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An efficient method of *Yarrowia lipolytica* immobilization using oil- and emulsion-modified bacterial cellulose carriers

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ABSTRACT

Background: *Yarrowia lipolytica* is a nonconventional, dimorphic yeast with multiple biotechnological applications. Considering the size of *Y. lipolytica* cells and a plethora of its morphological forms (spherical cells or hyphae and pseudohyphae), it is highly difficult to select a suitable carrier for this useful microorganism. Bacterial cellulose (BC) is currently considered one of the most promising immobilization carriers. In the current study, the usefulness of oil- and emulsion-modified BCs as a carrier for *Y. lipolytica* immobilization was investigated. Static and agitated cultures were conducted in media supplemented with oil or emulsion to improve carrier porosity.

Results: It was found that the application of oil- and emulsion-modified BCs correlated with significantly higher efficiency of *Y. lipolytica* immobilization and hence higher yield than the yield achieved with an unmodified carrier. Increased efficiency of immobilization correlated with BC porosity-related parameters, which, in turn, depended on the size of oil droplets introduced into the culture medium. Moreover, changes in porosity-related parameters caused by the addition of oil or emulsion to the medium were observed when the cultures were conducted only under static conditions and not under agitated conditions.

Conclusion: The application of oil- and emulsion-modified BCs as carriers significantly increased the efficiency of *Y. lipolytica* immobilization as compared to unmodified BC. The addition of oil or emulsion to the culture medium can be a simple but effective method to modify the porosity of BC-based carriers.

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1. Introduction

Yarrowia lipolytica is a nonconventional, dimorphic yeast with multiple biotechnological applications. This microorganism is used in biotechnological and industrial processes such as production of a variety of acids (including citric and isocitric acids), enzymes (proteases, lipases, and RNase), aromatic compounds (γ-lactone, green notes, and epoxy geraniol), or chemicals (dicarboxylic acids) [1,2]. It can also be used in the production of Single Cell Oil (SCO) from agriculture feedstock, in biosurfactants, or in bioremediation [3,4,5].

Microorganisms with a high ability to secrete useful products, such as *Y. lipolytica,* are often immobilized on various types of carrier to increase the efficiency of the conducted bioprocess and to prevent toxic effects of substrates, by-products, and final products on the productive cells [6,7,8]. Therefore, support/carrier selection is one of the crucial decisions to be made during the preparation of the immobilization process [9]. For immobilization of living cells, the material used as a support needs to meet numerous criteria; for example, it should be insoluble and nontoxic and have high mechanical and chemical stability [10]. Additionally, the properties of the carrier need to be individually matched to the type of immobilized cell and application [11]. Because of the large cell size of *Y. lipolytica* and plethora of its morphological forms (spherical cells or hyphae and pseudohyphae) [2], it is highly difficult to select a suitable carrier for this microorganism.

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Bacterial cellulose (BC) is a nanobiopolymer produced by the nonpathogenic bacterium *Komagataeibacter xylinus* [12]. Conventionally, BC has been produced by the static or the agitated culture method [12, 13]. In the static culture method, BC is formed on the water–air interface and characterized by highly ordered fibril arrangement, high crystallinity, and high polymerization degree [12]. In contrast, in the agitated culture method, BC is produced in the form of fibrous suspension, irregular mass, pellets, or spheres and characterized by irregular fibril arrangement, high porosity, lower mechanical strength, low crystallinity, and low polymerization degree [13,14,15].

In recent years, interest in this polymer has significantly increased owing to its unique properties and possibilities of wide application [13, 16,17,18]. Recent studies have shown the considerable potential of BC as a carrier for immobilization of enzymes [19], antibacterial substances [20], and a wide spectrum of microorganisms including *Lactobacillus* bacteria [21] and *Saccharomyces cerevisiae* yeast [22]. Compared to plant-derived cellulose, BC presents superior mechanical properties including tensile strength, high water-holding capacity, crystallinity, and biocompatibility [12,13,14,16]. Additionally, modification of the BC nanostructure is a relatively easy process that can be performed by addition of compounds such as ferrofluid, hemicellulose, chitosan, or carboxymethylcellulose to the culture medium and application of a rotating magnetic field during BC synthesis [19,23,24,25,26]. Modification of the BC nanostructure can also be achieved by simply altering *K. xylinus* cultivation conditions [12] such as the chemical composition of the culture medium used for BC production [27].

In our previous study, we demonstrated a novel, simple, and inexpensive method based on the addition of vegetable oil to the culture medium, which allows to insignificantly increase BC yield and to modify the physicochemical properties of BC [28]. We showed that an oil-modified BC displays significantly higher water swelling capacity and mechanical strength than the control BC obtained under standard conditions. Water-related properties of BC (directly related to porosity) and mechanical strength are among the most important parameters influencing the effectiveness of immobilization [9], especially during size variability of microbial cells [9,10]. Therefore, in the present study, it was assumed that an oil-modified BC can serve as an excellent carrier for the immobilization of large cells such as *Y. lipolytica*. It should be noted that in our previous study, we did not use oil emulsion, but we simply added raw oil onto the medium surface. However, taking into account that oil emulsions are commonly used to generate highly porous inorganic and organic materials in material engineering [29], in the current study, we decided to use not only oil but also oil emulsion. To the best of our knowledge, oil- and emulsion-modified BCs have never been used as carriers for the immobilization of any microorganism.

Taking into account the results of our previous work [28] and the high *Yarrowia* biotechnological potential, the aim of the current study was to investigate the impact of porosity parameters of BC obtained under static or agitated conditions and cultured in a medium supplemented with oil or emulsion on the effectiveness of *Y. lipolytica* immobilization.

2. Material and methods

2.1. Microorganisms

For BC production, the reference strain of *K. xylinus* (Deutsche Sammlung von Mikroorganismen und Zellkulturen – German Collection of Microorganisms and Cell Cultures DSM 46604) was used. Before the experiment, *K. xylinus* was cultivated in the Hestrin-Schramm (H-S) medium at 28°C for 7 days under stationary conditions.

For immobilization, the reference strain of *Y. lipolytica* DSM 3286 was used. *Y. lipolytica* was cultivated at 28°C for 48 h in the yeast extract-peptone-glucose (YPG) broth medium composed of yeast extract (1 w/v%), bacto-peptone (2 w/v%), and glucose (2 w/v%).

2.2. Microscopic examination of microorganisms

The microorganism suspension was first centrifuged at 3,300 x *g* at 4°C for 15 min and then washed and suspended in phosphate-buffered saline (PBS, Sigma-Aldrich, USA). The morphology of the unstained, live microorganisms was assessed using a phase-contrast microscope (Delta Optical Evolution 100 TP, magnification x 1000).

2.3. BC biosynthesis

A *K. xylinus* suspension (2 x 10^5 CFU/mL) was used to inoculate an H-S medium containing 1% of rapeseed oil and an H-S medium containing 1% of emulsion [28]. To prepare the emulsion, the solution of H-S medium, 1% of rapeseed oil, and 0.01% of Triton X-100 (Sigma-Aldrich, Germany) were mixed for 60 min at 30°C using a magnetic stirrer (1500 rpm, ChemLand, Poland) [30]. As a control, a standard H-S medium without additives was used.

The static cultures were carried out for 7 days at 28°C in 50 mL plastic tubes (3.8 cm diameter) (polypropylene conical centrifuge tube; Becton Dickinson and Company, USA) containing 25 mL of medium, whereas agitated cultures were performed 4 days at 28°C in 50 mL glass flasks with 25 mL medium on an orbital shaker (140 rpm, ES–20/60, Biosan, Latvia).

2.4. BC purification

The Soxhlet extraction method was used to remove the oil and emulsion from the modified BC [28]. To remove bacterial cells and medium components, BC samples were treated with 0.1 mol l^{-1} NaOH at 70°C for 90 min [22]. Both purification procedures were also applied for control unmodified BC samples. After purification, all BC samples were washed with double distilled water and autoclaved (121°C/100 kPa/15 min) [22,24].

2.5. Support characterization

2.5.1. Scanning Electron Microscopy analysis

After oil extraction and NaOH purification process, wet BC samples were fixed in glutaraldehyde (POCH, Poland) for 7 days and dried in a critical point dryer. The dry samples sputtered with Au/Pd (60:40) were examined using a scanning electron microscope (SEM, Auriga 60, Zeiss, Oberkochen, Germany). A description of the aforementioned methods is given in detail in our earlier study [20].

2.5.2. Porosity analysis

The porosity-related parameters were measured directly using the N_2 adsorption/desorption method (Micromeritics ASAP 2010 M instrument, USA), and SEM images were analyzed with ImageJ software (NIH, Bethesda, USA) and indirectly by determination of the swelling ratio. The total specific surface area was calculated by the Brunauer-Emmett-Teller method. Additionally, the average pore volume and average pore diameter were also calculated (Barrett-Joyner-Halenda method). The swelling ratio was calculated considering the weight of dry and swollen BC after oil extraction and purification. The results are shown as the percentage of swelling ratio (SR) calculated using the formula shown in Equation 1:

$$SR\ [\%] = \frac{(W_{wet} - W_{dry})}{W_{dry}} * 100\% \quad [1]$$

where W_{wet} is the weight of the swollen BC and W_{dry} is the dry weight of the sample.

2.5.3. Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

IR spectra of BC were recorded using an ALPHA FT-IR Spectrometer (Burker Co., Germany). The total crystallinity index of the BC samples was determined by the ratio of absorbance values for peaks 1430/900 I.C. I and 1370/2900 I.C. II as described by Fijałkowski et al. [24]. The area of the peaks at *A*710 (710 cm^{-1}) for *Iβ* and at *A*750 (750 cm^{-1}) for *Iα* was determined from the spectra deconvoluted in PeakFit software. The percentage of *Iα* was calculated according to the formula shown in Equation 2:

$$I\alpha\ [\%] = \frac{A750}{(A710 + A750)} * 100\% \quad [2]$$

2.6. Immobilization

Immobilization of *Y. lipolytica* in BC was performed using the "adsorption-incubation" method described by Żywicka et al. [31] with slight modification. The yeast strain was separated from the medium by centrifugation at 3,300 x *g* at 4°C for 15 min, washed, and suspended in the YPG medium to obtain a yeast concentration of 2 x 10^8 CFU/mL. In the next step, the BC pellicles were transferred into the yeast suspension (20 g of BC per 100 mL of yeast suspension) and incubated for 5 h at 30°C under agitated conditions (200 rpm) (ES-20/60, Biosan, Latvia). Next, the BC samples with adsorbed cells were transferred to sterile Erlenmeyer flasks and incubated for 48 h at 28°C.

After the immobilization procedure, all carriers were digested with cellulase (Sigma-Aldrich, Germany) in 0.05 M citrate buffer (pH 4.8), and the number of immobilized yeast cells was determined by quantitative plating on the YPG agar medium after 48 h of incubation at 28°C [22].

2.7. Statistical analysis

The results are presented as mean values ± standard error of the mean (SEM) and were analyzed using one-way analysis of variance (ANOVA). The Tukey–Kramer test was used for multiple comparisons of results obtained for control and oil-modified samples. The analyses were conducted in triplicates, and all experiments were repeated at least three times. Differences were considered significant at a level of $P < 0.05$. All statistical analyses were performed using Statistica 9.0 (StatSoft, Poland) software.

3. Results and discussion

Previously used carriers for the immobilization of *Y. lipolytica* were based mainly on polyelectrolyte microcapsules [6], methyl polymethacrylate [8], or calcium alginate [7]. In our previous studies, we have shown that BC can also be a suitable material for immobilizing these microorganisms [31,32]. However, to the best of our knowledge, an oil- or emulsion-modified BC has never been used for this purpose. Therefore, in the current study, BC was produced in a medium supplemented with oil or oil emulsion. Such a supplemented culture medium allowed to improve the porosity of the obtained biomaterial. The cultures were conducted under static and agitated conditions, which also influenced BC properties.

The method used for immobilization was previously developed by Nguyen et al. [9] for the yeast *S. cerevisiae*. According to Nguyen et al. [9], maximal efficiency of *S. cerevisiae* immobilization on 1 g of BC equals 5.8 x 10^8 colony-forming units (cfu). In the current study, we obtained similar *Y. lipolytica* immobilization efficiency when control (unmodified) BC (C-BC) was used as a carrier (5.3 x 10^8 CFU/g BC). In terms of morphology, *S. cerevisiae* cells (round to ovoid) are similar to the spherical cells of *Y. lipolytica* (Fig. S1), which can explain the similar efficiency of immobilization of both yeasts. Such findings can also be related to the composition and electromagnetic potential of the cell wall of both yeasts [33]. Because of the fact that the adsorption of cells is associated with physical interactions such as with electrostatic bonds, it can be assumed that microorganisms with the same composition as that of the cell wall, for example, different strains or species of yeast, and similar electrostatic potential could be immobilized in a similar way [33,34]. Therefore, our results may be applied not only for *S. cerevisiae* and *Y. lipolytica* but also for other yeast species. Furthermore, when the oil-modified BC (O-BC) or emulsion-modified BC (E-BC) was used as a carrier, the efficiency of immobilization increased significantly reaching, respectively, 29% (6.9 x 10^8 CFU/g BC) and 24% (6.6 x 10^8 CFU/g BC) as compared to the C-BC (Fig. 1).

As demonstrated by several authors, increased efficiency of immobilization (a higher number of immobilized cells) usually produces a larger amount of product [6,9,10]. Similar to our previous study, we also confirmed that a higher number of immobilized *S. cerevisiae* yeast cells resulted in a higher amount of ethanol produced during fermentation [22]. Therefore, it can be suggested that application of O-BC or E-BC as a carrier for *Y. lipolytica* immobilization will increase the amount of product synthesized during fermentation, for example, citric acid, enzymes, or aromatic compounds [2,3,4,5,6].

Several authors demonstrated that BC obtained from agitated culture was characterized by a loose structure and a high porosity level [13,14]. According to Yao et al. [10], the highly porous and loose structure of BC obtained in the agitated culture can cause difficulties for cell immobilization. However, in the current study, we revealed that the average number of immobilized yeast cells on the C-BC obtained from agitated cultures was 17% higher than that on the C-BC obtained from static cultures (Fig. 1). Our results are in line with those of our previous study, in which we showed that the efficiency of *Lactobacillus* spp. immobilization was significantly higher when BC beads obtained from agitated cultures were used as a carrier than when BC pellicles from static cultures were used [21]. The current study also showed that the efficiency of immobilization on the O-BC and E-BC obtained from static cultures was at a similar level as that in case of C-BC obtained from agitated cultures. There were also no statistically significant differences in the number of immobilized yeast cells regardless of the use of O-BC or E-BC from static or agitated cultures as carriers (Fig. 1). As the efficiency of immobilization is strictly dependent on the porosity of the carrier [31,32], it can be suggested that the O-BC and E-BC from static cultures were characterized by a similar porosity as that of the BC obtained from agitated cultures. However, it should be emphasized that the BC obtained from agitated cultures, although characterized by high porosity, shows lower mechanical strength, crystallinity, and polymerization degree than the BC obtained from static cultures; therefore, it is less valuable in terms of using it as a carrier for immobilization [14,15]. In the current study, the total crystallinity index (TCI), calculated as the ratio of the absorbance of the bands at 1370 cm^{-1} (CH bending) to that at 2900 cm^{-1} (CH and CH_2 stretching), and the content of *Iα* fraction in all BC samples obtained from agitated cultures were statistically significantly lower than those in the samples obtained from static cultures ($P < 0.05$) (Table 1). Moreover, there were no significant differences between TCI and the content of *Iα* fraction calculated for the C-BC and O-BC or E-BC obtained from agitated cultures ($P < 0.05$). Similarly, the TCI and content of *Iα* fraction were at a similar level also in the case of O-BC or E-BC and C-BC obtained from static cultures (Table 1). These results also confirmed that because of the use of oil or emulsion, it is possible to obtain BC with high porosity, which is comparable to that of BC from agitated cultures but with much better mechanical properties. In our previous study, we confirmed that oil-modified BC had not only higher porosity but also significantly higher mechanical strength than the unmodified BC [28].

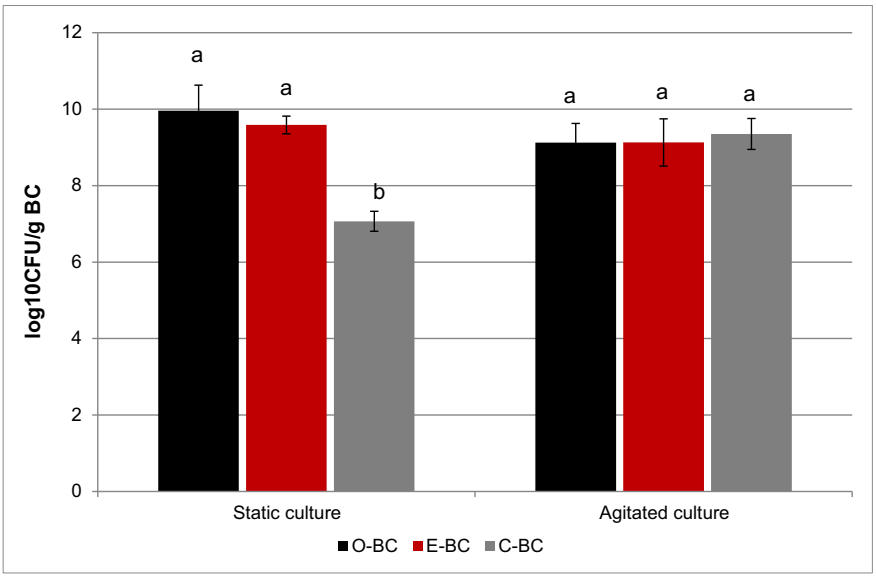

Fig. 1. Number of immobilized *Y. lipolytica* cells. O-BC: oil-modified BC; E-BC: emulsion-modified BC; C-BC: control BC (C-BC). Data are presented as mean CFU of immobilized yeast cells per gram BC (CFU x 10^8/g BC) ± standard error of the mean (SEM). Values with different letters show significant difference ($P < 0.05$).

Because there were no significant differences in the number of immobilized yeast cells between oil- and emulsion-modified and also control, unmodified BC carriers obtained from agitated cultures ($P < 0.05$) (Fig. 1) and because there was also no statistically significant differences in porosity-related parameters between these samples (Table 1, Table S1), further analyses described below were performed only for the BC obtained from static cultures.

A suitable porosity of the carrier provides appropriate surface area for cell adsorption and the space required for cell multiplication during fermentation [35]. However, considering the size of yeast cells, most of the available carriers demonstrate an insufficient number of pores and/or pore sizes for proliferation of these cells [9,10]. Results of the current study showed that the efficiency of immobilization calculated for samples from static cultures was strictly connected to their porosity-related parameters. It was also shown that total surface area, average pore diameter, and maximum pore size of O-BC and E-BC obtained from static cultures were statistically significantly higher than those of the C-BC ($P < 0.05$) (Table 2).

According to our previous investigation, oil droplets present in the culture medium adapt a funnel-type form within the cellulose, with the base of the funnel directed at the surface of the medium [28]. Such funnel-like oil droplets form spaces, which, after BC cleansing processes, create empty regions and, in turn, increase the porosity of O-BC. It has also been previously confirmed that the emulsion can be used to improve the porosity of synthetic and natural materials [29]. This kind of porous material is of particular interest in the preparation of catalysts, drug delivery systems, and sensors [36,37,38]. In the current study, we confirmed that this technique can also be applied to increase the porosity in nanobiopolymers such as BC.

It was also found that most of the porosity-related parameters (except average pore volume and minimum pore size) of the BC obtained from the oil-supplemented medium in static cultures were statistically significantly higher than those of the BC produced in an emulsion-supplemented medium ($P < 0.05$) (Table 2, Fig. 2). The oil added to the culture medium (1:100) did not cover the entire surface and formed large, irregular, unevenly distributed droplets. In the case of emulsion, addition of surfactant caused significant fragmentation of the oil droplets and hence were relatively equally distributed throughout the medium volume (Fig. 3, Fig. S2). After Soxhlet extraction, it was determined that the average amount of oil trapped inside BC produced in the oil-supplemented medium did not exceed 12% of the wet cellulose mass. In turn, in case of E-BC, the average amount of oil trapped inside the structure was estimated as ca. 3%. Consequently, it can be concluded that there is variation in size and distribution of oil and emulsion droplets trapped inside the BC structure, which determined the differences in the porosity-related parameters (Fig. 2, Fig. 3). Although the observed differences were statistically significant, they did not affect the number of immobilized cells (Fig. 1). It can be suggested that the differences in the porosity of O-BC and E-BC can have a greater significance in the case of immobilization of small bacteria or bioactive particles such as enzymes or antibacterial substances but not relatively large yeast cells.

In addition to nontoxicity, chemical stability, appropriate porosity, and high mechanical strength, low price of the carrier unit is of paramount importance from the economic point of view [10,12]. Thus far, the commercial use of BC remains limited owing to low production rates and high medium costs. Therefore, intensive research has been carried out by numerous research teams to develop a cheap and effective method for large-scale BC production. In our previous study, we confirmed that supplementation of the culture medium with 1% vegetable oil not only positively influences the porosity and mechanical strength of the obtained material but also increases the amount of BC produced [28]. In the present study, we also confirmed that not only the oil addition but also oil-in-water emulsion allowed to significantly increase the yield of BC ($P < 0.05$) (Fig. S3). The weight of dry O-BC and E-BC obtained from static cultures after oil extraction was higher by 49% and 47%, respectively, than that under control

Table 1
Iα crystalline phase amount and total crystallinity index of control and oil/emulsion-modified BC pellicles.

Samples	Static culture		Agitated culture	
	%Iα	TCI	%Iα	TCI
O-BC	46.40 ± 1.23^{ac}	1.59 ± 0.23^{ac}	41.93 ± 2.11^{ad}	0.78 ± 0.14^{ad}
E-BC	44.69 ± 2.01^{ac}	1.72 ± 0.17^{ac}	40.45 ± 1.65^{ad}	0.84 ± 0.51^{ad}
C-BC	47.67 ± 1.79^{ac}	1.47 ± 0.45^{ac}	40.07 ± 1.73^{ad}	0.88 ± 0.23^{ad}

O-BC - oil-modified BC; E-BC - emulsion-modified BC; C-BC - control BC. The results are presented as a mean ± standard error of the mean (SEM). Values with different letters are significantly different ($p<0.05$): a, b - statistically significant differences between the samples obtained in particular type of the culture, c, d - statistically significant differences between the samples obtained in static or agitated culture.

Table 2
Porosity related parameters of control and oil/emulsion-modified BC pellicle.

Porosity related parameters	Samples		
	O-BC	E-BC	C-BC
Total surface area [m^2/g]	18.62 ± 1.12^a	13.13 ± 1.56^b	7.77 ± 0.98^c
Average pore volume[cm^3/g]	0.047 ± 0.02^a	0.041 ± 0.01^a	0.038 ± 0.01^a
Average pore diameter [nm]	12.99 ± 0.92^a	8.53 ± 1.36^b	4.15 ± 1.03^c
Swelling ratio [%]	519.33 ± 14.68^a	470.03 ± 9.15^b	265.17 ± 13.61^c
Minimum pore size [nm]	53.22 ± 2.12^a	53.22 ± 2.12^a	53.22 ± 2.12^a
Maximum pore size [nm]	5261.16 ± 331.32^a	4705.07 ± 342.12^b	1768.91 ± 193.78^c

O-BC - oil-modified BC; E-BC - emulsion-modified BC; C-BC - control BC. The results are presented as a mean ± standard error of the mean (SEM). Values with different letters are significantly different ($p<0.05$).

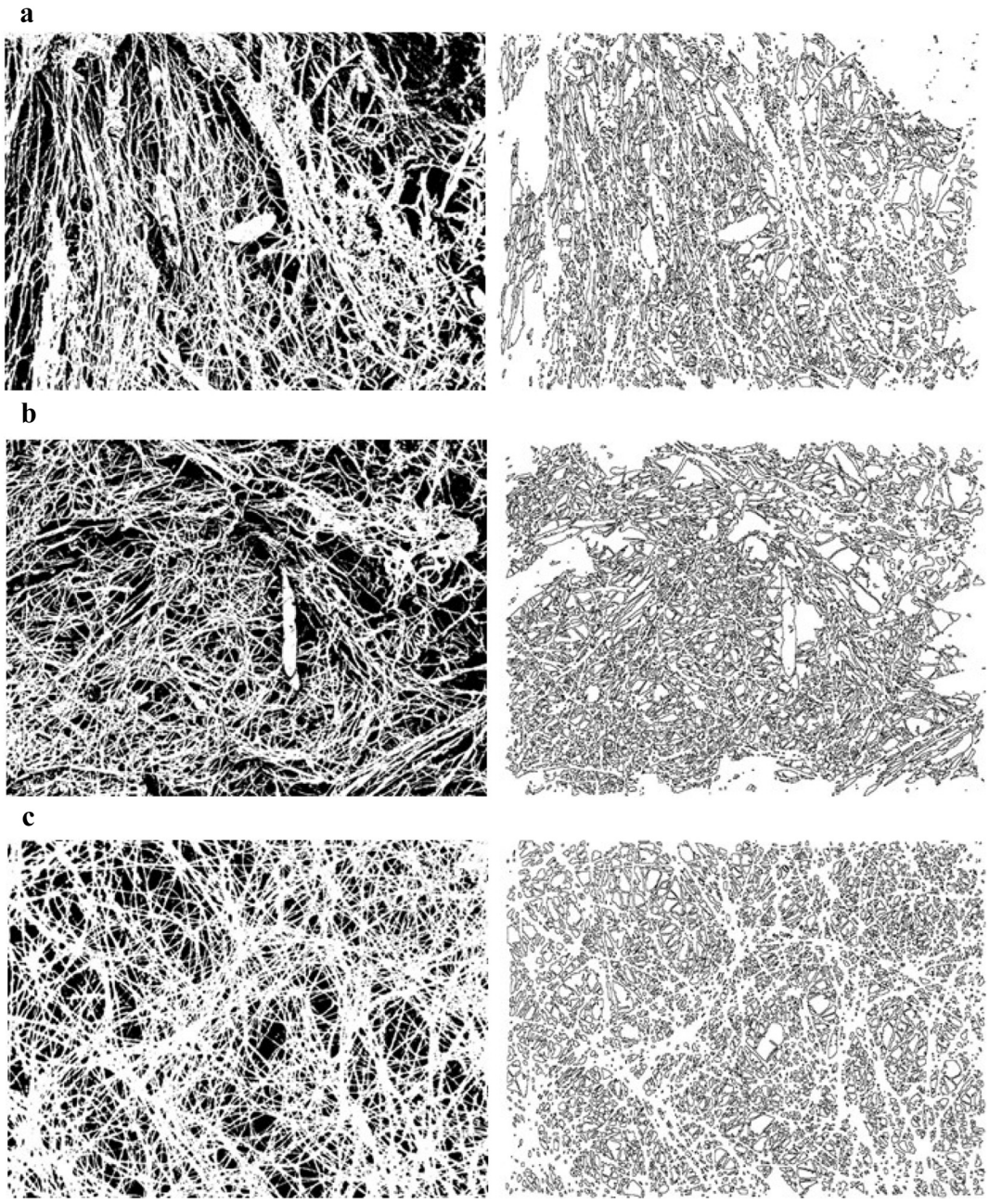

Fig. 2. Cellulose pores captured using Image J software for BC synthesized by *K. xylinus* in static culture: **(a)** H-S medium with 1% oil; **(b)** H-S medium with 1% emulsion; and **(c)** control H-S medium.

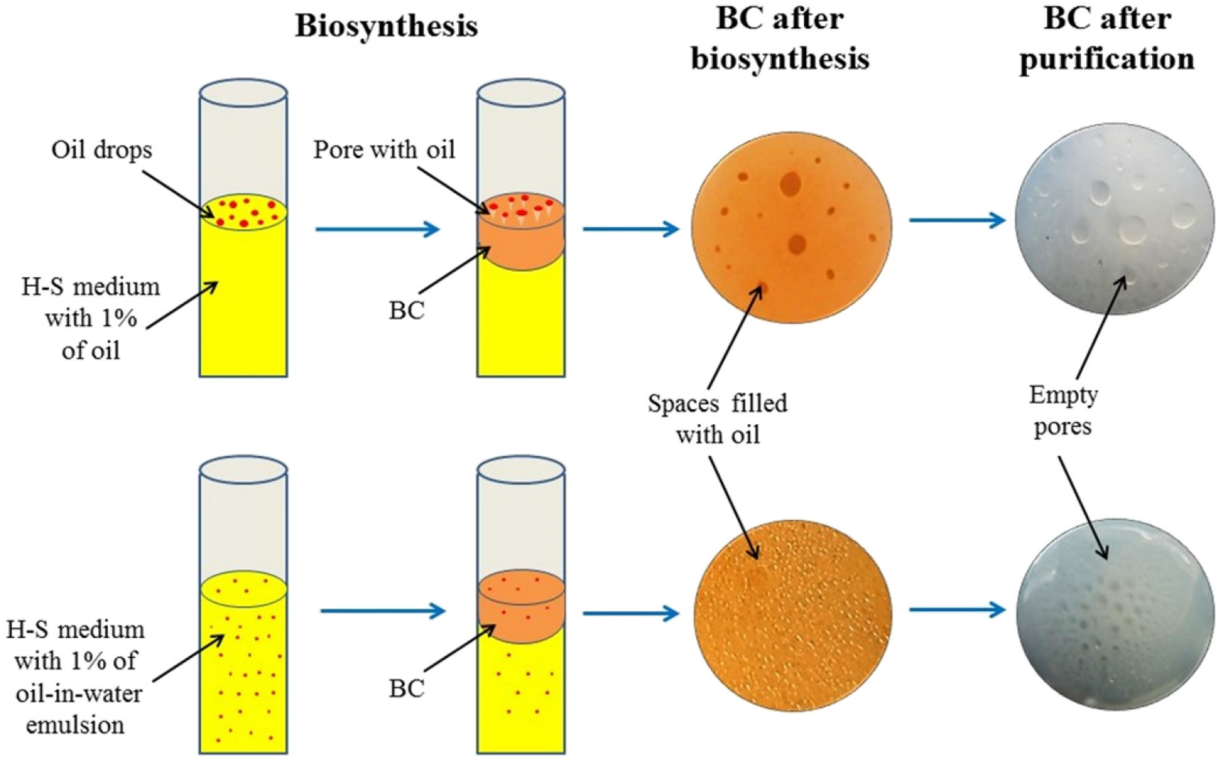

Fig. 3. BC pellicles synthesized by *K. xylinus* for 7 days in H-S medium supplemented with oil or emulsion.

culture conditions. In the case of agitated cultures, it was shown that addition of oil or emulsion to the culture medium did not increase the BC yield. There were no significant differences between weights of dry BC obtained from oil- or emulsion-supplemented medium as well as the BC obtained from control cultures (Fig. S3). The average amount of BC obtained was 2.43 mg of dry BC regardless of the type of medium modification.

As already reported [28], such a significant increase in BC yield obtained from static cultures is a result of complex interactions between *K. xylinus* biophysiology; chemical processes of BC synthesis; and physiochemical forces among BC membranes, oil, and culturing vessel walls. Such interaction between cellulose and culturing vessel walls is referred to as the "wall effect," which, according to Hornung et al. [12], is one of the strongest limitation factors of obtaining increased BC yield. In the case of agitated culture, the "wall effect" does not occur. Moreover, the addition of oil or emulsion to the culture medium did not have any additional impact on the improvement of substrate and oxygen transfer during BC biosynthesis. Therefore, it can be assumed that owing to the lack of "wall effect" as well as the constant availability of oxygen in both control and oil- and emulsion-modified samples in agitated culture, the higher production of BC could not be achieved.

4. Conclusion

The results of the current study reveal that the application of O-BC or E-BC leads to significantly higher efficiency of *Y. lipolytica* immobilization than the application of C-BC. The higher efficiency of immobilization was strictly related to porosity-related parameters. The differences in porosity-related parameters between O-BC and E-BC were connected to the size of oil droplets trapped inside BC during its synthesis. However, in contrast to the BC obtained from static cultures, addition of oil or emulsion to the culture medium did not influence the properties of the BC obtained from agitated cultures. The addition of oil or emulsion to the culture medium may be an efficient method to control porosity parameters in nanobiopolymers such as BC. Additionally, increased productivity of BC by supplementing the culture medium with oil or emulsion can allow for wider commercial use of BC, for example, as a carrier for immobilization.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Supplementary material

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