International Journal of Biological Macromolecules 108 (2018) 462–470

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

The application of magnetically modified bacterial cellulose for immobilization of laccase

Radosław Drozd [a,*], Rafał Rakoczy [b], Agata Wasak [a], Adam Junka [c], Karol Fijałkowski [a]

[a] West Pomeranian University of Technology in Szczecin, Faculty of Biotechnology and Animal Husbandry, Department of Immunology, Microbiology and Physiological Chemistry, 45 Piastów Avenue, 71-311 Szczecin, Poland
[b] West Pomeranian University of Technology in Szczecin, Faculty of Chemical Engineering and Technology, 42 Piastów Avenue, 71-065 Szczecin, Poland
[c] Department of Pharmaceutical Microbiology and Parasitology, Medical University of Wrocław, Borowska 211, 50-556 Wrocław, Poland

ARTICLE INFO

Article history:
Received 23 September 2017
Received in revised form
25 November 2017
Accepted 5 December 2017
Available online 6 December 2017

Keywords:
Rotating magnetic field
Immobilization
Fungal laccase
Bacterial cellulose

ABSTRACT

The usefulness of bacterial cellulose (BC), obtained from the cultures of *Komagataeibacter xylinus* exposed to rotating magnetic field (RMF), as a carrier for laccase immobilization was investigated in this study. It was found that the highest yield of laccase immobilization (>70%) was achieved in pH of 4.0 and this value was optimal in the case of both types of cellulose carriers applied. The pH equals 4.0 was also the optimal one with regard to immobilized enzymes' activity, while in case of free laccase, optimal pH value was 3.0. Process of immobilization had an impact on enzyme's optimal temperatures: while free laccase and laccase bound to RMF-unexposed cellulose was the most effective at 60 °C, optimal activity of enzyme immobilized on RMF-exposed carrier was reached at 70 °C. Laccase immobilized on both type of carriers had also better thermal stability at 70 °C compared to free laccase. After 8 cycles of use, laccase immobilized on RMF-exposed BC remained more active than laccase immobilized on RMF-unexposed BC (65% vs. 50% of initial activity, respectively). Our results indicate that RMF-modified BC may be successfully used as a carrier for the laccase immobilization.

1. Introduction

Laccase, i.e. p-diphenol:dioxygen oxidoreductase (EC 1.10.3.2), is commonly found and produced by fungi of *Trametes* genus, which use it to degrade lignin and other aromatic compounds' polymers in plant tissues [1]. Enzyme's ability to oxidize a wide range of organic compounds is of great potential in waste water treatment, bioremediation, and in the production of polymers displaying specific properties [2–5].

To ensure laccase's stability and high reusability, enzyme's immobilization process is performed. Carriers serving this purpose should be cheap, easy to obtain and environmental-friendly [6]. Bacterial cellulose (BC) is one of the natural carriers on which laccase immobilization may be performed on [7–12]. BC, synthesized by *Komagataeibacter xylinus* strains, may be considered a potentially versatile carrier for biocatalyst immobilization thanks to such unique properties as high mechanical strength, great capacity for water absorption, and low level of impurities. Bacterial cellulose can be obtained by a variety of methods which affect polymer's final physicochemical properties, such as water holding capacity or Young modulus [13,14]. The culture method determines such BC molecular properties as the degree of microfibril polymerization, the amorphous to crystalline ratio, or the Iα/Iβ ratio [15]. It also affects the density of BC microfibrils and pore dimensions, resulting in differences in water holding capacity and water release rate [16].

As reported earlier by Rakoczy and Masiuk [17], application of a rotating magnetic field (RMF) affects a mass transfer during fermentation process. It also results in changes in the rate of several metabolic conversions. With regard to BC synthesis, altered mass transfer may lead to *in situ* modification of this biopolymer [18,19]. Therefore, RMF exposure can be one of the factor changing properties of the BC membranes synthetized by *K. xylinus*. As previously reported, the BC wet pellicles obtained from RMF-exposed cultures are characterized by lower number of inter-associated microfibrils and reduced density of their cross-linking [18,19]. These changes affect directly wet and dry BC membrane porosity and, in result, biopolymer's water swelling and water holding capacity. Despite aforementioned changes of cellulose' properties, the structure of microfibrils of RMF-exposed BC, is retained on the molecular level

* Corresponding author at: Department of Immunology, Microbiology and Physiological Chemistry, West Pomeranian University of Technology in Szczecin, 45 Piastów Avenue, 71−311 Szczecin, Poland.
E-mail address: rdrozd@zut.edu.pl (R. Drozd).

https://doi.org/10.1016/j.ijbiomac.2017.12.031
0141-8130/

without any significant changes [18,20]. The altered properties of BC membranes in a result of RMF exposition may therefore have an impact on their properties with regard to immobilization of various micro- and macro-molecules, including such enzymes as described in this paper laccase and indirectly affecting enzymes' properties.

In the current study, the usefulness of RMF-exposed BC as a carrier of fungal laccase is described for the first time. Moreover, optimal conditions of this process as well as immobilized enzyme's parameters are evaluated.

2. Material and methods

2.1. Bacterial cellulose carrier preparation

For the experiment, 7 day culture of *Komagataeibacter xylinus* (Deutsche Sammlung von Mikroorganismen und Zellkulturen – DSM 46604) was agitated, and 100 μl of the obtained bacterial suspension used to inoculate 25 ml of Herstin-Schramm (HS) medium, in 50 ml plastic tubes with caps featuring 8 holes and a specific capillary pore filter membrane (CELLSTAR® CELLreactor™, Polypropylene Filter Top Tube, Greiner Bio-One, USA). Such prepared cultures were exposed to RMF of 18 mT at 50 Hz for 7 days at 28 °C, in a RMF supported bioreactor described by Fijałkowski et al. [18]. The obtained BC pellicles were purified in 0.1 M NaOH at 80 °C for 30 min. (this step was repeated three times). After alkaline treatment, the BC membranes were rinsed with water until the wash water reached neutral pH. Next, the cellulose pellicle was dried at 60 °C in a laboratory drier (EV-50, Trade Raypa, Spain) to a constant weight.

2.2. ATR-FTIR analysis of BC samples

IR spectra of the BC were examined by ATR-FTIR method, and ALPHA. The spectra were collected in the range from 400 cm^{-1} to 4000 cm^{-1}. For each of the samples 32 scans at a resolution of 2 cm^{-1} were performed. The spectra were collected using OPUS software and analyzed with Origin8pro software.

2.3. Scanning electron microscopy (SEM) and total optical porosity analysis of BC samples

The purified BC membranes were placed in a glutaraldehyde solution (3% v/v) for 1.5 h and then dehydrated for 5 min each in stepwise increasing concentrations of ethanol solution (10% – 99.5%). Next, the BC pellicles were dried at room temperature and examined in a Scanning Electron Microscope (TESCAN, VEGA SBU3, Czech Republic). The obtained images were analyzed by ImageJ macro jPOR software to estimate total visual porosity of the BC surface [21].

2.4. Enzyme source and purification

The source of laccase for the experiment was *Trametes* sp. (7/23/03/13) fungus from the collection of microorganisms at the Department of Immunology, Microbiology and Physiological Chemistry, West Pomeranian University of Technology in Szczecin. The fungus was cultivated for 7 days in a liquid culture (Potato Dextrose Broth), with the addition of $CuSO_4$ to a final concentration 1 mM to induce laccase secretion. Four days later, the induced culture was filtered and concentrated in a vacuum rotary evaporator. The concentrate was fractionated by three phase partitioning method with a 30%–60% ammonium sulfate saturation and 1:1 t-butanol ratio at 25 °C for 15 min. The fractions with a significant number of laccase units were pooled together and dialyzed in 20.0 mM phosphate buffer at pH 7.0. After dialysis, the sample was loaded onto a HiTrap DEAE sepharose column, equilibrated with 20.0 mM of phosphate buffer at pH 7.0. Proteins were eluted with a gradient from 0.0 M to 0.25 M NaCl. The fractions with laccase activity were pooled, concentrated, and loaded into a HiTrap AXN sepharose column, and conditioned in the same manner as the previous column. Finally, the fractions with laccase activity were pooled, concentrated and loaded on Sephacryl HR – 300 column equilibrated with 20.0 mM phosphate buffer, pH 7.0 with 10.0 mM NaCl. Fractions containing laccase activity were pooled together and concentrated. The purified enzyme was subjected to a series of studies for determination of optimal factors for its immobilization on BC and influence of this process on its catalytic properties.

2.5. Protein concentration determination

Protein concentration were assayed by Bradford method with bovine serum albumin as a standard [22].

2.6. Laccase activity assay

Free laccase activity was determined with 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate. A microplate reader (Infinite 200 PRO NanoQuant, Tecan, Switzerland) was used to evaluate the oxidation of 0.5 mM ABTS in 0.05 M sodium acetate buffer at pH 4.0. The reaction was started by adding 5 μl of enzyme solution to 100 μl of ABTS solution at 30 °C. The increase in absorbance at 420 nm ($\varepsilon = 36\ mM^{-1}\ cm^{-1}$) was measured after 2 min. One unit (U) of laccase oxidized 1 μmol of ABTS per minute. Activity was expressed as the number of units per mg of protein.

For determination of the activity of the immobilized laccase, the BC-laccase flakes were transferred to the substrate solution at a final concentration of 10 mg carrier per 1 ml of ABTS at 30 °C. The reaction was measured at 420 nm after 2 min from 100 μl of reaction mixture. One unit (U) oxidized 1 μmol ABTS per minute. Activity was expressed as the number of units per gram of carrier (U/g).

2.7. Laccase immobilization by absorption

The dried sample of BC was cut into 5 mm round flakes by a puncher. The obtained flakes of BC where then transferred into plastic tubes with appropriate binding buffer. After 30 min of equilibration the BC flakes were moved to plastic tubes filled with equal amount of laccase solution in binding buffer at the pH earlier characterized as most suitable for maximal binding. After overnight incubation at 4 °C the BC flakes were rinsed in binding buffer and used for further analysis. The optimal pH for laccase immobilization was tested with 50 mM acetate buffer at pH 4.0 and 5.0, and 50 mM phosphate buffer with a pH range from 6.0 to 8.0. For loading capacity, an equal dry mass of BC flakes was incubated with several concentrations of laccase units at optimal pH and 4 °C. The efficiency of immobilization was calculated from the equation (1).

$$Yield\,(\%) = 100x\frac{immobilized\ activity}{starting\ activity} \quad (1)$$

where immobilized activity is the difference between starting and remaining activity in the binding solution [23].

2.7.1. Determination of the immobilized enzyme's operational and catalytic properties

The effect of pH on free and immobilized laccase activity pH was measured in the range from 2.0 to 7.0 pH. 50 mM glycine buffer was used at pH from 2.0 to 3.0; 50 mM sodium acetate for pH from 4.0 to 5.0; and 50 mM phosphate for pH from 6.0 to 7.0. Cellulose flakes with immobilized laccase were pre-equilibrated in appropri-