Enhanced and Prolonged Activity of Enzymes Adsorbed on TEMPO-Oxidized Cellulose Nanofibers

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ABSTRACT: 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO)-oxidized cellulose nanofibers (TOCNs) have a width of about 4 nm and a very large specific surface area. TOCN is a negatively charged bionanomaterial having carboxy groups on the surface and promising physical properties. In particular, TOCN can be used as an adsorbent for biomolecules for biotechnological applications, but the adsorption behavior of biomolecules on the TOCN surface requires investigation. Thus, in this study, we investigated the adsorption behavior of pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) on TOCN and evaluated the activity, structure, and long-term stability of the adsorbed enzyme. Transmission electron microscopy observation revealed that the enzyme was aligned and adsorbed on the TOCNs, and circular dichroism measurements were used to determine the structure of the enzyme adsorbed on TOCN. Interestingly, the adsorbed enzyme showed higher activity after adsorption, resulting in long-term retention of enzyme activity, probably because the stability of PQQ-GDH was improved by adsorption. These results suggest that TOCN is an excellent biomolecule immobilization material. Our results can be used for the development of biomaterials using TOCN as a scaffold for the adsorption of enzymes with increased stability and activity.

INTRODUCTION

The modification of surfaces with biofunctional molecules, enzymes, and antibodies has various applications, such as the preparation of biomaterials and biosensors.1–6 The surface of materials modified with biofunctional molecules can act as a reaction field for enzymatic reactions and molecular recognition. Crucially, the design of the molecular layer on the material surface greatly affects the material characteristics. To date, numerous studies into the immobilization of biomolecules on surfaces have been carried out, and new methods for surface modification have been proposed.7–10 By exploiting this technology, a range of novel devices and materials have been developed.11,12

The structure of an adsorbed or immobilized biomolecule changes because of the interactions with the material surface, which can impair biomolecule function.13 Even if a biomolecule has good activity, its performance in a composite material cannot be guaranteed after immobilization on the material surface. However, a new material, TEMPO-oxidized cellulose nanofibers (TOCNs), shows promise for the immobilization of molecules without loss of activity.14 Thus, in this study, we investigated the use of TOCN as a substrate material for the adsorption of biomolecules. TOCN is an ultrafine fiber in which the hydroxy groups on the surface of the microfibrils are converted into carboxylic acid groups by oxidation with 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO).

The oxidation results in the disintegration of the fibrils by electrostatic repulsion and yields negatively charged TOCNs.15–18 These fibers are thin, measuring approximately 4 nm, and have excellent physical properties, for example, excellent thermal stability and strength.19 Currently, there is interest in exploiting the excellent properties of TOCN in various fields. For example, TOCN can be used to form nanocomposite materials with plastics19 and modify heavy metal ion adsorbents chemically20,21 as well as for drug delivery, bioimaging, and other biomedical applications.22

In this study, we focused on the fact that TOCNs are very thin (4 nm), their surface is negatively charged, and they have a large specific surface area. These properties suggest that TOCN could be a suitable material for the adsorption of biomolecules while retaining the biomolecular structure, which is crucial for retaining activity, after adsorption. Previous studies have reported the use of nanosized immobilization materials as materials for retaining the structures of immobilized biomolecules. Interestingly, it has been reported

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that the structure and activity of biomolecules can vary with the size of the nanoparticles. This is because smaller particles have a smaller contact area with the protein and, thus, fewer interactions.\textsuperscript{24,25} Consequently, the fine TOCNs can be expected to retain the structure of adsorbed proteins,\textsuperscript{14} as also observed for 15 nm silica nanoparticles.

Previously, we studied the interactions between TOCN and proteins by adsorbing proteins having different surface charges, including lysozyme and bovine serum albumin, to TOCN. The results showed that the positively charged proteins were adsorbed by electrostatic interactions with the negatively charged TOCNs.\textsuperscript{14} Crucially, the proteins that were adsorbed on the TOCN retained their secondary structures. However, the activities of the biomolecules adsorbed on the TOCN surface were not determined. Therefore, in this study, the enzyme pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) was immobilized on TOCN to investigate the effect of immobilization on the function and activity of the adsorbed biomolecules. PQQ-GDH is widely used as an anode component for glucose sensors and bio-based batteries.\textsuperscript{26} In this study, the structure of PQQ-GDH after immobilization was observed by transmission electron microscopy (TEM), and the structure and activity after adsorption were evaluated spectroscopically and using dichlorophenolindophenol (DCIP), respectively. This allowed us to determine whether TOCN is a suitable material for biomolecule immobilization.

\section*{RESULTS AND DISCUSSION}

**PQQ-GDH Adsorption on the TOCN Surface.** PQQ-GDH adsorption experiments were performed using TOCN concentrations of 0.18–0.0036 wt \%. When the concentration of TOCN was 0.18–0.018 wt \%, 94% or more of the PQQ-GDH (0.1 mg) in the solution was adsorbed on the TOCN (Figure 1). At a TOCN concentration of 0.0072 wt \% or less, the adsorption rate decreased because there was no space on the TOCN surface for PQQ-GDH adsorption, and the concentration of nonadsorbed PQQ-GDH increased. The TOCN adsorption capacity of 0.018 wt \% TOCN was 1.85 mg of PQQ-GDH per mg. To date, reported protein adsorption capacities have been less than or equal to 1 mg of protein/mg of adsorbent.\textsuperscript{28–30} Therefore, the values obtained for TOCN indicate that it has a high adsorption capacity, which is consistent with its large specific surface area. Further, protein adsorption occurs within 3 h, the protein being quickly adsorbed on the surface of the TOCNs dispersed in water. Subsequent experiments were carried out at concentrations of 0.18–0.018 wt \% TOCN, and almost no unadsorbed protein was present.

**TEM Observation.** Figure 2a shows a representative TEM image, revealing that the average fiber diameter equaled 5.5 ± 1.5 nm (n = 50). Cellulose microfibrils are known to have diameters of 3–4 nm, and TOCNs were therefore concluded to comprise one or two cellulose microfibrils. The adsorption state of PQQ-GDH on the TOCN surface was observed by TEM (Figure 2b). Particles of approximately 10 nm were observed on the surfaces of TOCNs. Meanwhile, in TEM images of TOCNs without PQQ-GDH, no particles were observed on the fiber surfaces (Figure 2a), indicating that the particles in Figure 2b are PQQ-GDH. Furthermore, the particle size is almost the same as the previously reported size of the PQQ-GDH enzyme, further confirming that the particles were PQQ-GDH. From the above results, it was confirmed by TEM that PQQ-GDH was adsorbed on the TOCN surface.

**Secondary Structure Analysis by CD Spectroscopy.** The secondary structure of PQQ-GDH adsorbed on the TOCN surface was evaluated by CD spectroscopy (Figure 3).

Figure 1. Adsorption efficiency of PQQ-GDH at different TOCN concentrations.

![Figure 1](https://dx.doi.org/10.1021/acsomega.0c01948)

Figure 2. (a, b) Transmission microscopy analysis of (a) TOCNs and (b) PQQ-GDH adsorbed onto TOCNs. The arrows indicate PQQ-GDH.

Figure 3. CD spectra of PQQ-GDH adsorbed onto TOCN at different TOCN concentrations.

The spectrum of the adsorbed PQQ-GDH was almost the same as that before adsorption, suggesting that the structural changes induced by adsorption were small. Because of the very small diameter of TOCN (5 nm), the protein–TOCN contact area is expected to be very small, thus facilitating the retention of the native structure of the protein. However, changes in the \( \alpha \)-helix content on adsorption have been observed for other proteins. Nevertheless, when the \( \alpha \)-helix ratio was calculated, we found that it was not affected by adsorption significantly (Table 1).

Despite the small differences, the \( \alpha \)-helix ratio was most like that of the unadsorbed PQQ-GDH structure at low TOCN...
concentrations. Thus, higher concentrations of TOCN in the solution may increase the number of TOCNs in contact with PQQ-GDH, thus increasing the protein—TOCN interactions and increasing the degree of structural change slightly.

**Enzyme Activity.** Adsorption experiments were carried out at pH 5 at TOCN concentrations of 0.045 and 0.018 wt %. Under these conditions, the structure of PQQ-GDH was retained when it was adsorbed on the TOCN surface. When the activity was evaluated immediately after adsorption, the PQQ-GDH activity adsorbed on 0.045 wt % TOCN was 110%, and the PQQ-GDH activity adsorbed on 0.018 wt % TOCN was 100% as the free PQQ-GDH activity was 100%. Therefore, the adsorbed PQQ-GDH showed almost the same activity as free PQQ-GDH (Figure 4). In case of the PQQ-GDH, activity adsorbed on 0.045 wt % TOCN was increased by 10%. More quantitative evaluations are needed to elucidate the mechanisms of this phenomenon. Combining this result with the information from the CD spectra, we determined that the enzyme activity was maintained because the three-dimensional structure was stable even when PQQ-GDH was adsorbed on the TOCN surface.

**Long-Term Stability.** To examine the change over time in the PQQ-GDH activity of the samples, the activity of the adsorbed PQQ-GDH was measured every day for 4 days and again after a week after the initial adsorption experiment (Figure 5). The activity immediately after the adsorption was defined as 100%, and the change in activity with time was evaluated. The adsorbed PQQ-GDH showed higher activity for longer periods than the free PQQ-GDH.

Thus, the activity of the enzyme immobilized on the material surface was maintained, as has been observed for other immobilized enzymes. In particular, because the structure and activity of PQQ-GDH adsorbed on TOCN were maintained for long periods, TOCN is a promising adsorbent for enhancing and maintaining the activity of other enzymes.

### CONCLUSIONS

In this study, we clarified the characteristics of a composite material comprising PQQ-GDH adsorbed on TOCN obtained by the TEMPO oxidation of bleached kraft pulp. The enzyme was adsorbed to the surfaces of the TOCNs by electrostatic interactions arising from the negatively charged carboxylate groups on the TOCN surface and the positive charges on the PQQ-GDH surface. In addition, TOCN has an enormous surface area because of its ultrafine fibers having a diameter of 4 nm or less. The TOCN adsorbent was able to adsorb 1.85 mg of PQQ-GDH/mg of adsorbent and had a higher adsorption capacity than other nanomaterials. The state of adsorption was observed by TEM to confirm the immobilization of the enzyme. On the basis of CD spectrum analysis, the adsorbed PQQ-GDH retained its secondary structure even after immobilization. In addition, the effect of the TOCN concentration on the immobilized PQQ-GDH structure was clarified. Furthermore, PQQ-GDH adsorbed on TOCN showed higher activity than before adsorption. In addition, it was found that the high activity was maintained for a long period. These results indicate that TOCN is a promising biomolecular adsorbent.

### EXPERIMENTAL SECTION

**Materials and Sample Preparation.** TOCN was obtained by the oxidation of bleached kraft pulp with TEMPO, and the TOCNs were as thin as 4 nm and had a very high specific surface area, as reported previously and shown by TEM image analysis (vide infra). Furthermore, TOCN has a high density of carboxylic acid groups on the surface, as reported previously. TOCN gel (2 wt %; RHEOCRYSTA I-2 SP; DKS Co., Ltd.) was diluted with ultrapure water to a concentration of 0.1 or 0.001 mg/mL. Then, 1 mL of the prepared TOCN solution was mixed with 300 μL of PQQ-GDH solution and 300 μL of 30 mM NaOH to adjust the pH to 7.4. The TOCN adsorbent was able to adsorb 1.85 mg of PQQ-GDH/mg of adsorbent and had a higher adsorption capacity than other nanomaterials. The state of adsorption was observed by TEM to confirm the immobilization of the enzyme. On the basis of CD spectrum analysis, the adsorbed PQQ-GDH retained its secondary structure even after immobilization. In addition, the effect of the TOCN concentration on the immobilized PQQ-GDH structure was clarified. Furthermore, PQQ-GDH adsorbed on TOCN showed higher activity than before adsorption. In addition, it was found that the high activity was maintained for a long period. These results indicate that TOCN is a promising biomolecular adsorbent.

**Adsorption of PQQ-GDH on the TOCN Surface.** The 2 wt % TOCN gel was diluted with ultrapure water to prepare 0.18–0.0036 wt % TOCN solutions. Then, 28 kHz ultrasonication for 2 h was used to disperse the TOCN. PQQ-GDH was dissolved in 40 mM pH 5 Britton–Robinson buffer and adjusted to 0.1 or 0.001 mg/mL. Then, 1 mL of the prepared PQQ-GDH solution and 300 μL of the TOCN solution were mixed and adsorbed on the TOCN surface.
mixed and allowed to stand at room temperature for 2 h to allow the adsorption of PQG-GDH on the surface of the TOCN.

**Evaluation of PQG-GDH Activity.** To examine the enzyme activity of PQG-GDH adsorbed on the TOCN surface, a 0.1 mg/mL PQG-GDH solution was dropped into a cell containing DCIP and 0.1 M glucose. When PQG-GDH oxidizes glucose, the released electron reduces DCIP, which is blue, and the solution becomes colorless. At this time, the change in the absorbance (450 nm) was measured for 60 s to determine the activity.

**Measurement of the Rate of Adsorption of PQG-GDH on TOCN.** Adsorption experiments were performed by mixing a 0.1 mg/mL PQG-GDH solution and a 0.18–0.0036 wt % TOCN solution. The mixed solution of TOCN + PQG-GDH was centrifuged at 9000g for 10 min to precipitate PQG-GDH adsorbed on the TOCN. The activity of the unadsorbed PQG-GDH remaining in the supernatant was measured in the same manner as described in the previous section. The adsorption rate of PQG-GDH was calculated using eq 1:

\[
\text{adsorption rate (\%)} = 100 \times \frac{(C_a - C_b)}{C_a}
\]  

Here, \( C_a \) is the PQG-GDH activity without TOCN and \( C_b \) is the activity of unadsorbed PQG-GDH after centrifugation.

**TEM Observation of PQG-GDH on TOCN.** TEM observation of the modified TOCN was carried out with samples treated by negative staining using phosphotungstic acid (FUJIFILM Wako Pure Chemical Corporation). A plasma-treated hydrophilized mesh (Nisshin EM Co., Ltd.) was sequentially treated with TOCN solution (added dropwise; 5 \( \mu \)L, 0.018 wt %) and phosphotungstic acid (5 \( \mu \)L, 0.5%) as a negative staining agent. Excess moisture was removed using a filter paper, and the mesh was thoroughly dried. TEM images of the negatively stained TOCN samples were obtained using an H-7650 TEM (Hitachi; acceleration voltage = 80 kV; magnification = 60,000x), and the individual fiber diameters were measured using ImageJ.

**Secondary Structure Analysis of PQG-GDH Adsorbed on TOCN.** Circular dichroism (CD) spectra of pristine PQQ-GDH and that adsorbed onto TOCN (at TOCN concentrations of 0.02 and 0.0016 wt %) were recorded at pH 4 using a CD spectrometer (J-765, Jasco). All measurements were carried out at a scanning speed of 100 nm/min and a loading number of 3. The \( \alpha \)-helix, a secondary protein structure, is known to exhibit a negative dichroic peak at 222 nm, and the relative content of \( \alpha \)-helices was calculated based on the ellipticity of the obtained CD spectra at 222 nm, as shown in eq 2:

\[
\alpha \text{ - helix (\%)} = \left( \frac{-\theta}{10 r [\text{protein}]} \right) - 2340 \right) / 30,300
\]  

Here, \( \theta \) is the ellipticity (mdeg) at 222 nm, \( r \) is the number of amino acid residues in the protein, \( I \) is the cell length (0.1 cm), and \([\text{protein}]\) is the protein concentration (M).

**Evaluation of the Long-Term Stability of PQG-GDH.** For these experiments, 0.045 wt % TOCN + PQG-GDH, 0.018 wt % TOCN + PQG-GDH, and free PQG-GDH were stored at 4 °C. The activities of these samples were measured every other day, and the changes in the activities with time were evaluated.

### References

1. Puleo, D. A.; Kissling, R. A.; Sheu, M.-S. A technique to immobilize bioactive proteins, including bone morphogenetic protein-4 (BMP-4), on titanium alloy. **Biomaterials** 2002, 23, 2079–2087.  
2. Gatenholm, P.; Klemm, D. Bacterial nanocellulose as a renewable material for biomedical applications. **MRS Bull.** 2010, 35, 208–213.  
3. Zhang, Z.; Lai, Y.; Yu, L.; Ding, J. Effects of immobilizing sites of RGD peptides in amphiphilic block copolymers on efficacy of cell adhesion. **Biomaterials** 2010, 31, 7873–7882.  
4. Kim, Y. S.; Raston, N. H. A.; Gu, M. B. Aptamer-based nanobiosensors. ** Biosens. Bioelectron.** 2016, 76, 2–19.  
5. Rocchitta, G.; Spanu, A.; Babudieri, S.; Latte, G.; Madeddu, G.; Galleri, G.; Nuvoli, S.; Bagella, P.; Demartis, M. I.; Fiore, V.; Manetti, R.; Serra, P. A. Enzyme biosensors for biomedical applications: strategies for safeguarding analytical performances in biological fluids. **Sensors** 2016, 16, 780.  
6. Patel, S. K. S.; Choi, S. H.; Kang, Y. C.; Lee, J.-K. Eco-friendly composite of Fe$_3$O$_4$-reduced graphene oxide particles for efficient enzyme immobilization. **ACS Appl. Mater. Interfaces** 2017, 9, 2213–2222.  
7. Sakamoto, H.; Ikeno, S.; Kato, T.; Nishino, N.; Haruyama, T. Smart immobilization of oligopeptides through electrochemical deposition onto surface. **Anal. Chim. Acta** 2007, 604, 76–80.  
8. Sakamoto, H.; Haruyama, T. Electrochemical preparation of junction between a molecule and solid surface through a metal coordinative peptide tag. **Colloids Surf., B** 2010, 79, 83–87.  
9. Asakawa, H.; Tahara, S.; Nakamichi, M.; Takehara, K.; Ikeno, S.; Linder, M. B.; Haruyama, T. The amphiphilic protein HBII1 as a genetically taggable molecular carrier for the formation of a self-organized functional protein layer on a solid surface. **Langmuir** 2009, 25, 8841–8844.  
10. Takatsuji, Y.; Yamasaki, R.; Iwanaga, A.; Lienemann, M.; Linder, M. B.; Haruyama, T. Solid-support immobilization of a...
“swing” fusion protein for enhanced glucose oxidase catalytic activity. Colloids Surf., B 2013, 112, 186–191.

(11) Klajn, R.; Stoddart, J. F.; Grzybowski, B. A. Nanoparticles functionalised with reversible molecular and supramolecular switches. Chem. Soc. Rev. 2010, 39, 2203–2237.

(12) Sun, X.-L.; Stabler, C. L.; Caizalis, C. S.; Chaikof, E. L. Carbohydrate and protein immobilization onto solid surfaces by sequential diels-alder and azide-alkyne cycloadditions. Bioconjugate Chem. 2006, 17, 52–57.

(13) He, C.; Liu, J.; Xie, L.; Zhang, Q.; Li, C.; Gui, D.; Zhang, G.; Wu, C. Activity and thermal stability improvements of glucose oxidase upon adsorption on core-shell PMMA-BSA nanoparticles. Langmuir 2009, 25, 13456–13460.

(14) Yamaguchi, A.; Sakamoto, H.; Kitamura, T.; Hashimoto, M.; Suye, S.-i. Structure retention of proteins interacting electrostatically with TEMPO-oxidized cellulose nanofiber surface. Colloids Surf., B 2019, 183, 110392.

(15) Nishino, T.; Matsuda, I.; Hirao, K. All-cellulose composite. Macromolecules 2004, 37, 7683–7687.

(16) Suchy, M.; Kontturi, E.; Vuorinen, T. Impact of drying on wood ultrastructure: similarities in cell wall alteration between native wood and isolated wood-based fibers. Biomacromolecules 2010, 11, 2161–2168.

(17) Saito, T.; Nishiyama, Y.; Pataux, J.-L.; Vignon, M.; Isogai, A. Homogeneous suspensions of individualized microfibrils from TEMPO-catalyzed oxidation of native cellulose. Biomacromolecules 2006, 7, 1687–1691.

(18) Saito, T.; Kimura, S.; Nishiyama, Y.; Isogai, A. Cellulose nanofibers prepared by tempo-mediated oxidation of native cellulose. Biomacromolecules 2007, 8, 2485–2491.

(19) Isogai, A.; Saito, T.; Fukuzumi, H. TEMPO-oxidized cellulose nanofibers. Nanoscale 2011, 3, 71–85.

(20) Liu, P.; Milletto, C.; Monti, S.; Zhu, C.; Mathew, A. P. Design of ultrathin hybrid membranes with improved retention efficiency of molecular dyes. RSC Adv. 2019, 9, 28657–28669.

(21) Li, M.; Liu, Z.; Wang, L.; James, T. D.; Xiao, H.-N.; Zhu, W.-H. A glutamic acid-modified cellulose fibrous composite used for the adsorption of heavy metal ions from single and binary solutions. Mater. Chem. Front. 2017, 1, 2317–2323.

(22) Zhou, J.; Butchosa, N.; Jayawardena, H. S. N.; Park, J.; Zhou, Q.; Yan, M.; Ramström, O. Synthesis of multifunctional cellulose nanocrystals for lectin recognition and bacterial imaging. Biomacromolecules 2015, 16, 1426–1432.

(23) Xu, J.; Liu, S.; Chen, G.; Chen, T.; Song, T.; Wu, J.; Shi, C.; He, M.; Tian, J. Engineering biocompatible hydrogels from bicomponent natural nanofibers for anticancer drug delivery. J. Agric. Food Chem. 2018, 66, 935–942.

(24) Roach, P.; Farrar, D.; Perry, C. C. Surface tailoring for controlled protein adsorption: effect of topography at the nanometer scale and chemistry. J. Am. Chem. Soc. 2006, 128, 3939–3945.

(25) Shang, W.; Nuffer, J. H.; Dordick, J. S.; Siegel, R. W. Unfolding of ribonuclease A on silica nanoparticle surfaces. Nano Lett. 2007, 7, 1991–1995.

(26) Yehezkeli, O.; Tel-Vered, R.; Raichlin, S.; Willner, I. Nano-engineered flavin-dependent glucose dehydrogenase/gold nanoparticle-modified electrodes for glucose sensing and biofuel cell applications. ACS Nano 2011, 5, 2385–2391.

(27) Sasmal, M.; Bhowmick, R.; Islam, A. S. M.; Bhuiya, S.; Das, S.; Ali, M. Domain-specific association of aphenanthrene-pyrene-based synthetic fluorescent probe with bovine serum albumin: spectroscopic and molecular docking analysis. ACS Omega 2018, 3, 6293–6304.

(28) Ma, Z. Y.; Guan, Y. P.; Liu, X. Q.; Liu, H. Z. Synthesis of magnetic chelator for high-capacity immobilized metal affinity adsorption of protein by cerium initiated graft polymerization. Langmuir 2005, 21, 6987–6994.

(29) Qi, C.; Zhu, Y.-J.; Chen, F. Microwave hydrothermal transformation of amorphous calcium carbonate nanospheres and application in protein adsorption. ACS Appl. Mater. Interfaces 2014, 6, 4310–4320.

(30) Du, P.; Zhao, J.; Mashayekhi, H.; Xing, B. Adsorption of bovine serum albumin and lysozyme on functionalized carbon nanotubes. J. Phys. Chem. C 2014, 118, 22249–22257.

(31) Yuan, X.; Iijima, M.; Oishi, M.; Nagasaki, Y. Structure and activity assay of nanomolecules prepared by the coimmobilization of practically useful enzymes and hydrophilic block copolymers on gold nanoparticles. Langmuir 2008, 24, 6903–6909.

(32) Arola, S.; Tammelin, T.; Setälä, H.; Tullila, A.; Linder, M. B. Immobilization-stabilization of proteins on nanofibrillated cellulose derivatives and their bioactive film formation. Biomacromolecules 2012, 13, 594–603.

(33) Kim, J.; Grate, J. W.; Wang, P. Nanostructures for enzyme stabilization. Chem. Eng. Sci. 2006, 61, 1017–1026.

(34) Fernandez-Lopez, L.; Pedrero, S. G.; Lopez-Carrollés, N.; Gorines, B. C.; Virgen-Ortíz, J. J.; Fernandez-Lafuente, R. Effect of protein load on stability of immobilized enzymes. Enzyme Microb. Technol. 2017, 98, 18–25.