Role of Culture Medium in Bacterial Cellulose Biosynthesis: Details

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Abstract

Bacterial Cellulose biosynthesis is one of the most important applied biochemical processes in biology. In order to explain the detailed molecular events of bacterial cellulose biosynthesis, we discuss in this work, the different steps required for bacterial cellulose formation and crystallization from sugar cane and honey. The potential of organisms to produce biocellulose fibers was analyzed. After fermentation bioprocess change new morphological and thermal properties were obtained.

Keywords: *applied biotechnology; bacterial cellulose production; fermentation process; nanobiocomposites.*

1)Introduction

Cellulose is found in groups of microorganisms like fungi, bacteria, and algae. In green algae, cellulose, xylan, or mannan may serve as structural cell wall polysaccharides. Cellulose is found, although in small quantities, in all of the brown algae (Phaeophyta), most of the red algae (Rhodophyta), and most of the golden algae (Chrysophyta (Chrysophytes)). It was also reported to be present in some fungi.However, cellulose has traditionally been sourced from plants, but needs refining of plant cellulose typically involves harsh, aggressive processing to remove non cellulose materials such as lignin and hemi-cellulose.[Olyveira et al., 2011]

Bacterial cellulose (BC), which is produced by some strains of the bacterial genera *Acetobacter, Agrobacterium, Gluconacetobacter, Rhizobium*, and *Sarcina*, represents a potential alternative to plant-derived cellulose. Due to its high water-holding capacity, high crystallinity, high tensile strength and fine web-like network structure, which means that it can be formed into any size or shape, BC is being used as a promising nanofiber biomaterial.[Basmaji et al., 2011]

Microbial cellulose is an exopolysaccharide produced by various species of bacteria, such as those of the genera *Gluconacetobacter* (formerly *Acetobacter*), *Agrobacterium*, *Aerobacter*, *Achromobacter*, *Azotobacter*, *Rhizobium*, *Sarcina*, and *Salmonella*[Olyveira et al., 2011].

Cellulose synthesis by *Acetobacter* is a complex process and involves the polymerization of glucose residues into linear *β*-1,4-glucan chains, the extracellular secretion of these linear chains, and crystallization of the glucan chains into hierarchically composed ribbons.[Costa et al.; 2012].It is well known that cellulose fiber networks – as in the case of paper – provides good mechanical properties because of the degree of hydrogen bonding obtained between the fibers in the network. The greater the hydrogen bonding, the stronger the paper material. BC synthesised extra-cellularly by the *Acetobacter xylinum* is of nano-size, as a result of which hydrogen bonding between fibrils is greater than with plant cellulose in normal paper. The hydrogen bonds due to the hydroxyl group give rise to properties such as a high degree of crystallinity, high water-holding capacity and high tensile strength.[Olyveira et al., 2011a] Many Gram-negative bacteria secrete extracellular polysaccharide material, but only a few have been shown to produce cellulose. *A. xylinum* has been applied as model microorganism for basic and applied studies on cellulose. It is most commonly studied source of bacterial cellulose because of its ability to produce relatively high levels of polymer from a wide range of carbon and nitrogen sources.[Olyveira et al., 2011b]

The structural features of microbial cellulose, its properties and compatibility of the biomaterial for regenerative medicine can be changed modifying its culture medium[Costa et el., 2012] or surface modification by physical[Olyveira et al.,2013; Costa et al., 2012a] and chemical methods[Valido et al., 2012; Olyveira et al., 2013a] to obtain a biomaterial with less rejection with cellular contact and blood contact cells interation. Costa et al. obtained several morphological properties and thermal properties with bacterial cellulose just changing bacterial culture medium with cane sugar and honey[Costa et al., 2011]. In this work, bacterial cellulose biosynthesis are explain in details and connection is established between bc biosynthesis, culture medium, surface morphology and thermal properties.

2) Materials and Methods

2.1)Materials

Bacterial cellulose were supplied from Innovatecs-Produtos Biotecnológicos Ltda, Brazil . Sugar cane extract were purchased from Hangzhou New Asia International Co. Honey samples were purchased from Zhejiang Jiangshan Bee Enterprise Co., Ltd.

2.2) Synthesis and Fermentation of Bacterial Cellulose Nanocomposites

The acetic fermentation process is achieved by using the sugar as carbohydrate source. Different carbon sources can be used for the cellulose synthesis, namely glucose, fructose, cane sugar and honey. Results of this process would be vinegar and a nanobiocellulose biomass. The modified process is based on the addition of sugar cane or honey (1% w/w) to the culture medium before bacteria are inoculated. After being added to the culture medium the medium is autoclaved at 100 celsius degree. Then, bacterial Cellulose (BC) produced by Gram-negative bacteria Gluconacetobacter xylinus can be obtained from the culture medium in the pure 3-D structure consisting of an ultra fine network of cellulose nanofibers.

2.3) Bionanocomposites characterization

Scanning Electron Microscopy (SEM)- Scanning electronic microscopy images were performed on a PHILIPS XL30 FEG. The samples were covered with gold and silver paint for electrical contact and to perform the necessary images.

Surface morphology of nanocellulose was observed using atomic force microscopy, NanoScope IVa, Multimode SPM (Veeco Inc) in tapping mode.

Differential scanning calorimetry(DSC)- To analyze the crystalline and thermal behavior of biocomposites, calorimetric experiments were carried out with the help of differential scanning calorimetry (DSC 822 Mettler Toledo, Switzerland).The measurements were done at the heating rate of 10 celsius/min and the temperature range was 25°C –700°C.

TGA- Thermogravimetric analysis (TGA) was carried out for biocomposites using a NETZSCH TG 209F1in Helium environment, with a heating rate of 30°C/min**.** The temperature range scanned was from 50°C to 700°C. The weight of all specimens was maintained around 10 mg.

3)Results and Discussion

A. xylinum cultures are characterized by a thick cellulosic surface mats, called a pellicle, in which the embedded cells of this aerobe microorganism has direct contact with the liquid/air interface [Barbara et al., 1996; Dong and Wang,2002; Gilardi and Fantuzzi, 2001]. Bacterial cellulose contains a thin peptidoglycan layer adjacent to the cytoplasmic membrane. In addition, contains an outer membrane composed by phospholipids and lipopolysaccharides with invaginations of the cell membrane, which can be either simple folds as vesicular or tubular structures .Several functions have been attributed such as: role in cell division and respiration. In general, aerobic respiration more efficiently use glucose to produces more energy (i.e. electrons) than fermentation.

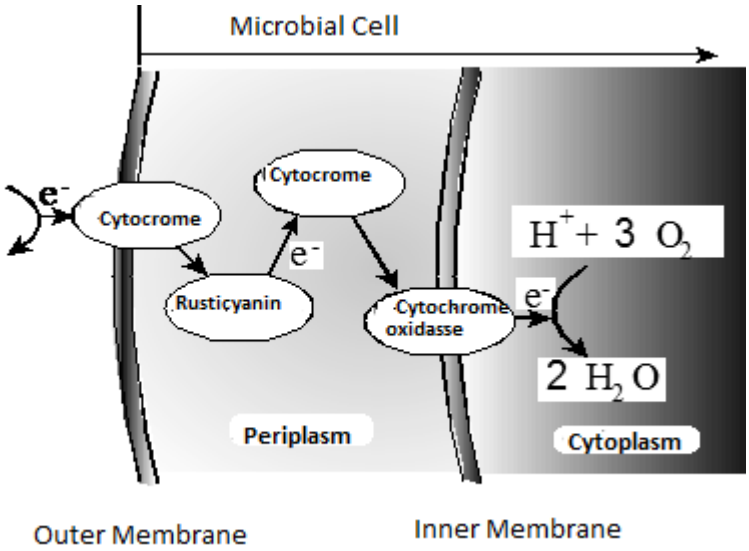

Fig.1. Tubular Fibrils positioned inside microbial cells

It is believed that the tubular fibrils become positioned within tunnelling distance of the cofactors with little consequence to denaturation. The combination of symbiose with redox active enzymes would appear to offer an excellent and convenient platform for a fundamental understanding of biological redox reactions like illustred in figure 1.[O´Connell and Guilbault, 2001;Wang, 1999; Wang, 2002].

The biosynthesis of the sugar of the various sugar with cell structures begin by the synthesis of the units of sugar nucleotides. The supply of the sugars for the biosynthesis is dependent on the intracellular sugar nucleotide levels that are influenced by the activities of the intracellular enzymes involved in their biosynthesis.[Svancara et al., 2001]

The size of the cellulose molecule is normally expressed in terms of their degree polymerization (DP), ie, the number of anhydroglucose units present in a chain. However, the conformational analysis of cellulose indicates that cellobiose (4-O-α-D-glucopyranosyl-α-D-glucopyranose) is its basic structural unit.[Kotayama et al.;1997]. The conformation of the repeating unit of cellulose can be explained if we consider the model proposed for the biosynthesis of glucose.

The active site of the enzyme cellulose synthase, responsible for the synthesis of cellulose, contains two consecutive sites of binding to uridine-diphosphoglucose precursor (UDP-glucose), positioned at 180 ° from each other, and a binding site the non-reducing end of β-glucan . The hydroxyl at C-5 of glucose residues linked to these sites are activated by a mechanism of general base catalysis by promoting the dephosphorylation of UDP-glucose units and establishing new links β (1-4). The resulting β-glucan and had little affinity binding sites for UDP-glucose, moves to a better place, the

binding site for β-glucan. Two new units of UDP-glucose can then be added, continuing the synthesis.[Ramos, 2003].

Hydroxyls present in the structure of cellulose are distributed equatorially in a quasi-planar arrangement that allows the formation of a linear chain , in which adjacent chains are aligned to form crystalline structures stabilized by hydrogen bonds inter-and intramolecular. These links lead to a crystalline structure compact and extremely stable having on its surface hydrophilic, but hydrophobic inside, which makes cellulose insoluble in water. Thermodynamic limitations on the growth of these structures make occur, alternating with these crystalline regions, amorphous regions where the molecules are randomly distributed. The process includes the formation of UDP-Glucose, which is the precursor in the formation of cellulose, followed by glucose polymerization into the β (1-4) glucan chain and a nascent chain which forms ribbon-like structure of cellulose chains formed by hundreds or even thousands of individual cellulose chains, their extrusion outside the cell, and self-assembly into fibrils.[Zugenmaier, 2001].

Pathways and mechanisms of uridine diphosphoglucose (UDP-Glucose) synthesis are relatively well known, whereas molecular mechanisms of glucose polymerization into long and unbranched chains still need exploring. [Zugenmaier, 2001].

When food is plentiful, " the survival of the fittest " generally means the survival of those that divide quickly . Under proper conditions, a simple prokaryotic cell can divide every 20 minutes, giving rise to 5 billion cells (approximately equal numbers to the human population of the earth) in just under 11 hours.

Bacterial cellulose has cell division and DNA synthesis within cells. DNA is organized into long structures called chromosomes, during cell division these chromosomes are duplicated in the process of DNA replication, providing each cell its own complete set of chromosomes, then it will subsequent assembly of the *β*-1,4-glucan chains outside the cell in a precise, hierarchical process.

3.1) Bacterial Cellulose mats

Bacterial cellulose mats were characterized by SEM and AFM. Fig. 2 shows, as an example, SEM image of bacterial cellulose formation from a) sugar cane, b) honey respectively. These results confirm that the relationship between BC biosynthesis, culture medium and differents surface morphology.

The morphological changes such as size and bacterial cellulose fibers surface can be observed by AFM from each BC production. Clearly, AFM images shows that by changing the culture medium of bacterial cellulose results in an excellent dispersion of nanofibers with high aspect ratio and these fibers has thickness from 30-40 nm. Otherwise, these alterations resulting in surface roughness changes too. It is known that the surface roughness is related to the recoverable strain of the material and consequently the optical properties presented in the film. A high roughness is related to the presence of large, coarse spherulites, leading to greater opacity of the film.[Guerrini et al; 2004] So, clearly, in figure 3 it can be concluded that figure 3a and 3b has different roughness.

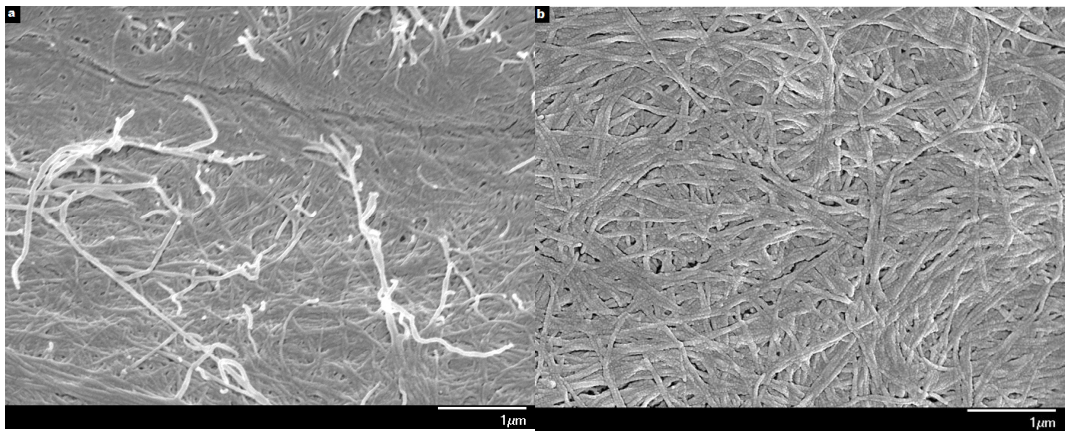

Fig.2. Scanning electron microscopy (SEM) of Bacterial cellulose from: a) cane sugar, b) honey respectively.

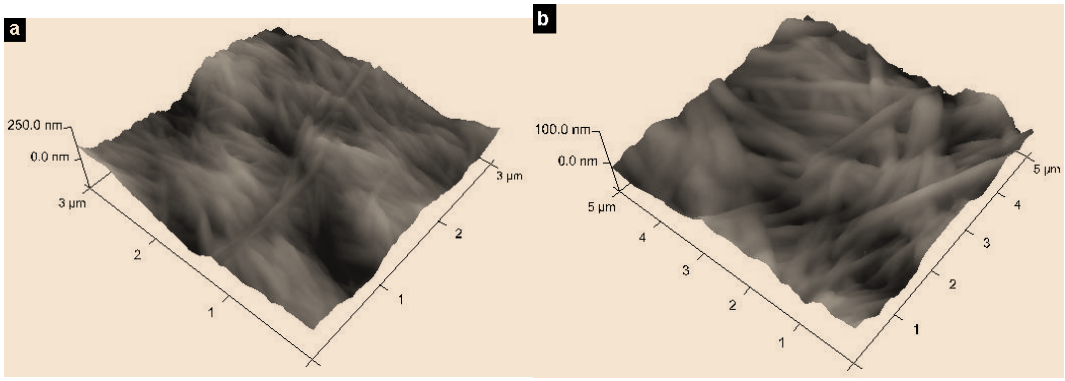

Fig.3. Force Atomic Microscope (AFM) of Bacterial cellulose from: a) cane sugar, b) honey respectively.

3.2)DSC- The crystallization behavior in the bionanocomposites was carried out by DSC tests. It can be observed in Figure 4, heating curves of bionanocomposites. From the course of the curve, the first transformation is related to the evaporation of water at an endothermic maximum of 85 °C. According to literature, at a temperatures of 80 - 140 °C, there is an know transformation related to the melting of the crystalline phase of cellulose[George et al.; 2005]. The next transformation occurs only at the temperature of approx. 355 °C, leading to the decomposition of the sample.

All systems analyzed presents differents thermal behavior. It can be observed that cane sugar addition cause peak broading probably caused by the presence of crystals with different thicknesses and varying degrees of perfection because the addition of filler.Besides,it can be observed in figure 4 that system has fusion peaks shifts to lower temperatures and with less crystals to merge , characteristic of a system with lower crystallinity. Otherwise, honey addition facilitated the mobility in crystallization process, with obtained symmetrical crystals and crystallization occurred at higher temperatures, characteristic of a system with higher crystallinity.

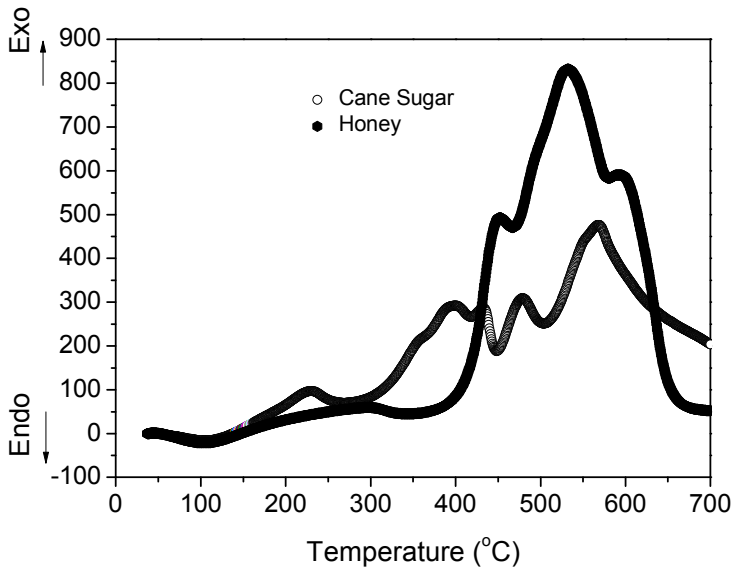

Fig.4. DSC curves of Bacterial cellulose from: cane sugar and honey respectively.

3.3)TGA- In order to analyze thermal behavior for bionanocomposites are characterized typical weight loss verses temperature plots. The TG spectrum (Figure 5) shows a weak loss of weight due to the evaporation of water (at temp. 85°C) and also quick drop in weight at a temperature of approx. 300 °C is mainly attributed to thermal depolymerization of hemicellulose and the cleavage of glycosidic linkages of cellulose [Manfredi et al.,2006; Ouajai and Shanks, 2005], complete degradation of cellulose take place between 275 and 400 ºC [Alvarez and Vazquez,2004; Deepa et al.; 2011]. The TG curve shows that the maximum rate of degradation occurs at temperature of approx. 370 °C for bacterial cellulose/cane sugar. However, bacterial cellulose/honey has higher degradation at temperature of approx 450°C mainly because of higher crystallinity rate.These results clearly evidence higher thermal behavior with developed bionanocomposites than pure bacterial cellulose mats.

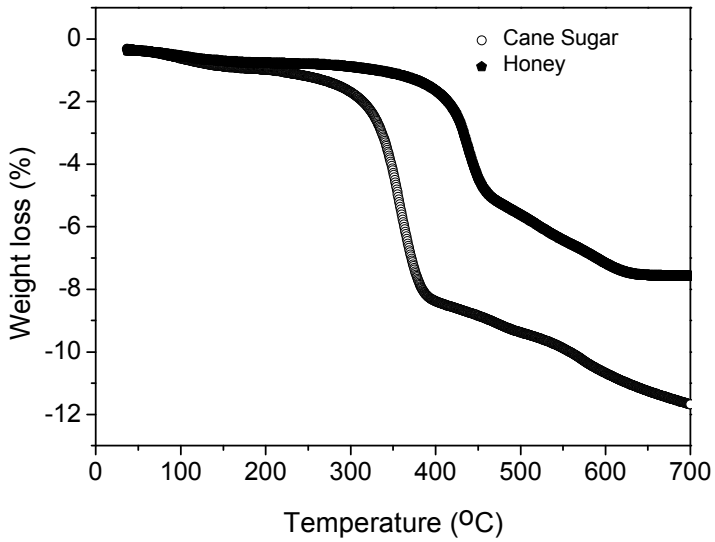

Fig.5. TGA thermogram of Bacterial cellulose from: cane sugar and honey respectively.

4)Conclusion

Bacterial cellulose with its characteristics like nanofibers size and distribution, mechanical properties, compatibility and ability to mold is a biomaterial indispensable in health area. It was the intention of this work to broaden knowledge in this subject area and stimulate the practical application of bacterial cellulose with new materials and biocomposites obtained with fermentation control for potential applications in medicine, food packing and sensor applications. It can concluded after fermentation bioprocess change that SEM and AFM images showed differents surface morphology. DSC and TGA showed higher thermal properties and change crystallinity of the developed bionanocomposites.Honey/bacterial cellulose sample presents higher crystallinity and cane sugar/bacterial cellulose sample presents lower crystallinity in studied system.

5)References

Alvarez,V.A.; Vazquez, A., 2004. Degradation of sisal fibre/Mater Bi-Y biocomposites buried in soil. Polym Degrad Stab ;84;13-17.

Barbara, P.F; Meyer, T.J.; Ratner, M.A, 1996. Contemporary Issues in Electron Transfer Research. J. Phys. Chem., 100 (31), 13148–13168

Basmaji, P.; Olyveira, G.M.; Costa, L.M.M, 2011. Nanoskin for Medical application. Proceeding of Nanotechnology 2011: Bio Sensors, Instruments, Medical, Environment and Energy,(2011), June 13 - 15, Boston – USA.

Costa, L.M.M.; Olyveira, G.M.; Basmaji, P.; Filho, L.X, 2011. Bacterial Cellulose Towards Functional Green Composites Materials. Journal of Bionanoscience, 5, 167–172.

Costa, L.M.M.; Olyveira, G.M.; Basmaji, P.; Filho, L.X.,2012. Bacterial Cellulose Towards Functional Medical Materials. J. Biomater. Tissue Eng;. 2,185-196.

Costa, L.M.M.; Olyveira, G.M.; Basmaji, P.; Filho, L.X, 2012a. Nanopores structure in electrospun bacterial cellulose. J Biomater Nanobiotech, 3, 92-96.

Deepa,B.; Abraham,E.; Cherian, B.M.; Bismarc,A.; Blaker, J.J.; Pothan, L.A.; Leao, A.L.; Souza, S.F.; Kottaisamy, M, 2011. Structure, morphology and thermal characteristics of banana nano fibers obtained by steam explosion. Bioresour Technol ;102;1988-1991.

Dong, S.J.; Wang, B.Q.;2002. Electrochemical Biosensing in Extreme Environment. Electroanalysis, 14, 7-16.

George , J.; Ramana, K.V.; Sabapathy, S.N.; Jagannath, J.H.; Bawa, A.S., 2005.Characterization of chemically treated bacterial (Acetobacter xylinum) biopolymer- Some thermo-mechanical properties. Int J Biol Macromol ;37;189-193.

Gilardi, G and Fantuzzi,A , 2001. Manipulating redox systems: application to nanotechnology. Trends Biotechnol, 19, 468–476.

Guerrini, L.M.; Filho,P.P.; Bretas, R.E.S.; Bernardi, A., 2004.Correlação entre propriedades reológicas, ópticas e a morfologia de filmes soprados de LLDPE/LDPE. Polímeros:Ciência e Tecnologia ;14;38

Koyama, M.; Helbert, W.; Imai, T.; Sugiyama, J.; Henrissat, B, 1997. Parallel-up structure evidences the molecular directionality during biosynthesis of bacterial cellulose. Proc. Natl. Acad. Sci. USA, 94, 9091-9095.

Manfredi, B.L.; Rodriguez, E.S.; Wladyka-Przybylak, M.; Vazquez, A., 2006. Thermal degradation and fire resistence of unsaturated polyester modified acrylic resins and their composites with natural fibers. Polym Degrad Stab ;91;255-260.

O´Connell, P.J.; Guilbault, G.G.;2001. Future trends in biosensor research. Anal Lett 34(7),1063–1078.

Olyveira,G.M.; Costa,L.M.M, Góis, P.B.P., Basmaji,P., Filho, L.X, 2011.Novel Natural Transdermal Patch for Osteoporosis Treatment- Journal of Nanotechnology in Engineering and Medicine, 2, 031011-0311016.

Olyveira, G.M.; Costa, L.M.M; Basmaji, P.; Filho, L.X, 2011a. Bacterial Nanocellulose for medicine regenerative . J.Nanotech. Eng. Med., 2, 3, 34001-34009.

Olyveira,G.M.; Valido, D.P.; Costa, L.M.M; Gois,P.B.P; Filho, L.X.; Basmaji, P, 2011b. First Otoliths/Collagen/Bacterial Cellulose Nanocomposites as a Potential Scaffold for Bone Tissue Regeneration. J Biomater Nanobiotech ;2,239-243.

Olyveira,G.M.; Costa, L.M.M; Basmaji, P., 2013. Physically Modified Bacterial Cellulose as Alternative Routes for Transdermal Drug Delivery. J. Biomater. Tissue Eng. 2, 3, 1-6.

Olyveira, G.M.; Acasigua,G.A.X; Costa, L.M.M.; Scher, C.R.; Filho, L.X.; Pranke, P.; Basmaji, P, 2013a.Human dental pulp stem cell behavior using natural nanotolith/bacterial cellulose scaffolds for regenerative medicine. Journal of Biomedical Nanotechnology, 9, 1-8.

Ouajai, S.; Shanks, R.A, 2005. Solvent and enzyme induced recrystallization of mechanically degraded hemp cellulose. Polym Degrad Stab;89;327-330.

Ramos, L. P., 2003. The chemistry involved in the steam treatment of lignocellulosic materials. Química Nova, 26, 863-871.

Svancara I., Vytras K., Barek J., Zima J.,2001. Carbon paste electrodes in modern electroanalysis. Crit. Rev. Anal. Chem.;31,311–345.

Valido, D.P.; Costa, L.M.M; Olyveira, G.M; Góis, P.B.P; Júnior, R.L.A.C; Filho, L.X.; Basmaji, P, 2012. Novel otoliths /bacterial cellulose nanocomposites as a potential natural product for direct dental pulp capping. J Biomaterials and Tissue Engineering, 2, 48–53.

Wang, J., 1999. Amperometric biosensors for clinical and therapeutic drug monitoring: a review. J. Pharm. Biomed. Anal. 1947-53.

Wang,J.,2002. Electrochemical detection for microscale analytical systems: a review. Talanta,56, 223-231.

Zugenmaier, P., 2001. Conformation and packing of various crystalline cellulose fibers. Progress in Polymer Science, 26, 1341-1417.