# Nanocellulose from Oil Palm Biomass to Enhance Microbial Fermentation of Butanol for Bioenergy Applications

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Nanocellulose made by 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-catalyzed oxidation, described as TEMPO-oxidized cellulose nanofibers (TOCNs), has a high density of negative charges on its surface. Its use in microbial fermentation systems is expected to benefit microbial process stability. In particular, microbial stability is strongly required in acetone–butanol–ethanol (ABE) fermentation associated with the solvent-extraction process of butanol production. Here, TOCNs derived from oil palm empty fruit bunches pulp were added to extractive ABE fermentation media containing glucose as a main source, which can be potentially obtained from biomass by saccharification. Then, microbial fermentation was carried out using free or immobilized bacterial cells, to produce butanol from glucose. The presence of TOCNs induced higher total butanol production in broth by improving the growth environment of *Clostridium saccharoperbutylacetonicum* N1-4, which was used as the butanol-producing strain. Microscopic analysis revealed that the spider-web-like TOCN network helped to entrap bacterial cells in alginate beads, by ionic crosslinking of TOCNs and alginates *via* $Ca^{2+}$ ions, to increase stability of bacterial cells in the composite gel beads. The addition of TOCNs to fermentation media had significant positive effects on the total butanol yield.

*Keywords: Nanocellulose; Fermentation; Biobutanol; Alginate; Cell immobilization; Oil palm empty fruit bunch*

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## INTRODUCTION

Cellulose is the key material for the forthcoming sustainable society because it is renewable and the most abundant biomass on Earth (Sadeghifar *et al.* 2017). Nanocellulose, a crystalline bundle of cellulose macromolecules with nanometer-order width, has recently emerged as a promising nanomaterial for various practical applications

because of its physicochemical properties such as high aspect ratio, elasticity, transparency, thermal stability, chemical durability, and biodegradability (Fukuzumi *et al.* 2009, 2010; de Mesquita *et al.* 2010). While woody biomass is the major cellulose resource, non-woody biomass, such as oil palm empty fruit bunches (OPEFB), a large-scale byproduct of oil palm plantations in Southeast Asia, is another attractive raw material for production of nanocellulose because it contains 60% cellulose (Azrina *et al.* 2017). Various types of nanocellulose are produced from cellulosic raw materials by physical disintegration, chemical/enzymatic treatment, or combined processing (Jonoobi *et al.* 2011; Nechyporchuk *et al.* 2016; Chen *et al.* 2017; Hastuti *et al.* 2018). Nanocellulose produced by 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-catalyzed oxidation, namely "TEMPO-oxidized cellulose nanofibers" (TOCNs), have attracted considerable attention since they require low energy for production and afford the narrowest reported nanofibers (Isogai *et al.* 2011). TOCNs bear anionic carboxylate groups on their surfaces as the result of TEMPO-mediated selective oxidation of surface-exposed primary alcohols to carboxylates (Saito and Isogai 2004). These carboxylates induce a zeta potential as low as –70 mV on the solid surfaces, resulting in high dispersibility of TOCNs in water by electrical repulsion (Okita *et al.* 2010), and high dispersant effects for various suspended solid materials (Li *et al.* 2015).

Although nanocellulose has been extensively studied for developing reinforcing agents in composite plastics and films (Goetz *et al.* 2009; Yan *et al.* 2017), enhancers in emulsion systems (Kalashnikova *et al.* 2012; Hu *et al.* 2015), functional hydrogels for environmental remediation (Jin *et al.* 2015; Dwivedi *et al.* 2017), and carriers for metal catalysts and enzyme immobilization (Azetsu *et al.* 2013; Sulaiman *et al.* 2014; Uddin *et al.* 2017), its application in microbial systems remains limited. Yu *et al.* (2016) reported the effects of the size of nanocellulose on microalgal flocculation and lipid metabolism; cellulose nanofibrils effectively induced microalgal flocculation *via* a mechanical interaction based on geometric properties such as nanocellulose morphology and hydrogen bonding. Sun *et al.* (2014, 2015) observed aggregation of *Pseudomonas fluorescens* and *Escherichia coli* K12 in the presence of nanocellulose; they found that bacterial aggregation and adhesion to solid surfaces were significantly affected by the surrounding solution chemistry. The electrostatic interaction promoted by the charged cellulose nanocrystals could give rise to clustering, phase separation, and rapid aggregation of negatively charged bacteria (Larsen *et al.* 2009; Sun *et al.* 2012, 2014). Clustering and phase separation of bacteria are very important for product recovery in microbial biofuel production.

Practical applications of microbial systems in the production of biofuels from renewable resources are attracting much attention for environmental and economic reasons (Zheng *et al.* 2009). Biobutanol produced by microbial fermentation is of significant interest as demand for butanol as an industrial intermediate is rapidly increasing, and it can be used directly in gasoline engines without any modification and/or substitution (Xue *et al.* 2017). Butanol is the most attractive biofuel alternative to ethanol because it has numerous desirable properties, including lower vapor pressure, higher calorific value, less corrosive properties, and a non-hygroscopic nature (Dürre 2007). Butanol can be produced from renewable resources through acetone–butanol–ethanol (ABE) fermentation (Lee *et al.* 1995) using *Clostridia* as a high-performance butanol-producing strain (Tashiro *et al.* 2004). The metabolic pathway of typical ABE fermentation of butanol-producing *Clostridia* strain (Jones and Woods 1986) is summarized in Fig. 1.

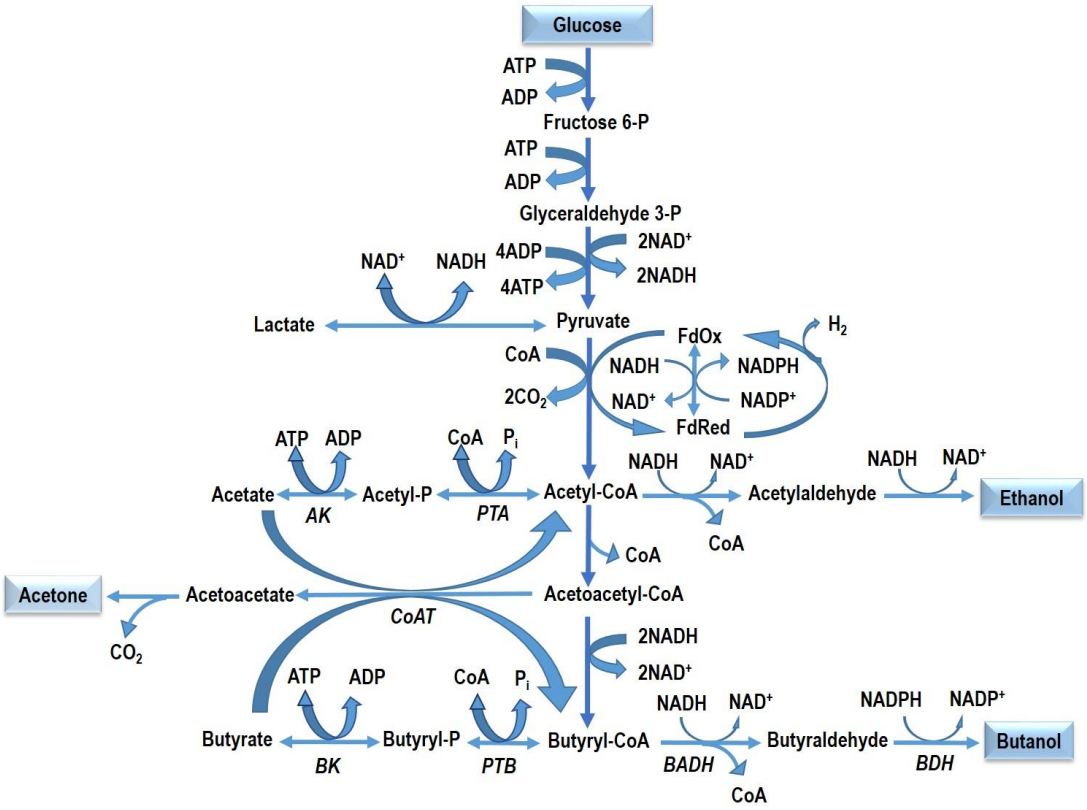


**Fig. 1.** Metabolic pathway of acetone–butanol–ethanol (ABE) fermentation in butanol-producing *Clostridia* strain. Several enzymes involved in ABE fermentation are described in italics. These abbreviations are: *AK*, acetate kinase; *PTA*, phosphotransacetylase; *CoAT*, CoA transferase; *PTB*, phosphotransbutyrylase; *BK*, butyrate kinase; *BADH*, butyraldehyde dehydrogenase; *BDH*, butanol dehydrogenase.

However, microbial butanol production has suffered from severe product inhibition in fermentation processes and high cost of product recovery, resulting in low butanol productivity. Studies have been conducted to overcome these obstacles by integrating a butanol separation step, such as cell immobilization, extractive fermentation, pervaporation, or perstraction (Barton and Daugulis 1992; Qureshi and Maddox 1995, 2005). Among these, extractive fermentation (liquid-liquid extraction) has great potential to increase the product titer if one can determine the most appropriate solvent for butanol selectivity that is compatible with the cells producing the butanol (Qureshi and Maddox 1995; Ishizaki *et al.* 1999). In extractive fermentation, the broth is in contact with an extracting solvent; therefore, some inhibitory products become dissolved in the solvent, resulting in reduction of inhibitory effects on the culture (Roffler *et al.* 1987). However, the efficiency of this method depends on the affinity of solutes for the extraction solvent and the mixing ratio of the phases (de Jesus *et al.* 2019). Extractive fermentation may result in inactivation of cells due to the extensive exposure of cells to extraction solvents and product toxicity (Ishii *et al.* 1985). Therefore, advanced technology to immobilize cells has been investigated to overcome such problems by using silica gel, pumice, and Ca-alginate as microbial carriers and has been applied in microbial fermentation (Napoli *et al.* 2010; Pereira *et al.* 2014). However, to the best of our knowledge, no study has reported an effective strategy using nanocellulose to improve microbial stability to enhance butanol productivity.

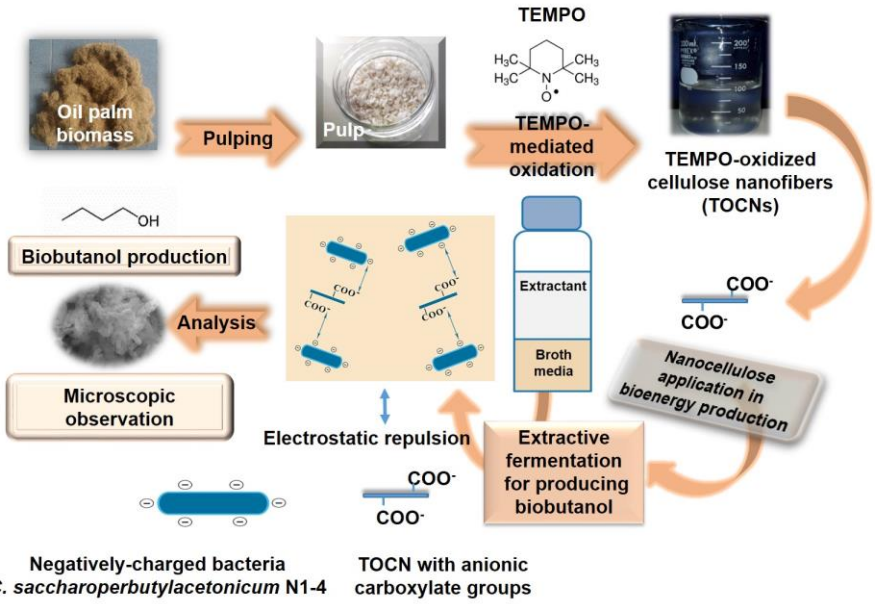


**Fig. 2.** Overview of use of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-oxidized cellulose nanofibers (TOCNs) in microbial biobutanol production.

Herein, microbial stability in biobutanol production was addressed by using in-broth TOCNs, which were produced from OPEFB waste. The characteristics of raw pulp OPEFB and the resultant TOCNs are described in Table S1 and Figs. S1–S4 in the Appendix. TOCNs have surface anionic carboxylate groups with high density (up to 1.5 mmol $g^{-1}$) and aspect ratio more than 40. The characteristics of TOCNs from company made from wood are described in Fig. S5; these can promote electrostatic repulsion of negatively charged bacteria producing biobutanol, which is important in microbial system stability by preventing bacterial aggregation and then enhancing microbial dispersibility (Fukuzumi *et al.* 2009; Sun *et al.* 2012). The strategy is illustrated in Fig. 2. The addition of TOCNs improved microbial stability and increased butanol production. The addition of TOCNs in extractive fermentation of biobutanol significantly improved cell growth and the total butanol concentration after 96-h fermentation. This new application of nanocellulose obtained from the low-cost agricultural residue OPEFB in bioalcohol production is expected to expand the potential of natural nanomaterials from biomass.

## EXPERIMENTAL

### Materials

*Microorganism inoculation*

*Clostridium saccharoperbutylacetonicum* N1-4 ATCC 13564 was used in this study. One milliliter of spore suspension of *C. saccharoperbutylacetonicum* was transferred from sand stock and refreshed in 9 mL of fresh potato glucose medium (10% *v/v*) (Ishizaki *et al*. 1999). The spore suspension was heat-shocked in a water bath at 100 °C for 1 min, and then refreshed at 30 °C for 24 h anaerobically using an Anaeropack (Mitsubishi Gas Chemical, Co., Inc., Tokyo, Japan). The refreshed culture broth was inoculated into tryptone–yeast extract–acetate (TYA) medium (Tashiro *et al.* 2004). After

inoculation, the culture broth was incubated anaerobically at 30 °C for 15 h using an Anaeropack, and then used as a seed culture.

## Methods

*Extractive fed-batch fermentation with free cells*

Fed-batch extractive fermentation with free cells was performed in a 25-mL portion of TYA broth and a 50-mL portion of extractant, which was composed of a 1:1 (*v*/*v*) mixture of oleyl alcohol and tributyrin; the volume ratio of extractant ($V_e$) to broth ($V_b$) was thus 2.0 ($V_e/V_b = 2.0$). In brief, seed culture (2.5 mL) was inoculated into a 25-mL portion of TYA medium, containing glucose (2.5 mL of 50 g/L solution), calcium carbonate (2.5 mL of 3 g/L solution), and TOCNs (5.0 mL of 0.5% *w*/*v* aqueous dispersion, 25 mg of TOCNs in dry weight; see the Supporting Information for preparation of TOCNs) in a 500-mL Erlenmeyer flask closed with a rubber seal, as an aqueous phase. The flask was then sparged with nitrogen gas for 10 min to obtain anaerobic conditions. The fed-batch cultures were grown at 30 °C in a shaker (100 rpm) and by feeding 1 g of glucose powder at 24, 48, and 72 h after seeding and maintained the anaerobic condition by sparging with $N_2$ after glucose feeding.

*Extractive fed-batch fermentation with immobilized cells*

A solution of sodium alginate (4% *w*/*v*) was prepared in boiling water and autoclaved at 115 °C for 15 min. Precultured cells (2.5 mL) and TOCNs (0.5% *w*/*v*, 25 mg dry weight) were added into sterilized saline water (20 mL, 0.85% NaCl). The resultant suspension was mixed with an equal volume of 4% sodium alginate solution (final concentration of sodium alginate, 2%). The mixture was dropped into a 3% $CaCl_2$ solution using a syringe with continuous stirring to form alginate beads containing cells and TOCNs. The resultant beads were recovered by filtration. Cell-containing alginate beads without TOCNs were also prepared as a control. The diameters of beads were approximately 5 mm. Extractive fed-batch fermentation with immobilized cells was performed as described above (extractive fed-batch fermentation with free cells).

*Analytical methods*

Cell density was determined by measuring the optical density of the suspension at 562 nm ($OD_{562}$) using a UV/vis spectrophotometer (Bio-Spec, Shimadzu, Kyoto, Japan) after diluting the samples. The dry cell weight (DCW) was calculated as previously reported (Yoshida *et al.* 2012),

$$DCW = 0.301 \times OD_{562} \times D - 0.0008 \quad (1)$$

where $D$ is the dilution ratio.

The total butanol concentration $[BuOH]_{Total}$ was defined as the total amount of butanol produced in all the phases per broth volume (g/L-broth), and calculated as follows (Darmayanti *et al.* 2018),

$$[BuOH]_{Total} = [BuOH]_b V_b + [BuOH]_c V_c + [BuOH]_e V_e) / V_b \quad (2)$$

where $[BuOH]_b$ and $V_b$ are the butanol concentration (g/L) in the broth and the volume (L) of the broth, $[BuOH]_c$ and $V_c$ are the butanol concentration in the cell beads and the volume of the beads, and $[BuOH]_e$ and $V_e$ are the butanol concentration in the extractant and the volume of the extractant, respectively. $V_c$ the volume of alginate beads, is 1.5 mL for every measurement because six beads were crushed in aqueous sodium citrate solution in a total volume of 1.5 mL.

The concentration of glucose was measured in the supernatant liquid obtained by centrifugation of broth by using a high-performance liquid chromatograph (US-HPLC-1210, JASCO, Tokyo, Japan) equipped with a refractive index detector and SH-1011 column (Shodex, Tokyo, Japan). Aqueous $H_2SO_4$ (0.05 mM) was used as the mobile phase (1.0 mL/min, 50°C), using an injection volume of 20 µL. The concentration of butanol was measured in supernatant obtained by centrifugation of both extractant and broth using a gas chromatograph (6890A, Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector and a 15-m capillary column (INNOWAX 19095N-121, Agilent Technologies) in previously reported conditions (Tashiro *et al.* 2004).

The distribution coefficient ($K_d$) of butanol between the extractant (oil phase) and broth (aqueous phase) was calculated using Eq. 3.

$$K_d = [BuOH]_e / [BuOH]_b \quad (3)$$

The treatments of free TOCNs and the presence of TOCNs were evaluated with analysis of variance (ANOVA) reported as *p*-values. Findings with *p*-values less than 0.05 suggested that differences were statistically significant.

*Microscopic analysis*

Samples for scanning electron microscopy (SEM) analysis were prepared as follows. Samples of free cells were prepared by collecting a 1.5-mL portion of broth, which was centrifuged at 120 rpm for 20 min to obtain cells as the precipitate. For immobilized cells, several alginate beads were collected from the broth and cut into smaller pieces. A 500-µL portion of broth from free cells or the cut beads containing immobilized cells were fixed by 2% formaldehyde (300 µL) and phosphate buffer (1 mL) at pH 5.5 and 4 °C, overnight. Half of the fixed cells were collected and washed with deionized water and centrifuged at 120 rpm for 10 min.

The precipitate was stained with 1% $OsO_4$ solution for 4 h and washed with deionized water. The specimen was washed successively with 50, 70, 80, and 99.5% ethanol, each for 5 min. The specimens were freeze-dried and observed using an SU-8000 apparatus (Hitachi, Tokyo, Japan) at the Center of Advanced Instrumental Analysis, Kyushu University.

The cells were observed using a confocal laser scanning microscope (LSM 700, Carl Zeiss AG, Oberkochen, Germany). A 500-µL portion of culture medium containing free cells was collected in a 1.5-mL plastic tube and washed with phosphate buffer at pH 5.5 by centrifugation.

The precipitate was stained with 4',6-diamidino-2-phenylindole (DAPI), and washed again with phosphate buffer by centrifugation. The cells in alginate beads were treated in the same manner after destroying the beads by treatment with 500 µL 0.2 M sodium citrate for 2 h. A 20-µL portion of prepared cells in phosphate buffer was put on an observation glass and observed under a 405-nm laser.

## RESULTS AND DISCUSSION

### Extractive Fed-batch Fermentation with Free Cells

The fermentation behavior of free cells was monitored in terms of DCW, glucose consumption, and butanol production for 96 h in the presence of OPEFB-derived TOCNs (op-TOCN), and compared with the results using wood-derived TOCNs (w-TOCN), and in the absence of TOCNs (control). As shown in Fig. 3, the cells drastically increased by 48 h and then stayed in a stationary phase until 96 h in the presence of both op-TOCN and w-TOCN; in contrast, cell growth was almost negligible over 96 h in the absence of TOCNs (Fig. 3A).

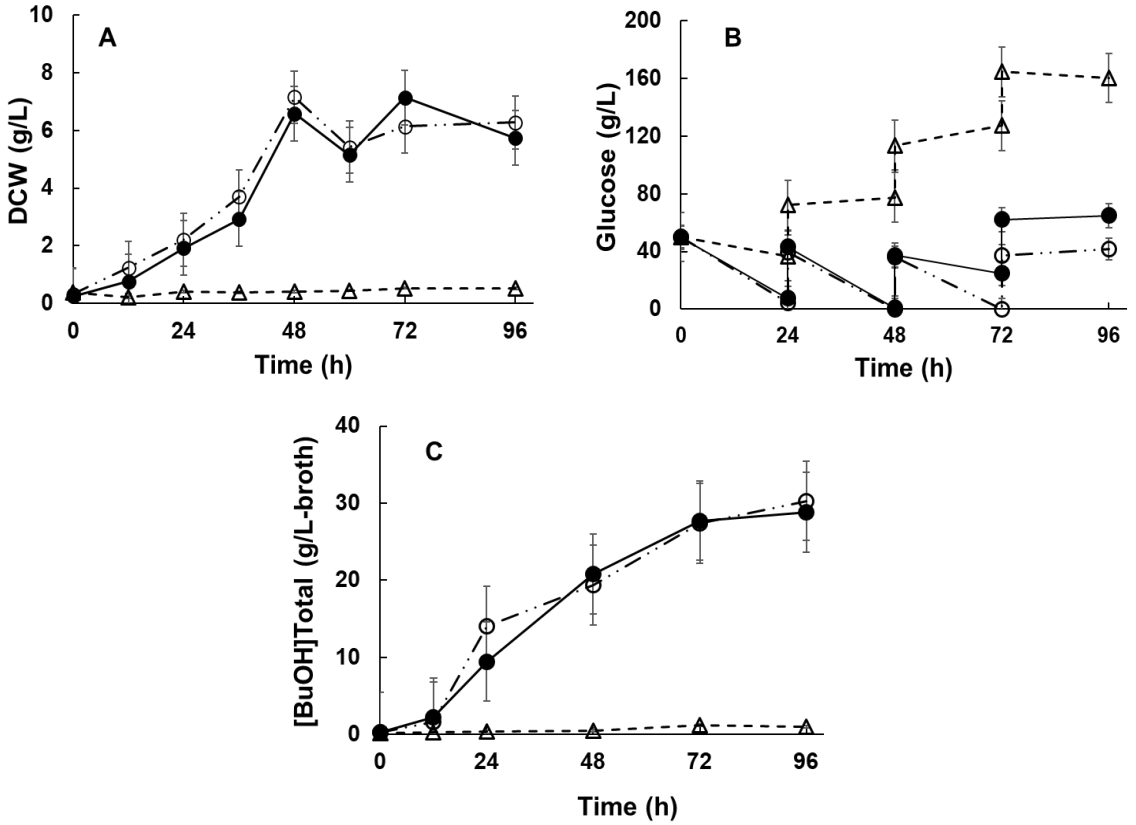


**Fig. 3.** Time course of extractive fermentation using free *Clostridium saccharoperbutylacetonicum* N1-4 cells. Glucose was fed every 24 h. (A) Dry cell weight (DCW), (B) glucose concentration, and (C) total butanol concentration. Filled circles: oil palm empty fruit bunches (OPEFB)-derived TOCNs (op-TOCN); open circles: wood-derived TOCNs (w-TOCN); triangles: control, no TOCNs.

An increase of cell density in the presence of op-TOCN and w-TOCN is in good accordance with the glucose consumption, in which fed glucose was efficiently consumed over 48 h in the presence of op- and w-TOCNs (Fig. 3B). Glucose consumption became slower after 48 h of fermentation, corresponding to the stationary phase of the cultures. In contrast, very little glucose was consumed in the absence of TOCNs. Butanol production was drastically improved in the presence of both op- and w-TOCNs, reaching 30 g/L total butanol concentration after 96-h fermentation, while the butanol concentration was almost negligible in the absence of TOCNs (Fig. 3C). It is noteworthy that the total butanol concentrations in the presence of op- and w-TOCNs were higher (28.8 and 30.3 g/L-broth

after 96 h, respectively) than that reported in previous work (24.2 g/L-broth) (Darmayanti *et al.* 2018) in which extractive fermentation was carried out by the free cell method using a large ratio of extractant to broth ($V_e/V_b$ = 5.0) and *C. saccharoperbutylacetonicum* N1-4 was used as butanol-producing strain. The distribution coefficients of butanol ($K_d$) in the present TOCN systems were also higher (3.98 for op-TOCN and 4.97 for w-TOCN) than in the previous work ($K_d$ = 3.14).

The time course of cell growth is in good accordance with the general features of the metabolic pathway of butanol production by *C. saccharoperbutylacetonicum* N1-4, which consists of two phases, acidogenesis and solventogenesis (Jones and Woods 1986). The growth behavior in the first 48 h corresponds to acidogenesis, during which cells rapidly grow while producing acetic acid and butyric acid. Solventogenesis occurs in the stationary phase, during which the cells reassimilate the previously excreted acids to form acetone, butanol, and ethanol (Shinto *et al.* 2007).

Enhancement of butanol production with TOCNs could be explained by increased dispersibility of bacteria during fermentation, which improves the microenvironment of cells. According to the Derjaguin–Landau–Verwey–Overbeek theory, dispersibility of suspended solids such as bacteria is dominated by repulsive electrical double layer forces (Larsen *et al.* 2009). The surface of both TOCNs and bacterial cells are negatively charged through carboxylate and phosphate groups, respectively. Therefore, their anionic nature causes improved colloidal stability of the bacterial system by preventing flocculation between bacteria (Sun *et al.* 2012). In another aspect, carboxylate groups in TOCNs were possibly involved in calcium binding, resulting in keeping repulsion between bacterial cells, even in the presence of $Ca^{2+}$ ions. In this work, the increased dispersibility presumably enhanced bacterial growth, as illustrated in Fig. 4, resulting in higher butanol production.

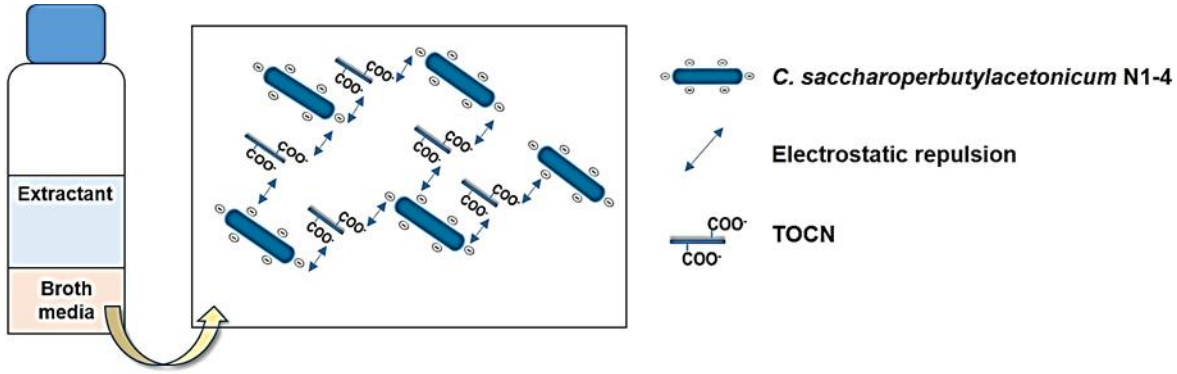


**Fig. 4.** Schematic illustration of nanocellulose (TOCN) in extractive fed-batch fermentation with free cells

## Extractive Fed-batch Fermentation using Immobilized Cells

The present strategy of TOCN-enhanced butanol production was further explored in immobilized-cell fermentation. This method has great advantages since immobilized cells can be elaborated into practical flow-reactor systems, easily separated to obtain products from cell suspension, and protected from toxic products (Gotovtsev *et al.* 2015). Alginate hydrogels have demonstrated high applicability as a support for cell immobilization, because they provide mild gelation conditions, transparency for microscopic observation, a gel pore network that allows the diffusion of nutrients, and a

gentle environment for the entrapped materials (Smidsrod and Skjak-Braek 1990; Andersen *et al.* 2015).

Cell immobilization in alginate beads was applied to the present extractive fermentation for biobutanol production by introducing bacterial cells and TOCNs together into alginate beads. Fermentation was conducted using cells immobilized with op- and w-TOCNs, and cells immobilized without TOCNs served as a control. The time courses of DCW, glucose consumption and butanol production for immobilized-cell fermentation are shown in Fig. 5. Interestingly, the DCW with op-TOCN continued to increase for 72 h, and remained at a high level up to 96 h. However, DCW with w-TOCN and in controls decreased after 48 h of fermentation (Fig. 5A).

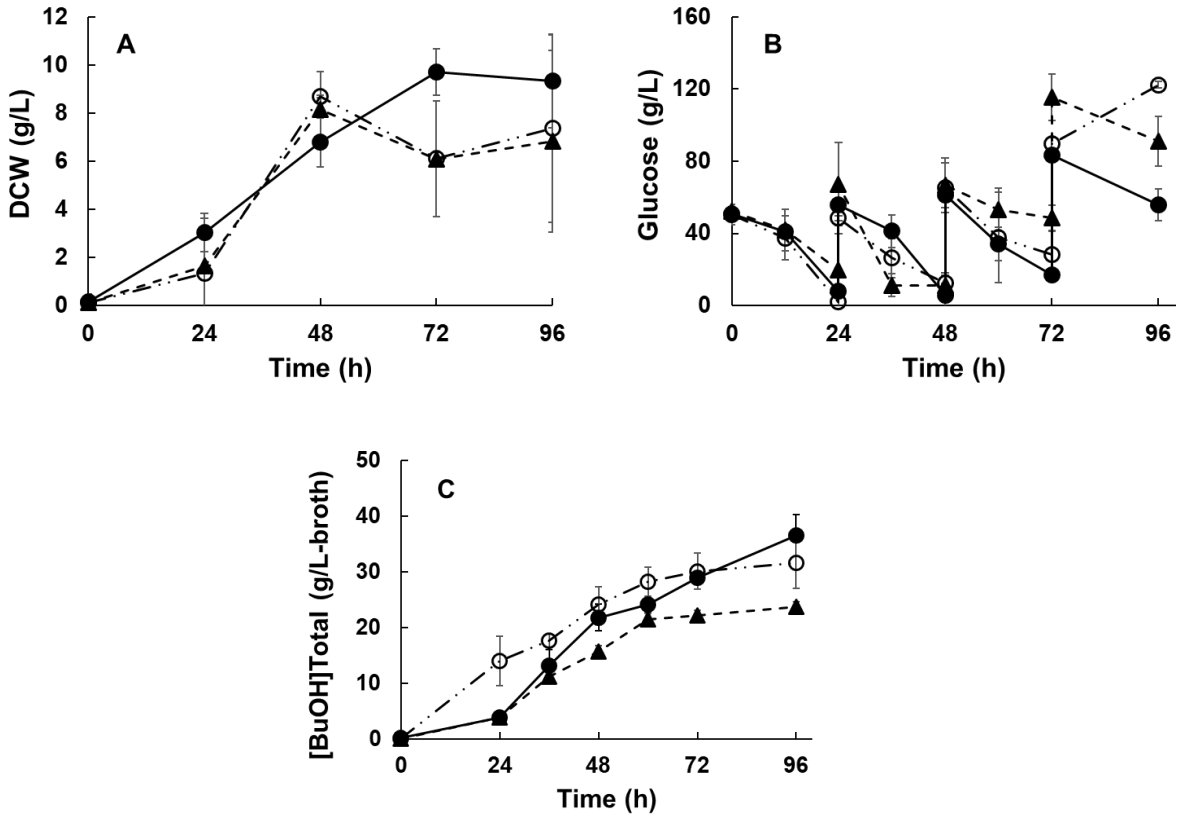


**Fig. 5.** Time course of extractive fermentation using immobilized *C. saccharoperbutylacetonicum* N1-4 cells. Glucose was fed every 24 h. (A) DCW, (B) glucose concentration and (C) total butanol concentration. Filled circles: op-TOCN; open circles: w-TOCN; triangles: control without TOCNs

The residual glucose was lower with op-TOCN than with w-TOCN and in controls even after 96 h, implying satisfactory cell growth. Higher glucose accumulation inhibits the fermentation process, which is related to catabolic flux (Jones and Woods 1986). The higher glucose accumulation for w-TOCN and TOCN-free conditions indicated that the substrates were not completely used. Therefore, to operate at the best glucose use efficiency, appropriate dilution ratios and concentration control of glucose feedstock are required (Yen and Li 2011). The lower amount of residual glucose in op-TOCN conditions can be attributed to a greater number of active cells in alginate beads, resulting in higher consumption of glucose (Table S2) as the energy source in butanol-producing bacterial metabolism. On exhausting glucose in the fermentation broth, the fermentation was

immediately stopped (Zhao *et al.* 2019). Besides, solvent extraction generally enhances glucose consumption, and the solvent production (butanol) in the presence of op-TOCNs appears to be the highest among the conditions tested (Figs. 5A–C) (Ishizaki *et al.* 1999).

The total butanol concentrations in the immobilized-cell treatments were 36.59 g/L-broth for op-TOCN, 31.58 g/L-broth for w-TOCN, and 23.71 g/L-broth for controls (TOCN-free) (Fig. 5C). These results were comparable with, but higher than, those for the free-cell method, which ranged from 29 to 30 g/L-broth in the presence of TOCNs. The slight increase of total butanol concentration after 96-h fermentation indicated that immobilization contributed to the extractive fermentation by altering cell density (in the alginate beads), but did not alter the metabolic pathway (Yen and Li 2011). It is noteworthy that the total butanol production in the presence of TOCNs ($V_e/V_b$ = 2.0) was higher than that reported for an immobilization method using a large ratio of extractant, $V_e/V_b$ = 5 (30.9 g/L-broth) (Darmayanti *et al.* 2018).

The $K_d$ of butanol (extractant to aqueous phase) for immobilized cells using alginate beads with op-TOCN with w-TOCN, and without TOCN, was 4.45, 5.10 and 4.68, respectively (Fig. 6). These values are significantly higher than the $K_d$ in the free cell method, which was related to the higher total butanol production obtained from immobilized cells. The higher total butanol production by immobilized cells was achieved by continuous extraction from the aqueous phase by extractant, which maintained the butanol concentration in the aqueous phase at a low level. Nevertheless, fermentation yields mainly depend on the strain and the culture conditions, which affect ABE metabolism (Tashiro and Sonomoto 2010). In addition, the immobilization method would also increase plasmid stability by creating a protective microenvironment for bacterial cells (Willaert 2007). This advantage is important for the growth of butanol-producing strain.

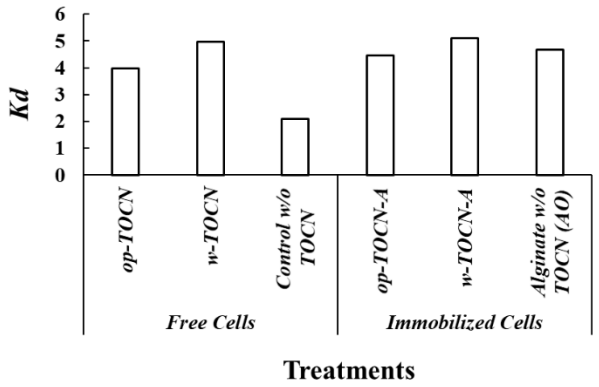


**Fig. 6.** Distribution coefficient ($K_d$) of butanol between the extractant and the aqueous phase.

Statistical analysis revealed that immobilized cells produced significantly more butanol in the presence of TOCNs (op-TOCN and w-TOCN) compared with alginate only (*i.e.*, no TOCNs) ($P < 0.01$). Immobilized cells in alginate (in the absence of TOCNs) are protected from the disturbance of the cellular metabolic activity such as an inhibitory effect of produced butanol (Häggström and Molin 1980). In addition, the diffusion of butanol into the beads is strongly affected by the pore size. Effective pore size may be modified by electrostatic interaction of the negatively charged alginate matrix with other charged species (Smidsrod and Skjak-Braek 1990), including TOCNs which have a high density of

anionic carboxylate groups. The presence of TOCNs safeguards the alginate beads from swelling, prevents the loss of calcium ions because of the tightly packed nanofibrous structure, maintains the spherical shape of beads, retains a high level of cell viability, and preserves the ability of the cells to proliferate (Park *et al.* 2015). In addition, carboxylate groups in TOCNs were possibly involved in calcium binding. A schematic image of the crosslinking among TOCNs, alginates and $Ca^{2+}$ ions (from $CaCl_2$) is provided in Fig. 7.

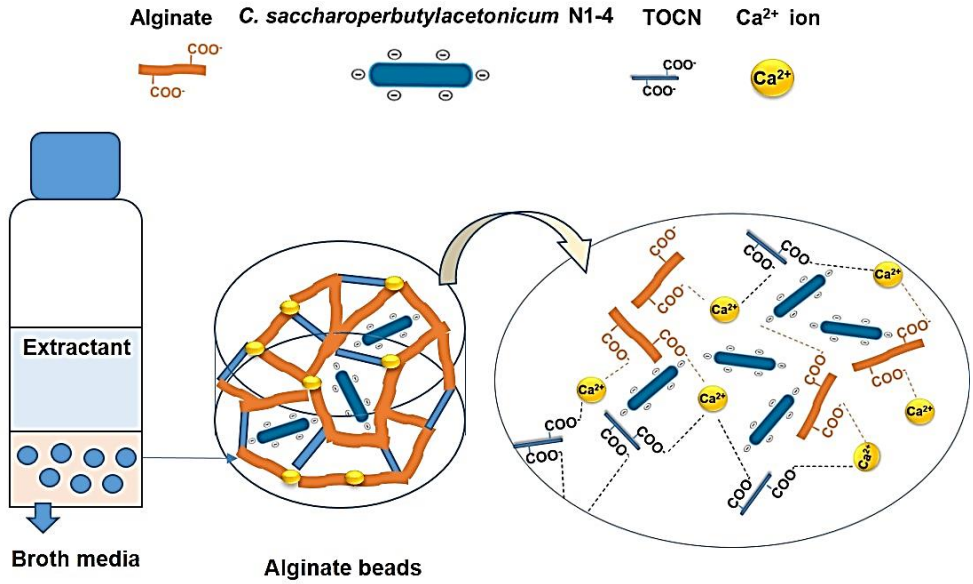


**Fig. 7.** Schematic image of the crosslinking among TOCNs, alginates and $Ca^{2+}$ ions in extractive fed-batch fermentation using immobilized cells

The free-cell and immobilized-cell methods were significantly different in terms of butanol yield ($P < 0.01$) in the presence of op-TOCN and w-TOCN (Fig. 8). This is the first report of TOCN addition in extractive fermentation, and it showed that TOCNs enhance microbial biobutanol production.

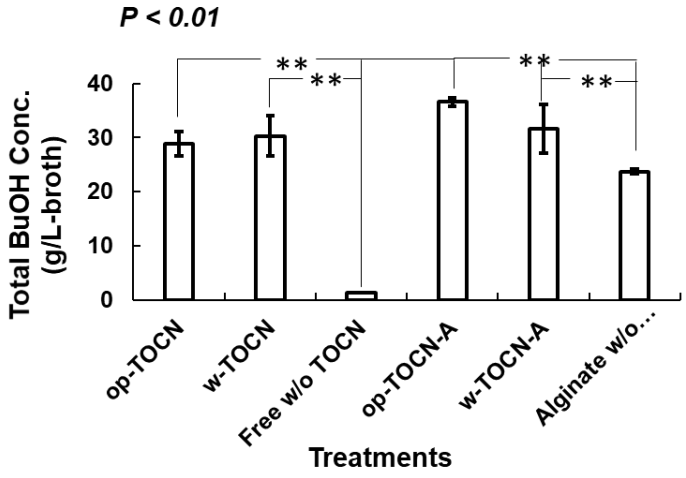


**Fig. 8**. Statistical analysis for different TOCN treatments in microbial biobutanol production.

## Microscopic Analysis

Microscopic analysis was conducted to observe the morphological differences in cultures in the presence and absence of TOCNs (Fig. 9). The TOCN network was similar to a spider's web after drying, as reported previously (Nemoto *et al.* 2012). The spider-web-like structure was not seen in Fig. 9A, which shows material from the broth for free cells without TOCNs. In contrast, the presence of the spider-web-like structure was clearly observed in Figs. 9B and 9C, when bacterial cells were cultured in the presence of TOCNs. Incorporation of alginate and TOCNs possibly contributed to the mechanical and chemical stability of alginate beads. Cells that were immobilized in the alginate beads in the presence of op- and w-TOCN were more viable and proliferated better compared with the control (without TOCNs), due to the 3D fibrous TOCNs, which would be expected to perform as an extracellular matrix with morphological similarity to nanofibrous collagen, glycoproteins, and acidic polysaccharides (Park *et al.* 2015).

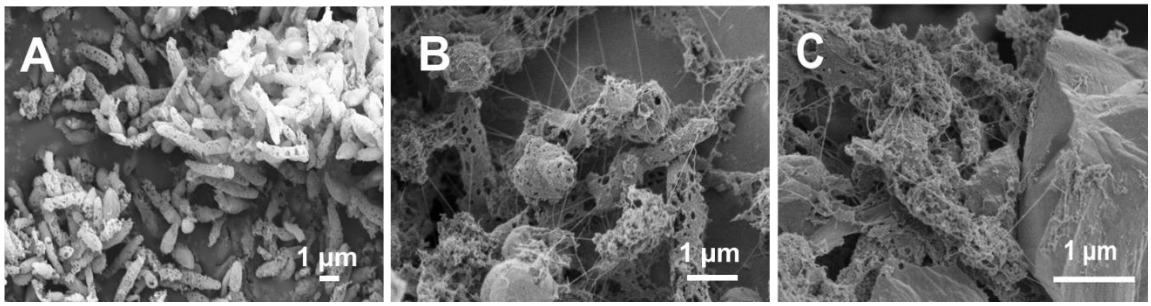


**Fig. 9.** Scanning electron microscopy images of *C. saccharoperbutylacetonicum* N1-4 cells cultured (A) without TOCNs, (B) with op-TOCN, and (C) with op-TOCN in immobilized cells conditions

Optical microscopic images of *C. saccharoperbutylacetonicum* N1-4 cells were acquired using a confocal laser scanning microscope after staining by DAPI in order to compare morphological differences between living cells. The blue fluorescence of single bacteria appeared to be aggregated to some extent in free cells without TOCNs (Fig. 10A). In contrast, bacterial cells were dispersed in the presence of TOCNs (Fig. 10B).

Figures 10D and 10E show that immobilized cells in alginate beads (in the presence of TOCNs) were trapped compared with those in alginate beads without TOCNs (Fig. 10C). One problem of cell entrapment inside the porous matrix of a polysaccharide gel like alginate is the ability of cells on the outer surface of the beads to multiply and be released from the beads, as shown by Fig. 10C (Kourkoutas *et al.* 2004). The presence of TOCNs in alginate beads overcomes this disadvantage by interactions between the alginate and TOCNs. The carboxyl groups on the surfaces of TOCNs can participate in the formation of a crosslinked network in alginate beads through $Ca^{2+}$ ions, and add structural and mechanical stability to the beads. Both cellulose and alginate belong to the polysaccharide family, and their chemical structures should provide good compatibility for the resultant crosslinked beads (Lin *et al.* 2012). This crosslinking reduced cell release from the beads. The cells entrapped in the alginate–TOCN beads retained a high level of viability and ability to proliferate.

The presence of TOCNs with free cells or immobilized cells during extractive fermentation has been demonstrated. In the free-cell method, the presence of TOCNs induced bacterial dispersibility by electrostatic repulsion among anionic carboxylate

groups of TOCNs and negatively-charged *C. saccharoperbutylacetonicum*. This dispersibility induced higher contact of bacteria with substrate and yielded a higher total butanol concentration.

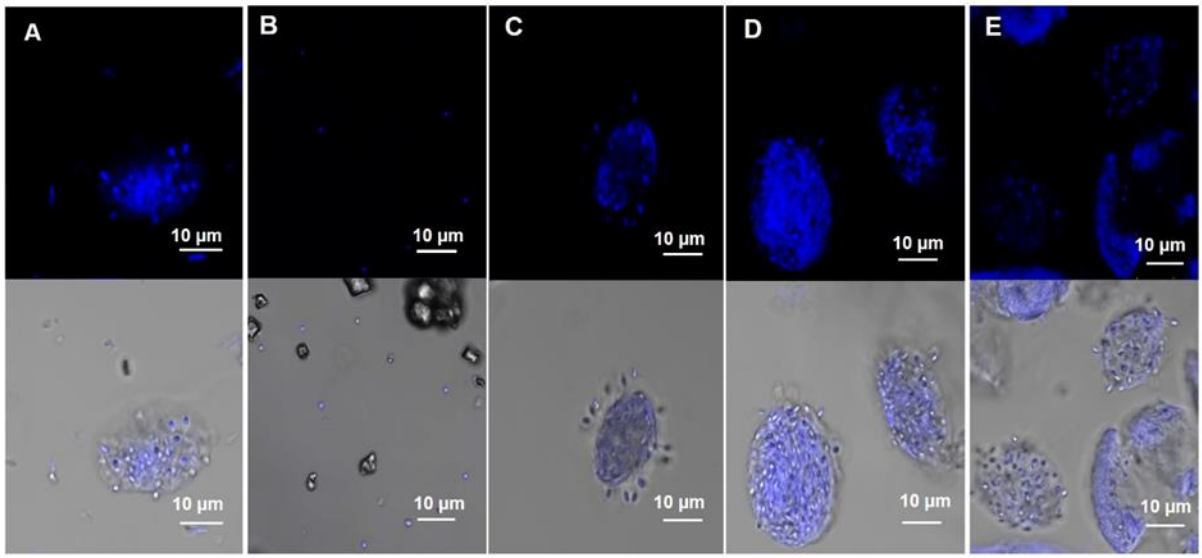


**Fig. 10.** Fluorescent (top) and phase contrast (bottom) images of bacterial cells in (A) aqueous medium without TOCNs (free cells); (B) in aqueous medium in the presence of op-TOCN (free cells); (C) in alginate beads without TOCNs; (D) in alginate beads in the presence of op-TOCN; and (E) in alginate beads in the presence of w-TOCN.

The presence of TOCNs in the immobilization method induced mechanical and structural stability of alginate as a cell immobilizer by crosslinking of the acidic groups of TOCNs and alginate *via* $Ca^{2+}$ ions, resulting in the formation of a 3D network for cell entrapment while maintaining high viability of bacteria. An increase of butanol concentration, particularly in the presence of op-TOCN, indicated that the op-TOCN contributed to the formation of a better microenvironment without altering the metabolic pathway of the butanol-producing strain. This finding opens up the advanced use of low-cost agricultural residues in bioalcohol production.

## CONCLUSIONS

1. Nanocellulose, having carboxylate groups on its surface, was successfully applied with extractive fermentation in microbial biobutanol production.
2. TEMPO-oxidized cellulose nanofibers (TOCNs) from OPEFB (op-TOCN) and wood (w-TOCN) resulted in better micro-environmental conditions during fermentation because of electrostatic repulsion between anionic carboxylate groups on the surface of TOCNs and negatively-charged bacterial cells. This induced good bacterial dispersibility in the medium, improving the total butanol production in broth.
3. Higher DCW and higher total butanol concentration were achieved, up to 28–30 g/L-broth for free cells and 32 to 37 g/L-broth for immobilized cells, in the presence of TOCNs.

4. Microscopic analysis revealed that the presence of TOCNs improved physical cell entrapment when alginate beads were used as an immobilizer. This finding opens up the advanced use of nanomaterials derived from low-value agricultural residues in wider applications in microbial systems.

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# APPENDIX

## SUPPLEMENTARY INFORMATION

### Materials

Bleached kraft pulp of oil palm empty fruit bunches (OPEFB) was kindly supplied by the Biomaterial Research Institute, Indonesian Institute of Sciences (Bogor, Indonesia). Softwood-derived 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO)-oxidized cellulose nanofiber was kindly supplied by Nippon Paper Industries Co., Ltd. (Tokyo, Japan). TEMPO, sodium bromide, sodium hypochlorite, and sodium borohydride were purchased from Sigma-Aldrich (Tokyo, Japan) and used without further purification. The water used in this study was purified with an Arium Ultrapure Water System (Sartorius Co., Ltd., Tokyo, Japan).

### Preparation of TEMPO-oxidized Cellulose Nanofibers (TOCNs) from OPEFB

The bleached kraft pulp of OPEFB was soaked in a 0.01 M HCl solution for 30 min for demineralization. TOCN was prepared using a TEMPO/NaBr/NaClO system. In brief, a 2.5-g portion of demineralized OPEFB pulp (about 85% cellulose content) was suspended in water (250 mL) containing TEMPO (16 mg/g-cellulose) and NaBr (100 mg/g-cellulose). Oxidation was initiated by adding 2 M NaClO aq. (20 mmol/g-cellulose), and the pH of the suspension was maintained at 10 by adding 0.5 M aqueous NaOH with a pH titrator (Mitsubishi Chemical Analytech, Yamato, Japan) during the reaction. After 2 h, the oxidation was quenched by adding ethanol (2 mL), followed by the addition of $NaBH_4$ (100 mg/g-cellulose), and the resultant mixture was stirred for 1 h. The obtained suspension was thoroughly washed using deionized water and then centrifuged at 4000 × *g* for 10 min (five times) and sonicated using an ultrasonic homogenizer US-300E (Nihonseiki Ltd., Tokyo, Japan) at the maximum level for 5 min. Remaining floating fibers were removed by further centrifugation at 12000 × *g* for 10 min. Obtained TOCN was kept at 4°C until further use, and named TOCN-OPEFB (op-TOCN).

### Characterization of TEMPO-oxidized Cellulose Nanofibers (TOCNs)

Elemental analysis was performed with an Organic Micro Analyzer CHN CORDER MT-6 (Yanaco Ltd., Tokyo, Japan). The surface morphology of OPEFB pulp and TOCN-OPEFB were observed using a scanning electron microscope (SEM SU-3500, Hitachi Ltd., Tokyo, Japan) at the Center of Advanced Instrumental Analysis, Kyushu University. Samples were mounted on carbon tape; the machine was operated at an acceleration voltage 15 kV and the vacuum was set at 30 Pa. The length and width of TOCN-OPEFB were observed by transmission electron microscopy (TEM) (JEM 2100-HC, JEOL Ltd., Tokyo, Japan) operated at an acceleration voltage of 120 kV at the Ultramicroscopy Research Center, Kyushu University. The size was measured using Image-J software version 1.51s. X-ray diffraction, Fourier-transform infrared spectroscopy and thermogravimetric analysis of TOCN were conducted as described in our previous work (Hastuti *et al.* 2018). The carboxylate content of TEMPO-oxidized cellulose nanofibers was determined by conductometric titration (Saito and Isogai,2004).

TOCNs from the company were characterized for TEM analysis and carboxylate content only.

**Table S1.** Elemental Analysis of Obtained TOCNs

| Sample | H % | C% |
|---|---|---|
| Raw pulp | 6.38 | 40.13 |
| TOCNs OPEFB | 5.57 | 37.77 |

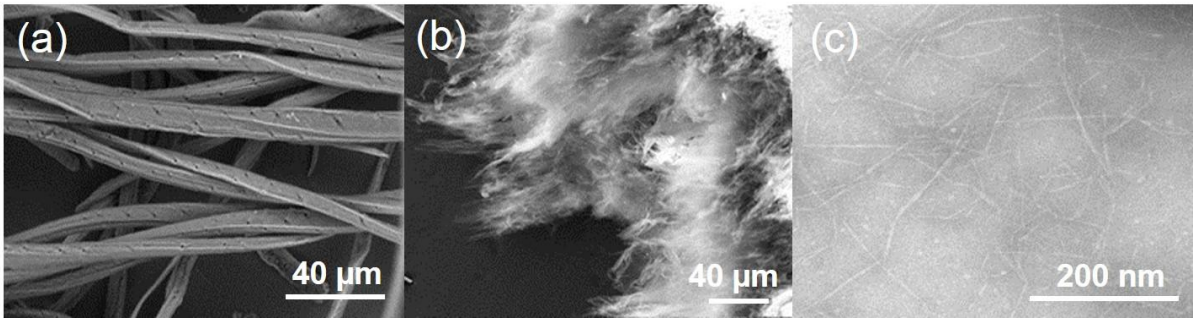


**Fig. S1.** Scanning electron microscopy images of: (a) raw OPEFB pulp; (b) TOCNs from OPEFB and (c) transmission electron microscopy image of TOCNs from OPEFB; carboxylate content 1.5 mmol/g; aspect ratio 41±14.

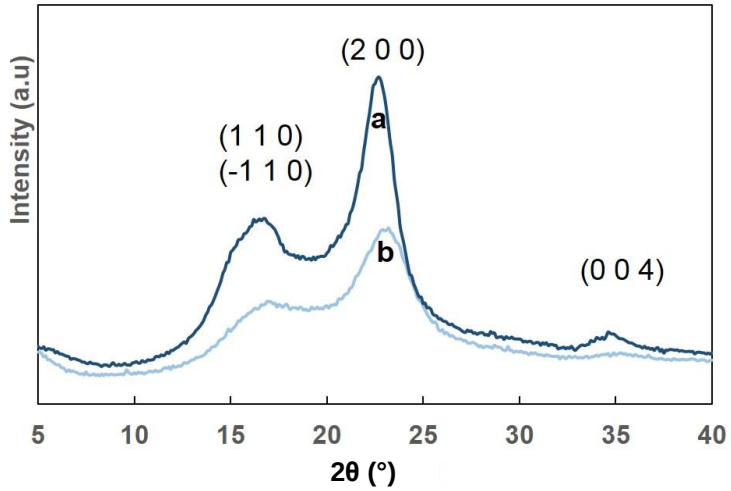


**Fig. S2.** X-Ray diffraction patterns of: (a) raw OPEFB pulp (b) TOCNs from OPEFB

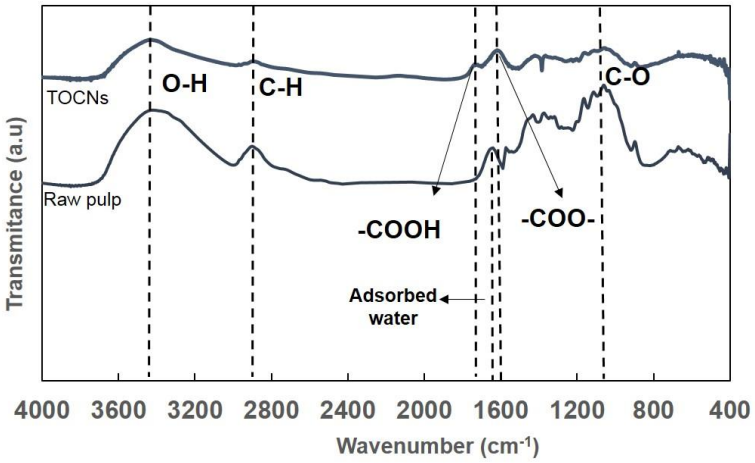


**Fig. S3.** Fourier-transform infrared profiles of raw OPEFB pulp and TOCNs from OPEFB

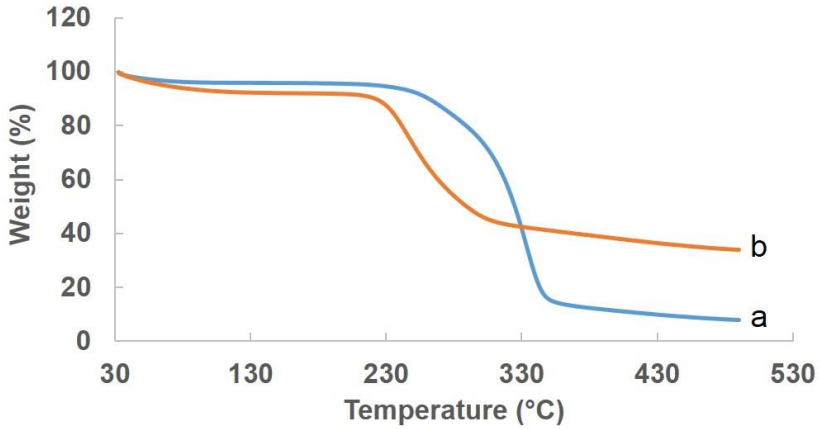


**Fig. S4.** Thermogravimetric curves of raw OPEFB pulp (a) and TOCNs from OPEFB (b)

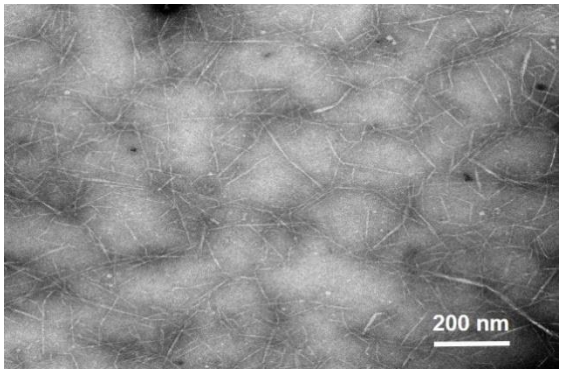


**Fig. S5.** Transmission electron microscopy of TOCNs from company; carboxylate content: 1.43 mmol/g; aspect ratio 44±16

**Table S2.** Extractive Fermentation Properties for Free Cells and Immobilized Cells

| | Free cell | | | Immobilized cell | | |
|---|---|---|---|---|---|---|
| **Parameter** | op-TOCN | w-TOCN | Control | op-TOCN | w-TOCN | Alginate without TOCNs (control) |
| Total butanol concentration (g/L) | 28.82 | 30.30 | 1.04 | 36.59 | 31.58 | 23.71 |
| Total consumed glucose (g) | 2.97 | 2.54 | 0.17 | 3.30 | 2.60 | 3.27 |
| Butanol yield to consumed substrate (C-mol/C-mol) | 0.39 | 0.48 | 0.23 | 0.45 | 0.49 | 0.29 |

## REFERENCES CITED IN APPENDIX

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