Development of an antimicrobial bioactive paper made from bacterial cellulose

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Highlights

- Bacterial cellulose paper is suitable for physical adsorption of lysozyme
- Lysozyme retained activity after incorporation in BC paper matrix
- Lys-BC biopaper presented antimicrobial and antioxidant activity
- Enzymatically active paper showed high stability and durability
- Lys-BC biopaper could be suitable for active packaging

Abstract

Bacterial cellulose (BC) has emerged as an attractive adsorptive material for antimicrobial agents due to its fine network structure, its large surface area, and its high porosity. In the present study, BC paper was first produced and then lysozyme was immobilized onto it by physical adsorption, obtaining a composite of lysozyme-BC paper. The morphology and the crystalline structure of the composite was similar to that of BC paper as examined by scanning electron microscopy and X-ray diffraction, respectively. Regarding operational properties, specific activities of immobilized and free lysozyme were similar. Moreover, immobilized enzyme showed a broader working temperature and higher thermal stability. The composites maintained its activity for at least 80 days without any special storage. Lysozyme-BC paper displayed antimicrobial activity against Gram-positive and Gram-negative bacteria, inhibiting their growth by 82 % and 68 %, respectively. Additionally, the presence of lysozyme increased the antioxidant activity of BC paper by 30 %. The results indicated that BC is a suitable material to produce bioactive paper as it provides a biocompatible environment without compromising the activity of the immobilized protein. BC paper with antimicrobial and antioxidant properties may have application in the field of active packaging.

Keywords: Bacterial cellulose paper; enzyme immobilization; active packaging

1. Introduction

Food packaging is one of the most critical steps in terms of food safety. Packaging which, apart from offering a barrier to the outside environment, performs some other role, is defined as active packaging [1]. An active food-packaging can provide antioxidant and antimicrobial properties through the direct interaction of compounds with the food as well as help to remove some negative factors, such as oxygen or water vapor [2], thus improving food stability [3]. Loading of natural antimicrobial substances is receiving considerable attention as a means of inactivating bacterial cells, slowing the growth rate of microorganisms, and maintaining food quality and safety [4,5]. Lysozyme is an enzyme with antimicrobial activity that can be abundantly found in nature and is produced by plants, fungus, bacteria, birds and mammals [6]. Because of its selective activity against the cell walls of a wide variety of Gram-positive and Gram-negative bacteria [7,8], lysozyme is extensively used in the food industry and classified as GRAS [9–12]. Lysozyme has been shown to be effective as a preservative of cheeses, cow's milk, beer [13], fresh fruits and vegetables, fish and meat [14],and wine. However, direct addition of free lysozyme to food may lead to some loss of its activity because its high sensitivity and quick inactivation under different environmental conditions [15,16]. Enzymes immobilized into a polymeric matrix usually gain stability against pH, temperature, and other environmental factors [17]. An usual way to prepare antimicrobial packaging is through chemical immobilization of antimicrobial agents to the packaging material [18]. Even though an effective enzyme immobilization can be achieved by covalent binding or cross linking [19,20], these methods often require chemical modifications of the matrix and/or the use of chemical linkers that complicate the procedure, limit the functionality of the resulting composite, and generate residues harmful for the environment [21]. On the contrary, physical methods as direct adsorption are the most simple, cost-effective, and environmentally friendly techniques for enzyme immobilization [22,23].

Among the available packaging materials, cellulose-based matrices have attracted increasing interest as good carriers for a wide range of antimicrobial agents [9,24]. Bacterial cellulose (BC) – also called bacterial nanocellulose - is synthetized as an exopolysaccharide by aerobic bacteria, such as acetic acid bacteria of the genus *Komagataeibacter* [25]. Compared to plant cellulose, BC displays superior structural and mechanical properties [26,27] and has become a new basic material for advanced applications, including artificial skin, wound dressings, and scaffold for tissue engineering [28–33]. Additionally, BC is inert and biocompatible under a wide range of conditions. Owing to its large surface area, high porosity and fine network structure, BC is able to easily entrap different types of molecules [20,34,35]. Moreover, from the original produced BC, diverse matrices can be obtained showing different physical and mechanical properties that allow different applications [36–38]. Enzyme immobilization by physical adsorption onto BC has been already described for lipase onto BC nanocrystals [39] and for nisin, laccase and lipase onto BC membranes [40–42]. Lysozyme has been successfully immobilized onto BC nanofibres in suspension [16] and onto other polymeric supports as plant cellulose materials and chitosan [43–45].

Recently, it has been described the production of paper from bacterial cellulose pulp in the form of thin sheets that combine the characteristics of BC nanofibres with the stiffness and physical properties of paper [46,47]. Moreover, this material is a suitable matrix to immobilized biologically active molecules by physical adsorption [48]. Composites of metals and BC paper with antimicrobial activity has been successfully obtained [46,49]. However, to our knowledge, this is the first description regarding the combination of BC paper and the antimicrobial enzyme lysozyme. The main goal of this work was to produce a Lysozyme/BC bioactive paper and evaluate its antimicrobial properties and operational characteristics under different conditions. This study could lead to the design of a new active packaging material.

2. Material and methods

2.1. Materials

Bacterial cellulose was produced by *Komagataeibacter intermedius* JF2, a strain previously isolated in the laboratory [50]. Antimicrobial activity was tested against *Staphylococcus aureus* CECT 234, and *Escherichia coli* CECT 515. Strains were obtained from the Spanish Type Culture Collection (CECT). Lysozyme from chicken egg white (L6876) was purchased from Merck.

2.2. Production of Lysozyme/Bacterial Cellulose Paper (Lys-BCP)

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. After 7 days of static 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 to remove the bacteria. Then, the BC membranes were thoroughly washed in deionized water until the pH reached neutrality. Finally, membranes were mechanically disrupted with a blender and homogenized (Homogenizing System UNIDRIVE X1000) to obtain a BC pulp containing a suspension of BC fibres. The amount of BC was determinate by drying samples at 60 ºC until constant weight was reached. This pulp 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) of a grammage of 70 g/m^2. Adsorption of lysozyme to BCP was conducted immersing pieces of 1 cm^2 into a lysozyme binding solution (10 g/L in 20 mM KH_2PO_4, pH 6), and incubated at room temperature with slight shaking for 18 h. Then, samples were washed twice in buffer solution (20 mM KH_2PO_4, pH 6), air-dried and stored at room temperature.

2.3. Protein determination

Initial and final concentrations of protein in the lysozyme binding solution were measured according to the Bradford's protein assay [51] to determinate the content of protein in the Lys-BCP biopaper, taking into account also the residual protein in the washing solutions. Protein loading was calculated using **equation (1)** [35]**.**

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

2.4. Scanning electron microscopy (SEM)

Dried samples of Lys-BCP 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.

2.5. X-Ray Diffractometry (XRD)

Dried samples of Lys-BCP were subjected to XRD analysis (PANalytical X'Pert PRO MPD Alpha1 powder diffractometer). The samples were analysed at the radiation wavelength of 1.5418 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 bacterial cellulose was calculated based on **equation (2)** [52]:

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

(2)

where I_c is the maximum intensity of the lattice diffraction and I_{am} is the height of the intensity at the minimum at 2Θ between 18º and 19º, which corresponds to the amorphous part of cellulose.

2.6. Operational properties of the immobilized lysozyme

Lysozyme hydrolytic activity was analysed by measuring the release of MUF (methylumbelliferone) from MUF-derivate β-D-N,N',N'-triacetylchitotrioside hydrate substrate (Merck). Stock solution of MUF-substrate was prepared at 12,5 mM in dimethyl formamide (DMF): water (1:1). The working solution contained 250 µM of MUF-substrate in 20 mM KH_2PO_4, pH 6. MUF was measured using a Varian Cary Eclipse spectrofluorometer (Agilent Technologies) equipped with a microplate reader (λex = 360 nm, λem = 465 nm). One unit of activity was defined as the amount of enzyme that released one µmol of MUF per minute under the assay conditions. The specific activity and the recovery of lysozyme activity were calculated using **equation (3)** and **equation (4)**.

$$\text{Specific activity of immobilized lysozyme } \left(\frac{\text{mU}}{\text{mg protein}}\right) = \frac{\text{Activity of immobilized enzyme } \left(\frac{\text{mU/ml}}{\text{g BC}}\right)}{\text{Protein loading } \left(\frac{\text{mg protein/ml}}{\text{g BC}}\right)}$$

(3)

$$\text{Recovery of lysozyme activity } (\%) = \frac{\text{Specific activity of immobilized lysozyme}}{\text{Specific activity of free lysozyme}} \text{ x } 100$$

(4)

The determination of the operational properties of Lys/BCP biopaper was carried out immersing pieces of 1 cm^2 into 1 mL of the appropiated buffer at the conditions being analysed. Assays of free lysozyme activity were run at the same time. Optimum

temperature of free and absorbed lysozyme was determined by the analysis of the activity over a range from 30 ºC to 90 °C at pH 6. Long-term thermal stability was based on the residual activity of lysozyme measured after incubation at room temperature in 20 mM KH_2PO_4 pH 6 for a determinate period of time. Optimum pH of free and absorbed lysozyme 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).

2.7. Antimicrobial activity

The antimicrobial properties of Lys-BCP biopaper were tested against the Gram-positive bacteria *Staphylococcus aureus* and the Gram-negative *Escherichia coli* using the dynamic contact conditions test, adapted from ASTM E2149–01 (*Standard test Method for determining the antimicrobial activity agents under dynamic contact conditions*). To obtain the inoculum, the strains were grown overnight in TSB at 37°C in shaking conditions. The overnight cultures were centrifuged for 4 minutes at 14000 rpm in an Eppendorf® 52424 centrifuge, and the pellet suspended in 20 mM KH_2PO_4. Pieces of 1 cm^2 were immersed in 1 mL of a suspension of a known concentration of the microorganism and incubated at room temperature while slighty stirred. A control was run with samples of BCP under the same conditions. The viable cells on the suspension were determined at different times, and the percentage of reduction was calculated by **equation (5)**.

$$\%\ \text{cell viability reduction} = \frac{\text{viable CFU at } t_0 - \text{viable CFU at } t_x}{\text{viable CFU at } t_0} \text{ x } 100 \quad \textbf{(5)}$$

where t_0 is the time 0 h and t_x is the time at which the percentage of reduction is calculated.

2.8. Antioxidant activity

The antioxidant activity was assessed by a procedure consisting in the determination of the antioxidant capacity of insoluble components by quantification of the inhibition of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid radical ($ABTS^{+}$) [53–55]. Firstly, the ABTS* radical was pre-formed adding 140 mM potassium persulfate to 7 mM ABTS. Then, samples of 1 cm^2 of Lys-BCP and BCP were placed in microcentrifuge tubes and 1 mL of $ABTS^{+}$ was added. After that, the tubes were centrifuged at 14000 rpm in an Eppendorf® 52424 centrifuge and incubated in the darkness for 30 minutes. Finally, 900 µL of the liquid was pipetted in a cuvette and the final absorbance at 730 nm was measured in a T92+ UV Spectrophotometer (PG Instruments). A tube without sample was used as the blank. The percentage of inhibition, corresponding the percentage of antioxidant activity, was calculated according to **equation (6)**.

$$ABTS^{*}inhibition\ (\%) = \frac{A_i - A_f}{A_i}\ x\ 100 \quad \textbf{(6)}$$

where A_i is the ABTS* absorbance value of the blank, and A_f is the ABTS* absorbance value after contact with the antioxidant compound.

2.9. 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 one-way single factor or more analysis of variance (ANOVA) in STATGRAPHICS Centurion XVIII software (Statgraphics.Net, Madrid), considering a value of $p \leq 0.05$ statistically significant. Scheffe's multiple range

test was used to detect differences among mean values. 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.

3. Results and discussion

3.1. Adsorption of lysozyme and specific activity of the biopaper

Factors as the ionic strength and the charge density play an important role in the formation of complexes between proteins and polysaccharides [56]. Lysozyme adsorption was performed with a 20 mM KH_2PO_4 binding solution at pH 6. The lysozyme has an isoelectric point of 11.5 [57], therefore, at pH 6 the protein is positively charged and adsorbs on the BCP anionic surface [58]. Moreover, 6 is the optimum pH for lysozyme activity [13] which allowed maintaining the same working conditions in the determinations of enzymatic activity. In addition, it has been described that low ionic strength enhances the formation of the protein/polysaccharide composites [59]. In these conditions, lysozyme was blended by physically adsorption onto BCP at a rate of 80.39 μg/mg of BC fibres, equivalent to 0.5 mg/cm^2 of paper sheet, obtaining a Lysozyme/BCP composite (Lys-BCP) (**Table 1**).

The content of lysozyme bounded to BC was higher than previously reported for lysozyme immobilized onto chitosan supports [43,60]. Furthermore, the lysozyme absorbed in the paper made from BC showed enzymatic activity indicating that the Lys-BCP composite was biologicaly active (Table 1). Regarding specific activity, the immobilised enzyme retained over 92 % of the activity of the free enzyme, even though a decrease of specific activity is a common phenomenon described for immobilized enzymes due to a lower accessibility of substrate to active sites [61]. However, BC large surface area results in more available hydroxyl groups [62], which highly

enhances protein loading onto its fibres, displaying a higher absorption capacity when compared with wood pulp cellulose [39,63]. In addition, three-dimensional structure provided by BC nanofibres would help to retain the enzyme [48]. The results indicated that BCP is a suitable support for lysozyme binding, leading to the maintenance of a specific activity very similar to the free enzyme.

3.2. Characterization of the Lys-BCP biopaper

The morphological structure of BCP and Lys-BCP was investigated by SEM analysis (**Fig. 1)**. SEM images of the BCP surface (**Fig. 1 A**) showed a dense and homogeneous matrix of nanofibres with a diameter of 50-70 nm. Pores randomly distributed through the matrix would provide microchannels to entrap protein molecules [35]. The structure of Lys-BCP was very similar (**Fig. 1 B**). However, even though the nanofibres arrangement barely changed by the immobilization of lysozyme, a smooth layer could be observed on the surface, perhaps due to the coating of BCP by the enzyme. In the literature, other authors had also reported weak irregularities after protein immobilization, as Zhijiang et al. (2011) [64], who detected the same type of layer on the surface of a collagen/BC composite.

XRD patterns were measured to analyse microstructural changes on the BC due to the immobilization of lysozyme. **Fig. 2** shows diffraction peaks at 2Θ angles around 18,4 º and 22,7 º, the main diffraction peaks of the typical profile of cellulose in crystalline form [65]. With the introduction of lysozyme, the profile did not change. Moreover, the estimated degree of crystallinity index (equation 2) was 93 % for both of them, suggesting that crystallinity is not affected during the process of adsorption. These findings contrast with those of Bayazidi et al. [16], who registered a dramatic decrease of the degree of crystallinity after immobilizing lysozyme on BC nanofibres suspension. However, BC nanofibres suspended in aqueous medium and BCP are matrices with different fibre density and porous structure. During the process of papermaking, the

fibres of cellulose undergo dehydration through evaporation of water. This loss of water molecules produces irreversible formation of new hydrogen bounds between the hydroxyl groups of adjacent glucan chains holding the structure strongly [66]. Results indicated that no changes in the crystalline structure within the cellulose fibres did occur during the incorporation of lysozime by physical adsorption, suggesting that characteristics as mechanical strength and interfacial properties of the cellulose fibre were not modified [67].

3.3. Operational characterization the Lys-BCP biopaper

The effect of temperature, thermal stability and pH on the enzymatic activity is reported in **Fig. 3.** The optimal catalytic temperature of Lys-BCP was higher than that of free form (**Fig. 3A**): free enzyme had its optimum temperature at 37 ºC while Lys-BCP nanocomposite retained its maxim activity between 50 - 60 ºC with statistically significant differences. Changes of physical and chemical properties of the immobilized enzyme could be related with this increase of thermal stability [35]. However, food storing is usually performed at room temperature or at 4 ºC [11,18,60]. For this reason, and for taking into account for foreseeable applications, it should be pointed out that Lys-BCP had not only more relative activity but a higher specific activity than the free enzyme in the range 20 – 30 ºC. Moreover, as can be seen in **Fig. 3B**, room temperature stability was highly enhanced by the adsorption of lysozyme onto BCP: Lys-BCP residual activity was about 70 % after 2 h of incubation up to 24 h, as corroborated by the statistical analysis, whereas for the same time, free lysozyme conserved over 40 % of the activity. At 72 h, inactivation of free enzyme took place, while Lys-BCP was still active, retaining 10 % of initial activity. Lysozyme immobilized onto BCP is more stable as the restricted conformational mobility of the molecule embedded in the BCP matrix would delay the rate of inactivation [41,68].These results suggested that lysozyme would be more effective as a food preservative in an immobilization state than in a soluble bulk state. Free and immobilized lysozyme

showed similar activity at pH 6 and 7, being pH 6 the optimum. However, the immobilized enzyme was less active at pH 5 and 8 than the free enzyme (**Fig. 3C**). The stability of some enzymes at different pH can be coupled to conformational changes in the molecule [69]. Results suggested that the binding to BC nanofibres would restrict the conformational mobility of lysozyme, decreasing its activity when was not operating at optimum pH. It is worth noting that no disintegration or erosion of the biopapers was observed after 72 hours in the aqueous medium at pH 6 and room temperature.

3.4. Leaching of lysozyme from the Lys-BCP biopaper

To determinate the stability of immobilized lysozyme onto BCP, samples of the biopaper were incubated in buffer solution at room temperature. Enzymatic activity in the Lys-BCP biopaper, and enzymatic activity and protein contain in the surrounding aqueous medium, were measured at different times (**Fig. 4**). After 2 h of incubation, 20 % of the inicial Lys-BCP activity was found in the medium. This value remained nearly constant up to 48 h. The protein content in the medium also increases during the first 2 hours of incubation, corroborating activity values, and continues to increase slightly until 48 h. At that time, the amount of protein released to the medium was about 8% of the total protein absorbed in the paper. Most of the release of enzyme took place during the first 2 h in contact with the aqueous solution and could be attributed to the molecules that were loosely attached to the BC nanofibres. On the other hand, Lys-BCP biopaper retained 70 % of its original activity after 48 hours of incubation in aqueous medium. Although physical adsorption is the simplest method of immobilization, it is not always feasible. Often, as in the case of cellulose of plant origin, the interactions between the enzyme and the support are not strong enough for immobilization and prevention of leakage of the attached biomolecules [22]. In this study, only a small fraction of the lysozyme migrated from the biopaper, suggesting that

the high concentration of hydroxyl groups and the nanofibre network of the bacterial cellulose matrix was able to stabilize and retain the enzyme.

3.5. Antimicrobial activity

Lys-BCP was tested for its antimicrobial activity against Gram-positive and Gram-negative bacteria. As Sjollema et al. (2018) [70] suggested, in the evaluation of antimicrobial surface designs, a challenge concentration in line with the intented application should be applied. Considering that Lys-BCP design is most intended for prophylactic use, antimicrobial activity was evaluated at maximum challenge numbers of 1000 CFU per cm^2. In **Table 2**, is clearly shown the antimicrobial effect of Lys-BCP biopaper on the viability of *S.aureus* and *E.coli*, which showed about 82 % and 68 % reduction, respectively, after 24h. *S.aureus* was more sensitive to lysozyme than *E.coli*, probably due to the well-known differences in structure and composition of the Gram-positive and Gram-negative bacteria cell wall. Biopaper's capability to kill microorganisms in aqueous surroundings is a relevant characteristic for applications, such as food packaging design to avoid microbial spoilage [71].

3.6. Antioxidant activity

Antioxidant activity of BC paper and Lys-BCP bioactive paper was assessed**.** BCP showed some antioxidant activity (**Fig. 5**). Previous studies have reported the presence of aldehyde groups in BC [46] and its antioxidant capability [72]. Moreover, when lysozyme is immobilized onto BCP, the antioxidant activity of the bioactive paper increased about 30%, being statistically significant. Lysozyme antioxidant activity could be attributed to NH_2 residues which have the capacity to scavenge radicals [73]. This is an interesting property in materials used for active packaging applications. Antioxidant agents in coatings and packaging preserve the nutritional value and extend the shelf-life of packaged food [74].

3.7. Storage and durability

The functionality of the Lys-BCP active paper was tested over a period of 80 days of storage (**Fig. 6**). Results indicated that the biopaper could be stored at room temperature for several weeks without any statistically significant loss of enzymatic activity, and retained over 70 % of residual activity after 80 days. This high storage stability can be related to the biocompatibility and nano network structure of BC which, in a water-free environment, allowed the preservation of lysozyme without the need of special storage, such as low temperature.

4. Conclusions

In this work, a functional active paper made from BC with antimicrobial activity against Gram-positive and Gram-negative bacteria, and oxidative scavenging capacity was generated. The immobilization by physical adsorption of lysozyme onto BC paper had no negative effect on the specific activity of lysozyme or on the morphology and crystallinity of the paper. Lysozyme was stabilized and well retained in the matrix of nanofibres. Lys-BCP biopaper was active in a wide range of temperatures, showing higher stability than the free enzyme at room temperature. Additionally, it was easily stored at room temperature without any decrease in enzymatic activity for several weeks. Moreover, owning the intrinsically nature of its components, Lys-BCP active paper is biodegradable and biocompatible. Lys-BCP biopaper presents attractive features to be part of the design of new active packaging materials. It is foreseeable that other biomolecules can be physically absorbed into paper made from bacterial cellulose to obtain active papers with different functionalities.

Acknowledgments

This work was financed by the Spanish Ministry of Economy, Industry and Competitiveness, grants CTQ2017-84966-C2-2-R, FILMBIOCEL (CTQ2016-77936-R)

(funding also from FEDER) and MICROBIOCEL (CTQ2017-84966-C2-1-R), and by the Pla de Recerca de Catalunya, grant 2017SGR-30, and by the Generalitat de Catalunya, “Xarxa de Referència en Biotecnologia” (XRB). C. Buruaga-Ramiro acknowledges an APIF predoctoral grant from the University of Barcelona. Special thanks are due to the Serra Húnter Fellow to Cristina Valls.

Declaration of interest: none

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Tables

Table 1. Characteristics of lysozyme immobilized onto BCP

	Adsorbed lysozyme		Specific activity	Recovered activity
	(mg/cm^2 BCP)	**(µg/mg BC)**	**(µU/mg protein)**	**(%)**
Free lysozyme	-	-	153 ± 5	-
Lys-BCP	0.5 ± 0.13	80.39 ± 21	141 ± 16	91.9

Table 2. Viable cell counts (CFU/mL) and cell viability reduction (%) of microorganisms in contact with Lys-BCP

		CFU/mL	% reduction
Staphylococcus aureus	t_0	$2.52 \cdot 10^3$	0
	t_4	$1.46 \cdot 10^3$	46
	t_{24}	$4.50 \cdot 10^2$	82
Escherichia coli	t_0	$1.10 \cdot 10^3$	0
	t_4	$5.70 \cdot 10^2$	48
	t_{24}	$3.50 \cdot 10^2$	68

Captions to illustrations

Fig. 1. Scanning electron microscopic (SEM) images of BCP (A) and Lys-BCP (B) at two different magnifications (up 10.000 x; down 20.000 x)

Fig. 2. XRD patterns of BCP (black line) and Lys-BCP biopaper (grey line).

Fig. 3. Operational characteristics of the Lys-BCP biopaper. Data are presented as average ± standard deviation and were statistically analysed by analysys of variance (ANOVA, considering a value of $p \leq 0.05$ statistically significant. (A) Lysozyme activity at different temperatures. Activity was expressed in relative values, with the highest activity denoting 100%. Solid bars = free lysozyme, dot bars = Lys – BCP. (B) Thermal stability at room temperature under different times of incubation, where residual activity was expressed as percentage of the initial activity at time zero. Solid line = free lysozyme, dot line = Lys – BCP. (C) Effect of pH. Activity was expressed in relative values, with the highest activity denoting 100%. Solid bars = free lysozyme, dot bars = Lys – BCP

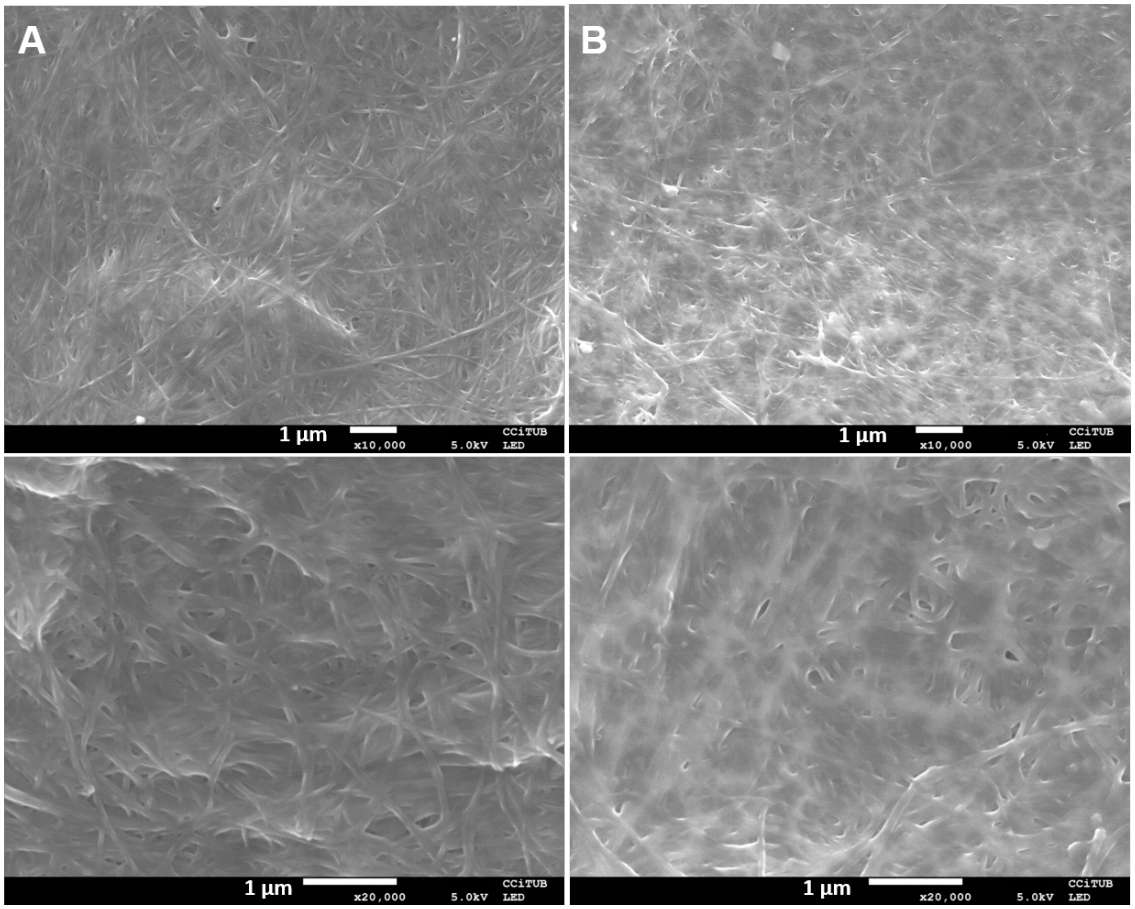
Fig. 4. Leaching of lysozyme from Lys-BC biopaper. Bars = Lys – BCP enzymatic activity; dots line = medium enzymatic activity; triangle line = medium protein concentration

Fig. 5. Antioxidant activity of BCP and Lys-BCP supports. Data are presented as average ± standard deviation and were statistically analysed by analysys of variance (ANOVA, considering a value of $p \leq 0.05$ statistically significant

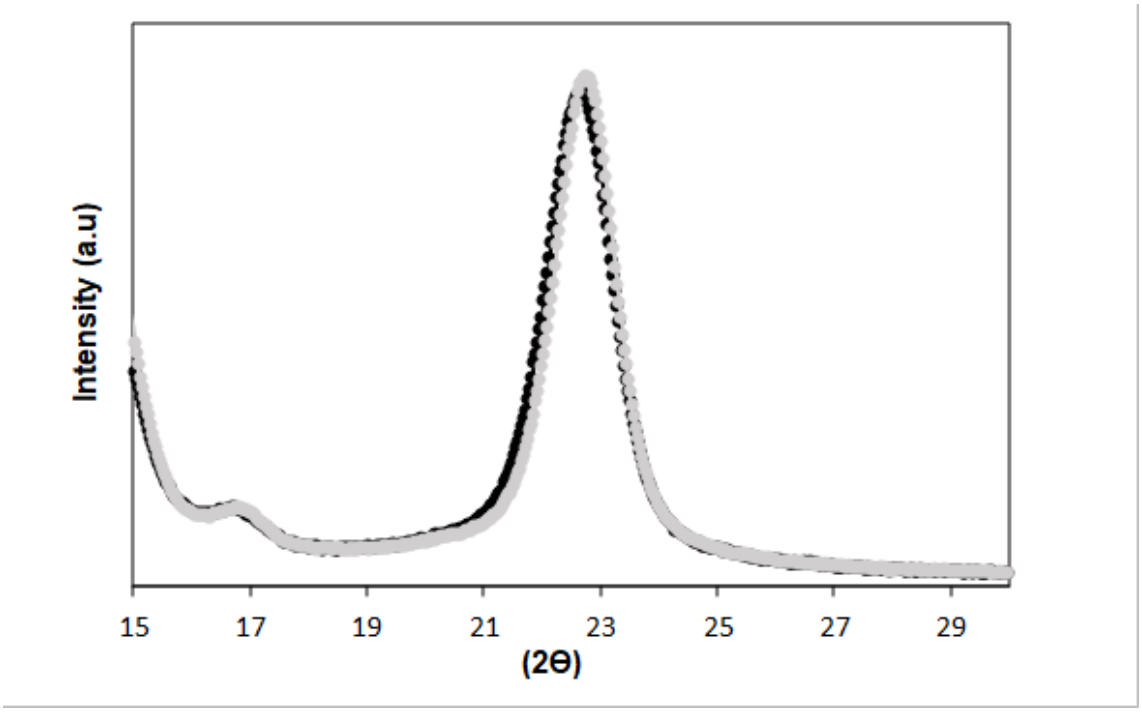
Fig. 6. Stability of Lys-BCP active paper stored at room temperature. Residual activity was expressed as percentage of the initial activity at time zero. Data are presented as

average ± standard deviation and were statistically analysed by analysys of variance (ANOVA, considering a value of $p \leq 0.05$ statistically significant

Illustrations



International Journal of Biological Macromolecules. Carolina Buruaga-Ramiro, Susana V. Valenzuela, Cristina Valls, M. Blanca Roncero, F.I. Javier Pastor, Pilar Díaz, Josefina Martinez. Fig. 1



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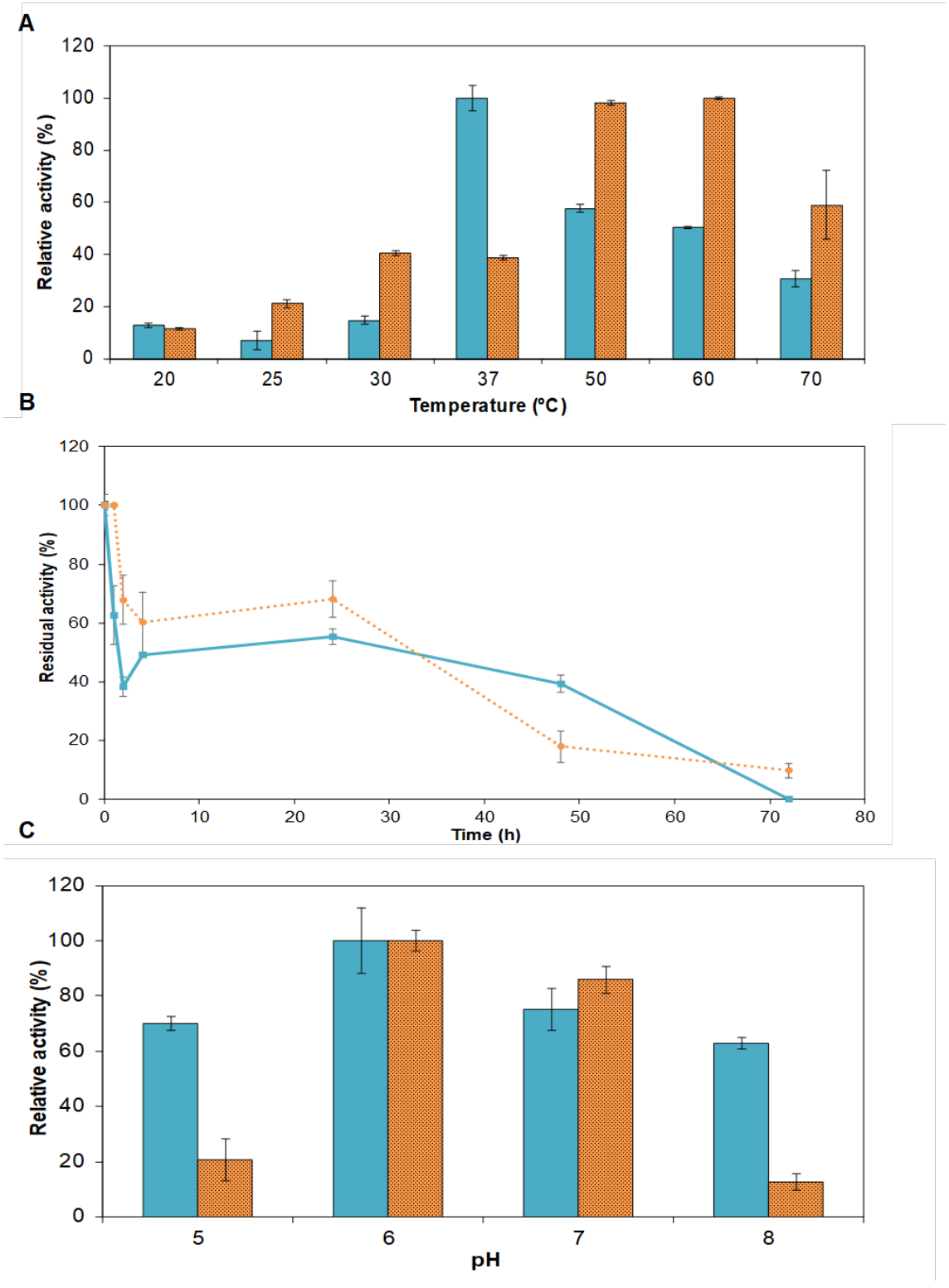

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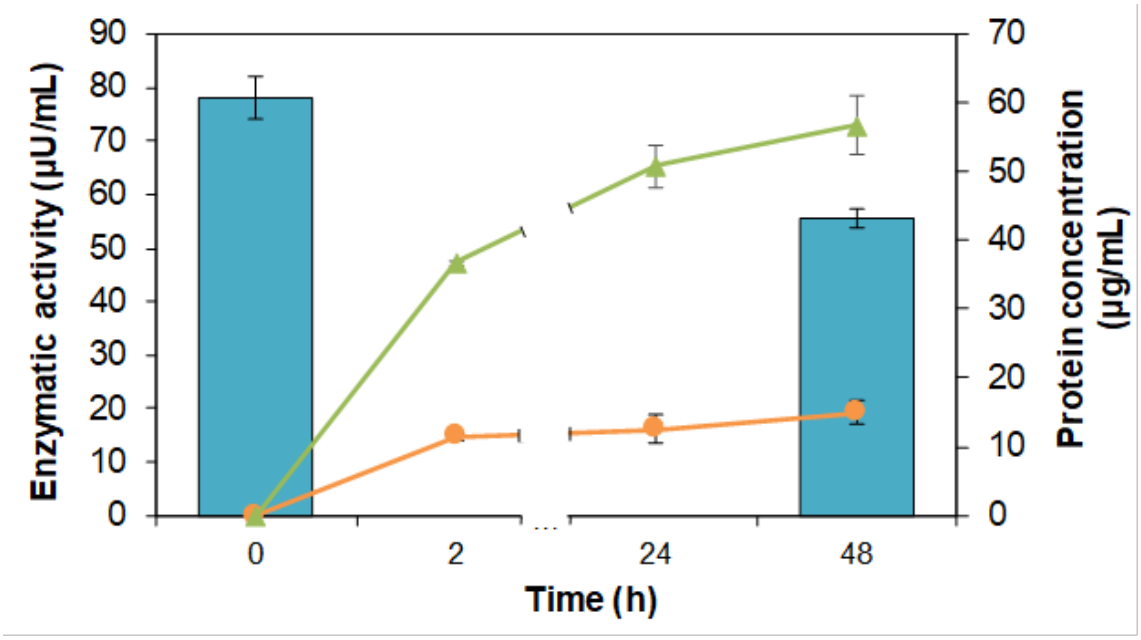

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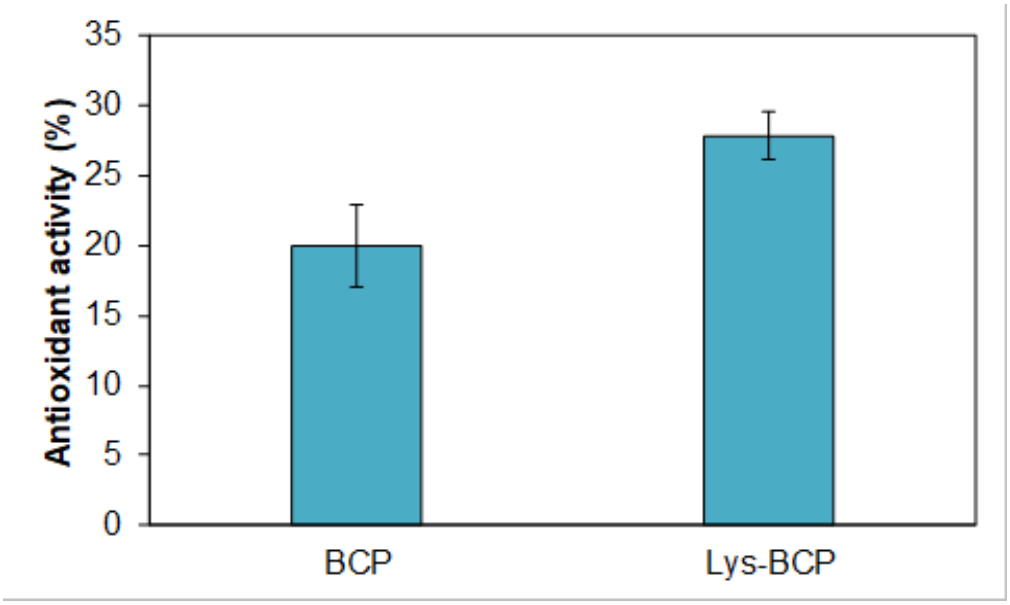

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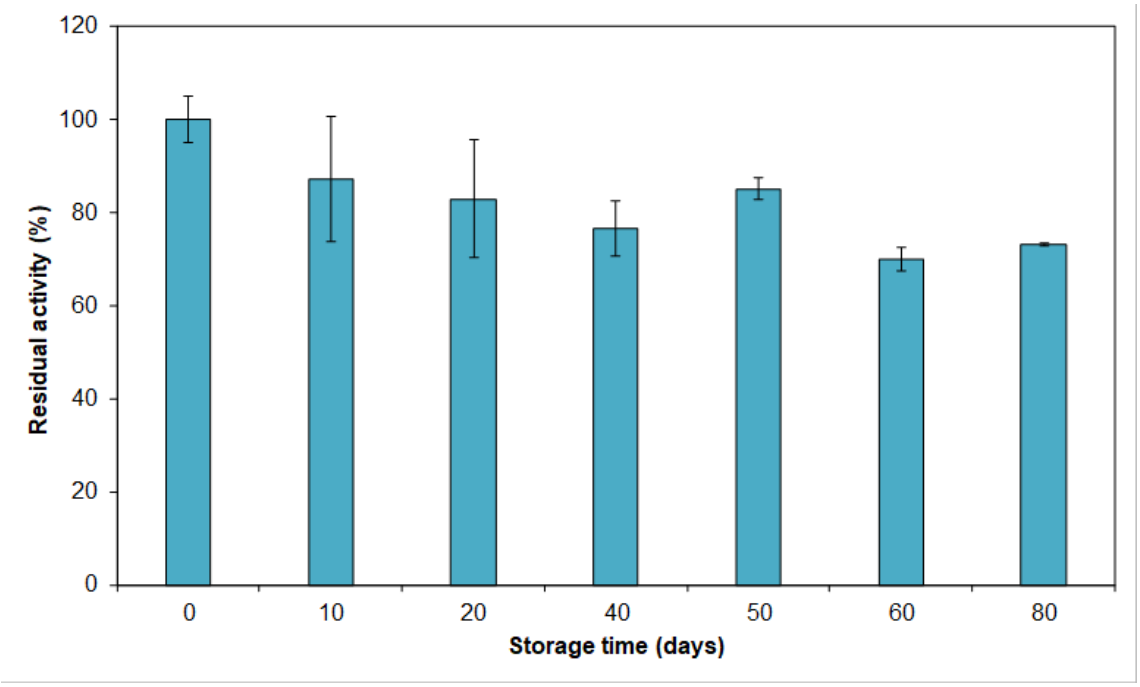

International Journal of Biological Macromolecules. Carolina Buruaga-Ramiro, Susana V. Valenzuela, Cristina Valls, M. Blanca Roncero, F.I. Javier Pastor, Pilar Díaz, Josefina Martinez. Fig. 6