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Study of Nano-fiber Cellulose Production by *Glucanacetobacter xylinum* ATCC 10245

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Abstract: Bacterial Celluloses (BC) are gaining importance in research and commerce due to numerous factors affecting the bacterial cellulose characteristics and application in different industries. The aim of the present study was to produce bacterial cellulose in different media using different cultivation vessels. Bacterial cellulose was produced by static cultivation of *Glucanacetobacter xylinum* ATCC 10245 in different culture media such as Brain Heart Agar, Luria Bertani Agar /Broth, Brain Heart Infusion, Hestrin-Schramm and medium no. 125. Cultivation of bacterium was conducted in various culture vessels with different surface area. The cellulose membrane was treated and purified with a 0.1 M NaOH solution at 90°C for 30 min and dried by a freeze- drier at -40°C to obtain BC. The prepared bacterial cellulose was characterized by scanning electron microscopy (SEM), Fourier transform infrared (FT-IR) spectroscopy and X-ray diffraction (XRD). The amount of produced BC was related directly to the surface area of culture vessels.

Key words: Bacterial cellulose, *Glucanacetobacter xylinum,* nano-fiber membranes, surface area, extraction media

INTRODUCTION

Cellulose is the most abundant natural polysaccharide found in nature with the chemical formula $(C_6H_5O_{10})_n$ (Chawla *et al*., 2009). The hydroxyl groups in cellulose are able to combine partially or completely with other chemicals to produce useful cellulosic derivatives. Cellulose biopolymer is formed by four methods: A isolation of cellulose from plants through lignin and hemicellulosic process which is a normal process (Tarchevsky *et al*., 1991; Klemm *et al*., 1998). biosynthetic (Vandamme *et al*., 1998; Jonas and Farah, 1998) enzymatic and (Kobayashi *et al*., 1991, 1992) chemical synthesis (Nakatsubo *et al*., 1996). Biological synthesis of cellulose is brought about by various microorganisms such as algae (*Vallonia*), fungi (*Saprolegnia, Dictystelium* and *Discodium* species), bacteria (*Acetobacter, Achromobacter, Aerobacter, Agrobacterium, Pseudomonas, Rhizobium, Alcaligenes, Saecina* and *Zoogloea* species). The above mentioned bacterial species are not able to synthesize fibrous cellulose extracellularly (Sun *et al*., 2007). *Gluconobacter xylinum* (formerly known as *Acetobacter xylinus* or *Acetobacter xylinum*) is Gram negative, rod shape, non pathogen, obligatory aerobic bacterium which belongs to the family of Acetobacteracea. A notable feature of this bacterium is its ability to produce extracellular cellulose as a pure, ultra fine fibrous network, possessing high crystallinity, water absorption and mechanical stability. Thus the produced cellulose network is known as pellicle (Klemm *et al*., 2001). The bacteria of genus *Acetobacter* are obligatory aerobic and are often found in fruits, vegetables, vinegar, fruit juice and alcoholic beverages. The mechanism of synthesizing bacterial cellulose helps the bacterium as follows: (a) provides oxygen at the surface of cultivation medium, (b) protect the bacteria against ultra violet rays and (c) maintains the humidity of cultivation medium. (Sun *et al*., 2007). Bacterial cellulose is gaining research interest due to properties like, fine fiber network, high water holding/absorption capacity, high mechanical strength (Hong *et al*., 2006; Putra *et al*., 2008) . Since the bacterial cellulose fiber's size is much smaller than that of plant cellulose, this makes the bacterial cellulose to be unique. High water maintenance capacity, high elasticity, high stability and compatibility of bacterial cellulose can be characteristics of cellulose produced by bacteria rather than isolated from plants (Czaja *et al*., 2006). Growth of bacteria under static or agitated conditions, media compositions will give rise to bacterial cellulose of different morphological characteristics (Ross *et al*., 1991). Bacterial cellulose produced under different cultural condition with various morphological characteristics and sizes, can be applied as stent coatings, for dura-mater substitution in tumor or trauma cases or as skin protection

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in cases of burning, deep wounds, periodontal tissue recovering (Rambo *et al*., 2008). In this study, attempts are made to produce bacterial cellulose in different media using different cultivation vessels.

MATERIALS AND METHODS

Microorganism: Glucanacetobacter xylinium ATCC 10245 was obtained from Iranian Research Organization for Science and Technology (IROST). The ampoule containing the bacteria was opened aseptically at Laminar Air Flow cabinet as per supplier's instruction. The revived microorganism was propagated and stocks were prepared. Following media were used and inoculated with *Glucanacetobacter xylinium* to produce bacterial cellulose:

- The 100 mL Luria Bertani broth in 500 mL flask with the composition of peptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 5 g L^{-1} with pH adjusted to 7
- The 100 mL Brain Heart Infusion Broth in 500 mL flask with the pH of 7
- The 100 mL Hestrin-Schramm broth in 500 mL Rough bottle (large surface area), with the following composition per liter: peptone 5 g, yeast extract 5 g, citric acid 1.15 g, disodium hydrogen phosphate 2.7 g and glucose 20 g with pH adjusted to 6 (Hestrin and Schramm, 1954)
- Test tube (small surface area) and Petri plate (larger surface area than slant) containing Brain Heart infusion agar
- Petri plate containing solid LB medium with composition as mentioned above. 2% agar was added to solidify the medium
- Solid medium of *Gluconobacter oxidans:* The medium consisted of g L^{-1} yeast extracts 10 g, calcium carbonate 20 g, glucose 100 g, agar 15 g, pH of the medium was adjusted to 6.8 (recommended by the supplier of organism designated as medium No.125)

The flasks and plates were incubated at 28-30°C for 2-7 days at static condition. After the cultivation period, the formed gel at the liquid surface and between the pin array templates was removed, washed with deionized water and dried at 50°C. Subsequently, the cellulose membrane was treated with a 0.1 M NaOH solution at 90°C for 30 min to remove bacterial impurities and eventual cell debris. The membrane was again washed in deionized water to reach to neutral pH and then dehydrated by a freeze-dryer at -40°C.

Characterization: The prepared bacterial cellulose was characterized by Scanning electron microscopy (SEM), Fourier transform infrared (FT-IR) spectroscopy and X-ray diffraction (XRD) studies.

Scanning Electron Microscopy (SEM) analysis: SEM images of the samples were taken with a microscope (SEM, Philips, XL-30) to study the morphological changes. For the observations, BC membranes were freeze dried and placed over an aluminum support and sputtered with gold.

Fourier Transform-Infrared Spectrum (FTIS) analysis: FT-IR spectra were obtained using a BRUKER-Equinox 55 FT-IR spectrophotometer for the evaluation of chemical structures using a KBr pellet.

X-ray diffraction: XRD patterns were recorded on an X-ray diffractometer (Phlips PW 1140) by using Cu K_α ($\lambda = 1.45$ nm) radiation at 40 kV and 30 mA. The diffraction angle ranged from 5 deg to 40 deg. X-ray diffractometry was used to identify the phases of the cellulose membrane.

RESULTS AND DISCUSSION

Bacterial cellulose production: Table 1 reveals the production of bacterial cellulose in different cultivation media and type of vessels employed. As it can be seen from the Table 1, the surface area is an important factor and the production of bacterial cellulose occurs at the interface of air/liquid or solid surface. Bacterial cellulose has quite different structure from that of plant cellulose due to different synthesis procedure. During cultivation, the bacteria synthesize fine sub-elementary cellulose fibrils which are extruded from terminal enzyme complexes into the culture medium. Nascent cellulose extending from terminal enzyme complexes is initially amorphous and is gradually crystallized to cellulose. The obtained bacterial cellulose is dried by freeze-dryer. Figure 1 and 2 are typical images of bacterial cellulose synthesized by *G. xylinum* (ATCC 10245) under the static condition. Observation made by SEM indicates that the bacterial cellulose dried by freeze-drier contains more homogenous pores than those dried by hot air. Some isolated fibrils have less than 100 nm width. Pores with diameters lower than 100 μm are suitable for wound contracture (Czaja *et al*., 2006). They prolong the fibroblast migration through the porous template. Beside the use of thin pore membranes in medical applications, membranes with pore sizes in the range of 10-100 μm are interesting materials to

Table 1: Production of cellulose in different medium

Medium	Cellulose production	Vessel
Brain Heart Agar	Solid medium/air interface cellulose production	Petri plate
Brain Heart Agar	No production of cellulose	Test tube
Luria Bertani Agar	No production of cellulose	Test tube
Luria Bertani broth	Air/liquid interface cellulose production	Erlen Meyer
Brain Heart Infusion	-do-	-do-
Hestrin- Schramm	-do-	Rough bottle
Medium No. 125	Solid medium/air interface cellulose production	Petri plate

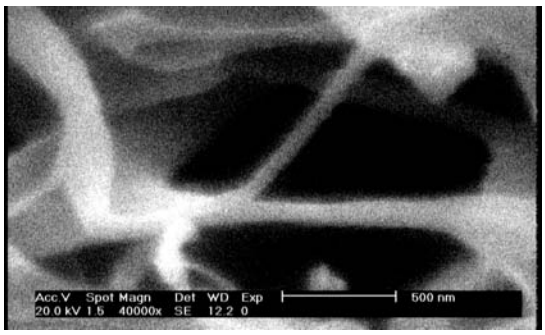

Fig. 1: SEM image of produced BC with Gluconacetobacter xylinum ATCC 10245 in static culture, 2500x magnification

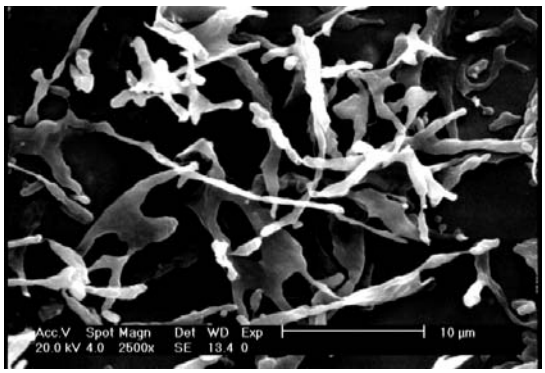

Fig. 2: SEM image of produced BC with *Gluconacetobacter xylinum* ATCC 10245 in static culture, 40000x magnification

promote selective cell migration through the pores for specific cell cultures, with different cell sizes (Rambo *et al*., 2008). The sub-elementary fibrils are approximately 2-4 nm in diameter and are assembled into micro fibrils. These micro fibrils are then bundled to form ribbon-shaped fibrils of approximately 4 (thickness) × 80 (width) nm (Cai and Kim, 2010). The fibril length is in micro scale and some micro fibril-ends are not apparent in figures (Retegi *et al*., 2010). Cellulose biosynthesis is characterized by unidirectional growth and crystallization, where glucose molecules are linear bonded by β (1→4)-glycosidic bonds. The union of glycosidic chains forms oriented micro fibrils with intermolecular hydrogen bonds. The cellulose is crystallized outward the organisms, particularly in *A. xylinum* that synthesizes cellulose chains by introducing glucose units to the reducing ends of the polymer. The growth mechanism during bacterial activity determines the morphology of the final cellulose (Rambo *et al*., 2008).

Table 2: FT-IR spectra band assignments

Assignment, related bond	Wave number (cm^{-1})
(O-H)'s stretching vibration, H-bond between the molecules	3400
(C-H)'s stretching vibration of sugar ring	2927
(-CONH-)'s stretching vibration	1652
(C-H)'s bending vibration	1465/52
(C-O-C and C-O-H)'s stretching vibration of sugar ring	1065

Figure 3 depicts the FT-IR spectra of bacterial cellulose sample. FT-IR spectral band assignments of the sample are clearly listed in Table 2. In this case, a broad band at 3400 cm^{-1} is attributed to O-H stretching vibration. The band at 2927 cm represents the aliphatic C-H stretching vibration. A sharp and steep band observed at 1065 cm^{-1} is due to the presence of C-O-C stretching vibrations. The carbonyl amide group at 1652 cm^{-1} in the FT-IR spectra of BC is due to the proteins and bacterial cells of BC's suspension that is not easily wiped off after the NaOH treatment.

The morphological changes of ribbons are supposed to be related to the changes in microstructures such as crystallinity, I_α fraction and hydrogen bonding between the molecules (Sun *et al*., 2007). For pure BC, three main peaks located at 14.2, 16.6 and 22.4 deg can be identified in spectra for (110) (110) and (200) reflex ion planes of cellulose I (Kim *et al*., 2010). Like plant cellulose, BC has cellulose I structure. In the X-ray diffraction pattern of dry BC, peaks appear at 2θ = 14.5°, 16.5° and 22.5° for the (110) (110) and (200) reflex ion planes, respectively. However, during the cultivation, the (110) plane has a selective uniplanar orientation and the (110) peak becomes sharp and large. It was reported that, cellulose crystals become preferentially oriented in the (110) plane when water is removed from BC pellicle (Takai *et al*., 1975). Also, replacement of the water in BC pellicle with organic solvents prior to drying affects the orientation of

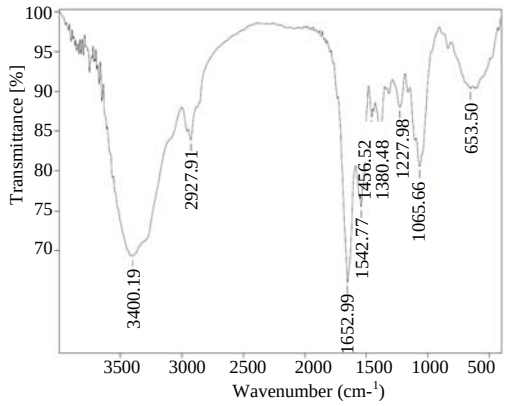

Fig. 3: FT- IR spectra of freeze- died BC synthesized under static culture condition

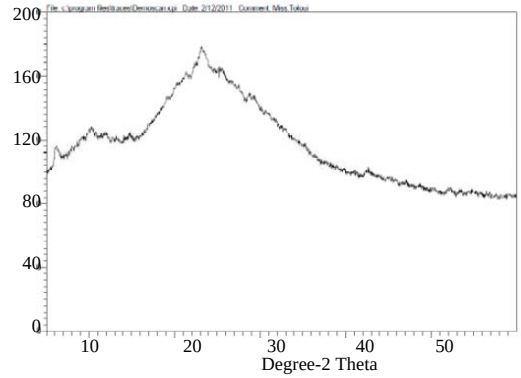

Fig. 4: XRD pattern for produced BC

the (110) plane. Polar solvents such as acetone and pyridine gave a higher selective uniplanar orientation, resulting in an increase in the intensity of the (110) peak, whereas non- polar solvents such as carbon tetrachloride and cyclohexane disturb the orientation and the intensity of the (110) peak decreases. Figure 4 shows the X-ray diffraction patterns of BC sample dried in the air. This produced BC is not pure and it should be purified and recrystallized.

CONCLUSION

BC synthesized by *G. xylinum* ATCC 10245 is in twisting network structure with many nano-fiber ribbons having the diameter of about 30 nm to 1 μm or more. Based on FT-IR spectrum produced BC has some impurities and it should be purified and recrystallized. The pore size is different with a range of more than 1 μm. Bacterial cellulose is proving to be a very versatile material. It can be used in a wide variety of biomedical applications, from topical wound dressings to the durable scaffolds required for tissue engineering. Many scientists are trying to develop novel biomaterials through biotechnological process. These new materials could be used in many biomedical and biotechnological applications, such as tissue engineering, drug delivery and, wound dressing medical implants. However, much interdisciplinary research is needed in order to bring microbial cellulose products to successful commercialization. For example, a wide variety of mammalian cells need to be seeded onto BC membranes in order to assess their viability and proliferation. A number of clinical studies are necessary to prove its usefulness and functionality. If bacterial cellulose proves to be effective in wound repair and tissue engineering, then it should be produced on an industrial scale. Due to its simple fermentation process, large scale bacterial cellulose production appears to be quite feasible.

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