Bacterial Cellulose Production by Pure Culture of Acetobacter xylinum in Sweet Black Tea Medium

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Abstract: *Bacterial cellulose (BC) is a versatile biopolymer synthesized by bacteria through the conversion of carbon sources available in the culture medium into polysaccharides. In this study, the impact of inoculum size, medium composition, and cultivation conditions on BC production by Acetobacter xylinum was studied. The medium supplemented with 1.5 g/L $(NH_4)_2SO_4$ as nitrogen source and 1% ethanol as addition carbon source, significantly enhanced BC, and acid production up to 9.82±0.19 g/L and 6.75±0.28 g/L. Static fermentation conditions gave higher BC yield than agitated conditions. This study demonstrates that it was possible to produce BC using sweet black tea based medium, which is low-cost and may contribute to the cost-effective BC production at industrial scale.*

Keywords: Low-cost medium; Ethanol; Static fermentation conditions

1. INTRODUCTION

Bacterial cellulose (BC) is generated during fermentation, forming a thin membrane at the liquid-air interface. Bacteria use cellulose synthase to polymerize glucose into β-1,4 glucan chains. These linear chains are secreted extracellularly and, through organization and crystallization by hydrogen bonds and Van der Waals forces, create a robust three-dimensional structure known as microfibril. These microfibrils then assemble into cellulose ribbons, eventually forming the visible BC pellicle [1] Unlike plant cellulose, BC lacks undesirable compounds such as lignin, pectin, and hemicellulose, which reduces production costs and environmental impact [2-4]. BC is a biopolymer that possess distinct features such as unidirectional polarity and varying thickness [5-7]. BC is a promising biomaterial with several advantages over plant cellulose, including high chemical purity [8], a nanofibril structure ranging from 3 to 8 nm [9], and ease of isolation and purification [10]. Its excellent water absorption and retention capabilities, high crystallinity, mechanical strength, biodegradability, and biocompatibility make BC suitable for various applications in medicine, pharmacy, food, agriculture, textiles, and electronics. Additionally, crystalline BC has been applied for pollutant removal by encapsulating cationic industrial pollutants in aqueous solution [11] and applied in sensing technology [12].

Bacterial genera commonly known to produce BC include *Acetobacter* (now reclassified as *Komagataeibacter*), *Aerobacter*, *Achromobacter*, *Agrobacterium*, *Pseudomonas*, *Sarcina*, and *Rhizobium*. Among these, *Komagataeibacter* specifically the *Komagataeibacter xylinus* strain, which is extensively studied for its high BC production is the most frequently referenced genus [15,16]. Through cellular respiration fermentation, BC comprises intricate networks of pure cellulose nanofibrils measuring 2-4 nm in diameter and less than 100 nanometers in size [12-14]. These unique properties have garnered significant interest in BC for industrial applications.

Enhancing BC production involves employing both engineering and genetic strategies. The engineering aspect focuses on reactor design, selecting optimal nutrients, controlling processes, and optimizing conditions. Meanwhile, the genetic approach revolves around cloning the BC synthesis gene and genetically modifying relevant genes to boost production efficiency [14]. Despite its potential, the primary challenge in commercial BC production is the high cost of fermentation media and low production yields. The data obtained from the literature shows that tea leaves are rich in valuable bioactive substances such as polyphenols, proanthocyanidins, phenolic acids, alkaloids, catechins, oleic and lauric acids, saponins and polysaccharides [17-20]. Additionally, tea leaves contain 21-28% protein in its dry phase [21,22] and also amino acids rich in glycine, valine, lysine, leucine, alanine, and methionine in its structure [19,23]. Furthermore, the bioactive chemicals can also be formed during the production process of black tea. Therefore, black tea is an attractive, cheap, alternative nitrogen source for the optimization of cultivation conditions with the goal of achieving higher yields, lower costs, and shorter production times. This study aimed to produce BC using black tea-based medium added with low-cost nitrogen source. The medium composition and culture conditions were optimized.

2. MATERIALS AND METHODS

2.1 Inoculum preparation

Acetobacter xylinum used in this study was obtained from Faculty of Agro-Industry, Prince of Songkla University. Glucose yeast extract ethanol (GYE) medium was prepared as follows: 70 g/L glucose, 10 g/L yeast extract, 0.5 g/L Na_2HPO_4, 0.5 g/L KH_2PO_4, 0.05 g/L $MgSO_4$ and 2% (v/v) ethanol. The pH of the media was adjusted to 5.6 before being autoclaved at 121°C for 15 min at 15 psi. *A. xylinum* stock culture was added aseptically to the sterile GYE medium and incubated for 2 days and agitated 180 rpm at room temperature (28 °C). Prior to the use as inoculum, surrounding cellulose was

removed from the cells by adding cellulase (iKnowZyme, Reach Biotechnology, Thailand) and shaking at 180 rpm for 2 h [24]. The cell pellets were collected by centrifugation at 4,000 rpm for 15 min. The cell pellets were suspended with 40 mL of 0.85% NaCl to adjust optical density (OD) at 2 (8 log CFU/mL).

2.2 Bacterial cellulose production in sweet black tea medium by *A. xylinum*

Aliquot of the *A. xylinum* (7, 6.5, and 6 log CFU/mL) was cultured on sweet black tea broth medium (ST medium composed of black tea, 3.3 g/L and sucrose, 80 g/L), sweet black tea medium with nitrogen source (STN medium composed of black tea, 3.3 g/L; sucrose, 80 g/L; $(NH_4)_2SO_4$, 1.5 g/L), and sweet black tea medium added with ethanol and nitrogen source (STEN medium composed of black tea, 3.3 g/L; sucrose, 80 g/L; ethanol, 1% v/v; $(NH_4)_2SO_4$, 1.5 g/L). The cultures were cultured under static and agitated condition at 30 °C for 7 days (Fig. 1). The samples were collected to determine the OD, dry cell weight (DCW), pH, total acidity, and BC production.

2.3 Dry cell weight measurement

The samples after cellulose digestion were centrifuged at 4,000 rpm for 15 min at 4 ºC. The bacteria cell pellets were collected. The pellets were washed twice with deionized water. The supernatant which consisted of the remaining medium and impurities were removed carefully, leaving the washed cell pellets in the tubes. These procedures were conducted twice to ensure the maximum removal of impurities. The cell pellets were then dried in an oven at 60 °C for 24 h. The dried samples were placed in a desiccator for 15 min before being weighed using an analytical balance. The cell dry weight was calculated according to the formula given below.

$$\text{Cell dry weight (mg/mL)} = (W_a - W_b)/V$$

W_a: weight of dry sample in tube (mg)
W_b: weight of empty tube (mg)
V: volume of sample (mL)

2.4 Bacterial cellulose production and purification

BC produced in black tea media was harvested and subjected to centrifugation at 8,000 rpm for 15 min. Afterwards, the liquid above the sediment was removed, and the sediment itself was rinsed with distilled water. The BC was subjected to treatment with a 1 M NaOH solution at 80°C for 1 h to eliminate trapped cells. Subsequently, the BC was incubated in distilled water overnight to remove impurities and the color from the black tea. Finally, the purified cellulose was dried at 60°C for 24 h until a constant weight was achieved [24].

$$\text{Cellulose production (g/L)} = (W_{st} - W_t)/V$$

W_{st}: weight of dry sample in tube (mg)
W_t: weight of empty tube (mg)
V: volume of sample (mL)

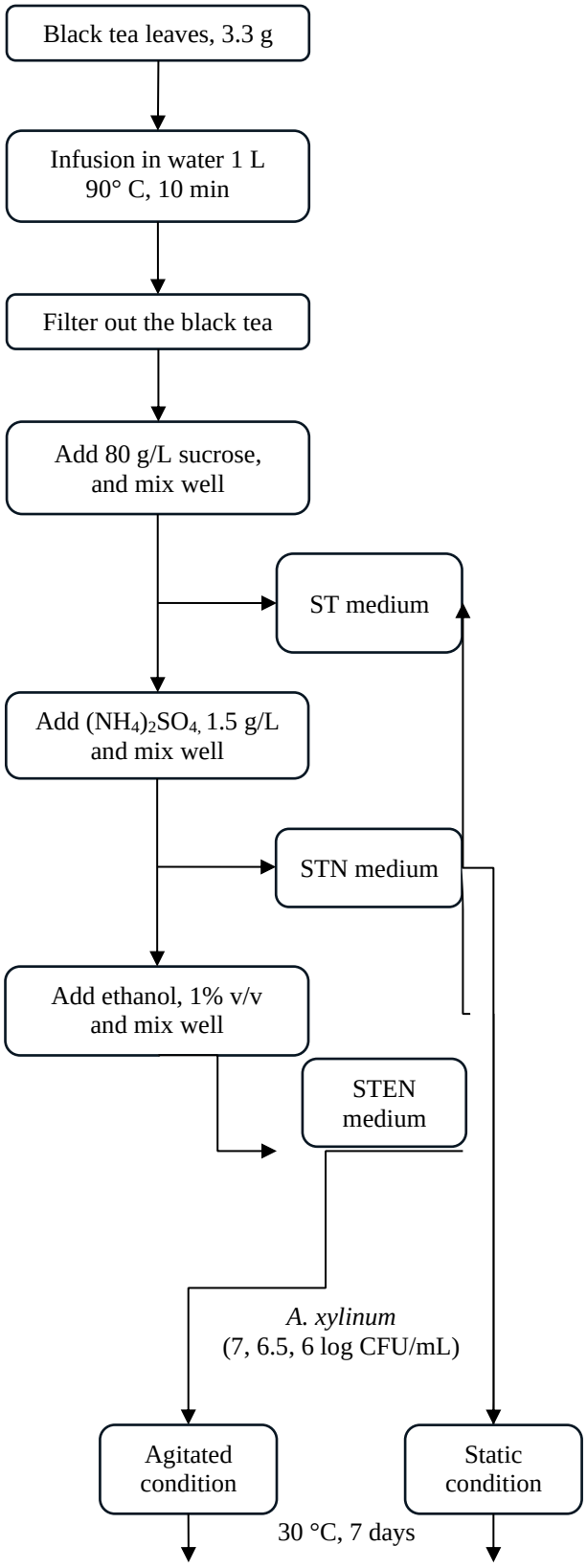

Fig.1 Flow diagram for bacterial cellulose production.

2.5 Measurements of pH and total acid (TA)

The pH values will be measured with a Mettler Toledo Five Easy pH meter coupled with a LE498 probe.

Total acidity will be determined by titration with 0.1 N NaOH (purity ≥ 98%) and 0.2% phenolphthalein as color indicator, with reagents purchased from Merck (Darmstadt, Germany).

$$TA\ (acetic\ acid) = (t * m * 60.05) / v$$

Where:

m = molarity of NaOH

t = titre of NaOH required (mL)

v = volume of sample used (mL)

3. RESULT AND DISCUSSIONS

3.1 Effect of inoculum size

Fig. 2a-c shows effects of inoculum size on optical density (OD), dry cell weight (DCW; g/L), pH, total acidity (TA; g/L) and bacterial cellulose (BC; g/L) production of *A. xylinum* in ST medium (a), STN medium (b), and STEN medium (c) under agitated condition. Under agitated conditions (Fig. 2a-c), there was no significant effect of inoculum size on OD, dry cell weight (DCW), total acid (TA) and bacterial cellulose (BC) production in ST and STN media. While in STEN medium TA and BC production became higher when increasing inoculum size to log 6.5 CFU/mL. Fig. 3 shows effects of inoculum size under static condition. Under static condition, inoculum size had no significant effect on OD, DCW, TA and BC production in ST and STN media, while in STEN medium, inoculum size at log 7 CFU/mL gave the highest TA and BC production.

Table 1 Results of total acid (g/L) and BC production (g/L) at 7 days

Culture modes	Medium	Inoculum (Log CFU/mL)	Total acid (g/L)	BC production (g/L)
Agitated	TS	6	0.51±0.08	0.15±0.04
		6.5	0.43±0.02	0.20±0.01
		7	0.66±0.08	0.07±0.06
	TSN	6	0.65±0.14	0.08±0.02
		6.5	0.61±0.03	0.07±0.02
		7	1.07±0.32	0.06±0.02
	TSEN	6	6.15±0.42	0.39±0.01
		6.5	7.08±0.29	1.36±0.24
		7	7.15±0.52	1.06±0.25
Static	TS	6	0.18±0.02	0.32±0.02
		6.5	0.33±0.05	0.47±0.12
		7	0.39±0.09	0.50±0.06
	TSN	6	0.58±0.09	0.22±0.02
		6.5	0.66±0.10	0.39±0.05
		7	0.72±0.17	0.45±0.05
	TSEN	6	3.61±0.37	5.10±0.33
		6.5	5.78±0.29	6.74±0.30
		7	6.75±0.28	9.82±0.19

3.2 Effect of culture medium

A. xylinum was cultivated on three culture media including ST, STN and STEN under agitated condition (Fig. 2). It can be observed that *A. xylinum* produced more BC and total acid in STEN medium compared to STN and ST media. The composition of the nutrient medium and strain activity are crucial for maximizing BC production. The commonly used medium for BC production contains glucose as a carbon source along with yeast extract and bactopeptone as nitrogen sources [24].

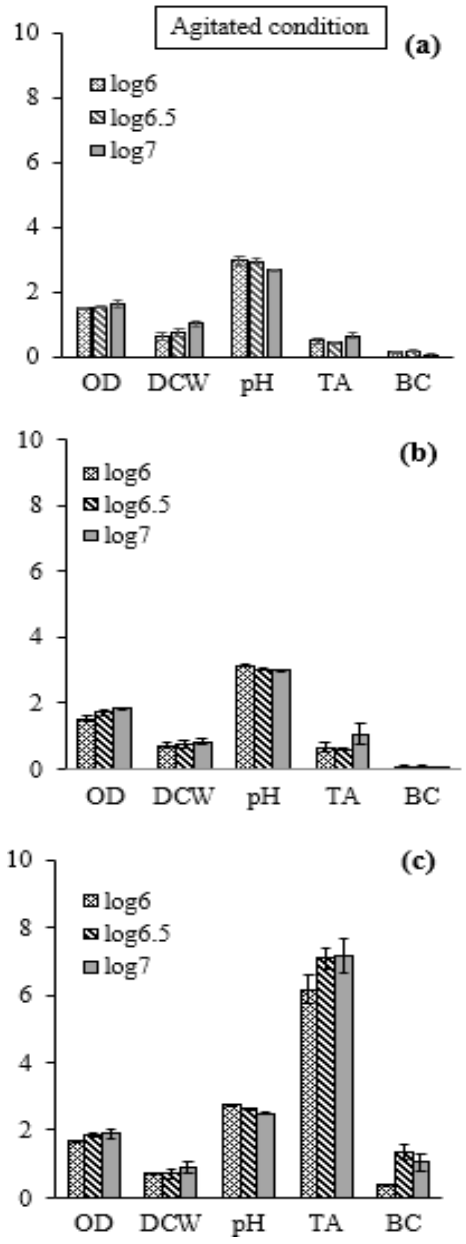

Fig. 2 Effects of inoculum size on optical density (OD), dry cell weight (DCW; g/L), pH, total acidity (TA; g/L) and bacterial cellulose (BC; g/L) production of *A. xylinum* in ST medium (a), STN medium (b), and STEN medium (c) under agitated condition.

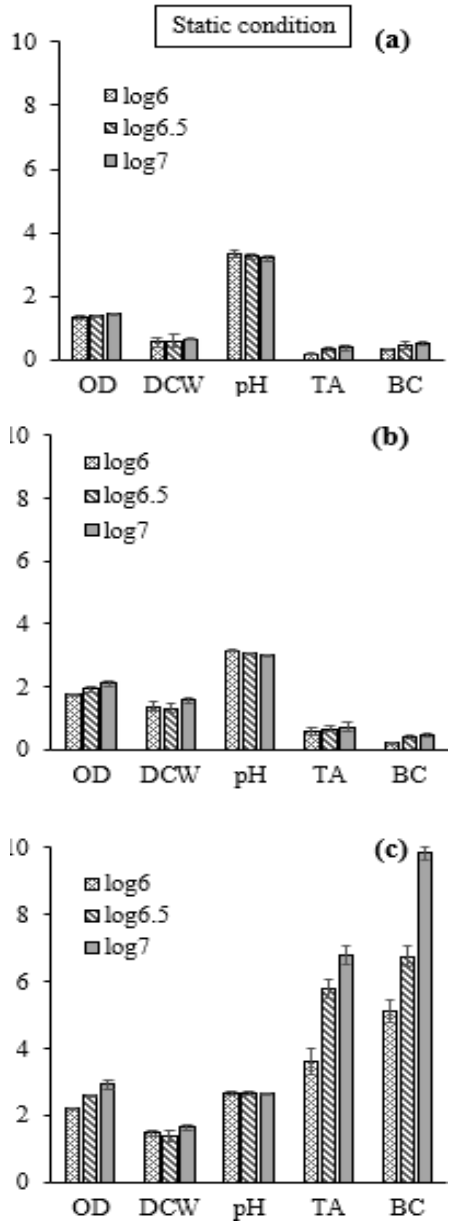

Fig. 3 Effects of inoculum size on optical density (OD), dry cell weight (DCW; g/L), pH, total acidity (TA; g/L) and bacterial cellulose (BC; g/L) production of *A. xylinum* in ST medium (a), STN medium (b), and STEN medium (c) those under static condition.

Research has expanded to explore various carbon sources beyond glucose, such as monosaccharides, disaccharides, polysaccharides, and other organic compounds like glycerol, which are processed via the gluconeogenesis pathway [25]. In addition, Krystynowicz et al. [26] and Cielecka et al. [27] demonstrated that adding 1% ethanol to standard medium for BC production notably improved BC production by serving as an alternative energy source to glucose [28, 29]. This also facilitates BC aggregation, which can help in mitigating shear stress [30]. In the case of STEN medium, which incorporates sweet black tea, the addition of 1.5 g/L $(NH_4)_2SO_4$ and 1% ethanol significantly increased both BC and total acid production. The pH decreased likely due to the production of acids [31]. When ammonium sulfate was added (STN, STEN), the dry cell weight and OD value increased while there was no obvious effect on BC production. It was also reported that *A. xylinum* could grow when ammonium sulfate was used as a sole nitrogen source, but there was no BC production [32].

3.3 Effect of static and agitated conditions

When comparing BC production under static and agitated conditions, *A. xylinum* produced BC higher under static condition (Fig. 4) possibly due to the shear stress exerted on bacteria during agitated culture results in the development of negative cellulose mutants, leading to reduced BC productivity. Therefore, a simple static culture without shear stress is the preferred method for BC production [33-36].

In contrast, total acid production exhibited greater levels under agitated condition. Other research performed BC production in a rotary incubator in order to overcome the mass transfer limitations in static culture [34]. In accordance with Fu et al. [37], the total acid production was found to be higher, and pH became lower during static fermentation. This can be explained by the fact that acidogenic bacteria directly use the dissolved oxygen in the fermentation liquid to oxidize ethanol into acetic acid [38]. Furthermore, DCW and OD value in static condition were higher than those under agitated condition.

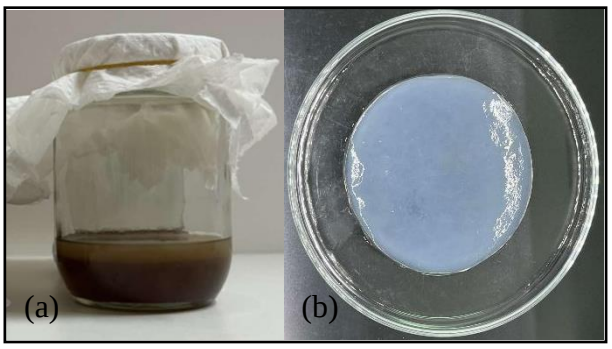

Fig. 4 Bacterial cellulose (BC) from inoculated the *A. xylinum* at 7 Log CFU/mL in TSEN medium 7 days (a) and purified bacterial cellulose (b).

4. CONCLUSIONS

This study demonstrated the optimization of BC production using *Komagataeibacter xylinus* in a cost-effective medium. Key findings include the positive impact of increased inoculum size on BC and total acid production, the superiority of the STEN medium (with 1.5 g/L $(NH_4)_2SO_4$ and 1% ethanol) over other media in boosting BC and total acid yield up to 9.82±0.19 g/L and 6.75±0.28 g/L and the advantage of static over agitated condition. These results highlight the potential of using sweet black tea as an economical medium for industrial-scale BC production.

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