# Bacterial Cellulose Matrices to Develop Enzymatically Active Paper

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# Abstract

This work studies the suitability of bacterial cellulose (BC) matrices to prepare enzymatically active nanocomposites, in a framework of more environmentally friendly methodologies. After BC production and purification, two kind of matrices were obtained: BC in aqueous suspension and BC paper. A lipase was immobilised onto the BC matrices by physical adsorption, obtaining Lipase/BC nanocomposites. Neither morphology nor crystallinity, measured by scanning electron microscopy (SEM) and X-Ray diffractometry (XRD) respectively, of the BC were affected by the binding of the protein. The activity of Lipase/BC suspension and Lipase/BC paper was tested under different conditions, and the operational properties of the enzyme were evaluated. A shift towards higher temperatures, a broader pH activity range, and slight differences in the substrate preference were observed in the immobilised lipase, compared with the free enzyme. Specific activity was higher for Lipase/BC suspension (4.2 U/mg) than for Lipase/BC paper (1.7 U/mg) nanocomposites. However, Lipase/BC paper nanocomposites showed improved thermal stability, reusability, and durability. Enzyme immobilised onto BC paper retained 60% of its activity after 48 h at 60 ºC. It maintained 100% of the original activity after being recycled 10 times at pH 7 at 60 ºC and it remained active after being stored for more than a month at room temperature. The results suggested that lipase/BC nanocomposites are promising biomaterials for the development of green biotechnological devices with potential application in industrials bioprocesses of detergents and food industry and biomedicine. Lipase/BC paper nanocomposite might be a key component of bioactive paper for developing simple, handheld, and disposable devices.



**Keywords**: Bacterial cellulose**;** lipase immobilization; physical adsorption; nanocomposite; bacterial cellulose biopaper

# Introduction

In recent years has been increasing interest in the design of functional nanocomposites for advanced biotechnological applications. Nanocomposites consist of the combination of two types of individual materials, the matrix and the material imbedded on it, being at least one of the two of nano size dimension. Often, the matrix acts as a scaffold and supports an organic molecule with biological activity (Mohamad et al. 2015). The matrix provides the physic-chemical characteristics to the composite, while the molecule in it imparts biological properties to the matrix. Over the last two decades, the study of cellulosic nanofibres as the supporting matrix in nanocomposites has become an increasingly topical subject (Ferreira et al. 2018).

Cellulose is very abundant in nature and the biopolymer of choice in many applications. Traditionally plants have been the main source of cellulose. However, plant-derived cellulose is always bound to hemicelluloses and lignin and, before further used, it needs to be purified by enzymatic, chemical and/or mechanical treatments that have a high economic and environmental impact (Abdul Khalil et al. 2012). Cellulose synthesized by bacteria is referred to as bacterial cellulose (BC), an extracellular polymer produced by some microorganisms, especially from the genera *Komagateibacter* (Bielecki et al. 2005). Apart from being chemically pure (Chawla et al. 2009), BC displays a higher degree of crystallinity, a higher tensile strength, a higher water-holding capacity and a finer three-dimensional nanofibre network, being all these features of relevant importance for practical applications (Yano et al. 2005; Lee et al. 2014). Its three-dimensional open porous network structure of nanofibres, with a large surface area, is suitable to hold a large amount of inorganic and organic molecules. Moreover, cellulose contains available hydroxyl groups in its surface that provide the possibility of molecular adsorption by the formation of hydrogen bonds and electrostatic interactions (Pahlevan et al. 2018). In fact,

BC has been used in the preparation of several composite materials for various applications, such as in electrical devices, batteries, biosensors, electromagnetic shielding, biomedical applications or electrochromic devices (Evans et al. 2003; Kim et al. 2011; Shi et al. 2012; Ul-Islam et al. 2012a, b; Hänninen et al. 2015; Zhou et al. 2019). Immobilization of enzymes has several advantages, such as easy separation of enzyme from products in the reaction mix, reusability of the enzyme and increased stability (Wang 2006; Omagari et al. 2009; Kadokawa 2012). These characteristics have promoted the widely utilization of enzyme immobilization in industry (Klemm et al. 1998; Božič et al. 2012). In biotechnology, immobilized enzyme-based biosensors have huge applications in various fields as biomedicine, the detection of environmental pollutants or the monitoring of food safety and industrial bioprocesses (Monosik et al. 2012; Nigam and Shukla 2015; Rocchitta et al. 2016). The use of nanomaterials as enzyme supports have expanded its applicability (Molinero-Abad et al. 2014). However, the development of new nanomaterials that are cheap, highly pure and non-toxic is needed (Kim et al. 2015). BC is an attractive biocompatible candidate as a carrier for the immobilization of enzymes. Its porous ultrafine network allows for high accessibility onto the active site, through low diffusion resistance and easy recoverability as well as potential applicability for continuous operations (Sulaiman et al. 2015). In addition, BC is considered not only safer but more environmentally friendly than other nanomaterials (Lu et al. 2013). An effective enzyme immobilization on BC can be achieved using methods such as covalent binding or cross linking (Yao et al. 2013; Lin and Dufresne 2014). However, these methods often require chemical modifications of the matrix and/or the use of chemical linkers that complicate the procedure limiting the functionality of the composite generating residues that are harmful for the environment (Castro et al. 2014). Physical methods for the immobilization of enzymes imply the attachment of the biomolecule to

the matrix through physical forces such as van der Waals, electrostatic or hydrophobic interactions, and hydrogen bounding (Credou and Berthelot 2014). They do not need chemical modification of either the matrix or the enzyme, allowing minimal configuration change of the enzyme (Choi 2004). Enzyme immobilization by physical adsorption has been described for lysozyme onto BC fibres in suspension (Bayazidi et al. 2018), for lipase onto BC nanocrystals (Kim et al. 2015) and for nisin, laccase and lipase onto BC membranes (Wu et al. 2017; Yuan et al. 2018; dos Santos et al. 2018). Nevertheless, obtaining an enzymatically active BC nanocomposite that had the physical characteristics and the handiness of the paper would be of great interest.

The aim of this paper was to prepare Enzyme/BC nanocomposites to evaluate the suitability of BC matrices as supports for enzyme immobilization by physical adsorption. Among the great variety of enzymes, lipases (EC 3.1.1.3, triacylaglycerol hydrolases) have gained much attention as the most powerful biocatalyst for applications in areas such as food technology, detergent formulation, flavour and drug production and biofuel synthesis, among others (Angajala et al. 2016). Therefore, a lipase was chosen due to its enormous relevance in the development of bioassays and biosensors (Pohanka 2019). Protein loading, hydrolytic activity and enzymatic stability of lipases immobilized on BC matrices were evaluated at different conditions of pH and temperature and compared to free lipase. Matrices of both BC suspension (BCS) and BC paper (BCP) were compared. To the best of our knowledge, this is the first description of enzyme immobilization in BC paper, a matrix that combine the high surface-to-volume ratio of the BC nanofibres with the stiffness and the mechanical properties of paper, and that could lead to the design of devices for high performance applications.

# Experimental section

## Materials

*Komagataeibacter intermedius* JF2, a bacterial cellulose producer, was previously isolated in the laboratory (Fernández et al. 2019). Callera™ Trans L, a commercial liquid formulation of *Thermomyces lanuginosus* lipase (Nordblad et al. 2014) was supplied by Novozymes. Lipase (37 kDa, pI 4.4) was purified (elution buffer: 20 mM TrisHCl pH 7, 500 mM NaCl and 0.02 % sodium azide) from the commercial preparation by ionic exchange chromatography using HiTrap™ Q HP (GE Healthcare) columns in an AKTA$^{TM}$ FPLC protein purification system.

## Preparation of bacterial cellulose matrices

To produce BC, *K. intermedius* JF2 was grown on the Hestrin and Schramm (HS) medium, containing 20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, 1.15 g/L citric acid, 6.8 g/L $Na_2HPO_4$, pH 6. The cultures were statically incubated at 25 – 28 °C for 7 days. After incubation, bacterial cellulose membranes generated in the air/liquid interface of the culture media were harvested, rinsed with water and incubated in 1% NaOH at 70 °C overnight. Finally, the BC membranes were thoroughly washed in deionized water until the pH reached neutrality. Membranes were mechanically disrupted with a blender and homogenized (Homogenizing System UNIDRIVE X1000) to obtain a BC paste containing a suspension of BC fibres. The amount of BC in the suspension was determinate by drying samples of known weight at 60 ºC until constant weight was reached. The bacterial cellulose paste was used to produce BC paper sheets using a Rapid–Köthen laboratory former (Frank–PTI) following the ISO–5269:2004 standard method, obtaining a bacterial cellulose paper (BCP) matrix of a weight of 70 g/m$^2$. The

BC in aqueous suspension (BCS) matrix was obtained diluting the BC paste at to 7 mg/ml. Procedures for the generation of CB matrices are schematized in **Fig. 1**.

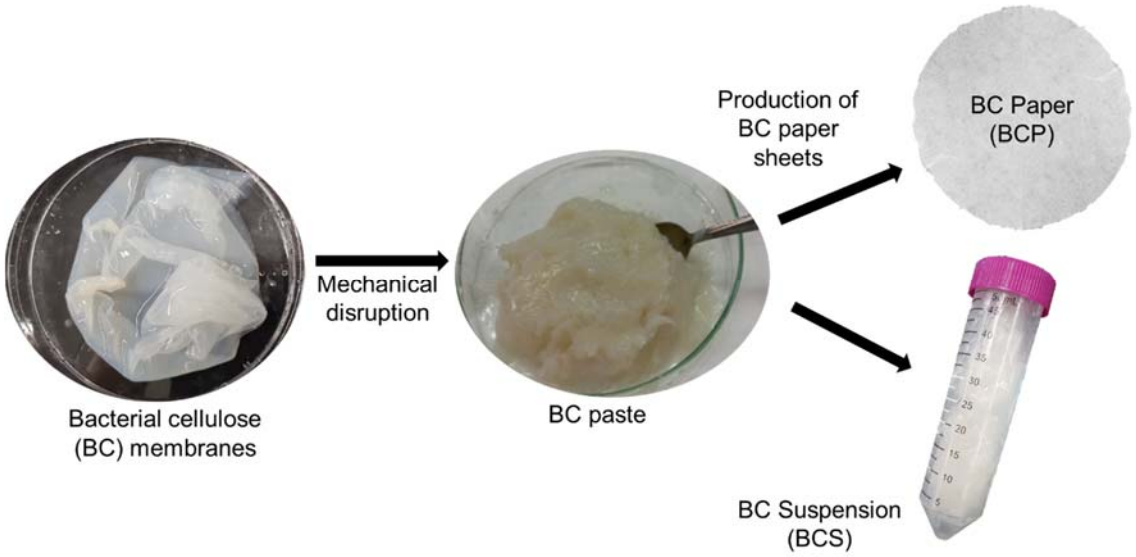


**Fig. 1** Schematic representation of the process to obtain bacterial cellulose matrices

## Preparation of Lipase/bacterial cellulose nanocomposites

Adsorption of lipase to BC matrices was conducted as follows: for BCP matrices, pieces of 1 $cm^2$ (7 mg $\pm$ 0.2) were immersed into the lipase binding solution (10 µg/ml, 20 mM TrisHCl pH 7), and incubated at 22 ºC with slight shaking for 18 h. Then, samples were removed, washed twice by dipping in buffer solution (20 mM TrisHCl, pH 7), air-dried and stored at room temperature. For adsorption of lipase in BCS matrices, a volume containing 7 mg of dry BC was centrifuged 5 min at 4000 rpm in an Allegra™ X-22R benchtop centrifuge (Beckman Coulter) to remove excess of water and resuspended in the same volume of lipase binding solution. After incubation at 22 ºC with slight shaking for 18 h, the solids were separated by centrifugation and washed twice with the buffer solution to remove the unbound enzyme. Finally, pellet was resuspended in the same buffer and stored at 4 ºC before used.

## Protein determination

The content of protein in the composites was determined comparing initial and final concentrations of protein in the lipase binding solution according to the Bradford's protein assay (Bradford 1976). The residual protein in the washing solutions was considered. Protein loading was calculated using **equation (1)** (Chen et al. 2015)**.**

$$Protein\ loading\ \left(\frac{\mu g}{gBC}\right) = [Total\ protein\ of\ free\ lipase\ (\mu g) - Total\ residual\ protein\ of\ free\ lipase\ after\ immobilization\ (\mu g)] / [Total\ mass\ of\ BC\ matrix\ (g)] \quad \textbf{(1)}$$

## Scanning electron microscopy (SEM)

Dried samples of Lipase/BC nanocomposites were analysed by SEM (JSM 7100 F) using a LED filter. Samples were graphite coated using a Vacuum Evaporator EMITECH K950X221. The diameter of the fibres was measured using the ImageJ software.

## X-Ray Diffractometry (XRD)

Dried samples of Lipase/BC nanocomposites were subjected to XRD analysis (PANalytical X'Pert PRO MPD Alpha1 powder diffractometer). The samples were analysed at the radiation wavelength of 1.5406 Å. Samples were scanned from 2º to 50°, 2Θ range. Samples were fixed over a zero background Silicon single crystal sample holder (pw1817/32), and the ensembles were mounted in a PW1813/32 sample holder. All the replicates of each sample were measured with the same Silicon holder. The

crystallinity index (CI) of produced bacterial cellulose was calculated based on **equation (2)** (Segal et al. 1959):

$$\mathrm{CI}(\%) = \frac{\mathrm{I_c} - \mathrm{I_{am}}}{\mathrm{I_c}} \times 100 \quad \textbf{(2)}$$

where $I_c$ is the maximum intensity of the lattice diffraction and $I_{am}$ is the intensity of the peak at 2Θ = 18°, which corresponds to the amorphous part of cellulose. The intensity of the peaks was measured as the maximum value obtained for the peak considering a baseline.

## Water Absorption Capacity

To assess the water absorption capacity of the BC paper, samples were weighted and immersed in deionized water for 24 h. After 24 h, excess of water was removed, and the weight was measured. The WAC was expressed according **equation (3)**:

$$\mathrm{WAC} = \frac{\mathrm{W_{wet-}W_{dry}}}{\mathrm{W_{dry}}} \quad \textbf{(3)}$$

where $W_{wet}$ is the weight of wet BC paper and $W_{dry}$ is the initial weight of the dried BC paper.

## Operational properties of the immobilized lipase

Lipase hydrolytic activity was analysed by measuring the release of MUF (methylumbelliferone) from MUF-derivate fatty acid (C4, C7, and C18) substrates (Sigma-Aldrich). Stock solutions of MUF-substrates were prepared at 25 mM in ethylene glycol methyl ester (EGME). The working solution contained 250 µM of MUF-substrates

in 20 mM TrisHCl pH 7. MUF was measured using a Varian Cary Eclipse spectrofluorometer (Agilent Technologies) equipped with a microplate reader, as previously reported (Panizza et al. 2013). One unit of activity was defined as the amount of enzyme that released one mmol of MUF per minute under the assay conditions. The specific activity and the recovery of lipase activity were calculated using **equation (4)** and **equation (5)**.

$$\text{Specific activity of immobilized lipase } \left(\frac{\text{U}}{\text{mg protein}}\right) = \frac{\text{Activity of immobilized enzyme } \left(\frac{\text{U/ml}}{\text{g BC}}\right)}{\text{Protein loading } (\frac{\text{mg protein/ml}}{\text{g BC}})} \quad \textbf{(4)}$$

$$\text{Recovery of lipase activity } (\%) = \frac{\text{Specific activity of immobilized lipase}}{\text{Specific activity of free lipase}} \text{ x } 100 \quad \textbf{(5)}$$

The determination of the operational characterization and properties of Lipase/BC nanocomposites was carried out with samples containing 7 mg BC (dry weigh)/ml. For Lipase/BCP, the nanocomposites were immersed into the appropriated buffer at the conditions being analysed. For Lipase/BCS nanocomposites, samples were centrifuged, and solids were suspended into the appropriated buffer, at the conditions being analysed. Assays of free lipase activity were run in parallel.

*Influence of temperature and thermal stability*

Optimum temperature of free and absorbed lipase was determined by the analysis of the activity over a range from 30 ºC to 90 °C at pH 7. Long-term thermal stability was

analysed based on the residual activity of lipase measured after incubation at 60 ºC in 20 mM TrisHCl pH 7 for a determinate period of time.

*Influence of pH*

Optimum pH of free and absorbed lipase was determined by analysis of the activity at various pH values with the appropriate buffers 20 mM: acetate buffer (pH 4 and 5), phosphate buffer (pH 6) and TrisHCl (pH 7, 8 and 9).

*Determination of kinetics constants*

The determination of the Michaelis-Menten constant (Km) and the maximum reaction rate (Vmax) of both free and immobilized lipase were carried out using MUF-butyrate as substrate, with initial concentrations varying from 50 µM to 1000 µM. The kinetic parameters were calculated by fitting hyperbolic Michaelis-Menten curves with GraphPad Prism 6 software (San Diego, California).

**Statistical analysis**

All determinations of enzyme activity were performed after two replicas of triplicates (6 determinations per sample). Experimental data were expressed as means ± standard deviations and were analysed statistically by the paired Student's *t*-test method and analysis of variance (ANOVA) in STATGRAPHICS Centurion XVIII software (Statgraphics.Net, Madrid) among more than two groups. Scheffe's multiple range test was used to detect differences among mean values. A value of $p \leq 0.05$ was considered statistically significant. Bartlett's test was used to test homogeneity of variance for all samples. Assumption that the residuals were normally distributed was tested with the Shapiro-Wilk test.

## Results and Discussion

### Adsorption of lipase in BC matrices and activity of the BC nanocomposites

pH plays a key role in the immobilization process by physical adsorption of proteins onto cellulose (Lin et al. 2015). The optimal pH for lipase immobilization into BC paper was tested in acetate buffer at pH 3 and 5, and TrisHCl buffer pH 7. Buffers concentration of the lipase binding solution was 20 mM, as it is described that lower ionic strength can enhance the formation of the protein/polysaccharide composites (Chai et al. 2014). The amount of protein adsorbed into BC paper at the different pHs is shown in **Table 1**. Results indicated that the highest efficiency of lipase adsorption was at pH 3, suggesting electrostatic bounding between the positive charged protein and the overall negative charge cellulose due to hydroxyl groups and molecular dipole. However, when enzymatic activity of the obtained Lipase/BC paper nanocomposites was measured under standard conditions, at pH 7, the enzyme immobilized at pH 3 showed less specific activity than the immobilized at pH 5 and at pH 7 (Table 1). These results suggested that the buffer at pH 3 used for the immobilization process, while favouring its binding, inactivated the enzyme. Therefore, and to maintain the same working conditions as in the determinations of enzymatic activity, further adsorption experiments were conducted with 20 mM Tris at pH 7, conditions that allowed both good adsorption and specific activity.

**Table 1**. Effect of the pH on the adsorption of the lipase onto BC paper and enzymatic activity of the adsorbed lipase.

| pH | Lipase adsorbed (μg/cm$^2$) | Specific activity (U/mg protein) |
|---|---|---|
| pH 3 | 5.29 ± 0.4 | 0.89 ± 0.03 |
| pH 5 | 2.90 ± 0.36 | 1.99 ± 0.02 |
| pH 7 | 2.65 ± 0.11 | 1.69 ± 0.11 |

Lipase was physically adsorbed onto BC cellulose fibres, both in aqueous suspension (BCS) and in paper (BCP) obtaining Lipase/BCS and Lipase/BCP nanocomposites, respectively (**Table 2**). BCS showed higher capacity to adsorb protein than BCP, which could be attributed to the difference in the density of their nanofibrils' network that influences the accessibility of the protein to the matrix of cellulose. Moreover, during the process of paper production to obtain BCP matrices, the fibres of cellulose undergo dehydration through evaporation of water. The loss of the molecules of water produce irreversible formation of new hydrogen bounds between the hydroxyl groups of adjacent glucan chains that would hinder the diffusion of the protein (Seves et al. 2001). To test if this structural modification would affect the adsorption properties of the BC matrix, its water absorption capacity (WAC) was measured (equation 3). BCP showed a WAC of 263 ± 28%, around 37 times its dry weight. As expected, WAC of BC paper was lower than that reported for never dried native BC membranes (Meftahi et al. 2010). However, Fernandez *et al.* (Fernández et al. 2019) reported values of WAC of only 10 – 20 % for dry films of BC membranes. The results obtained indicated that the dry BC paper matrices maintained enough WAC to carry out adsorption assays by immersing the paper matrix in the aqueous solution of the enzyme for its immobilization. Nevertheless, in BCP, probably most of the protein binding is taking place only in the most superficial layers of fibres of the matrix.

The obtained nanocomposites were enzymatically active, although specific activity of the immobilized enzyme decreased with respect to that of the free enzyme (Table 2). This is a common phenomenon described previously for a variety of enzymes and immobilizer supports (Lian et al. 2012). However, differences in the specific activity between the two types of Lipase/nanocomposites were observed. Enzyme bounded to BCS maintained about 68 % of activity respect to the free enzyme, while enzyme bounded to BCP

maintained only 28%, approximately (Table 2). The decrease of lipase activity after immobilization may be due to the changes in structural conformation of lipase and lower accessibility of substrate to its active sites (Kim et al. 2015). After the enzyme is entrapped and immobilized in the porous network of BC, more mass transfer resistance forms compared to the free enzyme, impairing the binding efficiency between the enzyme and the substrate (Chen et al. 2015). This effect is more accused for the lipase adsorbed onto BCP, which is a less porous matrix than BCS owing to its higher fibre density after water evaporation. Consequently, the lipase has less diffusional mechanisms, influencing the activity of the enzyme (Estevinho et al. 2014).

**Table 2**. Characteristics of lipase immobilized onto BCP and BCS matrices.

| Matrix | Adsorbed protein (μg/g BC) | Specific activity (U/mg protein) | Recovered activity (%) |
|---|---|---|---|
| Free enzyme | - | 6.13 ± 0.4 | - |
| Lipase/BCP nanocomposite | 416.37 ± 85 | 1.69 ± 0.11 | 27.6 |
| Lipase/BCS nanocomposite | 737.35 ± 106 | 4.15 ± 0.14 | 67.7 |

## Physical characterization of Lipase/BC nanocomposites

Lipase/BC nanocomposites were characterized in terms of morphology and chemical structure and crystallinity by SEM and XRD, respectively.

*Morphology observation by SEM*

SEM images of BCP and BCS matrices are shown in **Fig. 2**. In both of them, it could be observed a connected structure consisting of ultrafine cellulose fibrils with a diameter of about 50 - 70 nm, which results in large surface area. This high surface area and porous features of BC would provide microchannels to entrap enzyme and would improve the contact area exposed to protein molecules (Chen et al. 2015). BCP fibres disposition was

more flawless than in BCS fibres, where the fibres displayed a higher density. No changes were observed in the morphology or in the arrangement of the nanofibers after immobilization of the lipase.

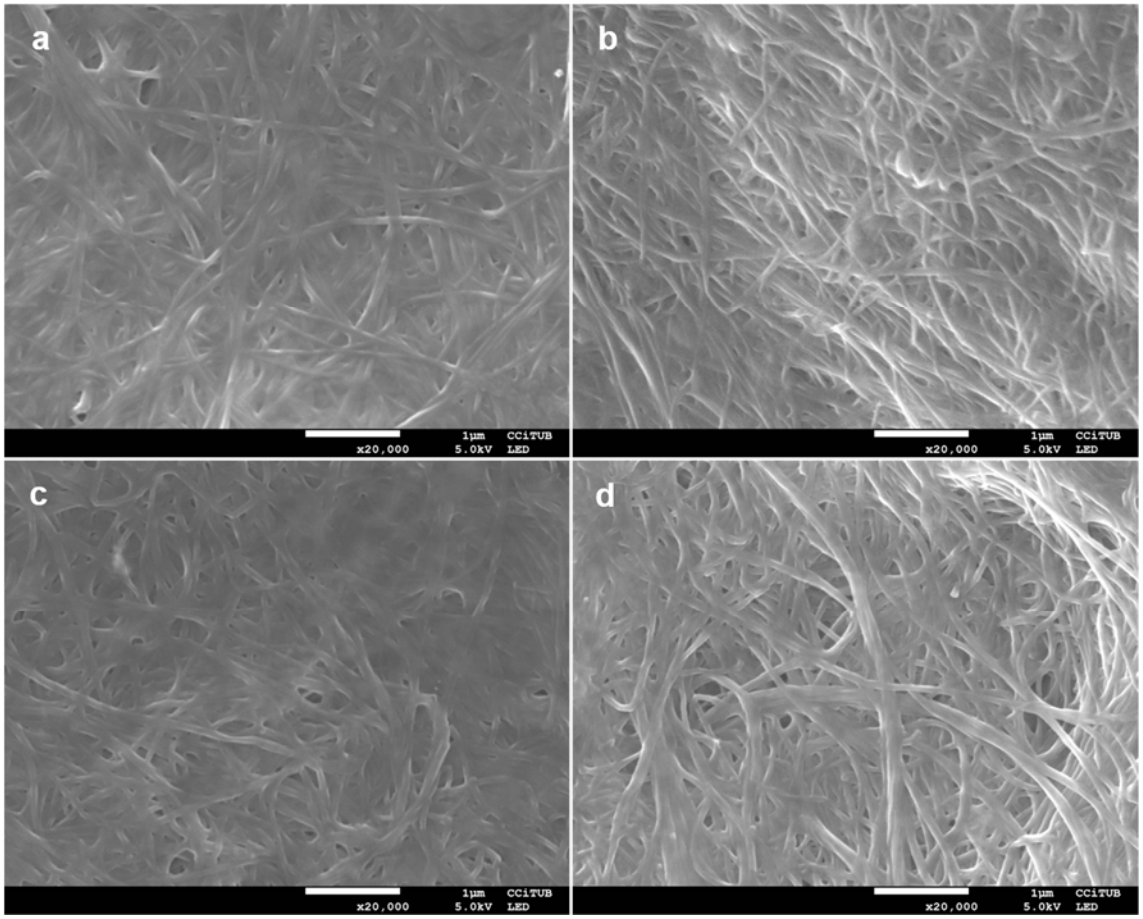


**Fig. 2** Scanning electron microscopic (SEM) images of BCP **(a)**, BCS **(b),** Lipase/BCP nanocomposite **(c)** and Lipase/BCS nanocomposite (**d**)

*Crystallinity*

XRD patterns of Lipase/BC nanocomposites were measured. **Figure 3** shows diffraction peaks at 2Θ angles around 18.4 º and 22.7 º; the presence of which were ascribed to the typical profile of cellulose I (natural cellulose) in crystalline form (Chen et al. 2015) for BCP and BCS matrices and their Lipase/nanocomposites. Even though immobilization of lipase caused a slight broadening of all peaks, intensities were not dramatically changed. The estimated degree of crystallinity index (equation 2) of the pure BC was 94% for BCP

and 93% for BCS. With the introduction of lipase, the crystallinity index did not change (94% for both nanocomposites). These results indicated that no changes in the crystalline structure within the cellulose fibres did occur during the incorporation of lipase by physical adsorption, suggesting that characteristics as mechanical strength and interfacial properties of the cellulose fiber were not modified (Huang et al. 2014).

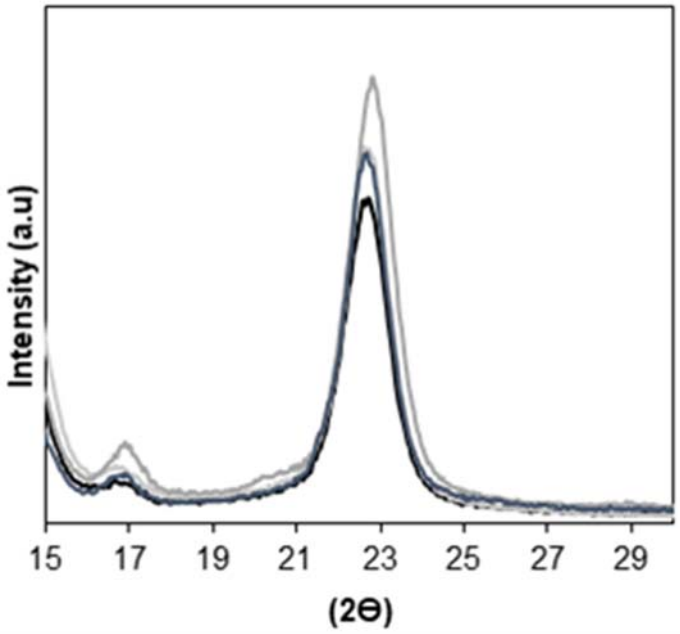


**Fig. 3** XRD patterns of BCP (black line), BCS (grey line), Lipase/BCP nanocomposite (light grey line) and Lipase/BCS nanocomposite (dark grey line)

## Operational properties of Lipase BC/nanocomposites

*Effect of the temperature and thermal stability*

The effect of temperature on the activity of free and immobilized lipase was studied in the temperature range of 30 – 90 ºC (**Fig. 4a)**. Free enzyme had its optimum temperature activity between 40 – 50 ºC, while Lipase/BCS nanocomposite retained its maxim activity at 50 ºC. Remarkably, Lipase/BCP nanocomposite shifted its optimal temperature to 60 – 70 ºC and, in addition, it broadened the range of temperature where the enzyme can be active. This shift of the optimum temperature suggested an increase in the thermal

stability of the immobilized lipase, and it could be related to the change of structural stabilisation of the immobilized enzyme (Chen et al. 2015). In fact, the thermal stability of lipase at 60 ºC was highly enhanced by the adsorption of lipase onto BCP (**Fig. 4b**). After 2 h of incubation, the residual activity of free lipase and Lipase/BCS nanocomposite was about 50 % whereas the lipase immobilized onto BCP retained more than 60 % of enzymatic activity after 48 h. These results highlight that the BCP matrix provided a framework of great stability for the activity of the lipase at elevated temperatures. This enhanced stability could be attributed to the restricted conformational mobility of the entrapped lipase molecules after immobilization (Frazão et al. 2014) onto cellulose matrix, delaying the rate of inactivation (Yuan et al. 2018), and it has been reported by other authors for other BC supports as BC membranes (Yuan et al. 2018) and BC nanocrystals (Kim et al. 2015).

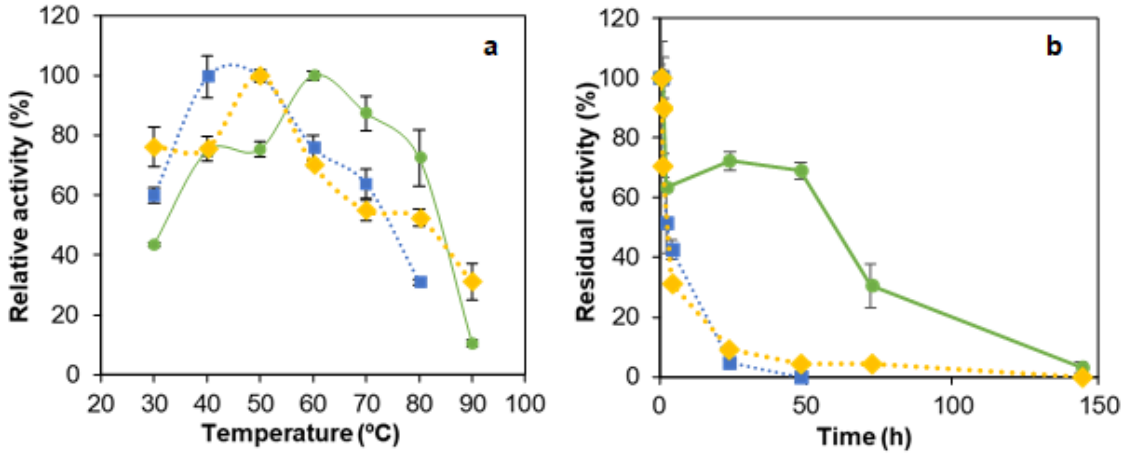


**Fig. 4** Lipase activity at different temperatures **(a)**. Activity was expressed in relative values, with the highest activity denoting 100%. **(b)** Thermal stability at 60 ºC under different times of incubation, where residual activity was expressed as percentage of the initial activity at time zero**.** Squares line = free lipase, dots line = Lipase/BCP nanocomposite, rhombus line = Lipase/BCS nanocomposite

*Effect of pH*

The effect of pH on the activity of free and immobilized lipase was tested under various pH (**Fig. 5**). The general profiles of the pH dependency were very similar; pH 8 was optimal for free enzyme and Lipase/BCP nanocomposites. Nevertheless, the higher activity of Lipase/BCS nanocomposites was preserved at a wider range of pH, showing the highest activity between pH 7 and 9. Very low activity was detected at pH lower than 6.

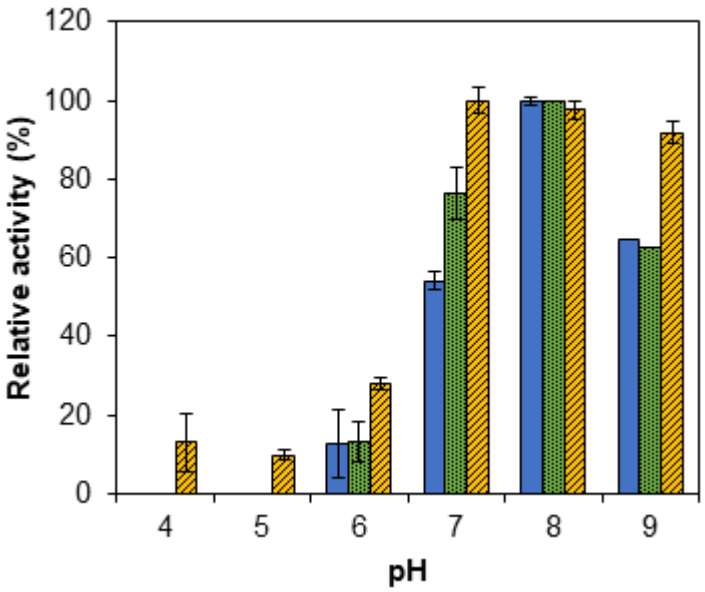


**Fig. 5** Effect of pH on free lipase and the Lipase/BC nanocomposites. Activity was expressed in relative values, with the highest activity denoting 100%. Solid bars = free lipase, dot bars = Lipase/BCP, line bars = Lipase/BCS

*Specificity of substrate length*

Specificity of substrate length of Lipase/BCS and lipase/BCP nanocomposites was tested on MUF-derivative fatty acid of different chain-length and compared with that of the free lipase. The free enzyme and the enzyme immobilized onto BCS and BCP nanocomposites showed activity on butyrate (C4) heptanoate (C7) and oleate (C18) (**Fig. 6**). Butyrate was

the optimum substrate, with significant differences regarding the other two assayed substrates. However, even if all of them demonstrated the same profile of relative activity, the most striking result to emerge from the data was that with oleate. Interestingly, Lipase/BCP nanocomposite showed higher activity with oleate than Lipase/BCS nanocomposite and free lipase, suggesting that the lowest water content matrix could better accommodate more hydrophobic substrates.

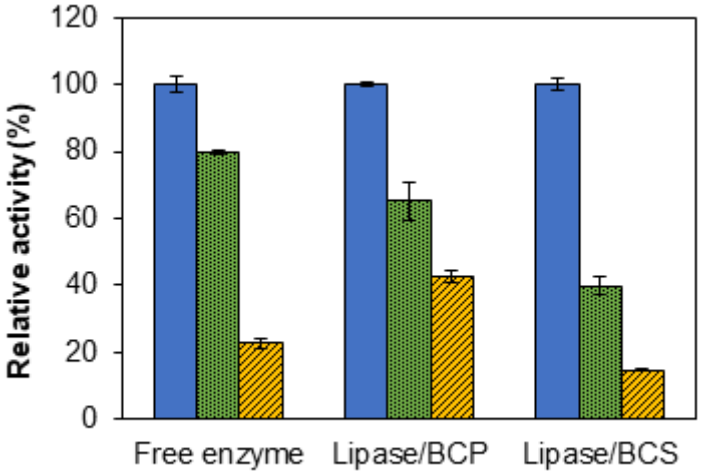


**Fig. 6** Specificity of substrate for the free lipase and the Lipase/BC nanocomposites: butyrate (solid bars), heptanoate (dot bars) and oleate (line bars). Activity was expressed in relative values, with the highest activity denoting 100%

*Kinetic constants*

Enzyme activity was measured at different substrate concentrations (50 – 1000 μM) with free and immobilized lipases. The kinetic data was fitted to the Michaelis-Menten equation and parameters were calculated. The kinetic parameters are summarized in **Table 3**. Both $K_m$ and $V_{max}$ were affected by immobilization process. $K_m$ has higher values in immobilized enzyme than the in the free one: in Lipase/BCP nanocomposite it was almost the double, whereas in Lipase/BCS nanocomposite an approximately 4-fold increase was observed, indicating a weaker attachment of substrate to enzyme.

Diffusional limitations due to the immobilization of the enzyme would cause a lower affinity for the substrate.

**Table 3**. Kinetic constants of free and immobilized lipase.

| | $K_m$ (µM) | $V_{max}$ (U/mg protein) |
|---|---|---|
| Free lipase | 169.9 ± 25.75 | 3.03 ± 0.18 |
| Lipase/BCP nanocomposite | 276.9 ± 53.02 | 3.31 ± 0.32 |
| Lipase/BCS nanocomposite | 659.0 ± 264.3 | 6.44 ± 1.7 |

During the process of immobilization by physical adsorption, the orientation of the immobilized lipase on the matrix was not a controlled process. Therefore, an improper fixation could hinder the active site for binding of substrates to the immobilized enzyme (Yang et al. 2010). The increasing of kinetic parameters correlates favourably with previous studies on enzyme immobilization (Bayazidi et al. 2018).

**Leaching of the lipase from the nanocomposites**

The stability of immobilized lipase onto BC matrices was determinated. Lipase/BC nanocomposites were incubated in buffer solution (20 mM TrisHCl pH 7) at room temperature and enzymatic activity was measured in the solution at several times. Lipase activity was not detected at times 0, 24 and 48 h. After 72 h, only about 4 % of the lipase activity was released from the Lipase/BCP nanocomposites, whereas for Lipase/BCS nanocomposites no leaching of activity was detected (**Table 4**). At this time, the activity that remained in the nanocomposites was measured. The results indicated that Lipase/BCP nanocomposites maintained 100 % of the activity, in accordance with the results obtained from the leaching of the activity. However, Lipase/BCS nanocomposites retained only 33 % of the activity, suggesting that the enzyme lost activity during the 72 h incubation at room temperature (Table 4).

It has been described that the interactions by physical adsorption between enzymes and plant cellulose supports would be not strong enough to ensure permanent immobilization and to prevent, consequently, the leaking of the biomolecules (Credou and Berthelot 2014). Nevertheless, in this study, the lipase adsorbed onto BCP matrices seemed to be strongly entrapped. Probably, the high density and specific surface area provided by BC nanofibers resulted in more available hydroxyl groups where the lipase can be adsorbed (Skočaj 2019). Moreover, the porous three-dimensional structure of nanofibers would help to retain the enzyme.

**Table 4.** Activity and leaching of lipase from BC nanocomposites

| | t = 0 h | t = 72 h | | |
|---|---|---|---|---|
| | mU/ml | mU/ml | % leaching | % remaining activity |
| Lipase/BCP | 5.6 ± 0.03 | 5.74 ± 0.90 | 3.9 | 100 |
| Lipase/BCS | 20.8 ± 0.4 | 6.93 ± 0.62 | 0 | 33 |

**Reusability of Lipase/BC nanocomposites**

To determine the reusability of the Lipase/BC nanocomposites activity was measured. Then, Lipase/BCP nanocomposites were rinsed twice by immersion in 20 mM TrisHCl pH 7 and allowed to air-dry before the following activity assay. Lipase/BCS nanocomposites were rinsed by centrifugation and resuspension of the pellet in 20 mM TrisHCl pH 7. A third centrifugation allowed the resuspension of the pellet in the reaction buffer for subsequent lipase activity assay. These operational cycles were repeated 10 consecutive times. Results are shown in **Fig. 7.** The activity of the BCS nanocomposites gradually decreased with the subsequent cycles, retaining 55 % of the original activity after five recycling times, although significant differences were already detected in the

second round of recycling. As for the Lipase/BCP nanocomposites, the activity did no decrease along the reusing cycles, without any significant difference. In comparison to other supports, as green coconut fibre, where a laccase was immobilized by physical adsorption, the composite lost 30 % of its initial activity in the second cycle (Cristóvão et al. 2011). Therefore, BCP would stand out as a matrix that allows a notable operational stability. Moreover, the efficiency of reusability of lipase on BC paper was higher of that described for a crosslinking-immobilized laccase on BC membrane, which showed 69 % of its original activity after seven recycling times (Chen et al. 2015). Good reusability of enzyme can lead to significant reduction of operational cost which is of utmost relevance for the industry (Silva et al. 2006) and for practical applications as biosensors (Nigam and Shukla 2015).

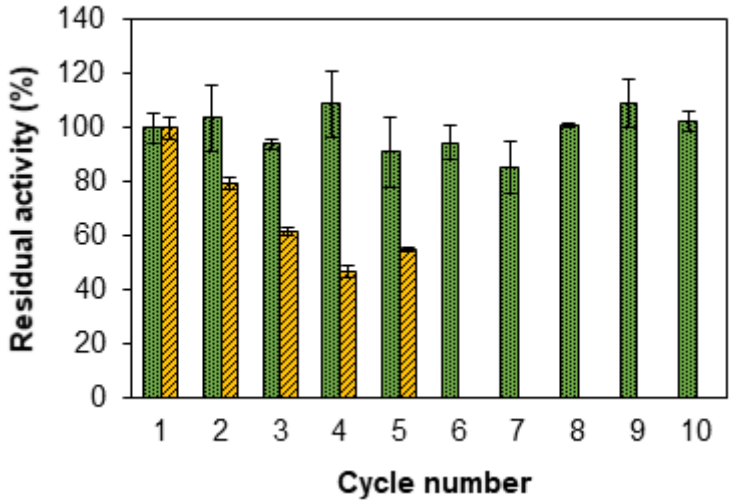


**Fig. 7** Reusability of the Lipase/BCP nanocomposites (dot bars) and the Lipase/BCS nanocomposites (line bars). The reusability was expressed as the percent of remaining activity where activity from the first run was taken as 100%

## Storage stability of the Lipase/BCP nanocomposites

The effect of storing time on the functionality of Lipase/BCP nanocomposite was studied during 75 days period. Results showed that nanocomposites with immobilized enzyme could be stored at room temperature during at least 20 days without any significant loss of activity, retaining 71 % after 45 days (**Fig. 8**). These results indicated that neither the enzyme activity nor the cellulose-

attachment of the enzyme were compromised under these conditions during several weeks. The biocompatibility and the three-dimensional network of nanofibers of the BC in conjunction with the water-free environment of BCP allowed the preservation of the activity of the enzyme without the need for special storage. Those are essential properties to be found in biopaper based devices (Crini 2005).

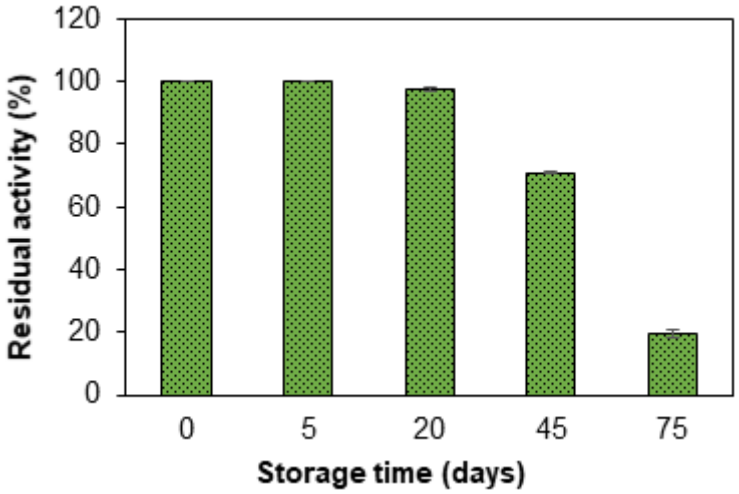


**Fig. 8** Stability of Lipase/BCP nanocomposite during a 75-days storage at room temperature. Residual activity was expressed as percentage of the initial activity at time zero.

## Conclusions

In the present work, the immobilization of lipase by physical adsorption, a cost-effective and environmentally friendly method, generated functional bacterial cellulose-based nanocomposites. BCS matrices showed higher protein adsorption capacity than BCP matrices. Likewise, Lipase/BCS presented higher specific activity than Lipase/BCP nanocomposites. However, enzyme immobilized onto BCP was able to operate at higher temperatures and showed greater thermal stability. Moreover, Lipase/BCP nanocomposites maintained their enzymatic activity after several weeks of storage at room temperature and for, at least, 10 reusing cycles. This study could be the first step in establishing a process to obtain bioactive BC paper, considering "BC paper" as the

material obtained from bacterial cellulose paste in the form of thin sheets that combine the characteristics of BC nanofibers with the stiffness and physical properties of paper. It is foreseeable that nanocomposites of BC paper with other enzymes could be obtained. Enzyme/BCP nanocomposites are of particular interest because they could be used as part of biosensors devices with applications in many fields including clinical diagnosis, environmental monitoring, and food quality control. Due to their operational properties, they could be suitable for point-of-use testing devices.

## Compliance with Ethical Standards

## Declaration of interest

Authors declare no conflict of interest


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## References


Abdul Khalil HPS, Bhat AH, Ireana Yusra AF (2012) Green composites from sustainable cellulose nanofibrils: A review. Carbohydr Polym 87:963–979. https://doi.org/10.1016/j.carbpol.2011.08.078

Angajala G, Pavan P, Subashini R (2016) Lipases: An overview of its current challenges and prospectives in the revolution of biocatalysis. Biocatal Agric Biotechnol 7:257–270. https://doi.org/10.1016/j.bcab.2016.07.001

Bayazidi P, Almasi H, Asl AK (2018) Immobilization of lysozyme on bacterial cellulose nanofibers: Characteristics, antimicrobial activity and morphological properties. Int J Biol Macromol 107:2544–2551. https://doi.org/10.1016/j.ijbiomac.2017.10.137

Bielecki S, Krystynowicz A, Turkiewicz M, Kalinowska H (2005) Bacterial Cellulose. In: Vandamme EJ, De Baets S, Steinbüchel A (eds) Biopolymers Online. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany

Božič M, Gorgieva S, Kokol V (2012) Laccase-mediated functionalization of chitosan by caffeic and gallic acids for modulating antioxidant and antimicrobial properties. Carbohydr Polym 87:2388–2398. https://doi.org/10.1016/j.carbpol.2011.11.006

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Castro C, Vesterinen A, Zuluaga R, et al (2014) In situ production of nanocomposites of poly(vinyl alcohol) and cellulose nanofibrils from Gluconacetobacter bacteria: effect of chemical crosslinking. Cellulose 21:1745–1756. https://doi.org/10.1007/s10570-014-0170-1

Chai C, Lee J, Huang Q (2014) The effect of ionic strength on the rheology of pH-induced bovine serum albumin/κ-carrageenan coacervates. LWT - Food Sci Technol 59:356–360. https://doi.org/10.1016/j.lwt.2014.05.024

Chawla PR, Bajaj IB, Survase S a., Singhal RS (2009) Microbial cellulose: Fermentative production and applications ( Review ). Food Technol Biotechnol

Chen L, Zou M, Hong FF (2015) Evaluation of Fungal Laccase Immobilized on Natural Nanostructured Bacterial Cellulose. Front Microbiol 6:. https://doi.org/10.3389/fmicb.2015.01245

Choi MMF (2004) Progress in Enzyme-Based Biosensors Using Optical Transducers. Microchim Acta 148:107–132. https://doi.org/10.1007/s00604-004-0273-8

Credou J, Berthelot T (2014) Cellulose: from biocompatible to bioactive material. J Mater Chem B 2:4767–4788. https://doi.org/10.1039/C4TB00431K

Crini G (2005) Recent developments in polysaccharide-based materials used as adsorbents in wastewater treatment. Prog Polym Sci 30:38–70. https://doi.org/10.1016/j.progpolymsci.2004.11.002

Cristóvão RO, Tavares APM, Brígida AI, et al (2011) Immobilization of commercial laccase onto green coconut fiber by adsorption and its application for reactive textile dyes degradation. J Mol Catal B Enzym 72:6–12. https://doi.org/10.1016/j.molcatb.2011.04.014

dos Santos CA, dos Santos GR, Soeiro VS, et al (2018) Bacterial nanocellulose membranes combined with nisin: a strategy to prevent microbial growth. Cellulose 25:6681–6689. https://doi.org/10.1007/s10570-018-2010-1

Estevinho BN, Damas AM, Martins P, Rocha F (2014) Microencapsulation of β-galactosidase with different biopolymers by a spray-drying process. Food Res Int 64:134–140. https://doi.org/10.1016/j.foodres.2014.05.057

Evans BR, O'Neill HM, Malyvanh VP, et al (2003) Palladium-bacterial cellulose membranes for fuel cells. Biosens Bioelectron 18:917–923. https://doi.org/10.1016/S0956-5663(02)00212-9

Fernández J, Morena AG, Valenzuela S V., et al (2019) Microbial Cellulose from a Komagataeibacter intermedius Strain Isolated from Commercial Wine Vinegar. J Polym Environ 27:956–967. https://doi.org/10.1007/s10924-019-01403-4

Ferreira FV, Dufresne A, Pinheiro IF, et al (2018) How do cellulose nanocrystals affect the overall properties of biodegradable polymer nanocomposites: A comprehensive review. Eur Polym J 108:274–285. https://doi.org/10.1016/j.eurpolymj.2018.08.045

Frazão CJR, Silva NHC, Freire CSR, et al (2014) Bacterial cellulose as carrier for immobilization of laccase: Optimization and characterization. Eng Life Sci 14:500–508. https://doi.org/10.1002/elsc.201400054

Hänninen T, Orelma H, Laine J (2015) TEMPO oxidized cellulose thin films analysed by QCM-D and AFM. Cellulose 22:165–171. https://doi.org/10.1007/s10570-014-0530-x

Huang Y, Zhu C, Yang J, et al (2014) Recent advances in bacterial cellulose. Cellulose 21:1–30. https://doi.org/10.1007/s10570-013-0088-z

Kadokawa J (2012) Preparation and Applications of Amylose Supramolecules by Means of Phosphorylase-Catalyzed Enzymatic Polymerization. Polymers (Basel) 4:116–133. https://doi.org/10.3390/polym4010116

Kim HJ, Park S, Kim SH, et al (2015) Biocompatible cellulose nanocrystals as supports to immobilize lipase. J Mol Catal B Enzym 122:170–178. https://doi.org/10.1016/j.molcatb.2015.09.007

Kim J, Cai Z, Lee HS, et al (2011) Preparation and characterization of a Bacterial cellulose/Chitosan composite for potential biomedical application. J Polym Res 18:739–744. https://doi.org/10.1007/s10965-010-9470-9

Klemm D, Philipp B, Heinze T, et al (1998) Comprehensive Cellulose Chemistry: Volume I: Fundamentals and analytical Methods. Compr Cellul Chem

Lee K-Y, Buldum G, Mantalaris A, Bismarck A (2014) More Than Meets the Eye in Bacterial Cellulose: Biosynthesis, Bioprocessing, and Applications in Advanced Fiber Composites. Macromol Biosci 14:10–32. https://doi.org/10.1002/mabi.201300298

Lian Z-X, Ma Z-S, Wei J, Liu H (2012) Preparation and characterization of immobilized lysozyme and evaluation of its application in edible coatings. Process Biochem 47:201–208. https://doi.org/10.1016/j.procbio.2011.10.031

Lin N, Dufresne A (2014) Nanocellulose in biomedicine: Current status and future prospect. Eur Polym J 59:302–325. https://doi.org/10.1016/j.eurpolymj.2014.07.025

Lin Q, Zheng Y, Wang G, et al (2015) Protein adsorption behaviors of carboxymethylated bacterial cellulose membranes. Int J Biol Macromol 73:264–269.

https://doi.org/10.1016/j.ijbiomac.2014.11.011

Lu H, Gui Y, Zheng L, Liu X (2013) Morphological, crystalline, thermal and physicochemical properties of cellulose nanocrystals obtained from sweet potato residue. Food Res Int 50:121–128. https://doi.org/10.1016/j.foodres.2012.10.013

Meftahi A, Khajavi R, Rashidi A, et al (2010) The effects of cotton gauze coating with microbial cellulose. Cellulose 17:199–204. https://doi.org/10.1007/s10570-009-9377-y

Mohamad NR, Marzuki NHC, Buang NA, et al (2015) An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. Biotechnol Biotechnol Equip 29:205–220. https://doi.org/10.1080/13102818.2015.1008192

Molinero-Abad B, Alonso-Lomillo MA, Domínguez-Renedo O, Arcos-Martínez MJ (2014) Malate quinone oxidoreductase biosensors based on tetrathiafulvalene and gold nanoparticles modified screen-printed carbon electrodes for malic acid determination in wine. Sensors Actuators B Chem 202:971–975. https://doi.org/10.1016/j.snb.2014.06.057

Monosik R, Stredansky M, Tkac J, Sturdik E (2012) Application of Enzyme Biosensors in Analysis of Food and Beverages. Food Anal Methods 5:40–53. https://doi.org/10.1007/s12161-011-9222-4

Nigam VK, Shukla P (2015) Enzyme Based Biosensors for Detection of Environmental Pollutants-A Review. J Microbiol Biotechnol 25:1773–1781. https://doi.org/10.4014/jmb.1504.04010

Nordblad M, Silva VTL, Nielsen PM, Woodley JM (2014) Identification of critical parameters in liquid enzyme-catalyzed biodiesel production. Biotechnol Bioeng 111:2446–2453. https://doi.org/10.1002/bit.25305

Omagari Y, Matsuda S, Kaneko Y, Kadokawa J (2009) Chemoenzymatic Synthesis of Amylose-Grafted Cellulose. Macromol Biosci 9:450–455. https://doi.org/10.1002/mabi.200800237

Pahlevan M, Toivakka M, Alam P (2018) Mechanical properties of TEMPO-oxidised bacterial cellulose-amino acid biomaterials. Eur Polym J 101:29–36.

https://doi.org/10.1016/j.eurpolymj.2018.02.013

Panizza P, Syfantou N, Pastor FIJ, et al (2013) Acidic lipase Lip I.3 from a Pseudomonas fluorescens -like strain displays unusual properties and shows activity on secondary alcohols. J Appl Microbiol 114:722–732. https://doi.org/10.1111/jam.12089

Pohanka M (2019) Biosensors and Bioassays Based on Lipases, Principles and Applications, a Review. Molecules 24:616. https://doi.org/10.3390/molecules24030616

Rocchitta G, Spanu A, Babudieri S, et al (2016) Enzyme Biosensors for Biomedical Applications: Strategies for Safeguarding Analytical Performances in Biological Fluids. Sensors 16:780. https://doi.org/10.3390/s16060780

Segal L, Creely JJ, Martin AE, Conrad CM (1959) An Empirical Method for Estimating the Degree of Crystallinity of Native Cellulose Using the X-Ray Diffractometer. Text Res J 29:786–794. https://doi.org/10.1177/004051755902901003

Seves A, Testa G, Bonfatti AM, et al (2001) Characterization of Native Cellulose/Poly(ethylene glycol) Films. Macromol Mater Eng 286:524–528. https://doi.org/10.1002/1439-2054(20010901)286:9<524::AID-MAME524>3.0.CO;2-B

Shi Z, Zang S, Jiang F, et al (2012) In situ nano-assembly of bacterial cellulose–polyaniline composites. RSC Adv 2:1040–1046. https://doi.org/10.1039/C1RA00719J

Silva CJSM, Zhang Q, Shen J, Cavaco-Paulo A (2006) Immobilization of proteases with a water soluble–insoluble reversible polymer for treatment of wool. Enzyme Microb Technol 39:634–640. https://doi.org/10.1016/j.enzmictec.2005.11.016

Skočaj M (2019) Bacterial nanocellulose in papermaking. Cellulose 26:6477–6488. https://doi.org/10.1007/s10570-019-02566-y

Sulaiman S, Mokhtar MN, Naim MN, et al (2015) A Review: Potential Usage of Cellulose Nanofibers (CNF) for Enzyme Immobilization via Covalent Interactions. Appl Biochem Biotechnol 175:1817–1842. https://doi.org/10.1007/s12010-014-1417-x

Ul-Islam M, Khan T, Park JK (2012a) Water holding and release properties of bacterial cellulose obtained by in situ and ex situ modification. Carbohydr Polym 88:596–603. https://doi.org/10.1016/j.carbpol.2012.01.006

Ul-Islam M, Khan T, Park JK (2012b) Nanoreinforced bacterial cellulose–montmorillonite composites for biomedical applications. Carbohydr Polym 89:1189–1197. https://doi.org/10.1016/j.carbpol.2012.03.093

Wang P (2006) Nanoscale biocatalyst systems. Curr Opin Biotechnol 17:574–579. https://doi.org/10.1016/j.copbio.2006.10.009

Wu S-C, Wu S-M, Su F-M (2017) Novel process for immobilizing an enzyme on a bacterial cellulose membrane through repeated absorption. J Chem Technol Biotechnol 92:109–114. https://doi.org/10.1002/jctb.4994

Yang J, Ma X, Zhang Z, et al (2010) Lipase immobilized by modification-coupled and adsorption–cross-linking methods: A comparative study. Biotechnol Adv 28:644–650. https://doi.org/10.1016/j.biotechadv.2010.05.014

Yano H, Sugiyama J, Nakagaito AN, et al (2005) Optically Transparent Composites Reinforced with Networks of Bacterial Nanofibers. Adv Mater 17:153–155. https://doi.org/10.1002/adma.200400597

Yao W, Wu X, Zhu J, et al (2013) In vitro enzymatic conversion of γ-aminobutyric acid immobilization of glutamate decarboxylase with bacterial cellulose membrane (BCM) and non-linear model establishment. Enzyme Microb Technol 52:258–264. https://doi.org/10.1016/j.enzmictec.2013.01.008

Yuan H, Chen L, Hong FF, Zhu M (2018) Evaluation of nanocellulose carriers produced by four different bacterial strains for laccase immobilization. Carbohydr Polym 196:457–464. https://doi.org/10.1016/j.carbpol.2018.05.055

Zhou D, Sun Y, Bao Z, et al (2019) Improved Cell Viability and Biocompatibility of Bacterial Cellulose through in Situ Carboxymethylation. Macromol Biosci 19:1800395. https://doi.org/10.1002/mabi.201800395