Production of nanocellulose in miniature-bioreactor: Optimization and characterization

Sepideh Khazeni, Ashrafalsadat Hatamian-Zarmi, Fatemeh Yazdian, Zahra Beagom Mokhtari-Hosseini, Bahman Ebrahimi-Hosseinzadeh, Behnam Noorani, Ghassem Amoabedini, and Mohammad Reza Soudi

Department of Life Science Engineering, Faculty of New Science and Technology, University of Tehran, Tehran, Iran; Department of Chemical Engineering, Faculty of Petroleum and Petrochemical Engineering, Hakim Sabzevari University, Sabzevar, Iran; Faculty of Chemical Engineering, College of Engineering, University of Tehran, Tehran, Iran; Research Center for New Technologies in Life Science Engineering, University of Tehran, Tehran, Iran; Department of Microbiology, Faculty of Biological Science, Alzahra University, Tehran, Iran

ABSTRACT

Bacterial cellulose (BC) is a very fascinating microbial biopolymer which is mainly produced by Gluconacetobacter xylinum. Optimization of BC production by G. xylinum was performed based on scale-down studies in miniature-bioreactor and response surface methodology in which the optimum pH value (6.5) and shaking rate (50 rpm) were obtained. The static culture condition for BC production has newly been defined. Nanostructure of BC includes nanofibers up to (60 nm) and nanoporosity up to (265 nm) was observed by scanning electron microscopy. By Fourier transform infrared spectroscopy study, the most expected BC interaction is nucleophilic interaction. MTT assay showed high biocompatibility. Appropriate mechanical strength (0.37 MPa) and Young's modulus (3.36 MPa) evinced BC scaffold utilization for skin tissue. The results indicate that BC sheets can be utilized in biomedical application and nanotechnology approaches.

Introduction

Cellulose is a very important and fascinating biopolymer that the design and development of renewable resources and innovative products for science, medicine and technology have led to a global revival of interdisciplinary research and utilization of this abundant natural polymer in recent decades. [1]

Microbial cellulose is produced extracellular in a considerable amount by an aerobic gram negative Gluconacetobacter xylinum, which is the most commonly used and studied sources of bacterial cellulose (BC). Bacterial cellulose is particularly appealing due to its purity and highly crystalline nanostructure. The BC has been applied for wound dressings, burn treatments, tissue regeneration, skin substitutes, catalyst-sensing materials, artificial blood vessels, and electronic devices. [2,3] BC’s micro fibrils are shaped in different ways depending on the degree of surface culture agitation, which is the result of ventilation and culture methods. [4,5] Various methods such as the static method which is capable of producing cellulose in the form of film on the air/liquid interface and the agitate method in which the surface of cultures is agitated and the grain shapes of cellulose are suspended in the culture. [6-8]

Although, bacterial cellulose was introduced by Brown for the first time in 1886, the commercial view of BC production was studied in 2008 by El-Saied et al. [9]. However, the processes of BC production have been studied mostly by Brown since 1976. [10] In the past decade, the studies approach to BC production for utilized in different industries [11] mostly for medical appliances such as biomedical engineering, [12] tissue engineering, [13] drug delivery, [14] and nanocomposites [15] and nanostructures. [16]

Medical and several other applications utilize BC films produced through static culture. Unfortunately, the low yield of the static production process has restricted the commercial efficacy of BC. Therefore, major goals of BC researches have been about development of the production yield. Studies show that BC grains, produced in agitated conditions, show a lower degree of polymerization, mechanical strength, and crystallinity than those BC films produced under static cultivation. However, because of better ventilation the production process is much faster during agitated culture than static culture. BC films obtained through static cultivation are usually utilized for composite synthesis, which can be effectively used in biomedical and other industrial applications. [17,18]

There is specific culture media for G. xylinum (used for both agitated and static conditions) which is called HS media because it was introduced by Hestrin and Scharm. [19] According to previous studies, an optimum temperature for HS medium is 30°C, but various pH values have been reported for example, Farah et al. reported optimum pH 4 that would decrease the risk of culture contamination [20] and Zuo et al. reported optimum pH value 5 with the highest yield of BC production 8 g/l [8] and Pourramezan et al. reported the range from 4 to 7 as optimum pH value. [21] Previously, shaking rates
are reported only for agitated conditions in laboratory scales, while the static culture needs ventilation as well. However, in this research we experimentally clarified the common abstract definition of static condition for BC production.

The productivity of BC depends on culture conditions, which include the cultivation method, composition of growth medium,[22] dissolved oxygen,[23] temperature, and pH value of the growth medium.[24] The design of experiment techniques help to identify the most effective operational parameters which influence the production of biopolymer.[25] The response surface methodology (RSM) based on the central composite design can be used to optimize culture conditions. RSM can be used for optimization of the culture conditions, which are found to significantly affect BC production from the other studies, with a minimum number of experiments.[26]

In this study, we evaluated the optimization of BC production by G. xylinum at static culture based on scale-down studies in miniature-bioreactor and central composite design (CCD) of experiment techniques. We also compared the optimum condition yield in our research to previous reports in the other studies, with a minimum number of experiments.[26]

In this study, we evaluated the optimization of BC production by G. xylinum PTCC 1734 obtained from Iranian Research Organization of Science and Technology was cultured. HS medium includes glucose %2 W/V (as a main carbon source), pepton %0.5 W/V, yeast extract %0.5 W/V, citric acid mono hydrate %0.15 W/V, and Na2HPO4 %0.27 W/V solved in water and after sterilization by autoclave used for G. xylinum culture. Incubation temperature during all stages of study was 30°C.

Media cultures of G. xylinum were examined in 13 different shaking rates from static condition up to 120 rpm. The physical characteristics of production in each culture were observed. To wash and remove bacterial cells, BC extraction from HS culture media has been done by the NaOH (alkaline) treatment method in which BC sheet was boiled in NaOH 1 N for 45 min and washed with normal saline as well as distilled water in all stages.

**Materials and methods**

**BC production condition and purification**

*Gluconacetobacter xylinum* PTCC 1734 obtained from Iranian Research Organization of Science and Technology was cultured. HS medium includes glucose %2 W/V (as a main carbon source), pepton %0.5 W/V, yeast extract %0.5 W/V, citric acid mono hydrate %0.15 W/V, and Na2HPO4 %0.27 W/V solved in water and after sterilization by autoclave used for *G. xylinum* culture. Incubation temperature during all stages of study was 30°C.

Media cultures of *G. xylinum* were examined in 13 different shaking rates from static condition up to 120 rpm. The physical characteristics of production in each culture were observed. To wash and remove bacterial cells, BC extraction from HS culture media has been done by the NaOH (alkaline) treatment method in which BC sheet was boiled in NaOH 1 N for 45 min and washed with normal saline as well as distilled water in all stages.

**Design of experiments for optimization of parameters**

The purpose of the application design of experiment techniques is to identify factors in the process of determining the optimal values. CCD is a technique that designs experiments in three levels which includes central point (0) and axial points (−1, +1). Two effective operational parameters in BC production in static condition for *G. xylinum* media were identified.

As *G. xylinum* is an aerobic microorganism, effective ventilation is required for its growth and BC production. Different shaking rates in the static ranges were observed and the highest axial point (+1) showed 50 rpm, central point (0) set to 40 rpm and the lowest axial point (−1) set to 30 rpm. Another parameter is the pH value of *G. xylinum* media which has been reported differently in different studies. Studies showed that under pH value 4 the enzymatic process of *G. xylinum* goes toward the production of gluconic acid which reduced BC production as well; therefore, the lowest axial pH value point (−1) was set to 4. As *G. xylinum* is an acidophil microorganism, the highest axial pH value point (+1) was set to 6.5 which is the boarder point of pH value between acid and neutral condition. Hence, the central pH value point (0) was set to 5.2. CCD, for these two parameters designed 13 experiments, which are shown in Table 1.

The RSM is a technique that evaluates the effects of the parameters on definite response and then statistically optimizes parameters. Here, two responses have been defined for each 13 designed experiments; optical density (OD) at 600 nm which showed the bacterial growth and cell density, and BC weight (g) which is the product mass.

**Miniature bioreactor**

Miniature bioreactor is a reactor that provides evaluation of operational parameters in low scales. This reactor belongs to New Technologies in Engineering and Biological Sciences Research Center of University of Tehran. Such a controlled system is based on scale-down studies.

Miniature bioreactor includes two parts of hardware (such as shaker–incubator, flasks, cables,) and software that provides parameter control and online monitoring. Four flasks can be compared at the same time in the same constant parameters and one or two alternatives. As all operational parameters are extremely controlled, the accuracy of results increased, so all experiments in miniature bioreactor are repeatable. Each flask in miniature bioreactor needs at most 25 ml of culture media, thus setting up experiments in miniature bioreactor would reduce the costs.

**FTIR spectroscopy**

To evaluate chemical features of extracted BC, FTIR spectroscopy was used. For preparation of extracted BC, samples were sterilized by Ethanol 70% and then air dried.
by Ethanol 96% at room temperature. Chemical analysis of BC was performed by ATR–FTIR (Nicolet Thermo IR100) spectroscopy over 400–4,000/cm at a resolution of 4/cm.

Scanning electron microscopy

Bacterial cellulose structure was observed by SEM. The BC sheets can be both air dried and freeze dried. The BC samples were prepared by both air drying and freeze drying which were then coated by gold and set to device. The BC specimen morphology was studied by the SEM probe.

MTT assay

The MTT assay is a colorimetric assay for assessing cell viability. MTT test methods used to evaluate the toxicity of the substances on cell survival which measured the number of viable cells that are capable of reducing the yellow tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble purple formazan form.[27] Cell lines used in this study were L929 mouse fibroblasts cultured at DMEM media.

Briefly, BC was placed into 96-well tissue culture polystyrene. The incubated suspension cells were pipetted at the same density (20,000 cells) into each well on the BC. A volume of 20 µl of MTT solution (5 mg/ml in PBS) was added to the cultures at determined time of 1, 3, and 5 days. Then, after 4 hr incubation at 37°C in a 5% CO2, the MTT solution was removed and 200 µl of dimethyl sulfoxide was added into culture well. Afterward, the OD value was measured by a spectrophotometer at 570 nm with an ASYS-Expert 96 ELISA reader (Austria).[28]

Tensile strength tests

The tensile properties of the BC scaffolds with optimized condition were determined using a Universal Testing Machine (Santam-Stm 2005) which was equipped with a 10 N load-cell. The BC samples with size of 25 × 10 mm² were tested by a cross-head speed of 1 mm/min. The machine-recorded data were used to represent the average tensile stress–strain curves of sample.[29]

Results and discussion

BC production condition

The physical characteristic of BC from medium cultures of G. xylinum in different shaking rates showed BC from 0 rpm, which is commonly known as static condition, up to 50 rpm is formed as BC sheets. The same physical features of BC sheets were observed in all these shaking rates. However, the sheet thickness was grown as shaking rates increased. This is concluded that from the rate of 0 rpm up to 50 rpm, not only there is no stress in the surface of G. xylinum media and the condition for BC production seemed to be static by BC sheets production but also the thickness of sheets can be grown by increasing shaking rates. Hence, static culture condition for BC production is now defined as a condition that has no agitation and stress in the surface of culture media. From 60 rpm up to 120 rpm, BC grains were seen floating in the culture media. The size of BC grains was diminished by increasing shaking rate.

Optimization analyzes

All 13 designed experiments have been set to miniature bioreactor and both two responses for each experiment have been evaluated. All data from experiments and their responses have been analyzed by RSM technique. The RSM results for OD response asserted that however, two-parameter pH value and shaking rate showed no significant interaction (AB; p-value > 0.05), they independently influenced OD response (A, B; p-value < 0.05) (Table 2).

The RSM 3D graph (Figure 1) showed that for the best OD response both parameters should lead to their highest axial points (+1).

Response surface methodology for BC mass response analyzed that the response is directly influenced by shaker rate (B; p-value < 0.05) (Table 3).

Thus, RSM analysis shows that the optimum condition for both growth OD and BC production in static culture is when two parameters of pH value and shaking rate lead to their highest axial points (+1) that the expected responses have been examined and approved (Table 4) which means that optimum pH value is 6.5 and optimum shaking rate is approximately 50 rpm.

Optimum condition in miniature bioreactor

The optimum condition, which was obtained from RSM, has been evaluated in miniature bioreactor. The most important part of G. xylinum culture is the last 48 hr in log phase when the BC layer is growing to maximum thickness. As the BC thickness increases, the oxygen transfer from the air surface to culture media decreases. Hence, if ventilation in this phase does not go well, G. xylinum leads to death phase in the case of oxygen deficiency. Optimum condition was set to miniature bioreactor and the last 48 hr in log phase of respiration was monitored online (Figure 2).

As Figure 2 shows, the respiratory quotient goes constantly as well as oxygen transfer rate (OTR) and CO2 transfer rate are constantly in the same level. That means in the optimum condition at the last 48 hr in log phase, bacterial cells are still active and respiring which exactly means ventilation is going well.

| Term                  | Sum of sq. | Mean sq. | F value | p-value |
|-----------------------|------------|----------|---------|---------|
| A (pH)                | 0.049      | 0.049    | 7.54    | 0.0287  |
| B (shaking rate)      | 3.65       | 3.65     | 565.30  | <0.0001 |
| AB                    | 9.604E-003 | 9.604E-003 | 1.48     | 0.2634  |
| A²                    | 5.381E-003 | 5.381E-003 | 0.83     | 0.3930  |
| B²                    | 0.81       | 0.81     | 125.22  | <0.0001 |

R-Squared = 0.99.

R² = 0.65 + 0.09 A + 0.78 B + 0.049 AB + 0.047 A² + 0.55 B².

RSM, response surface methodology; OD, optical density.
To compare the optimum pH value to other previous studies, the optimum pH value and other reported pH value was set to flask of miniature bioreactor and studied at the last 48 hr in log phase (Figure 3). Then, the yield of each flask would also be compared (Table 5).

The OTR graph is directly related to cell density which can be inferred that the highest OTR means the highest viable G. xylinum cells or cell density. As it is shown in OTR graphs of different pH values, in pH value 6.5 the highest OTR was observed; however, in pH value 4.8, the OTR graph is closed to pH value 6, but their production yields are different (Table 5).

**Scanning electron microscopy**

As it is shown in Figure 4, BC structure consists of porosity and fiber complex which are useful for biomedical appliances such as a microbial filter,[30] drug delivery and drug coating,[31] releasing control, and also in situ hybridization[32] for BC-based nanocomposite design.[33]

It is also shown in Figure 5 that the complex of fiber and porosity in BC structure showed different nanosize. Hence, it is better to mention that BC structure consists of the complex of nanofiber and nanoporosity.[34]

---

**Table 3.** RSM-evaluated BC mass response.

| Term   | Sum of sq. | Mean sq. | F value | p-value |
|--------|------------|----------|---------|---------|
| A (pH) | 0.077      | 0.077    | 0.86    | 0.3855  |
| B (shaking rate) | 1.31 | 1.31 | 14.51 | 0.0066 |
| AB     | 0.022      | 0.022    | 0.24    | 0.6324  |
| A²     | 1.712E-003 | 1.712E-003 | 0.019  | 0.8942  |
| B²     | 2.33       | 2.33     | 25.94   | 0.00014 |

R-Squared = 0.86.

R² = 2.34 – 0.11 A + 0.47 B + 0.075 AB – 0.014 A² – 0.91 B².

R² = 2.35 – 0.11 A + 0.47 B – 0.91 B².

RSM, response surface methodology; BC, bacterial cellulose.

**Table 4.** Optimum parameters and expected responses.

| A (pH) | B (shaking rate) | Predicted R1 (OD) | Predicted R2 (BC mass) | Experimented R1 (OD) | Experimented R2 (BC mass) |
|--------|------------------|-------------------|------------------------|----------------------|--------------------------|
| +1.00  | +1.00            | 2.163             | 1.851                  | 2.256                | 2.100                    |

OD, optical density; BC, bacterial cellulose.

To compare the optimum pH value to other previous studies, the optimum pH value and other reported pH value was set to flask of miniature bioreactor and studied at the last 48 hr in log phase (Figure 3). Then, the yield of each flask would also be compared (Table 5).
**FTIR spectroscopy**

Obtained BC FTIR diagram is shown (Figure 6). As in FTIR spectroscopy, each chemical bond shows the unique intensity in spectrograph on special wavenumber, chemical characteristic, and interaction of BC can be anticipated in the presence of other chemical compounds with known chemical groups in nanocomposites and other applications.\[16,35\]

Fourier transform infrared spectroscopy diagram peaks have been analyzed by IRPal 2 software (Table 6). It can be inferred from FTIR analyzed that BC has a diversity of organic functional groups which provide different expected interactions to other compounds especially in nanocomposite design.\[36\] The most expected interaction is nucleophilic interaction and/or H-bonds by BC electron donor groups such as C–O\(^{-}\) at the Carboxylic or Enol group.\[37\] Since the chemical group of BC has been known, chemical surface modification can be applied depending on biomedical utilization.\[38,39\]

**MTT assay**

To assay BC cytotoxicity, growth of L929 mice fibroblast in the presence of BC was assessed by MTT assay. The result shows (Figure 7) that after 5 days, approximately more than 90% of the mice fibroblast in the presence of BC showed the same growth in comparison to control wells. It is concluded that because of the BC nanofiber structure and its hydrophilic surface as well as low contact angles, fibroblast cells were grown on BC as a cell scaffold.\[40,41\] Hence, not only BC showed biocompatibility and no cytotoxicity for fibroblast cells\[42\] but also because of BC features it showed the ability to utilize as cell scaffold.\[28,43\]

In other studies to improve the biocompatibility of BC, different extracellular matrices (collagen, elastin, and hyaluronic) and growth factors (B-FGF, H-EGF, and KGF) were immobilized onto BC which exhibited desirable skin substitute characteristics that could be used as a deliver vehicle for therapeutic compounds during wound healing.\[44,45\]

**Tensile strength tests**

Mechanical properties of BC were studied by tensile strength test and calculate Young’s modulus. The stress–strain diagram of obtained BC from optimum condition is shown in Figure 8.

| pH  | BC yield (g/l) |
|-----|----------------|
| 6.5 | 10.5           |
| 4.8 | 9.7            |
| 5   | 9.2            |
| 4   | 8.6            |

BC, bacterial cellulose.

**Table 5.** Comparison of BC production yield in different pH values with constant condition of other variables.

Figure 3. OTR in different pH versus optimum pH. *Note:* OTR, oxygen transfer rate.

Figure 4. SEM of BC structure in optimum condition consisting of the complex of nanofiber and nanoporosity. *Note:* SEM, scanning electron microscopy; BC, bacterial cellulose.
Figure 5. SEM of BC structure in optimum condition consists of the complex of nanofiber and nanoporosity.

Figure 6. FTIR diagram of obtained BC.

Table 6. FTIR peak wavenumber analysis.

| Bond     | Wavenumber (/cm) | Expected group       |
|----------|------------------|----------------------|
| C–H R-CH₂-CH₃ | 2,850–3,000      | Alkan                |
|          | 1,375 and 1,450  | Methyl-group         |
|          | 1,465            | Methyl-group         |
| C–C      | 1,400–1,500      | C-cycle              |
| C–O      | 1,720–1,740      | Aldehydr             |
|          | 1,700–1,725      | Carboxylic acid      |
|          | 1,730–1,750      | Ester                |
| O–H      | 3,200–3,650      | Alcohol              |
|          | 2,500–3,300      | Carboxylic acid      |

FTIR, Fourier transform infrared spectroscopy.
The analyzed diagram and Young’s modulus show the BC mechanical properties (Table 7). As skin tissue has a low tensile strength and also low Young’s modulus, it can be inferred from BC mechanical properties that BC can be used as skin scaffolds for skin repairs and wound dressing in tissue engineering. In one study, BC scaffolds were used to repair, whereby it was shown that these scaffolds are good candidates for repairing wound because of 1.2 MPa tensile strength and 30% elongation.[46]

Conclusion
As G. xylinum is aerobic and acidophile bacteria and BC was produced in growth phase of G. xylinum, ventilation and pH value are the effective parameters in BC production. The BC sheets, which are utilized in medical and several other applications, are produced by G. xylinum in HS medium through static culture. Previously, static culture has been defined as a condition without any stress on the culture surface, unfortunately the low yield of the static production process has restricted the commercial efficiency of BC. However, in this study, static condition about BC production is newly defined as a condition that has no agitation on culture media surface which is the phase of forming BC sheets. This study concluded that up to 50 rpm shaking rates the culture condition is still static (no stress in surface). Previously, various pH values were reported for G. xylinum and HS culture media. This study designed experiments based on CCD with two-parameter pH value and shaking rates which were set to miniature bioreactor. While two responses as OD (600 nm) which showed the bacterial growth and BC mass (g) were defined for each experiments, RSM optimized the parameters and showed the expected responses which subsequently were approved. The optimum pH value 6.5 and shaking rate 50 rpm for static culture of G. xylinum to produce BC sheets were concluded. The optimum pH value in this study and other previous reports has been compared by OTR graphs in miniature bioreactor which showed that the maximum yield 10.5 g/l has been obtained in pH value 6.5.

The properties of obtained BC in optimum static condition were evaluated. For evaluating chemical properties of biopolymer FTIR spectroscopy was used and fiber morphology was studied by Fe-SEM which observed bacterial cellulose structure contains nanofiber and nanoporosity. These nanosstructural features of bacterial cellulose can be considered in biomedical engineering. Biocompatibility was assessed by the MTT assay method and showed that BC has features to utilize as cell scaffolds. Tensile strength tests (Young’s modules) showed that the biopolymer can be utilized as scaffolds for skin tissues. Wound dressing materials are required strong and flexible scaffolds with appropriate bending feature. Appropriate mechanical strength (0.37 MPa) and low Young’s modulus (3.36 MPa) make BC attractive as potential wound dressing. BC shape can be variable and controllable, which enables BC scaffold to contact with the skin completely.

References
[1] Hu, W.; Chen, S.; Yang, J.; Li, Z.; Wang, H. Functionalized Bacterial Cellulose Derivatives and Nanocomposites. Carbohyd. Polym. 2014, 101, 1043–1060.
[2] Chawla, P.R.; Bajaj, I.B.; Survase, S.A.; Singhal, R.S. Microbial Cellulose: Fermentative Production and Applications. Food Technol. Biotech. 2009, 47, 107–124.
[3] Scherner, M.; Reutter, S.; Klemm, D.; Sterner-Kock, A.; Guschlbauer, M.; Richter, T.; Wippermann, J. In vivo Application of Tissue-engineered Blood Vessels of Bacterial Cellulose as Small Arterial Substitutes. J. Surg. Res. 2014, 189, 340–347.
[4] Tahara, N.; Tabuchi, M.; Watanabe, K.; Yano, H.; Morinaga, Y.; Yoshinaga, F. Degree of Polymerization of Cellulose from Acetobacter xylinum BPR2001 Decreased by Cellulase Produced by the Strain. Biosci. Biotechnol. Biochem. 1997, 61, 1862–1865.
[5] Naritomi, T.; Kouda, T.; Yano, H.; Yoshinaga, F. Effect of Lactate on Bacterial Cellulose Production from Fructose in Continuous Culture. J. Ferment. Bioeng. 1998, 85, 89–95.
[6] Toyosaki, H.; Naritomi, T. Screening of Bacterial Cellulose-producing Acetobacter Strains Suitable for Agitated. Biosci. Biotechnol. Biochem. 1995, 59, 1498–1502.
[7] Kouda, T.; Yano, H.; Yoshinaga, F. Effect of Agitator Configuration on Bacterial Cellulose Productivity in Aerated and Agitated Culture. J. Ferment. Bioeng. 1997, 83, 371–376.

[8] Zuo, K.; Cheng, H.P.; Wu, S.C.; Wu, W.T. A Hybrid Model Combining Hydrodynamic and Biological Effects for Production of Bacterial Cellulose with a Pilot Scale Airlift Reactor. Biochem. Eng. J. 2006, 28, 81–90.

[9] El-Saied, H.; El-Diwany, A.I.; Basta, A.H.; Atwa, N.A.; El-Ghwas, D.E. Production and Characterization of Economical Bacterial Cellulose. Bioresources 2008, 3, 1196–1217.

[10] Brown, R.M. The Biosynthesis of Cellulose. J. Macromol. Sci. Chem. 1996, 33, 1345–1373.

[11] Keshk, S.M. Bacterial Cellulose Production and its Industrial Applications. J. Bioprocess. Biotech. 2014, 4, 1–10.

[12] Czaja, W.K.; Young, D.J.; Kawecki, M.; Brown, R.M. The Future Prospects of Microbial Cellulose in Biomedical Applications. Biomacromolecules 2007, 8, 1–12.

[13] Okamoto, M.; John, B. Synthetic Biopolymer Nanocomposites for Tissue Engineering Scaffolds. Prog. Polym. Sci. 2013, 38, 1487–1503.

[14] Almeida, I.F.; Pereira, T.; Silva, N.H.C.S.; Gomes, F.P.; Silvestre, A.J.D.; Freire, C.S.R.; Costa, P.C. Bacterial Cellulose Membranes as Drug Delivery Systems: An In Vivo Skin Compatibility Study. Eur. J. Pharm. Biopharm. 2014, 86, 332–336.

[15] Wang, S.; Cheng, Q.; Rials, T.G.; Lee, S.H. Cellulose Microfibril Nanofibril and its Nanocomposites, Proceedings of the 8th Pacific Rim Bio-based Composites Symposium, Kuala Lumpur, Malaysia, 2006, 20–23.

[16] Ma, H.; Burger, C.; Hsiao, B.S.; Chu, B. Fabrication and Characterization of Cellulose Nanofiber based Thin-film Nanofibril and its Nanocomposites. J. Membrane. Sci. 2014, 454, 272–282.

[17] Maneerrona, T.; Tokurab, S.; Rujiravanit, R. Impregnation of Silver Nanoparticles into Bacterial Cellulose for Antimicrobial Wound Dressing. Carbohyd. Polym. 2008, 72, 43–51.

[18] Shah, N.; Ul-Islam, M.; Khattak, W.A.; Park, J.K. Overview of Bacterial Cellulose Composites: A Multipurpose Advanced Material. Carbohyd. Polym. 2013, 98, 1585–1598.

[19] Hestrin, S.; Schramm, M. Synthesis of Cellulose by Acetobacter xylinum. 2. Preparation of Freeze-dried Cells Capable of Polymizing Glucose to Cellulose. Biochem. J. 1954, 58, 345–352.

[20] Jonas, R.; Farah, L.F. Production and Application of Microbial Cellulose. Polym. Degrad. Stab. 1998, 59, 101–106.

[21] Pourramezan, G.Z.; Roayaie, A.M.; Qezelbash, Q.R. Optimization of Culture Conditions for Bacterial Cellulose Production by Acetobacter sp. 4B-2. Biotechnology 2009, 8, 150–154.

[22] Keshk, S.; Sameshima, K. The Utilization of Sugar Cane Molasses with/without the Presence of Lignosulfonate for the Production of Bacterial Cellulose. Appl. Microbiol. Biotechnol. 2006, 72, 291–296.

[23] Kouda, T.; Naritomi, T.; Yano, H.; Yoshinaga, F. Effects of Oxygen and Carbon Dioxide Pressures on Bacterial Cellulose Production by Acetobacter in Aerated and Agitated Culture. J. Ferment. Bioeng. 1997, 84, 124–127.

[24] Noro, N.; Sugano, Y.; Shoda, M. Utilization of the Buffering Capacity of Corn Sweet Liqueur in Bacterial Cellulose Production by Acetobacter xylinum. Appl. Microbiol. Biotech. 2004, 64, 199–205.

[25] Montgomery, D.C. Designs and Analysis of Experiments, 6th ed.; John Wiley and sons Inc., 2006.

[26] Zeng, X.; Small, D.P.; Wan, W. Statistical Optimization of Culture Conditions for Bacterial Cellulose Production by Acetobacter xylinum BPR 2001 from Maple Syrup. Carbohyd. Polym. 2011, 85, 506–513.

[27] Mosmann, T. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. J. Immunol. Methods 1983, 65, 55–63.

[28] Lacn, N.T. Development of Biodegradable Antibacterial Cellulose based Hydrogel Membranes for Wound Healing. Int. J. Biol. Macromol. 2014, 67, 22–27.

[29] Czichos, H.; Saito, T.; Smith, L.R. Springer Handbook of Materials Measurement Methods; Springer Science Inc. 2006; pp. 303–304.

[30] Rathore, B.S.; Sharma, G.; Pathania, D.; Gupta, V.K. Synthesis, Characterization and Antibacterial Activity of Cellulose Acetate-tin (IV) Phosphate Nanocomposite. Carbohyd. Polym. 2014, 103, 221–227.

[31] Dorj, B.; Won, J.E.; Purevdorj, O.; Patel, K.D.; Kim, J.H.; Lee, E.J.; Kim, H.W. A Novel Therapeutic Design of Microporous-structured Biopolymer Scaffolds for Drug Loading and Delivery. Acta Biomaterial. 2014, 10, 1238–1250.

[32] Ruka, D.R.; Simon, G.P.; Dean, K.M. In Situ Modifications to Bacterial Cellulose with the Water Insoluble Polymer Poly-3-hydroxybutyrate. Carbohyd. Polym. 2013, 92, 1717–1723.

[33] Yeh, J.T.; Tsai, C.C.; Wang, C.K.; Shao, J.W.; Xiao, M.Z.; Chen, S.C. Ultra Drawing Novel Ultra-high Molecular Weight Polyethylene Fibers Filled with Bacterial Cellulose Nanofibers. Carbohyd. Polym. 2014, 101, 1–10.

[34] Sheykhnazari, S.; Tahara, T.; Asahi, A.; Shakeri, A.; Golalipour, M. Bacterial Synthesized Cellulose Nanofibers: Effects of Growth Times and Culture Mediums on the Structural Characteristics. Carbohyd. Polym. 2011, 86, 1187–1191.

[35] Wu, J.; Zheng, Y.; Song, W.; Luan, J.; Wen, X.; Wu, Z.; Guo, S. In Situ Synthesis of Silver-nanoparticles/Bacterial Cellulose Composites for Slow-released Antimicrobial Wound Dressing. Carbohyd. Polym. 2014, 102, 762–771.

[36] Pullawat, T.; Wilkinson, A.N.; Zhang, L.N.; Eichhorn, S.J. Deformation Micromechanics of All-cellulose Nanocomposites: Comparing Matrix and Reinforcing Components. Carbohyd. Polym. 2014, 100, 31–39.

[37] Khandelwal, M.; Windle, A. Origin of Chiral Interactions in Cellulose Supra-molecular Microfibrils. Carbohyd. Polym. 2014, 106, 128–131.

[38] Arrieta, M.P.; Fortunati, E.; Dominici, F.; Rayón, E.; López, J.; Kenny, J.M. Multifunctional PLA-PHB/Cellulose Nanocrystal Films: Processing, Structural and Thermal Properties. Carbohyd. Polym. 2014, 107, 16–24.

[39] Yu, H.Y.; Qin, Z.Y. Surface Grafting of Cellulose Nanocrystals with Poly(3-hydroxybutyrate-co-3-hydroxyvalerate). Carbohyd. Polym. 2014, 101, 471–478.

[40] Mabrouk, A.B.; Salon, M.C.B.; Magnin, A.; Belgacem, M.N.; Boufi, S. Cellulose-based Nanocomposites Prepared via Mini-emulsion Polymerization: Understanding the Chemistry of the Nanocellulose/Matrix Interface. Colloids Surf., A 2014, 448, 1–8.

[41] Pereira, R.F.; Bartolo, F.J. Degradation Behavior of Biopolymer-based Membranes for Skin Tissue Regeneration. Proc. Eng. 2013, 59, 285–291.

[42] Taokaew, S.; Phisalaphong, M.; Zhang Newby, B. In Vitro Behaviors of Rat Mesenchymal Stem Cells on Bacterial Celluloses with Different Moduli. Mater. Sci. Eng. C 2014, 38, 263–271.

[43] Zang, S.; Sun, Z.; Liu, K.; Wang, G.; Zhang, R.; Liu, B.; Yang, G. Ordered Manufactured Bacterial Cellulose as Biomaterial of Tissue Engineering. Mater. Lett. 2014, 128, 314–318.

[44] Li, Y.; Qing, S.; Zhou, J.; Yang, G. Evaluation of Bacterial Cellulose/ Hyaluronan Nanocomposite Biomaterials. Carbohyd. Polym. 2014, 103, 496–505.

[45] Lin, Y.K.; Chen, K.H.; Ou, K.; Min, L. Effects of Different Extracellular Matrices and Growth Factor Immobilization on Biodegradability and Biocompatibility of Macroporous Bacterial Cellulose. J. Biocat. Compat. Polym. 2011, 26, 508–518.

[46] Fu, L.; Zhang, J.; Yang, G. Present Status and Applications of Bacterial Cellulose-based Materials for Skin Tissue Repair. Carbohyd. Polym. 2013, 92, 1432–1442.