Properties, Morphology and Bioproduction of Bacterial Cellulose Using Static Fermentation

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Abstract: *Overview:* Cellulose is the world's major polymer of economic importance. It is the main component of wood and cotton. Cotton and wood are the major sources for most of cellulose products i.e. textiles, paper and construction compounds.

Aim of Study: The work is aimed to study the probability of underutilized Hestrin-Schramm (HS) medium for the production and optimization of bacterial cellulose (BC) by *Gluconacetobacter xylinus* RO-7 and to study the properties of the resulted BC polymer.

Methods and Results: Experiments were designed to enhance the bacterial cellulose yields along with environmental growth factors. Production of bacterial cellulose using static fermentation process was studied in HS medium at 30°C in 250 Erlenmeyer flasks by using *Gluconacetobacter xylinus* RO-7 isolated from local pickles markets. Results revealed that *Gluconacetobacter xylinus* RO-7 produced a bacterial cellulose yield of 18 gm/L. Several factors were test to increase the productivity of BC. Cellulose fibrils were subjected to thermal gravimetric, X-ray diffraction, FTIR analysis. Morphological characters of cellulose fibrils were also observed using Scanning electron microscopy.

Conclusion: Microbial cellulose has many applications as scientific and biomedical endeavors. Therefore it is concluded to use the biological methods for the production of BC.

Keywords: Bacterial cellulose, fermentation, *gluconacetobacter xylinus* RO-7, FTIR, TGA, XRD.

1. INTRODUCTION

Cellulose is the most copious biological molecules in the world. It forms a structural matrix of cell walls of several fungi, algae, and nearly all plants. It plays an important role in the integrity of cell walls of all plants. Wood is the most common commercial source of cellulose; however, it requires extensive processing to eliminate the impurities like hemicelluloses and lignin to obtain purified cellulose [1].

Bacterial cellulose has many advantages over plant cellulose. Bacterial cellulose fibrils are randomly oriented and the product is highly amorphous [2]. The distinct properties of BC are due to the highly organized network of fine fibers with a diameter of 0.1 ml, which is about one hundredth that of plant fibers [3].

Many species of bacteria, such as those in the genera Gluconacetobacter have been reported to produce extracellular cellulose [4].

One of the most advanced types of purple bacteria is the common *Acetobacter* [5, 6].

Production and isolation of bacterial cellulose (especially that produced by *Acetobacter* strain) are relatively simple when compared to those produced from wood pulp. There is no single system, which has emerged as an ideal system for the study of cellulose biosynthesis.

Gluconacetobacter xylinus produces large quantities of cellulose as microfibrils. The microfibrils could be fuse to form ribbon-like cellulose [7, 8].

The main purpose of this study was to use a simple fermentation process for the production of bacterial cellulose using static fermentation.

2. MATERIALS AND METHODS

2.1. Microorganism

Microorganisms were isolated from crude pellicle formed on the surface of pickles effluents (previously collected from local pickles markets of Qurayyat governorate, Al-Jouf, KSA). Pellicles were collected and washed with sterile distilled water. To obtain a cell suspension, the contents were serially diluted and streaked on Hestrin-Schramm (HS) agar plates [9]. Isolation and purification of the resulted microorganisms were carried out according to the method described by Khattak *et al*. [1].

2.2. Fermentation

HS medium (g/L) contained glucose, 20; yeast extract, 5; peptone, 5; di-sodiumhydrogen phosphate, 2.7; citric acid, 1.15; pH 4.5. The pellicles formed after incubation were removed carefully.

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2.3. Purification of Pellicle

Pellicle grown on the surface of fermentation media was harvested under sterile environment. The pellicle was washed with water, soaked in 1 N NaOH for one day at room temperature to remove the cells and other impurities embedded in the pellicle and rinsed thoroughly with water until a neutral pH was attained in the drained water. The pellicle was press dried in between the filter papers at 60°C till the film weight became constant [10].

2.4. Media Optimization

Based on HS medium as a standard medium, several factors (pH values, Temperature, incubation period and various nitrogen sources) were tested in sequence to determine the optimal conditions of culture medium for the production of BC. After one component was chosen, the test for its optimum concentration followed. The amounts of BC produced were measured in static culture conditions under the optimized medium.

The incubation under static fermentation at 30^{o}C for 14 days was tested first. Then, the incubation period was conducted from 1 to 16 days. The effect of different pH values (3, 4, 5, 6, 7 and 8), and temperature range (10, 20, 30, 40, 50 and 60^{o}C) on BC production was also conducted. The best nitrogen source was tested either in the presence of Peptone (control) or without it. Many nitrogen sources (w/v) were tested i.e. yeast extract, urea, tryptone, ammonium sulphate.

2.5. Fourier Transform Infrared Spectroscopy (FTIR/ATR)

Thin film with uniform thickness was used for obtaining the IR spectra of BC film using IR spectrophotometer (FTIR-RAMAN Nicolet 5700, USA). The measurement was investigated at 20°C in anhydrous condition with air as the background. For each sample, 32 scans at 2cm^{-1} resolution were collected in the scanning range of 4000-700cm^{-1} wavelength.

2.6. Mechanical Properties

2.6.1. X-ray Diffraction (XRD)

The structure of bacterial cellulose was analysed with X-ray Diffractometer to examine the crystallinity of freeze-dried BC after alkaline washing. XRD spectra were recorded by using Diffractometer (RIGAKU, Japan) at 40kV and 200mA. In addition, crystallinity of bacterial celluloses was determined by integration of each XRD peaks taking into account a baseline for each peak (area assigned to the crystalline part), and the total area under the diffractogram considering a straight line from 0 to 80° 2θ as baseline.

2.6.2. Thermogravimetric Analysis (TGA)

Dynamic thermogravimetric analysis of dried samples was conducted in a TGA-51instrument (SHIMADZU, INDIA). Temperature programs were run from 50 to 700°C at a heating rate of 10°C/min, under nitrogen atmosphere (30ml/min) in order to prevent thermoxidative degradation.

2.6.3. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) of the BC sample produced from fermentation process was performed using Jeol-JSM 5500 (JEOL LTD., JAPAN) electron microscopy. The samples were coated by gold sputter and examined using high vacuum mode.

4. RESULTS AND DISCUSSION

4.1. Identification of Isolates

Eleven single opaque colonies with creamy color and approximately 2.0 to 2.5mm in diameter were observed on HS-agar plates. Between these entire isolates only one (RO-7) was able to produce cellulose. This is in accordance to results of Thompson and Hamilton [2] who stated that the most studied producer of bacterial cellulose is *Acetobacter* sp. BC is produced as gel shaped film on the surface of culture media in static culture (Figure **1**). The thickness and size of BC film is increased continuously with culture time.

Figure 1: Production of BC by *Gluconacetobacter xylinus* RO-7.

Gram staining of the isolated culture proved that isolate was gram negative, being short bacilli. The culture was transferred onto CaCO3-agar plates for visualization of acid production. The colonies formed CaCO3 clear zones. These colonies were selected as acetic acid bacteria and were subjected to further analysis. The isolate was able to oxidize both lactate and acetate. This finding is regarded as a distinctive property of *Acetobacter, Acidomonas*and *Asaia* spp. [11, 12] which was also verified by positive response for cellulose production. The biochemical properties of the isolate capable of cellulose production are summarized in Table **1**. The identification was confirmed using Bacterial Identifier instrument Vitek 2 Compact (BioMérieux, USA). Comparison of the results for the succeeded isolate showed that it could most likely to be a species of *Gluconacetobacter xylinus RO-7*.

Table 1: Biochemical Identification of Cellulose Producing Isolate

Test Name	Result
Cellulose production	(+)*
Oxidase	(-)*
Catalase	(+)
Urea utilization	(-)
Sodium citrate utilization	(-)
Indole production	(-)
H_2S formation	(-)
Oxidation of acetic acid	(+)
Lactate oxidation	(+)
Oxidation of ethanol	(+)
Gelatin liquefaction	(-)
Growth on mannitol agar	(+)
Ketogenesis of glycerol	(+)
Growth on glutamate agar	(-)
Fermentation of common sugars	
D-glucose	(+)
Fructose	(-)
Galactose	(+)
Sucrose	(-)
Lactose	(-)
Maltose	(+)
Mannose	(+)
Xylose	(+)

*Positive = (+) and *Negative result = (-).

4.2. Effect of Environmental Factors

4.2.1. Temperature

Temperature is a crucial parameter that affects both growth and cellulose production. The effects of different temperatures (10–60°C) were investigated using fermentation medium. The maximal cellulose production was achieved at 17 g/land the optimum temperature for cellulose production was found to be 30°C (Figure **2**). The same result was observed by Chawla Prashant *et al*. [13] who stated that the maximal cellulose production was observed between 28 and 30°C. At 10°C as well as 60°C production of BC was not resulted.

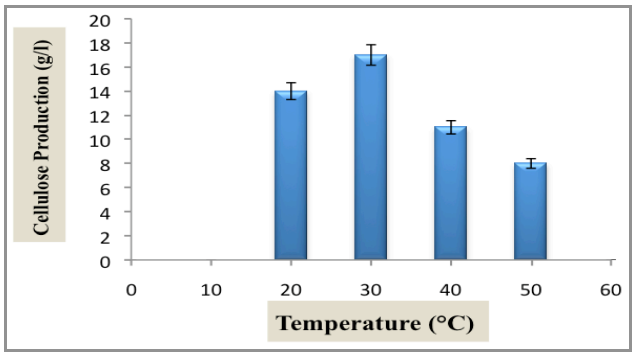

Figure 2: Effect of fermentation temperatures on BC production by *Gluconacetobacter xylinus*RO-7.

4.2.2. pH

The effect of pH is well documented in many studies [14-16]. The effect of initial pH on cellulose production was investigated in the range of 3.0-8.0. The investigated microorganism was found to produce cellulose over wide range of pH from 3.0 to 7.0 with optimum at pH 4 (Figure **3**). It is generally accepted due to the same pH range for cellulose production by Hungund and Gupta [4].

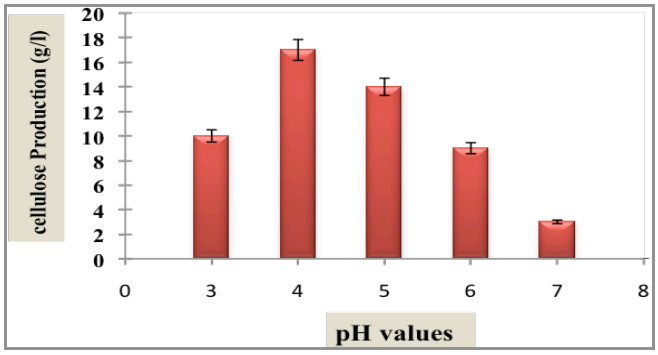

Figure 3: Effect of pH values on BC production by *Gluconacetobacter xylinus*RO-7.

4.2.3. Incubation Time

Microorganisms need time to accustom themselves to new fermentation condition. Results represented in

Figure **4** showed that the production of BC in the production media was observed for fourteen consecutive days in static cultures. A slow and relatively similar BC production was observed in the first 2 days of cultivation period. The initial low production of BC might be due to the lag growth phase of the bacterial cells and the presence of higher initial glucose concentration in the media. However, the rate of BC production was increased significantly after second day. Intense researches have been conducted to develop continuous fermentation. Ruka *et al*. [17] proposed 7-days of cultivation period which reached the highest BC productivity at approximately 14 days (10 g/l).

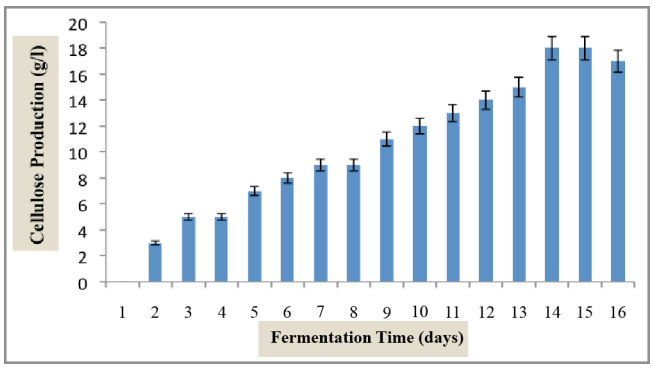

Figure 4: Effect of fermentation times on BC production by *Gluconacetobacter xylinus* RO-7.

4.2.4. Nitrogen Source

Several nitrogen sources were added to the fermentation medium at a level of 1% (w/v) to investigate the effect of nitrogen sources on the production of BC. As shown in Figure **5**, when peptone was added to the medium, the highest amount of BC (18 g/L) was obtained. The other nitrogen sources resulted in poor BC production. Through this result many researchers investigated different nitrogen sources for the production of BC. Peptone is considered as the most commonly used nitrogen source in BC production as it provides nitrogen and growth factors for microorganisms [18].

4.2.5. Fourier Transform Infrared Spectroscopy (FTIR)

Figure **6** shows the FTIR spectra collected for the bacterial cellulose produced by *Gluconacetobacter xylinus*RO-7. The bands were typical of cellulose I. The band centered at 1044cm^{-1} could be associated with ether C–O C functionalities [19]. The band at 1120cm^{-1} is assigned to cellulose C–O–C bridges [20]. The band at 1324cm^{-1} can be ascribed to C–H in-plane bending [21]. The band at 1432cm^{-1} could be associated with either CH_2 symmetrical bending or surface carboxylate groups. The band at 1647cm^{-1} is due to the H–O–H bending vibration of absorbed water molecules. The band centered at 2930cm^{-1} could be attributed to CH_2 stretching. The band at 3314cm^{-1} may indicate intermolecular and intra-molecular hydrogen bonds.

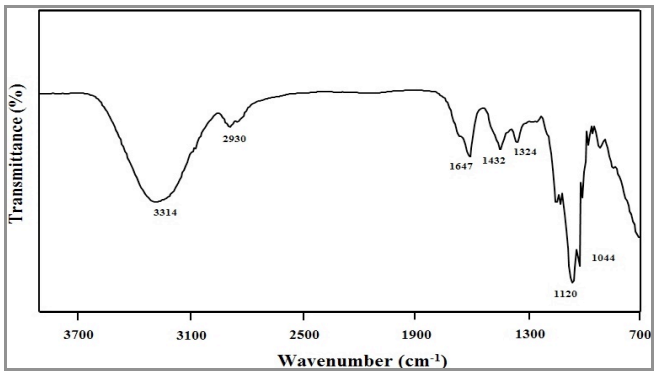

Figure 6: FTIR of BC produced by *Gluconacetobacter xylinus* RO-7.

4.3. Physico-Mechanical Analysis

4.3.1. Thermal Gravimetric Analysis

Thermal degradation behavior provides some evidence about the structural features and purity of BC.

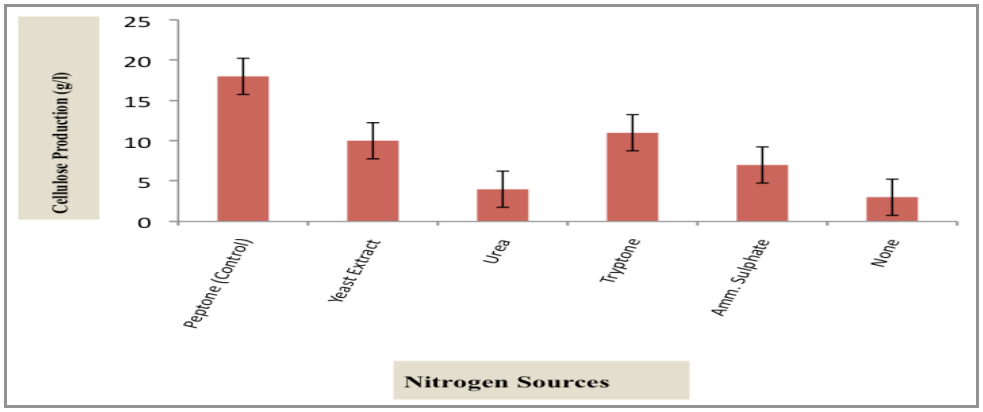

Figure 5: Effect of different nitrogen sources on BC production by *Gluconacetobacter xylinus* RO-7.

Figure **7** shows the percent weight of the original sample versus temperature. In all the samples three significant mass losses are observed. The first one takes place from room temperature to 230 ºC and it is assigned to membrane dehydration. Physically adsorbed and hydrogen bond linked water molecules can be lost at that first stage [22]. The second mass loss is observed from 230 to 375ºC, and is assigned to thermal decomposition of cellulose leading to the formation of carbonaceous char. These followed by weight loss within the temperature of 375-600^{o}C due to oxidation of charred product. The second and third processes regard the main degradation stages.

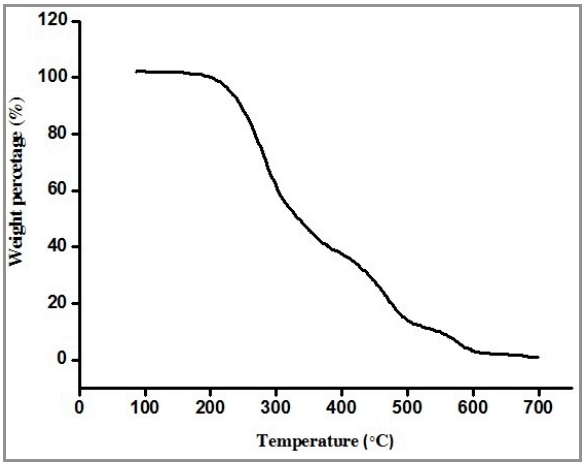

Figure 7: TGA of BC produced by *Gluconacetobacter xylinus* RO-7.

4.3.2. X-ray Diffraction (XRD)

Cellulose has several crystalline polymorphisms (I, II, III, IV). Cellulose I is the crystalline cellulose that is naturally produced by a variety of organisms, i.e. trees, plants, tunicates, algae and bacteria. The structure of cellulose I is thermodynamically metastable and can be converted to cellulose II or III. All the cellulose strands are ordered in a highly ordered parallel arrangement [23].

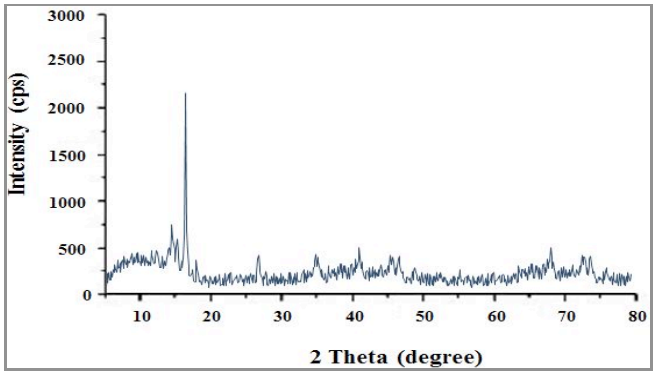

Figure 8: X-Ray Diffraction of BC produced by *Gluconacetobacter xylinus* RO-7.

Diffraction patterns obtained for BC obtained from glucose are shown in Figure **8**. One intense peak shown in Figure **8** at 2θ = 16.30^{o} confirmed that only cellulose I was present in BC samples [24-27]. No peaks, instead, are found at 2h = 12.1^{o} and 20.8^{o}, which are characteristic of cellulose II [28]. Cellulose I is the crystal structure with the highest axial elastic modulus [29].

4.3.3. BC Morphology

Morphological Features of BC are directly related with its physico-mechanical properties. The SEM morphology of the BC produced from *Gluconacetobacter xylinus* RO-7 was therefore visualized to investigate and compare their structural features. Figure **9** represents the surface of the BC produced from *Gluconacetobacter xylinus* RO-7. Surface morphology analysis shows that ribbon like microfibrils forming a highly fibrous network-like structure. Moreover, the arrangement may be caused by the presence of various additive materials in the media [30]. Similarly the medium viscosity also affects the cells activity and development of fibril network. Herein the viscosity and some additional soluble materials present in the media might have caused such variation in the morphological features of BC.

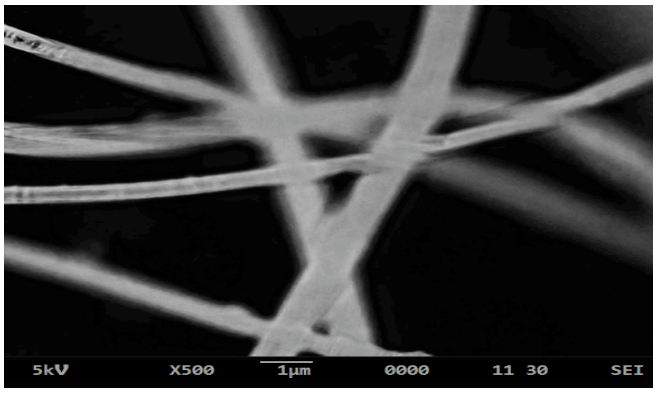

Figure 9: SEM of BC produced from *Gluconacetobacter xylinus* RO-7.

CONCLUSION

Microbial cellulose has a wide variety of applications as scientific and biomedical endeavors. This work produces a microbial strain for the production of microbial cellulose fibrils with tunable properties that can be applied for many medical applications as well as tissue engineering fields. The results obtained in this study revealed that some wastes such as pickles effluents may be used for the production of BC by *Gluconacetobacter* sp., which can bring down the cost of purchasing a high cost strains. Such utilization of

these wastes leads to the environmental management of some industries containing organic wastes. The analytical tools including FTIR, XRD and SEM confirmed the structural features and purity of BC. Hence, the study provides a broad idea about the potential of waste materials for ecofriendly BC production.

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