

Sonication Technique Application on Cellulose Producing Bacteria *Acetobacter xylinum*

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ABSTRACT

Cellulose is abundantly obtained from plant however the main drawback it has to be extracted from its source and purified by chemical separation process to remove the hemicellulose and lignin. Certain bacteria such as *Acetobacter xylinum* produce cellulose during fermentation process. This technique is more sustainable and the cellulose produced is pure which does not need any purification. Static fermentation methods using synthetic medium Shigeru Yamanaka usually take 14 days to produce bacterial cellulose (BC). Hence research is on-going to improve the fermentation process to be faster, more sustainable and economical. The objective of this study is to investigate the effect of ultrasonic wave on the *A. xylinum* as well as cellulose production and determine optimum sonication variables for improving yield of BC. Sonication process applies 40 kHz of frequency to the medium culture and glucose was supplied as the carbon source to *A. xylinum*. By adding ultrasonic wave to culture medium in static method the production of BC was improved with significant increment in yield. The production rate of BC was higher compare to non-sonicated medium however sonication should be at optimum time otherwise the bacterial cell is damaged after too long sonication. In this study the optimum time for sonication was 5 to 7 minutes. Sonication is like imitation of the agitation method but only apply small wave to the medium. It may shows effect on Gram negative bacteria like *A. xylinum* which have thin peptidoglycan layer. Sonication will enhance the transport process of nutrient in the medium to the bacterial cell.

Keywords: *Acetobacter xylinum*, bacterial cellulose, synthetic medium, sonication

1. INTRODUCTION

Cellulose that is synthesis by plant or bacteria can be defined as almost unlimited organic resource on earth. Thus, cellulose is now facing a high demand in production due to its environmental friendly and potential applications as alternative to petroleum base product [1]. Bacterial cellulose (BC) which is produced by some bacteria has been widely accepted as nano-biomaterial. According to [2] because of the resourcefulness of BC, it can be used in the food industry, textile industry, paper making industry and biomedicine. Due to its versatility and high demand, many researchers developed new methods in BC synthesis technique [3]. In general *Acetobacter xylinum* is a Gram-negative acetic acid bacteria having microfibrils from row of synthetic sites along the longitude axis of the cell [4]. The microfibrils merged with others to form a large ribbon-like cellulose associated cell tangle from floating pellicle [5]. BC is a fundamental structure of fibril with β -1 \rightarrow 4 glucan chain. The BC molecular formula is $(C_6H_{10}O_5)_n$. The glucan chains are formed together by hydrogen bonding [6]. *Acetobacter xylinum*, can also produce ribbon like polymer (cellulose I) and thermal

stable polymer [7]. During the synthesis process, the glucose chain called protofibril is scabble through cell wall. Then the ribbon forms the web-like shape structure of BC that creates the porous matrix. The synthesised BC has abundant surfaces of hydroxyl group [8,9]. The hydroxyl group makes the BC has the property like hydrophilicity, biodegradability and chemical modifying capability [10].

Sonication is the process of applying sound energy to vibrate the particles in a sample. The frequencies of >20 kHz are used. The process of applying ultrasonic frequencies is known as ultra-sonication. Ultrasound is a sound of frequencies greater than 20 kHz. Ultrasound is usually used in medical imaging and sonochemical processing [11]. Ultrasound is also useful in many food processing applications [12,13]. In medical field, medical imaging applications use only low power, at 1–10 MHz known as a diagnostic ultrasound. It requires high energy in the range between 20 to 100 kHz frequencies is used in many sonochemical processes. There is ultrasound equipment for processing large quantities of dry seed which has the frequency of 20 kHz and a vibrational amplitude of between 1 mm and 40 mm [12]. Phonoretic effect is the ultrasound that is applied on

cells to enhanced membrane permeation. This method has been used widely for cell disruption [14]. Ultrasound has been successfully used to induce transfer of genetic material into live animal [15] and plant cells [16]. At high power an input that creates high frequency called ultrasonication, would produce ultrasound that can rupture cells. This technique is used for cell disruption. A cell can be mollify by ultrasound at some point but less frequencies is needed than those that cause disruption [17]. Extreme ultrasound is known to damage macromolecules such as enzymes. Ultra-sonication can unfold and scrambling the protein chain and cause to break into small peptides. The objective of this study is to apply sonication towards fermentation using *Acetobacter xylinum* so that bacterial cellulose could be produced at shorter period of time at higher yield.

2. EXPERIMENTAL

2.1 Materials

Bacterial cellulose (BC) is produced from bacteria originated from many species. However, only the *Acetobacter* species could produce cellulose to fulfil the demand that BC base industry. The most studied species of the *Acetobacter* species is *Acetobacter xylinus*, formerly known as *Acetobacter xylinum* which has been used in this study as cellulose producer. It is gram negative bacteria that can be found naturally in ripened and rotten fruits. This bacterium was obtained from Malaysia Agricultural Research Development Institute (MARDI) Serdang, Selangor, Malaysia. Table 1 shows the list of chemicals for Shigeru Yamanaka medium meanwhile Figure 1 shows the steps for medium preparation.

Table 1 List of chemicals used in the bacteria medium

Chemicals	Manufacturer
Sucrose C ₁₂ H ₂₂ O ₁₁	Q-Rec
Yeast extract	Scharlau
Ammonium sulphate (NH ₄) ₂ SO ₄	Q-Rec
Pottasium dehydrogen phosphate KH ₂ PO ₄	Q-Rec
Magnesium sulphate MgSO ₄	Q-Rec
Agar powder	Q-Rec

2.2 Medium Preparation

About 100ml of Shigeru Yamanaka medium was prepared per 1 litre of distilled water containing 50g sucrose (QRec), 5g yeast extract (Bacto), 5g ammonium sulphate (QRec), 3g potassium dihydrogen phosphate (QRec) and 0.05g magnesium sulphate (QRec). The medium was mixed using stirrer until all ingredients dissolved. The medium initial pH was adjusted by using acetic acid to pH 5.5 by using pH meter. Then the medium was poured into a flask, closed with cotton, covered with aluminium foil and then put to autoclave at 121°C for 15 minutes. After cooled down to room temperature, 10 ml of *Acetobacter xylinum* obtained from MARDI, were added to the medium using aseptic technique. The solution was mixed apparently by shaking the flask slowly. Next, the flask was closed with cotton, covered with aluminium foil and placed in an incubator (Memmert 400, Germany) for 4 days at 28°C.

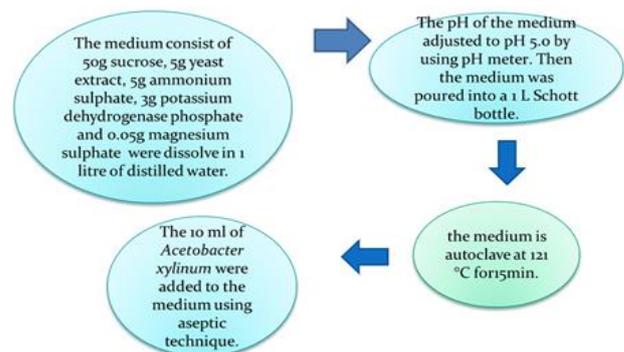


Figure 1 Schematic diagram for preparation medium

2.3 Methods

The sonicator LIR Biotech has ultrasonic power at 240 W and heating power of 200 W. Then it converts electrical power to high frequency electrical energy. This high frequency electrical energy was transmitted to the transducer within the converter, where it is changed to wave vibrations. The frequency was 40 kHz for this water bath model. The vibrations from the converter create ultrasonic waves in the water. This action creates a lot of microscopic bubbles during the sonication process. This scenario, known as cavitation only needs certain amount of energy to be released at certain point. Then it creates the powerful wave in the water bath. The larger the water bath sizes the more surface of sonication.

The prepared medium is pour into six 250 mL of Schott bottles. Each bottle is filled up with 45 mL of medium.

Then each of the bottles is pipetted with 5 mL of *Acetobacter xylinum* broth using aseptic technique. One bottle is the control (non-sonicate) and the other six bottles were sonicate at specific time which are 15, 12, 10, 7, and 5 minutes. After that the bottles were left at room temperature for fermentation process for 14 days. The white pellicle appeared as early as three days according to sonication time.

2.4 Wet Weight Measurement of Bacterial Cellulose (BC)

The BC formed from fermentation process was harvested and rinsed with distilled water. Then the cellulose was washed with NaOH solution. After that the cellulose was rinsed again this time with plenty of distilled water and dried with absorbent paper. The clean BC was weighed using Electronic Balance (DENVER) for the wet weight of microbial cellulose.

3. RESULTS AND DISCUSSION

3.1 Bacterial Cellulose Production from Fermentation by *Acetobacter xylinum*

Initially, the fermentation of bacterial cellulose by *Acetobacter xylinum* culture in 250 mL was sonicated for few minutes meanwhile the non-sonicated was taken as control. Obviously thicker BC was obtained from sonicated medium as observed after 7 days fermentation as showed in Figure 2.

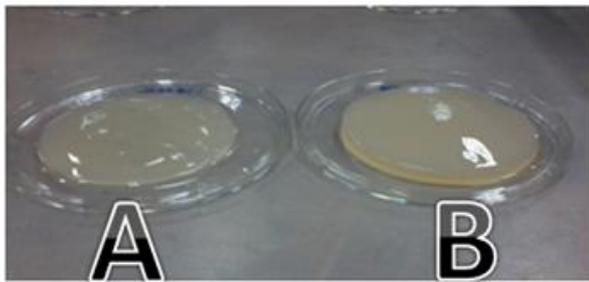


Figure 2 Bacterial cellulose harvested after 7 days fermentation from A) non-sonicated (control), and B) sonicated medium

Bacterial cellulose (BC) obtained from the fermentation is a fundamental structure of fibril that have $(\beta-1 \rightarrow 4)$ glucan chain (Figure 3). Generally BC molecular formula is $(C_6H_{10}O_5)_n$. The glucan chains were formed together by hydrogen bonding [6].

As shown in Figure 4, during synthesis process the glucose chain called micro fibril is scrubbed through cell wall of *A. xylinum*. The ribbon forms the web-like shape structure of BC. The hydroxyl group makes the BC has the property like hydrophilicity, biodegradability and chemical modifying capability.

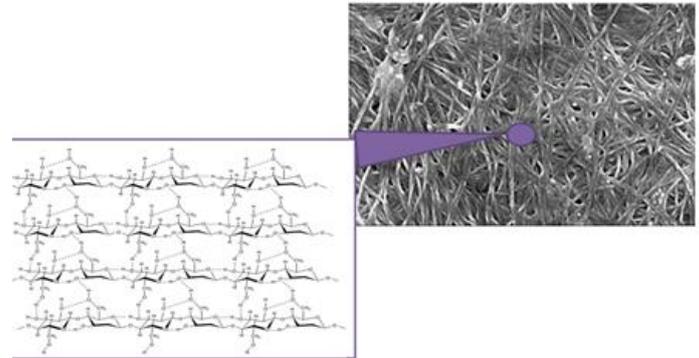


Figure 3 $\beta-1 \rightarrow 4$ glucan chain

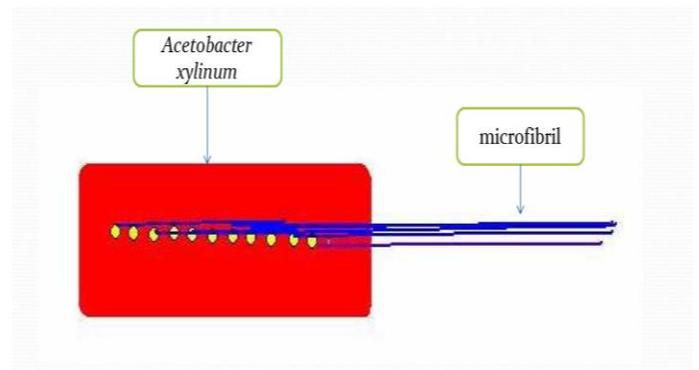


Figure 4 Illustration of *Acetobacter xylinum* produces bacterial cellulose

After 14 days the BC was harvested and rinsed with distilled water then washed with NaOH solution. From the result obtained the sonicated medium gave a weight of 15.31 g compared to the non-sonicated which gave a weight of 7.42 g. Hence sonication potentially enhances the BC production by up to twice the initial weight.

3.2 Effect of Different Sonication Time

From the preliminary study, sonication can enhance the bacterial cellulose production produced by *Acetobacter xylinum*. For further study, sonication was done in five different exposure time. A total of 6 media were prepared.

One for control (non-sonicated) and the other five sonicated at 15 minutes, 12 minutes, 10 minutes, 7 minutes and 5 minutes. All flasks were left for 14 days and the result obtained is shown in Figure 5 and Table 2.

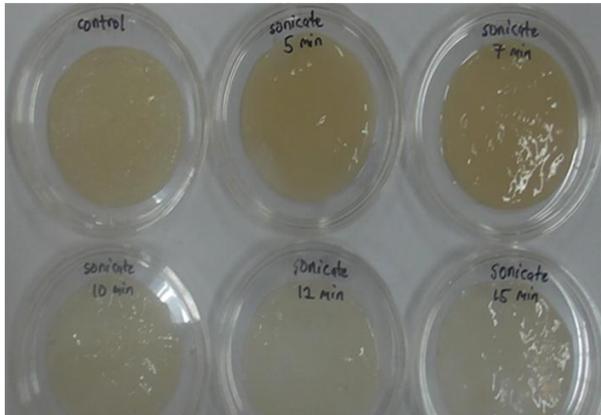


Figure 5 Wet bacterial cellulose obtained from fermentation with different sonication times (top left: control, then sonicated for 5, and 7 min; top below: 10, 12 and 15 min respectively)

From Figure 5 the result varies from each other. Different time of medium sonication affects the resultant BC weight and thickness. From Table 2, it shows that 5 minutes sonication gave better weight and thickness compare to control. For 7, 10, 12 and 15 minutes of sonicated medium, the obtained BC weight and thickness decreased gradually. From the result, sonication at five minutes gave

Table 2 Weight and thickness of BC obtained from fermentation using medium at different sonication time

Sonication Time (min)	BC Wet Weight (g)	BC Thickness (mm)
0 (control)	8.3562	3.92
5	10.2027	4.24
7	8.1149	3.82
10	4.0708	1.46
12	3.3597	1.25
15	3.0429	1.16

the most weight and thickness compare to the other time of sonication. In Table 2 optimum time of five minutes gave the most weight compares to others. Sonication at 5 minutes produced 21.4% cellulose which means more yield than non-sonicated medium.

The longer medium is sonicated, the less weight of BC obtained. This may be due to long period of exposing the medium to sonication may cause the bacterial cell rupture

which consequently decrease the population of the *Acetobacter xylinum* thus less weight of BC pellicle production. In this study, 5 minutes of sonication exposure enhanced the production of BC. Perhaps this due to phonophoretic effect as reported by Cock et al [18] after exposure to sonication, the bacteria behaviour was evaluated at low frequencies and at low intensities. The effects of ultrasound on microorganisms would increase the membrane permeability [19]. This low ultrasonic wave would allow the entire nutrient in medium to be absorbed efficiently through the bacterial membrane. This enables the *Acetobacter xylinum* produce more cellulose beyond its normal rate. Figure 6 shows how phonophoretic effect works.

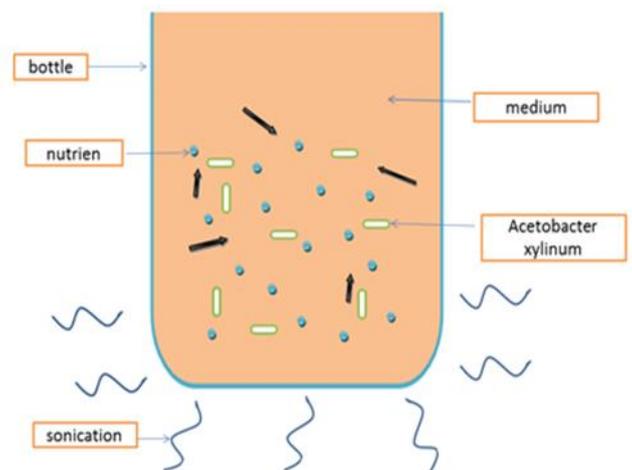


Figure 6 Phonophoretic effect to *Acetobacter xylinum* during sonication

3.3 Effect of Fermentation Factors from Sonication on the BC Production.

3.3.1 Medium pH

Table 3 shows the changes on pH of medium due to sonication exposure for different period. It was found that there was only very slight change towards more acidic in final pH of medium throughout the fermentation due to sonication effect. *Acetobacter xylinum* is an acidophilic organism which thrives under acidic conditions. It can live in low pH condition and the optimal pH for their growth is in the range 5.4 to 6.3 [20].

The pH scale measures hydrogen ion (H⁺) concentration and the hydrogen concentration give effects to the enzyme activity hence, microbial growth will be interrupted as well as the BC production [21]. Low pH correspond

with high concentrations of hydrogen ion, while neutral pH means concentration of hydrogen and hydroxyl ions (OH⁻) are equal, and high pH correspond to low concentrations of hydrogen ion.

Table 3 Effect of sonication time on the initial and final pH of medium in BC fermentation

Sonication Time (min)	Initial pH of medium	Final pH of medium
0 (Control)	6.0	5.8
5	6.0	5.8
7	6.0	5.7
10	6.0	5.6
12	6.0	5.5
15	6.0	5.4

3.3.2 Dissolved Oxygen Concentration

Table 4 shows that there was only very slight change towards lower oxygen concentration in final DO of medium throughout the fermentation due to sonication effect. However, it seems sonication allows better distribution and utilization of oxygen in the medium that could be assessed by bacteria.

Table 4 Effect of sonication time on the initial and final DO of medium in BC fermentation

Sonication Time (min)	Initial DO (mg/l)	Final DO (mg/l)
0 (control)	2.3	0.04
5	2.3	0.03
7	2.3	0.03
10	2.3	0.03
12	2.3	0.02
15	2.3	0.02

Acetobacter xylinum is an obligate aerobe that required oxygen as their metabolism pathway. It uses oxygen for aerobic respiration to oxidize substrates such as glucose to create energy and convert glucose into cellulose. As cellulose is produced at the air-liquid interface, the oxygen supply trapped between it. This is considered as the limiting factor for growth and cellulose formation. Oxygen tensions affect both the cellulose production and the membrane quality. Microbial cellulose that grows under lower oxygen tensions should have fewer ramifications than cellulose grow under higher oxygen tensions. It is recommended that 10% of oxygen tensions is able to go up

to a 25% higher cellulose production without any influence on the cell growth [22].

3.3.3 Fermentation Temperature

The *Acetobacter xylinum* is a mesophilic bacterium with optimum growth temperature between 25 to 30°C. In this study, the fermentation was carried out at fixed room temperature 26°C, and sonication exposure did not change the temperature significantly. At 37°C, *Acetobacter xylinum* failed to replicate in the medium even the temperature is optimum. In high temperature, the cell apparatus such as nucleic acid and protein will be denatured. Hence, temperature is an important factor that would give a large impact for bacterial growth [23].

3.3.4 Surface Area

In this study, the surface area was fixed at constant volume. The surface area has been proven that can be directly proportional to the surface area of air liquid interface [24]. The result suggests that surface area up to a certain extent has more dominant influences on cellulose production than absolute number of cells in the culture medium. Thus this parameter is very important in maximizing the production of microbial cellulose in film form.

4. CONCLUSION

In this study, effect of sonication on fermentation by *Acetobacter xylinum* for bacterial cellulose production in static culture had been studied. It was found that sonication time gives significant effect towards cellulose production. These results conclude that sonication time is important to provide better phonophoretic effect but at the same time, it should be controlled at the optimum level to ensure the sonication would not disrupt the bacteria cells. However, slight difference in time would cause cell disruption hence affect the BC production. Moreover, all fermentation had been run in static culture. Only sonication process at optimum amount of time and frequency will provide optimum nutrient supply to the culture. With optimum nutrient *Acetobacter xylinum* will produce significantly high yield of cellulose compare to medium without sonication. Sonication wave will induce the energy for bacterial activity hence improving the viability and rate of BC production for optimum period. Sonication wave produce phonophoretic effect would gave *Acetobacter xylinum* cell membrane a micro shockwave that open up the membrane pore hence efficiently absorb the nutrient for BC producing activities.

ACKNOWLEDGMENT

This work was financially supported by the Universiti Teknologi Malaysia under the Research University Grant (TDRG 06G55) and Ministry of Higher Education Malaysia. I would like to thank to my supervisor and colleagues that give support to my research work.

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