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Valorisation of *okara* Waste as an Alternative Nitrogen Source in the Biosynthesis of Nanocellulose

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Cellulose nanofibers biosynthesized by bacteria (BC) possess outstanding properties such as non-toxicity, high purity, high liquid holding capacity, high crystallinity, and high tensile strength. It is a more eco-friendly, bio-based material compared to cellulose isolated from plants via extraction/purification processes in which harsh chemicals are used. However, the low yield of BC limits the widespread production and usage of BC nanofibers. Hence, the purpose of this research was to study the effects of four different culture media, various carbon and nitrogen sources, initial pH, and static/dynamic cultivation conditions on BC yield. Suitable culture media formulations and cultivation conditions of cellulose-producing bacteria (*Gluconacetobacter xylinus*) were assessed. Moreover, BC production was examined in a modified *Hestrin-Shramm* (HS) medium by replacing the nitrogen source ingredients (yeast extract and peptone) with *okara*, an agricultural by-product from industrial soybean production. Nanocellulose yield and content in a hydrogel-like pellicle were compared. As a result, high BC pellicle yield (100-350 g/L) and nanocellulose content (3-12 mg/cm^3) were observed in HS and *Zhou* media at pH 5.0 under static and combined static-dynamic conditions by adding mannitol and corn steep powder as carbon and nitrogen sources, respectively. When *okara* was used as an alternative nitrogen source, the resultant BC exhibited high crystallinity (~90%) and high molecular weight (7-9×10^6 g/mol) over the range of tested concentrations. The highest BC pellicle yield (85 g/L) and cellulose content (3.4 mg/cm^3) were obtained when the culture was supplemented with 1 %w/v freeze-dried *okara*. The valorisation of non-valued *okara* waste through nanocellulose production can alleviate economic constraints around BC production and can simultaneously provide enhanced sustainability.

1. Introduction

Cellulose, a common polysaccharide in nature, is mainly found in cell walls to strengthen the structure of plants. It contains other natural impurities such as lignin and hemicellulose (Klemm et al., 2005). Chemical treatments to remove these impurities are required, which create several environmental problems (Ng et al., 2015). Consequently, there is an interest in reducing or replacing demand of plant cellulose with an alternative biocellulose synthesized by bacteria in an eco-friendly one-step production, so-called bacterial nanocellulose (BC) (Almeida et al., 2021; Klemm et al., 2005). The chemical structure of BC is analogous to that of plant cellulose i.e. many D-glucose units bound by 1,4-β-glycosidic bonds, but has superior properties such as high cellulose purity, an ultrafine nanofiber network, high surface area, and high-water holding capacity (Joseph et al., 2020; Taokaew et al., 2016). Due to an abundance of hydroxyl groups bonded inter and intramolecularly between parallel linear chains and adjacent glucose units, BC possesses highly crystallinity resulting in high tensile strength and these hydroxyl groups are available for modification and functionalization (Peter, 2021). These properties allow BC to be employed as a base material in foods, papers, medical uses, pharmaceutics, electronics, fuel cells, and other applications (Taokaew et al., 2016). However, the low yield of BC production limits its industrial applications. Therefore, various recent efforts have focused on increasing production of BC by optimizing the bacterial cultivation condition; for instance, carbon sources (mono- and disaccharides), nitrogen sources (proteins), minerals, pH, and air/oxygen content (Andriani et al., 2020).
The use of wastes e.g. fruit residues (Amorim et al., 2019) and corn steep liquor (Aragão et al., 2020) as alternative nutrient sources to produce BC have also been explored. The application of *okara*, an agricultural

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waste from industrial soybean production, as a nitrogen source for *G. xylinus* is rarely reported in scientific literatures. Hence, utilization of *okara* in the biosynthesis is the novel aspect of this study. Not only is the environmental impact minimized, and economic value gained, but also, the costly commercial protein in the bacterial culture is substituted. Commonly, the nitrogen source is expensive relative to the carbon source e.g. sugar. However, other studies focused on the carbon source to produce nanocellulose but retaining an expensive nitrogen source.

This study aims to biosynthesize BC in various culture media by adjusting carbon source, nitrogen source, and pH. The effects of cultivation conditions including static, dynamic, and the combination of both were also assessed. An optimum condition was applied in the *okara* experiment. The resultant BC was characterized in terms of yield, cellulose content, purity, crystallinity, molecular weight, morphology, and viscoelastic properties.

2. Experimental methods

2.1 Materials

Fresh *okara* (Nikko Food Cooperation, Japan) was purchased from a local food market in Niigata prefecture, Japan. The *okara* was freeze-dried using a freeze dryer (Eyela FDU-1200, Japan) for 24 h prior to use in the experiment. *Gluconacetobacter xylinus* BPR2001 ATCC® 700178™ (*G. xylinus*) was purchased from American Type Culture Collection (ATCC). Peptone and yeast extract were purchased from Becton, Dickinson and Company (BD), USA. Corn steep powder (CSP) was purchased from Oriental Yeast company, Japan. The 99% purity-grade dimethylacetamide (DMAc) was purchased from Nacalai Tesque Inc, Japan. Other chemicals used in this study were purchased from Wako Pure Chemical Industries, Ltd, Japan.

2.2 Cultivation of *G. xylinus*

To prepare the inoculum, *G. xylinus* was incubated in sterilized HS culture medium containing 2 %w/v glucose, 0.5 %w/v yeast extract, 0.5 %w/v peptone, 0.27 %w/v Na_2HPO_4, and 0.115 %w/v $(COOH)CH_2COOH{\cdot}H_2O$ (citric acid·H_2O) using coconut water (Malee®, Malee Group PCL, Thailand) as a solvent. The pH value of culture media was adjusted to pH 5 using acetic acid before sterilization at 121 °C for 15 min. This cell suspension was incubated at 30 °C for 7 days for using as an inoculum in the experiment. The four different culture media, which were yeast extract/glucose/calcium carbonate (YGC), HS, *Yamanaka* (Y), and *Zhou* (Z), having different ingredients (Table 1), were inoculated with 10 %v/v inoculum and incubated at 30 °C under a static condition for 7 days. Effects of various carbon (glucose, sucrose, fructose, and mannitol) and nitrogen (CSP, peptone, tryptone, and yeast extract) sources, initial pH (4-6.5), and static/dynamic conditions were evaluated in Z medium. The dynamic condition was performed in a horizontally shaking incubator at 160 rpm. The combined static and dynamic condition was also performed in a shaking incubator at 0 and 160 rpm for 3.5 days of each. Thereafter, peptone and yeast extract in HS medium were replaced by *okara* at the concentration of 0-3 %w/v using deionized (DI) water as a solvent. The medium (100 mL) was inoculated with 10 %v/v of the inoculum, and the cultivation was performed in a static condition. All the experiments were conducted at 30 °C. After 7 days, the hydrogel-like BC pellicles floating on the air-liquid medium interface were harvested and purified by washing with running tap water, rinsing with DI water, immersing in 1 %w/v NaOH for 24 h at room temperature (25±3 °C), and neutralizing with DI water. The samples were named as "BC*x*", in which *x* indicated concentrations of the freeze-dried *okara* supplemented in the culture media.

Table 1: Formulations of bacterial cell culture media in water (%w/v)

	YGC	HS	Y	Z
Glucose	5	2	5	2
Yeast extract	0.5	0.5	0.5	-
Peptone	-	0.5	-	-
CSP	-	-	-	2
$CaCO_3$	1.25	-	-	-
Na_2HPO_4	-	0.27	-	-
$(COOH)CH_2COOH{\cdot}H_2O$	-	0.115	-	-
$(NH_4)_2SO_4$	-	-	0.5	0.4
KH_2PO_4	-	-	0.3	0.2
$MgSO_4{\cdot}7H_2O$	-	-	0.005	0.04

2.3 Characterization

Yield and nanocellulose content were calculated according to Eq(1) and Eq(2), respectively.

$$Yield = \frac{W_{BC\ pellicle}}{V_{Media}} \quad (1)$$

$$Cellulose\ content = \frac{W_{BC}}{V_{BC\ pellicle}} \quad (2)$$

where $W_{BC\ pellicle}$ *and* W_{BC} are weights of hydrogel-like BC pellicle and freeze-dried BC, respectively. V_{media} and $V_{BC\ pellicle}$ are volumes of cell culture medium and BC pellicle, respectively. Analysis of elemental composition of the samples was performed by X-Ray Fluorescence spectrometer (XRF, Rigaku ZSX Primus II, Japan) using ZSX software to quantify the purity according to total mass of carbon and nitrogen elements. This spectrometer contains a 30-keV and 100-mA (Rh 4.0 kW) X-ray tube, providing the detection of diverse elements of the Periodic Table at 2θ of 63º. Prior to characterization, pressed pellet used for measurement were prepared by press machine under a pressure of 500 kgf/cm^2. X-ray diffractograms were obtained using X-Ray Diffractometer (XRD, Rigaku Smart Lab 3 kW, Japan) under operation conditions of 40 kV and 30 mA with Cu-Kα radiation. The relative intensity was recorded in steps of 0.01º and speed of 3.0º/min. Percent of crystallinity was determined by Eq(3).

$$\%\ crystallinity = \frac{I_{crys} - I_{am}}{I_{crys}} \times 100 \quad (3)$$

where I_{crys} denotes the maximum intensity of 200 crystalline planes and I_{am} denotes the minimum intensity of amorphous diffraction at ~18º (Nam et al., 2016). The crystallite size was obtained by *Rigaku PDXL2* software. Molecular weights of BC were determined using size exclusion chromatography equipped with refractive index detector (RID-10A, Shimadzu, Japan). Sample solution was prepared by dissolving in DMAc containing concentrated dehydrated LiCl at room temperature until the completed dissolution was observed. Thereafter, the sample was diluted to 0.1 %w/w of sample and 1 %w/w of LiCl using DMAc, and filtered through 0.45-μm membrane before injecting to an injector (7725i Rheodyne®, Sigma-Aldrich, Germany). The sample was fed at 1 mL/min into chromatographic column (KD-806 M, Shodex, USA) with the temperature controlled at 40 ºC. The molecular weight of the samples was determined by comparing with those of the polystyrene standards. Microscopic images of the samples were visualized by Scanning Electron Microscopy (SEM Hitachi TM3030 Plus, Japan). Freeze-dried BC was coated with a thin layer of gold using a gold sputter under a high-vacuum condition for 50 s before observation. The topographical information on the sample surface was then observed at a voltage of 15 kV using Charge-up reduction observation and Secondary Electron (SE) modes. The viscoelastic property of the hydrogel-like BC pellicles was acquired using a shear rheometer (Anton Paar Rheoplus, Austria). The pellicle was placed on the lower plate while temperature was controlled at 25 ºC. Complex modulus (G*) values were measured using a parallel plate having a diameter of 25 mm (PP25) at the frequency of 1 Hz and strains of 0.01-100%.

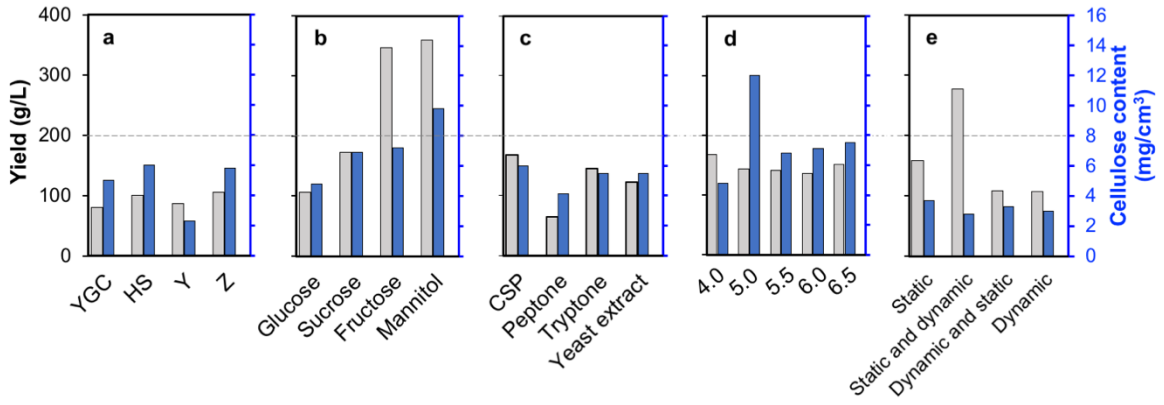

Figure 1: Yield of hydrogel-like BC pellicle (grey bar) and nanocellulose content in the pellicle (blue bar) after cultivating G. xylinus in different cell media (a), carbon sources (b), nitrogen sources (c), initial pH values of the cell media (d), and static/dynamic conditions (e).

3. Results and discussion

3.1 Optimization of culture media, carbon and nitrogen sources, pH, and static/dynamic conditions

Cultivation system of cellulose-producing bacteria, *G. xylinus*, was preliminarily optimized and assessed in terms of yield of BC pellicles and cellulose content in the pellicles. The yield and cellulose content were low, when YGC and Y media were used (Figure 1a), despite containing a high carbon source (5 %w/v glucose as listed in Table 1). In HS and Z culture media containing lower carbon source (2 %w/v) but higher nitrogen source (1-2 %w/v), yield and cellulose content were higher. This demonstrated that nitrogen source was critically important in cellulose biosynthesis. As seen in Figure 1b, the glucose-containing medium yielded the lowest cellulose production, whereas approximately three-time higher yields were obtained by using fructose and mannitol. This was because the pH of the media dropped to pH 3 on the second and seventh days of the cultivation, when glucose and sucrose were used as a carbon source (data not shown).This lower pH, more acidic, was a consequence of gluconic acid produced by metabolism of *G. xylinus* (Liu et al., 2015). In contrast, the pH of the media containing fructose and mannitol remained 4-5 throughout the culture period. As seen in Figure 1d, the optimal pH suitable for cellulose biosynthesis was pH 5. In Figure 1c, among various nitrogen sources, yield and cellulose content did not show an apparent difference. This might strengthen the case for potential replacement with protein from waste sources. In Figure 1e, the combined static and dynamic condition resulted the highest yield. However, higher cellulose content was observed in static condition (~4 mg/cm^3) than in other conditions (~3 mg/cm^3). Although the dynamic system enhanced solubility of oxygen/air into the culture medium preferable for aerobic bacteria like *G. xylinus*, the cellulose production was relatively lower since the motive cell media induced formation of spontaneous cellulose-nonproducing mutant cells, which dominated in the culture (Krystynowicz et al., 2002).

From this preliminary optimization, HS media at pH 5 under a static condition was adopted for further study using *okara* as a nitrogen source. According to the economic potential of the nanocellulose production (5.5 US$/g), sucrose was supplemented in HS media since it was cheaper (1.14 US$/kg) comparing with other tested carbon sources.

3.2 Effects of *okara* as a nitrogen source in nanocellulose biosynthesis

Concentrations of *okara* in the culture media influenced BC production. *Okara* at the concentration of 1% in the culture medium yielded the highest production of nanocellulose (sample BC1) as shown in Table 2, which doubled the yield of BC0 produced in an absence of *okara* in the medium. At higher *okara* concentrations, the increased medium viscosity was observed, which might not be suitable for motile bacteria resulting in lower cellulose production. Hence, this suggested that the optimal concentration of *okara* in the culture medium was 1%. The cellulose content in all BC samples reached similar values of about 3 mg/cm^3, in spite of the high pellicle yield of BC1. This could be explained by the higher thickness of BC1 providing a higher volume of BC pellicle. In comparison with other nitrogen sources as described in Figure 1c, cellulose content was slightly lower (1-3 mg/cm^3), when *okara* was added in the cell culture medium. Elemental analysis was performed to assess sample purity. The result showed that all BC samples had a high purity of ca. 100% quantified by total mass percentages of carbon (~45%) and oxygen (~55%), which were the major elements in carbohydrate molecules. For crystallinities of BC samples as summarized in Table 2, BC0.5, BC1, BC2, and BC3 exhibited crystalline material with crystallinity of about 90%, whereas BC0 possessed lower crystallinity (70%). This was caused by the difference in orientation of the polymer chains in the samples and incomplete growth of crystallite during the polymerization/regeneration process (Castro et al., 2011; Shanshan et al., 2012). In relation to % crystallinity, the increase in crystallite size at 2θ of 16^o from 63 to 76 nm was noticed as the *okara* concentrations increased from 0 to 0.5 and 1% (BC0.5, and BC1), and slightly dropped to 68 nm when the *okara* concentrations increased to 2 and 3% (BC2 and BC3).

Table 2: Yield of BC pellicle, cellulose content in BC pellicle, purity, crystallinity, crystallite size at 2θ of 16.8^o, weight average molecular weight (M_W) and polydispersity index (PDI)

	Yield (g/L)	Cellulose content (mg/cm^3)	Purity (%)	Crystallinity (%)	Crystallite size (nm)	M_W (10^6 g/mol)	PDI
BC0	43.6±1.1	3.0±0.0	99.9±0.1	70.4	63.2	5.8±0.9	2.3
BC0.5	57.1±5.2	3.3±0.3	99.9±0.0	89.7	70.6	6.8±2.3	2.3
BC1	84.7±6.7	3.4±0.2	99.8±0.0	91.2	76.8	9.4±0.9	2.3
BC2	72.7±5.9	3.2±0.0	99.8±0.1	89.4	68.3	7.8±0.0	2.2
BC3	61.1±1.6	3.0±0.0	99.8±0.1	90.8	68.4	7.55±2.0	2.9

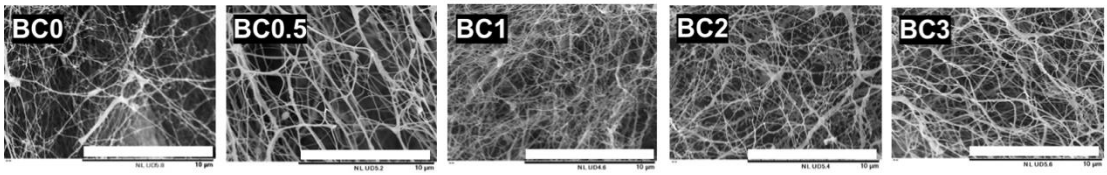

Figure 2: SEM images of freeze-dried BC samples at the magnification of 10000×. Scale bars represent 10 μm

The biosynthesis of cellulose in 0% *okara* resulted in a relatively low molecular weight of BC0, which appeared to be almost half of molecular weight of BC1. The polydispersity index (PDI) indicating the breadth of molecular weight distribution of the polydisperse polymer i.e. cellulose was in the intermediate range of about 2 for all the samples. This meant that the weight average molecular weights were two times higher than the number average molecular weights.

SEM images in Figure 2 visualize nanofibrous cellulose (~100 nm) with differences in density relating to the yield (Table 2). The lowest yield of BC0 brought about a loose fiber network with large vacant spaces between fibers. Unlike BC0, the high compactness of finer cellulose fibers with a uniform size of BC1 having the highest cellulose content was observed. SEM results additionally confirmed that nanocellulose biosynthesis was achieved using *okara* as a nitrogen source in the culture system of *G. xylinus*. This was the consequence of protein in *okara* containing various essential amino acids such as glutamic acid, aspartic acid, serine, glycine, tyrosine, alanine, proline, valine, lysine, isoleucine, and phenylalanine, supporting cell growth and bacterial products (Taokaew et al., 2022). These SEM results, revealing the interior nanofiber density and arrangement in each BC sample, explained the observed impact resistance against an external shear force. Viscoelastic properties of the hydrogel-like BC pellicles were characterized in terms of complex shear modulus (G*) and tangent phase angle (Tan δ) tested over the entire range of strains from 0.01 to 100% strain as seen in Figure 3. At strains ranging from 0.01-0.1%, all the moduli of BC samples were rather constant, and then gradually decreased to nearly 0 at the 100% strain due to the deformation of the BC pellicle (Figure 3a). As considered before the deformation occurred (in such constant region), G* of BC1 pellicles (24 kPa) was almost five times higher as compared to that of BC0 (5 kPa) due to the yield of BC1 being nearly twice that of BC0. The other samples, BC0.5, BC2, and BC3, had G* of 16, 20 and 17 kPa, respectively. In Figure 3b, Tan δ of greater than 1 indicated the phase change or deformation caused by the applied shear force. BC0 and BC1 deformed at 1 and 3% strains, respectively, while other samples deformed at about 2%. This meant that BC0 exhibited a lower resistance to deformation against the external shear force, than BC biosynthesized in the culture medium containing *okara*.

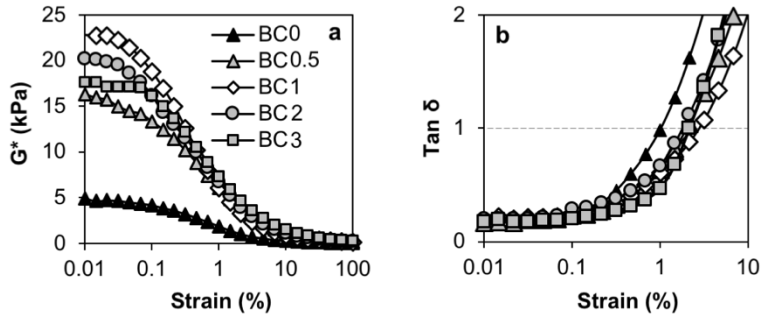

Figure 3: Complex shear modulus (a) and tangent phase angle (b) of hydrogel-like BC pellicles

4. Conclusions

Difference in formulations of cell culture media, carbon sources, nitrogen sources, initial pH values of the culture media, and static/dynamic conditions resulted different nanocellulose yield. The optimized cultivation system consisted of *Hestrin-Shramm* medium, sucrose as a carbon source, pH 5, and a static condition. The effects of nitrogen source type were not pronounced compared with the effects of different carbon sources. *Okara* waste was a promising nitrogen source for biosynthesis of high purity nanocellulose by *Gluconacetobactor xylinus*. The various concentrations of *okara* supplemented in the culture medium influenced the nanocellulose properties. With the *okara* as a nitrogen source, higher yield, crystallinity, molecular weight, and better viscoelastic properties were achieved, as compared to the cultivation without *okara*. Therefore, valorisation of the non-valued *okara* waste in the biosynthesis of nanocellulose let to enhanced opportunity for economic profit and had enhanced sustainability.

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