Carboxylated-cellulose nanofibers from oil palm empty fruit bunches enhanced extractive fermentation in microbial bio-
butanol production

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Abstract. Nanocellulose produced 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 be beneficial. In particular, microbial stability is required in acetone–butanol–ethanol (ABE) fermentation. Here, TOCNs derived from oil palm empty fruit bunches pulp were added to extractive ABE fermentation media, followed by microbial fermentation. The results showed that 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. Electrostatic repulsion between anionic surface carboxylate groups of TOCNs and negatively-charged bacteria made a positive contribution to the microenvironment for bacterial growth. The addition of TOCNs to fermentation media had significant positive effects on the total butanol yield density cell weight (DCW).

1. Introduction
Oil palm (Elaeis guineensis) is one of the major plantation commodities in Indonesia [1]. Total area of oil palm plantation in 2017 reached 13.20 million Ha, with the total production of crude palm oil...
(CPO) around 34.47 million ton and the total export value reached US$ 21 billion [2]. The process of fresh fruit bunches (FFBs) into CPO results in biomass wastes in large amount of oil palm empty fruit bunches (OPEFB). This OPEFB, which is wastes in palm oil factories, is quite abundant, are often incinerated at the plant site which causes some air pollution [3]. Along with the unprecedented increase in the palm oil industry in Indonesia, the generation of OPEFB is increasing every year [4]. The palm oil industry must dispose about 1.1 ton of OPEFB for every ton of CPO produced [1]. In addition, recent study showed that raw OPEFB containing about 59% of cellulose [5]. This relatively high cellulose content of non-woody materials is potential to be used as nanocellulose. Nanocellulose extraction can be employed by several chemical methods by breaking inter-fibrillar hydrogen bonds using mechanical defibrillation [6], acid hydrolysis [7], 2,2,6,6-tetramethylpyperidine-1-oxyl (TEMPO) mediated oxidation [8], or combination among chemical and mechanical methods [9].

The study about nanocellulose extraction comes mostly from woody materials. Meanwhile, nanocellulose preparation derived from OPEFB have been reported by a few researchers. Nanocrystalline cellulose was successfully extracted from microcrystalline cellulose of empty fruit bunches via a sono-assisted TEMPO oxidation [10]. Nanocellulose produced by TEMPO-catalyzed oxidation, namely “TEMPO-oxidized cellulose nanofibers” (TOCNs), have been the subject of growing attention because their surface reactivity by having carboxylate groups on their surfaces as the result of TEMPO-mediated selective oxidation of surface-exposed primary alcohols to carboxylates [8]. These carboxylates groups could bring electrostatic repulsive forces and make TOCNs in the stable, well dispersed suspension [11] and high dispersant effects for various suspended solid materials [12].

Utilization of nanocellulose derived from oil palm biomass had been reported by Elias and co-authors as reinforcing agent in chitosan-nanocellulose beads to immobilize Candida rugosa lipase for efficient production of butyl butyrate [13,14]. The use of nanocellulose in microbial system also had been reported by Sun and co-authors by using rod-shaped cellulose nanocrystals which cause depletion flocculation of bacteria [15]. Bacteria aggregation is one of the most critical issue in controlling colloidal stability which involves bacteria in the system. The reduced repulsive electrostatic forces may lead bacterial cells to approach each other or a solid surface [16]. Microbial-butanol production via extractive fermentation is one of microbial system which requires the bacterial stability and dispersibility. Therefore, at the first time, we have applied the TOCNs from OPEFB in extractive fermentation of butanol production. Bacteria dispersibility can be improved by assuming the presence of negatively-charged bacteria and anion carboxylate groups of TOCNs in colloidal system. The butanol yield and microscopic analyses were carried out to evaluate the effect of TOCNs-OPEFB addition.

2. Materials and Methods

2.1 Materials

Bleached kraft pulp of oil palm empty fruit bunches (OPEFB) was kindly supplied from Biomaterial Research Institute, Indonesian Institute of Sciences (Bogor, Indonesia). 2,2,6,6-tetramethylpyperidine-1-oxyl (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). Clostridium saccharoperbutylacetonicum N1-4 ATCC 13564 was used in this study. TOCNs from Nippon Paper Company were used as comparison and denoted as TC.

2.2 Methods

Preparation of TOCNs – OPEFB (TO) as described by Saito and Isogai [8]. Microscopic characterization using transmission electron microscopy (TEM) of TOCN-OPEFB and TOCN from
Nippon Paper Company as described in our previous paper [17]. Carboxylate content was measured using conductimetric titration as previously described. Samples for scanning electron microscopy (SEM) analysis were prepared as described in previous paper [18]. 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. Microorganism inoculation, extractive fermentation experimental, total butanol concentration, density cell weight (DCW) and glucose concentration analysis as described in previous paper and according to Darmayanti and co-authors [18,19] with the initial glucose feeding 50 g/L and 1 g/L for every 24 h. Extractive fermentation was conducted for 96 h with the sampling analysis every 24 h by measuring butanol yield, glucose consumption and DCW.

3. Results and Discussion

3.1 Characterization of TOCNs

OPEFB-derived TOCNs (TO) use in this experimental was TOCNs with the carboxylate content of 1.5 mmol g\(^{-1}\) with aspect ratio 41±14. Microscopy analysis of TO as described in Figure 1. In addition, TOCN from Nippon Paper Company (long and short TOCN) were used as comparison. Short and long TOCN were denoted as TC-S and TC-L, respectively (Figure 1). The carboxylate contents of TC-S and TC-L were: 1.61 mmol/g and 1.43 mmol/g, respectively with the aspect ratio of TC-S was 23±6 and TC-L was 44±16. The higher carboxylate content of short TOCNs is caused by more oxidized groups being formed on the microfibril surfaces with the smaller widths [20].

![Figure 1](image1.png)

**Figure 1.** Transmission electron microscopy of TOCN OPEFB (left); short TOCN (middle) and long TOCN (right) from Nippon Paper Company

3.2 Extractive Fermentation Analysis

Butanol yields, glucose consumption and density cell weight were observed during 96 h fermentation. The results as shown in Figure 2. The addition of TOCNs (TOCNs from OPEFB and TOCNs from paper company) in broth media of extractive fermentation induced higher density cell weight (DCW) compared to the control (Figure 2). Higher DCW indicate higher living cells in broth media. These living (active) cells were consuming glucose as a source of energy and co-substrate of *C. saccharoperbutylacetonicum* N1-4 for producing butanol [21]. Therefore, lower residual glucose (in the presence of TOCNs) yielded higher butanol yield (Figure 2). The DCW after 96 h of fermentation in the presence of TOCNs-OPEFB (TO) was about 5.7 g/L. In addition, the DCW in the presence of TOCNs from paper company, for short and long TOCNs were 1.2 g/L and 6.3 g/L, respectively.

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 like bacteria is dominated by repulsive electrical double layer forces caused by anionic carboxylate groups of TOCNs and negatively-charged bacteria [22]. Their anionic nature causes improved colloidal stability of the bacterial system by preventing flocculation between bacteria [15]. The bacteria dispersibility is
important to induce higher contact of bacteria with the substrate and yielded a higher total butanol concentration.

![Graphs showing DCW, glucose consumption, and butanol concentration over time.](image)

**Figure 2.** Parameters of extractive fermentation of microbial biobutanol production: density cell weight (DCW); glucose consumption and total butanol concentration

Microscopic observation also revealed that the presence of 3D fibrous network of TOCNs in broth media (Figure 3). This fibrous network contribution of TOCN was similar to extracellular matrix (ECM) which is important to cellular growth as a function of sequestration and storage site for growth factors and protecting them from degradation [23,24].

![SEM images of broth media: (A) without TOCN and (B) with TOCN.](image)

**Fig. 3.** SEM images of broth media: (A) without TOCN and (B) with TOCN

The length of TOCNs was assumed resulting the different performance in extractive fermentation of microbial biobutanol production. The long TOCNs seemed to be preferred to yield higher butanol
concentration (28.8 g/L-broth for TO and 30.3 g/L-broth for TC-L) concentration than short TOCNs (23.3 g/L-broth). The lower total butanol yield of short TOCNs was assumed that higher carboxylate content of short TOCNs tends to cause the microenvironment was more acidic and influence the bacteria system. Strains are basically negatively charged at neutral pH and becomes positively charged at acidic ones. Differences in zeta potential and isoelectric point with regard to surface charge of bacteria and other components in colloidal system, may destabilize the system, inducing coagulation and flocculation [25]. However, this assumption has not been elucidated in this study. More importantly, the use of carboxylated-cellulose nanofibers of OPEFB showed the effective way to improve bacteria dispersibility by providing anionic carboxylate groups which occurred during TEMPO-mediated oxidation.

4. Conclusions
Carboxylated-cellulose nanofibers from oil palm empty fruit bunches (OPEFB) pulp had been produced successfully via TEMPO-mediated oxidation at pH 10 with the carboxylate contents of 1.5 mmol.g⁻¹. The addition of this carboxylated-cellulose nanofibers (TOCNs) in broth media of extractive fermentation of microbial butanol production induced higher total butanol concentration and density cell weight (DCW). The performance of TOCNs from OPEFB was comparable with the TOCNs from paper company. The length of TOCNs indicated the performance differences in total butanol, DCW and glucose consumption. The long TOCNs was assumed to be preferred than short TOCNs in regard to induce better microenvironment of butanol-producing strain to yield higher butanol concentration.

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