAR; MARTIN; et al., 2012; TOME; PINTO; TROVATTI; et al., 2011).

An increasing number of approaches have explored the fabrication of transparent nanobiocomposites based on bacterial nanocellulose (BNC) and renewable biopolymer sources (CHEN, CHUCHU; LI; DENG; et al., 2012; FERNANDES; OLIVEIRA; FREIRE; et al., 2009; NOGI; YANO, 2008a; OKAHISA; YOSHIDA; MIYAGUCHI; et al., 2009a). To date, many studies have been focused on transparent BNC membranes (KLEMM; SCHUMANN; KRAMER;

et al., 2006; NOGI; YANO, 2008b; OKAHISA; YOSHIDA; MIYAGUCHI; et al., 2009b; YANO; SUGIYAMA; NAKAGAITO; et al., 2005). However, the best of our knowledge, there were no studies revealing a BNC membrane with high transparency besides considering BNC in association with other components. In order to achieve optically transparent biomaterials using BNC, all studies and patents reported in the literature improved BNC transparency through the association with two- or more-components, either with resins, with polymers such as nanocomposites based on chitosan/BNC (FERNANDES; OLIVEIRA; FREIRE; et al., 2009), composites of epoxidized soybean oil (ESO)/BNC (RETEGI; ALGAR; MARTIN; et al., 2012), nanocomposites of BNC in a matrix of poly(hydroxyethyl methacrylate) (PHEMA) (DAHMAN; OKTEM, 2012; XINSHENG; WANKEI; J, 2009), poly (lactic acid) (PLA)/BNC (TOME; PINTO; TROVATTI; et al., 2011), poly (vinyl alcohol)/BNC (WANG, JIEHUA; GAO; ZHANG; et al., 2010), nanocomposite films composed of poly-urethane (PU) based resin/BNC (UMMARTYOTIN; JUNTARO; SAIN; et al., 2012), boehmite-siloxane nanoparticles/BNC (BARUD; CAIUT; DEXPERT-GHYS; et al., 2012; CAIUT; BARUD; MESSADDEQ; et al., 2011), and BNC in lithium chloride/N,N-dimethylacetamide solution (YONGJUN; YANG; XIN; et al., 2014). All those studies showed composites with considerable transparency, flexibility and good mechanical properties that can be used as optically functional materials. Other investigations about the development of transparent biomaterials based on BNC have been patented and were described in Table 4.6. The patents described presented composites developed for electronic optical devices in biomedical applications, contact lenses, photovoltaic materials and/or optic components for biosensors (CAIUT; BARUD; MESSADDEQ; et al., 2011; DAGANG; QIAOYUN; YAN, 2013; XINSHENG; WANKEI; J, 2009; YONGJUN; YANG; XIN; et al., 2014).

Table 4.6 – Patents related to transparent biomaterials based on bacterial nanocellulose

PATENT TITLE	NUMBER, YEAR AND APPLICANT
Optically transparent composites based on bacterial cellulose and boehmite, siloxane and/or a boehmite-siloxane system.	Number: WO2012100315 Year: 2011 Applicant: UNIV. ESTADUAL PAULISTA - BRAZIL
Transparent bacterial cellulose nanocomposite biofilms	Number: US20130011385 Year: 2009 Applicant: AXCELON BIOPOLYMERS CORPORATION - CANADA
Transparent reproductive bacterial cellulose reproductive membrane as well as preparation method and application thereof	Number: CN104587516 Year: 2014 Applicant: SHENZHEN INSTITUTE OF ADVANCED TECHNOLOGY, CHINESE ACADEMY OF SCIENCES – CHINA
Bacterial cellulose nanometer optical transparent film preparation method	Number: CN103396569 Year: 2013 Applicant: NANJING FORESTRY UNIVERSITY - CHINA
Bacterial cellulose base optical thin film and preparation method thereof	Number: CN103217727 Year: 2013 Applicant: DONGHUA UNIVERSITY) - CHINA

To achieve optically transparent biomaterials using BNC, all those studies and patents reported in the literature included the addition of other substances. In this work, we developed BNC–Minimal membranes with high transparency using a defined minimal culture medium (DMCM). We discovered the capability of *K. hansenii* ATCC 23769 bacteria to synthesize BNC in DMCM supplemented with 25 mM of glucose and 10 mM of NH_4Cl. The defined minimal medium allowed the synthesis of BNC membranes optically transparent and without addition of other substances. Here, we proposed a novel culture medium to synthesize transparent BNC membranes with unique properties to several applications in the biomedical and industrial fields.

5. CONCLUDING REMARKS AND FUTURE PROSPECTS

Basically, this thesis aims to provide contributions to understanding the bionanocellulose synthesis through the development of a novel RNA extraction method and a defined culture minimal medium to produce bionanocellulose. Some gaps not answered in the literature so far were covered in this document. As stated in Section 1.1, the questions we aimed to answer are as follows:

1. How to obtain a high-quality RNA from bacterial cells living/producing BNC biofilms?
2. Will the developed extraction method be suitable to obtain RNA samples from biofilm and planktonic states with enough quantity and quality for transcriptome sequencing?
3. Can the bacteria under minimum nutrient requirements produce bacterial nanocellulose?
4. Which combination of carbon and nitrogen sources in minimal medium allows BNC synthesis?
5. Will the modification of the medium change the BNC properties?

Let's critically analyze, then, if this thesis was able to properly answer such questions and discuss implications derived from the work reported herein.

In Chapter 3 we had two main objectives:

- To evaluate and compare different techniques and methods for RNA extraction of *K. hansenii* cells in BNC biofilms to guarantee high-quality RNA samples.
- To obtain high concentration and integrity of RNA from *K. hansenii* cells in biofilms and planktonic states suitable for RNA-based analysis.

Our results achieved those aims, proposing a novel method for RNA extraction of *Komagataeibacter hansenii* cells living/producing nanocellulose biofilms and planktonic states. To the best of our knowledge, the method outlined here is the first to allow the extraction of high-quality RNA from *K. hansenii* cells in BNC biofilms.

The developed method is rapid and it has the ability to process many samples at a time with no concerns of cross-contamination and it

is applicable for extracting large quantities of high purity RNA from bacterial cells in BNC biofilms. The combination of shaking with metal beads as a disruption technique and the modifications of phenol-chloroform extraction was suitable to obtain a high concentration of RNA. The method was able to destroy the bacterial cell walls and nanocellulose fibers to separate RNA from cell debris and polysaccharides. Also, this method provided high yields of total RNA from small quantities of starting material (biofilms). RNA extraction method was also able to extract RNA from bacterial cells living in planktonic state. The modifications of DNase treatment were critical for DNA removal, resulting in RNA samples with no genomic DNA. Using the described method, high yields of total RNA were extracted, as confirmed by spectrophotometric and electrophoretic analyses. The values of the ratios $A_{260/280}$ and $A_{260/230}$ indicated that RNA samples were pure and effectively separated from protein, polysaccharides and other metabolites. The raw sequences (*reads)* obtained by RNA-Seq showed high-quality during the pre-processing of the reads and consequently confirms the quality of the RNA samples sent for sequencing. In conclusion, RNA extracted were of excellent quality and applicable for downstream applications.

Furthermore, with the high-quality bacterial RNA extraction method developed in this study it is now possible to compare differential gene expression of biofilm and planktonic cells through transcriptome assembly and to determine the genes regulated under each condition, contributing to the elucidation of the molecular mechanisms involved in BNC biosynthesis. We further suggest that the method may have wider applicability for RNA extraction of animal cells cultured on BNC biofilms.

In Chapter 4 we had two main objectives:

- To develop a defined minimal medium with specific carbon and nitrogen sources that enables *K. hansenii* to synthesize bacterial nanocellulose membranes;
- To characterize the membranes produced under defined minimal culture medium (BNC−Minimal) and compare it with the matrix (BNC−Mannitol) synthesized in complex medium.

Based on those aims, a defined minimal culture medium (DMCM) was developed to synthesize BNC. Although several studies

have investigated the effects of altering growth conditions to maximize BNC yield, this study explored the carbon and nitrogen sources concentrations as components of a defined minimal culture medium which provided the exact amount of nutrients for BNC production. To our knowledge, this is the first report revealing a defined minimal culture medium for bionanocellulose production. DMCM containing 25 mM of glucose and 10 mM of NH_4Cl showed the best combination of carbon and nitrogen sources concentration in terms of inducing *Komagataeibacter hansenii* to produce nanocellulose membranes.

Moreover, the developed DMCM culture medium can now be utilized to obtain experimental data to support future *in silico* metabolic investigations for the better understanding of bionanocellulose production by identifying the metabolic capacities and the behavior of the bacterium in terms of growth, nutrient demand and generation of extracellular metabolites.

The morphological and physicochemical characterization of BNC−Minimal membranes were compared to the usual matrix (BNC−Mannitol) to evaluate how DMCM will influence the properties of BNC−Minimal membranes. The membranes synthesized in DMCM showed a previously unknown transparency, which has not been reported yet without the addition of other substances.

Besides the transparency, the characterization of BNC–Minimal membranes revealed important improvements in some properties, such as higher water holding capacity, greater surface area and better elasticity than the usual BNC–Mannitol membranes, thus increasing their range of applications such as:

- the transparency and higher water holding capacity of the BNC–Minimal membranes can be valuable for the development of a new wound healing platform that can be easily monitored without removing the dressing;
- the distinct mechanical properties such as greater elasticity is desirable for the design of blood vessels and skin substitutes that require expansive movements and flexibility;
- the high porous BNC–Minimal membranes associated with their high transparency property could avoid the background normally present in the investigations of 3D biofilms-cells microscope analyses for tissue engineering and regenerative medicine (TERM) applications;

- the high transmittance of BNC-Minimal membranes is a necessary property for optical applications such as contact lenses or corneal substitutes;
- the BNC–Minimal membranes could be a transparent 3D platform that allows the visualization and monitoring of several animal cells interacting within a 3D chemically inert microenvironment.

The potential commercialization of the transparent nanocellulose membranes using the defined minimal culture medium lies in the innovation of their unique properties and the need for a low-cost BNC production. DMCM presented several advantages, such as low production cost, greater process consistency; better control and monitoring and improvement in product quality and reproducibility.

In summary, this study developed new methods for high-quality bacterial RNA extraction and for biofilm production that will allow the use of transcriptomic and metabolomic analysis that leads to the elucidation of the molecular mechanisms involved in BNC biosynthesis.

- An optimized RNA extraction method was developed to produce RNA samples with high quantity, purity, and integrity of *K. hansenii* cells living/producing BNC biofilms. From high-quality RNA samples of *K. hansenii* in biofilms and planktonic states, a transcriptome assembly can be performed to identify target genes that have a high impact on BNC synthesis.
- The defined minimal culture medium showed the ability of *K. hansenii* to synthesize BNC with strict limitation of nutrients. DMCM can provide precise metabolic analysis and greater experimental reproducibility in comparison with complex media. As an additional unexpected result, we come upon a cheap and easy pathway to produce highly transparent nanocellulose membranes with unique properties of significant interest for various applications in the biomedical and industrial fields.

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7. APPENDIX I

7.1 EVALUATION INDICATORS

In this section are listed some evaluation indicators achieved during the doctoral period.

Participation in the team of elaboration and execution of projects:

PROPELE - *Engenharia Tecidual e Avaliação Pré-Clínica e Clínica de Pele Biopolimérica Imunoativa.* Edital: Encomendas MCT/FINEP - COBIO Coordenador: Luismar Marques Porto Processo: 550009/2014-0
CELSYS - *Engenharia Genômica de Nanofibras de Celulose.* Edital: Chamada Nº 28/2013 MCTI/CNPq/CT- Biotec - Engenharia de Sistemas Biológico Coordenador: Luismar Marques Porto Processo: 402901/2013-4
BIÔMICA - *Tecidos e Órgãos Bioartificias- Engenharia de Tecidos Moles e Duros, Protótipos Funcionais e Cirurgia Robótica.* Edital: Encomendas MCT/FINEP - COENG Coordenador: Luismar Marques Porto Processo: 550084/2014-2

Participation in congress organization:

COBEQ (Congresso Brasileiro de Engenharia Química) 2014	Organizing Committee

Awards

2013 – Best Poster International Conference of AB3C - X-meeting BSB.	Draft reconstruction of the core metabolic network in *Komagataeibacter hansenii*
2014 – CAPES scholarship	CAPES - DS Start:01/04/2014 End: 28/02/2018
2015 – Speaker Invitation 3rd International Conference on Integrative Biology. Valencia - Spain	Lecture - Systems Biology of nanocellulose synthesis: new approach to build a core metabolic model based on computational analysis.

Presentations, courses and publications

Course 2012 **UFSC**	Course in Stem Cells and Cell Therapy
Course 2012 **FIOCRUZ**	International Systems Biology Course.
X-Meeting 2013	Draft Reconstruction of the core metabolic network in *Komagataeibacter hansenii* ATCC 23769
Course 2013 **UNESP**	Course in Application of Biology Computational Tools
CBAB 2014	FERRAMENTAS DE BIOINFORMÁTICA APLICADAS ÀS ANÁLISES DE SEQUÊNCIAS TRANSCRIPTÔMICAS
COBEQ 2015	SOUZA, S. S.; PORTO, L. M.; "RECONSTRUÇÃO DA REDE METABÓLICA DA *Gluconacetobacter hansenii* – UM MODELO SIMPLIFICADO", p. 197-204. Blucher Chemical Engineering Proceedings, v.1, n.2. 2015.

COBEQ 2016	SOUZA, S. S.; BERTI, F. V. ; SCHROEDER, C. ; CESCA, K. ; NASCIMENTO, F. X. ; PORTO, L. M. Optically transparent bacterial nanocellulose scaffolds for tissue engineering. In: XXI Congresso Brasileiro de Engenharia Química, 2016, Fortaleza. Anais do Congresso Brasileiro de Engenharia Química, 2016. v. 1.
BMC Proceedings	SOUZA, S. S.; PORTO, L. M. System biology of bacterial cellulose production. BMC Proceedings 2014, 8(Suppl 4): p256.
Brazilian Journal of Chemical Engineering	SOUZA, S. S.; CASTRO, J.V.; PORTO, L. M. Modeling the core metabolism of Komagataeibacter hansenii ATCC 23769 to evaluate nanocellulose biosynthesis. Accepted. 2017
Applied Biochemistry and Biotechnology	SOUZA, S. S.; OLIVEIRA, K. P. V.; PELISSARI, C.; BERTI, Fernanda Vieira ; PORTO, LUISMAR . High-quality RNA extraction from bacterial cells in nanocellulose biofilms. Submitted. 2017.

Partnerships and selection process

Group	***Publications***
GESAD – Grupo de Estudos em Saneamento Descentralizado Departamento de Engenharia Sanitária e Ambiental (ENS) -UFSC	PELISSARI, C.; GUIVERNAU, M.; VIÑAS, M.; SOUZA, S.S.; GARCÍA, J.; SEZERINO, P. H.; ÁVILA, C. Unraveling the active microbial populations involved in nitrogen utilization in a vertical subsurface flow constructed wetland treating urban wastewater. Science of the total environmental. v. 584, p. 642-650, 2017. PELISSARI, C.; GUIVERNAU, M.; VIÑAS, M.; SOUZA, S.S.; ROUSSO, Z.B.; GARCÍA, J.; ÁVILA, C.; ARMAS, D.R.; SEZERINO, P. H. Deciphering metabolically active microbial populations involved in nitrifying-denitrifying processes in unsaturated and partially saturated vertical subsurface flow constructed wetlands. Submitted.2017.

2014 - Processo Seletivo Simplificado do Departamento de Física - FSC, instituído pelo Edital N 42/DDP/2015.	Classificação: 2º lugar Área/Subárea de Conhecimento: Mecânica e Eletricidade
2016 - Processo Seletivo Simplificado do Departamento de Engenharia Química e Engenharia de Alimentos - EQA, instituído pelo Edital N 12/DDP /2016.	Classificação: 3º lugar Assumi a vaga em Março/2017 Área/Subárea de conhecimento: Engenharia Química/ Tecnologia Química.