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Immobilization of *Burkholderia cepacia* lipase on crosslinked chitosan-based support for the synthesis of geranyl acetate

Douglas Weber[a,b], Maria da Graça Nascimento[a], Alexandre Luis Parize[b,*]

[a] Laboratório de Biocatálise, Departamento de Química, Universidade Federal de Santa Catarina, CEP: 88040-900, Trindade, Florianópolis, SC, Brazil
[b] Grupo de Estudos em Materiais Poliméricos (POLIMAT), Departamento de Química, Universidade Federal de Santa Catarina, CEP: 88040-900, Trindade, Florianópolis, SC, Brazil

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ABSTRACT

In this study, glutaraldehyde-crosslinked chitosan beads were prepared and characterized for the immobilization of *Burkholderia cepacia* lipase (BCL, 23,000 U/g). The crosslinking time and the concentration of glutaraldehyde were evaluated. The morphological modifications and the chemical interaction between the chitosan beads and the crosslinking agent were investigated by Fourier transformed infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and ninhydrin assay. In general, both the concentration of glutaraldehyde (1.25 and 5.0% v/v) and crosslinking time (6 and 12 h) showed a minor influence on the intensity of the interaction between the crosslinking agent and chitosan. The immobilized BCL was employed in the transesterification of geraniol with vinyl acetate in an organic medium. The effects of the temperature (25–40 °C), reaction time (24–72 h), lipase mass used for the immobilization (20–100 mg) and type of organic solvent were evaluated along with the reusability of the biocatalyst. The lipase (BCL) remained stable and active under mild reaction conditions (35 °C for up to 72 h of reaction). The immobilized lipase showed greater stability in non-polar solvents (log $P > 3.0$), allowing its reuse, with a gradual loss in the catalytic activity for up to 120 days of storage. The results demonstrated that this immobilization process is efficient and of low cost.

1. Introduction

Enzymes are the catalysts evolved in nature that are involved in achieving the speed and coordination of a multitude of reactions necessary to develop and maintain life. As catalysts, enzymes bind to substrates, through multiple, predominantly non-covalent interactions, at an active site, thus lowering the activation energy of the reaction (Drauz, 2012).

Since the landmark publication by Zaks and Klibanov (1984), which described an enzyme-catalyzed process at 100 °C in organic medium, organic synthesis has been carried out with the aid of enzymes, contradicting the notion that enzymes are active only in aqueous medium. Subsequently, the number of researches and publications in this field of study has grown progressively, evolving from an academic curiosity to an industrially attractive technology area (Dwevedi, 2016).

Furthermore, enzymatic processes can be more environmentally friendly, cost-effective and sustainable than traditional chemical routes. The use of enzymes generally obviates the need for functional group protection and/or activation, affording synthetic routes that are more step-economic, generate less waste and are more energy efficient than conventional organic synthesis procedures (Sheldon and Pereira, 2017; Sheldon and Van Pelt, 2013).

Lipases (E.C. 3.1.1.3), for example, are hydrolytic enzymes of animal, microbial or plant origin and they constitute one of the most commonly used groups of enzymes in several synthetic processes (Faber, 2010). Besides being highly specific for the hydrolysis of triacylglycerides into fatty acids and glycerol, lipases exhibit a considerable enzymatic versatility, which gives them the ability to catalyze other types of reactions, such as esterification, transesterification, interesterification, and epoxidation in non-aqueous media. This remarkable feature of lipases has enhanced their importance, which has resulted in a large number of applications, such as in the production of food, detergents, pharmaceutical products, textiles, cosmetics, each other's (Kapoor and Gupta, 2012; Khan and Rathod, 2015; Pandya et al., 2014).

However, for the successful industrial application of lipases, they must be stable and fully functional under process conditions. The reaction conditions in an industrial surrounding often differs from the natural environment of enzymes in terms of, for instance, the temperature and pH conditions and the presence of organic co-solvents and

* Corresponding author. Departamento de Química, Universidade Federal de Santa Catarina, CEP: 88040-900, Florianópolis, SC, Brazil.
E-mail address: alexandre.parize@ufsc.br (A.L. Parize).

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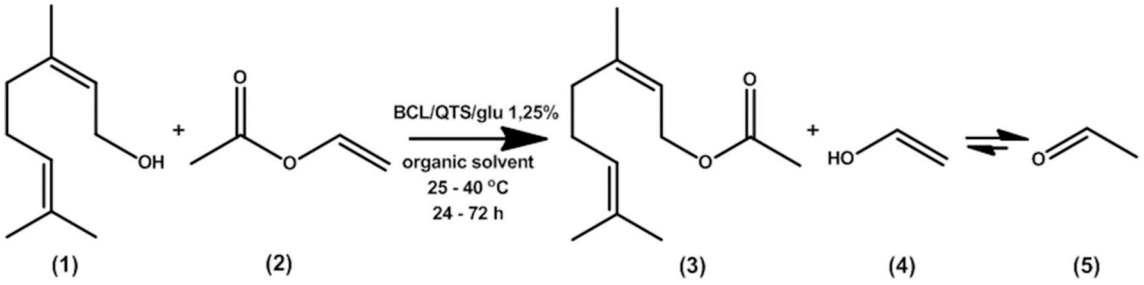

Scheme 1. Transesterification reaction of geraniol with vinyl acetate catalyzed by BCL immobilized on the chitosan-based support.

non-natural substrates and products. Consequently, physicochemical tools, such as chemical modification or enzyme immobilization, are of special interest in this context (Dwevedi, 2016; Faber, 2010; Liese and Hilterhaus, 2013).

The immobilization of enzymes is a process which consists of the confinement of the catalyst in or on a solid support (Mateo, 2007). In general, immobilization facilitates the efficient recovery and reuse of the enzyme, thus enabling its cost-effective use in, for example, repetitive batch or continuous reaction modes, e.g., using plug-flow reactors. A further benefit often encountered is enhanced stability, under both storage and operational conditions, e.g., towards denaturation by heat or organic solvents or by autolysis, although this is often combined with reduced activity compared to the soluble enzyme. Nevertheless, improved enzyme stability and repeated reuse are reflected in higher catalyst productivities (kg product per kg enzyme), which, in turn, determine the enzyme costs per kg of product (Dwevedi, 2016; Sheldon and Van Pelt, 2013; Hanefeld et al., 2009).

Furthermore, immobilization of an enzyme entails the interaction of two species: the enzyme and the carrier. The surface properties of both entities are therefore important. An essential requirement for any carrier is a large surface area. This can be achieved by using materials with small particle size, although this can hinder the separation, or with highly porous materials with pores of sufficiently large dimensions that do not limit diffusion of the substrates. Moreover, the material needs to be chemically and mechanically stable (Hanefeld et al., 2009; Vaghari et al., 2016).

Considering all these aspects, chitosan emerges as a promising alternative in the context of biocatalysis. It is a natural, non-toxic, renewable and biocompatible polymer which is suitable for applications in enzyme immobilization. Specifically, the chitosan chain is comprised of distinct functional groups that can present different reactivity characteristics. These reactive sites are versatile in terms of chemical modification, placing chitosan in the group of biomolecules with important biotechnological, biomedical and pharmaceutical applications. Indeed, the chitosan structure can be manipulated and modified by inserting new functional groups, giving to its derivatives the desired features based on the applications of interest (Dekamin et al., 2013; Singh et al., 2011).

In this context, glutaraldehyde has found widespread use in enzyme immobilization as a crosslinking agent, mainly because of its commercial availability and low cost, in addition to its high reactivity. It reacts rapidly with amine groups at around neutral pH and it is more efficient than other aldehydes in generating thermally and chemically stable crosslinks (Migneault et al., 2004).

Therefore, the combination of immobilization with chemical modification (after or before immobilization) could provide a source of new biocatalysts with even greater modifications to their properties (Rodrigues et al., 2013). In fact, enzyme immobilization continues to be a subject of immense interest, in both industry and academia. The commercial viability of industrial biotransformation is greatly influenced by the cost of the enzyme involved. Thus, immobilization is an enabling technology that, in addition to providing an active and stable biocatalyst, should involve a relatively simple operation, not requiring the preparation of a high purity enzyme or an expensive support that may not be commercially available (Sheldon and Van Pelt, 2013).

In this context, in this study, glutaraldehyde-crosslinked chitosan beads were prepared and characterized in terms of their structural and chemical modifications. *Burkholderia cepacia* PS-SD lipase (BCL) was immobilized on the beads to carry out a transesterification reaction among geraniol **1** and vinyl acetate **2** to prepare the terpenic ester geranyl acetate **3** and vinyl alcohol **4,** which tautomerizes to acetaldehyde **5**. The ester **3** is widely used as a flavor and fragrance compound in the food, drug and cosmetic industries (Khan and Rathod, 2015). Some experimental parameters such as time, temperature, enzyme loading, the influence of different organic solvents and the reusability of the immobilized lipase over several reactional cycles were evaluated (Scheme 1).

2. Materials and methods

2.1. Materials

Chitosan (99% purity), with a deacetylation degree of approximately 81% (determined by the method described by Brugnerotto et al. (2001)) and a medium molecular weight of 122.3 kg/mol), was purchased from Sigma-Aldrich. The BCL (EC 3.1.1.3, from *Burkholderia cepacia* PS-SD, activity of 23,000 U/g) was provided by Amano Enzyme Inc. (Nagoya, Japan). Geraniol (99% purity) was purchased from Sigma-Aldrich. Glutaraldehyde (50% purity) was purchased from Vetec. All other chemicals and solvents were of analytical grade.

2.2. Preparation of crosslinked chitosan beads

Chitosan powder was completely dissolved in a 2% (v/v) acetic acid solution resulting in a 2% (w/v) chitosan solution. This solution was added dropwise, using a syringe needle, into a coagulant bath of 2 mol/L sodium hydroxide solution under constant and vigorous stirring to form spherical beads. The solution was left to stand for 1 h and the spherical beads were then removed by filtration and rinsed with distilled water until neutrality was reached. Dried chitosan beads were obtained by drying at room temperature for more than 24 h.

The crosslinking process was carried out by the method described by Cavello et al. (2014), with small modifications. In this procedure, an aliquot of chitosan beads (300 mg) was incubated with varying concentrations of glutaraldehyde (1.25 and 5.0% v/v) for 6 and 12 h, at 35 °C, under constant stirring. After this time, the excess glutaraldehyde was removed by washing with distilled water and the solution left to dry at room temperature. The microparticles obtained were stored for characterization and reuse for lipase immobilization.

2.3. Characterization of crosslinked chitosan beads

The chitosan beads and crosslinked chitosan beads were characterized after being ground, in KBr pellets, by infrared spectrophotometry (FTIR, Prestige-21 Shimadzu spectrophotometer). The morphology and the internal appearance of the microparticles were

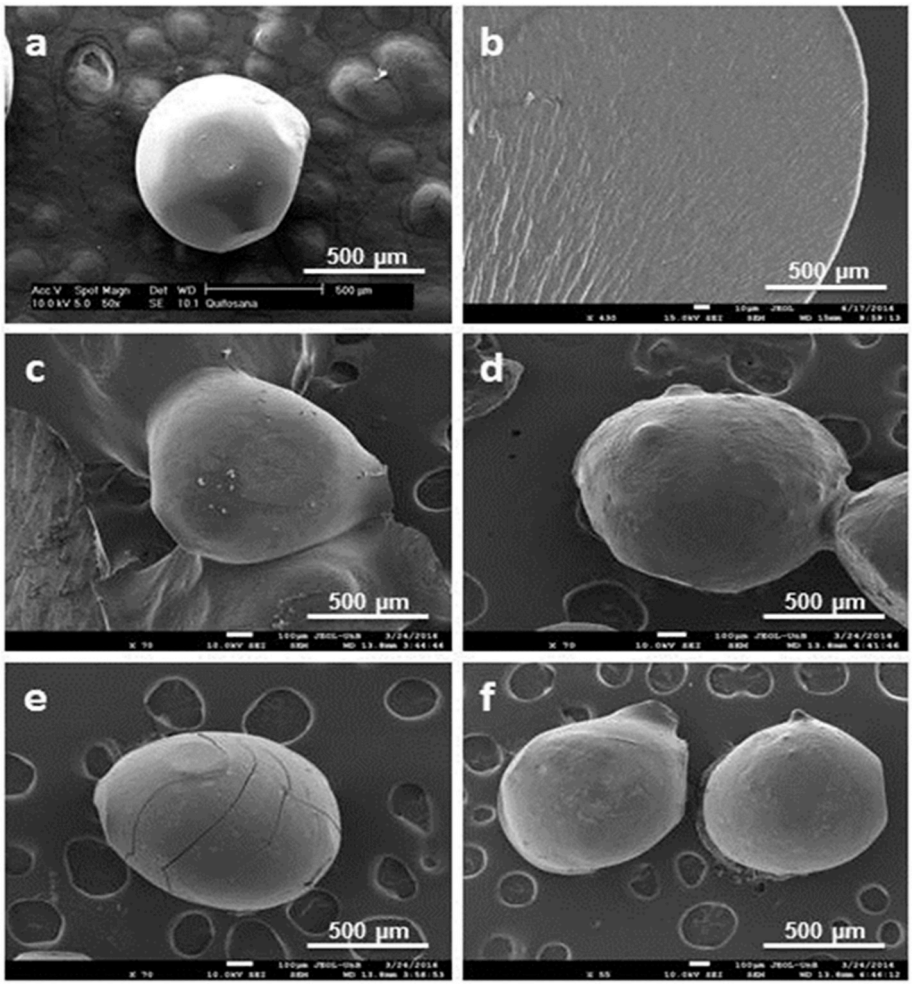

Fig. 1. SEM images of the chitosan-based supports used in this study: (a) external and (b) internal images of an uncrosslinked chitosan bead; (c) and (d) chitosan beads crosslinked with 1.25% (v/v) glutaraldehyde for 6 and 12 h, respectively; (e) and (f) chitosan beads crosslinked with 5.0% (v/v) glutaraldehyde for 6 and 12 h, respectively (Scale bar: 500 µm).

analyzed by scanning electronic microscopy (SEM, JEOL model JSM-7001F).

The residual free amino group after crosslinking was determined by the ninhydrin assay following the method described by Yuan et al. (2007) with small modifications, in which the percentage of free amino groups in the crosslinked chitosan beads was defined. The percentage of free amino groups was then calculated according to Eq. (1):

$$\text{Free amino group content } (\%) = [\text{Abs}_{\text{crosslinked}}/\text{Abs}_{\text{uncrosslinked}}] \times 100 \quad (1)$$

where $Abs_{crosslinked}$ is the mean absorbance value of the crosslinked chitosan beads, and $Abs_{uncrosslinked}$ is the mean absorbance value of the uncrosslinked chitosan beads.

The absorbance at 570 nm was measured on an 8452A Diode Array UV/VIS spectrophotometer, zero-set against a similarly treated blank of water. All experiments were performed in triplicate and the data reported are averages.

2.4. BCL immobilization

The immobilization of BCL on crosslinked chitosan beads was carried out by adding 300 mg of dried chitosan beads to a solution composed of 10 mL of a 0.1 mol/L sodium phosphate buffer solution (pH 7.2) and a known amount of BCL (20–100 mg). The immobilization process was conducted at 4 °C, without stirring, for 48 h. After this period of time, the immobilized BCL was separated by filtration and washed with distilled water to remove the excess of buffer solution. It was then dried at room temperature and kept at 4 °C for later use.

2.5. Protein content assay

The amount of protein was determined by the Bradford method at a wavelength of 595 nm using bovine serum albumin as the internal standard reference. This assay was performed in triplicate to minimize errors and the data reported are averages (Bradford, 1976). The amount of protein in the chitosan-based support before the immobilization of BCL was also determined and subtracted from the data obtained after immobilization.

2.6. Preparation of geranyl acetate

The enzymatic preparation of geranyl acetate with the immobilized lipase was conducted using geraniol and vinyl acetate as substrates. The conditions for the synthesis were as follows. In a typical reaction, 300 mg of chitosan-based support containing the immobilized lipase, 10 mmol of geraniol (1.7 mL), 10 mmol of vinyl acetate (0.9 mL) and 25 mL of organic solvent were placed in a 125 mL Erlenmeyer flask and kept in a thermostatic bath with orbital shaking (Technal TE-0532). The enzymatic reactions were performed applying different temperatures (25–40 °C) and times (24–72 h). A control reaction (with no enzyme) was performed under the same reaction conditions.

The influence of different solvents was also investigated. In this

study, 20 different organic solvents were used under the same reaction conditions described above (at 35 °C for 48 h).

The reusability was examined by repeated use of the immobilized lipase to catalyze the same reaction. The immobilized lipase was reused six times at 35 °C, for 48 h, using *n*-hexane as the solvent. After each reaction, the support was washed several times with organic solvent, to remove the substrates and product, and stored at 4 °C for later reuse.

The storage time was also evaluated with the use of the same support containing the immobilized lipase to synthesize geranyl acetate after a certain period of time following the first use of that support (30–120 days, 35 °C, for 48 h).

The conversion degrees in each study were calculated based on the amount of geraniol converted to geranyl acetate. The product was characterized by ^{1}H NMR (400 MHz, in $CDCl_3$), in which the formation of ester was quantified by comparing the relative areas on the images at 4.1 and 4.6 ppm, corresponding to the hydrogens of the $–CH_2$ group next to the hydroxyl and acetate groups, respectively.

3. Results and discussion

3.1. Preparation and characterization of the crosslinked chitosan beads

Chitosan is insoluble in water, but the presence of amino groups allows it to be soluble in acidic solutions below pH around 6.5 (Pospiskova and Safarik, 2013). In this study, an acidic solution of chitosan (2% w/v) with a deacetylation degree of 81% was added dropwise to a strong basic solution through a needle. The gel beads obtained were then neutralized and dried for later activation and the immobilization of BCL.

As shown on the scanning electron micrographs in Fig. 1, spherical and compact microspheres with a homogeneous morphology were obtained.

The mean diameter of the chitosan microspheres was 850 μm. The surface of the uncrosslinked chitosan beads was rigid and uniform, showing good sphericity. The same aspects were observed internally when a fraction of a chitosan bead was analyzed by SEM (Fig. 1a and b). The chitosan beads presented some structural modifications after crosslinking, as can be observed in Fig. 1c–f. The surface of the crosslinked chitosan beads was no longer uniform, showing some irregularities. In Fig. 1e, for example, it can be observed that the aspect of the beads that were crosslinked with 5.0% (v/v) glutaraldehyde for 6 h had changed significantly compared with their original aspect, presenting some fissures and a brittle form after the activation time. In addition, the color was also modified after crosslinking. The crosslinked beads were either red or yellow (when crosslinked with 1.25% or 5.0% glutaraldehyde (v/v), respectively), while the uncrosslinked beads were beige or very pale (results not shown).

Fig. 2 shows the FTIR spectra for the chitosan beads crosslinked with varying concentrations of glutaraldehyde for 6 h and 12 h at 35 °C and uncrosslinked. On these spectra, the main characteristic bands of the chemical groups present in the polymeric chain of uncrosslinked chitosan can be observed, as well as the interactions occurring between the chitosan and glutaraldehyde. These results characterize the formation of a new polymeric matrix.

In these spectra, four typical bands were highlighted, as shown in Fig. 2. The first is that observed at 1258 cm^{-1}, which is related to the protonated amino groups in the chitosan chain. A decrease in the intensity of this band after crosslinking is expected, suggesting that the amino groups have interacted with the functional groups of glutaraldehyde. A second band was observed at 1562 cm^{-1} and this is related to the ethylenic group of glutaraldehyde. In fact, Li et al. (2013), reported that an increase in glutaraldehyde in the modified chitosan caused a successive increase in the intensity of the ethylenic bond frequency at 1562 cm^{-1}.

A third band was present at 1593 cm^{-1} in the uncrosslinked chitosan spectrum, but this was less perceptible in the crosslinked chitosan samples. This band is related to the deformation vibration of the NH primary amine ($-NH_2$) and a decrease in intensity is expected in the crosslinked chitosan samples, verifying the chemical interaction between the biopolymer and glutaraldehyde (Silverstein et al., 2005).

Finally, the increase in the intensity of the band observed at 1659 cm^{-1} is related to the formation of a C=N bond (Schiff Base) between the amino groups of the chitosan and the carbonyl groups of glutaraldehyde. This band is of low intensity in the uncrosslinked chitosan spectrum (Fig. 2A) and it may be related to the carbonyl groups of the acyl groups that are still present in the polymeric chain of chitosan, since it is only 81% deacetylated. However, as can be observed in Fig. 2B–E, the intensity of this band increases, this being a strong indication of the formation of a new bond.

In this context, Knaul et al. (1999) found that a chitosan film which reacted with glutaraldehyde exhibited a strong absorbance at 1664 cm^{-1}, while Gupta and Jabrail (2006) reported that the infrared spectra for glutaraldehyde crosslinked chitosan microspheres showed a strong absorption band at 1660 cm^{-1}. Also, Andriani et al. (2015) reported that chitosan nanoparticles crosslinked with glutaraldehyde showed a band in 1627 cm^{-1}, which was related to an imine group, C=N. These data are consistent with the band observed at 1659 cm^{-1} for the chitosan beads crosslinked with varying concentrations of glutaraldehyde, confirming the interaction between them.

However, as can be also observed in Fig. 2B–E, the intensities of the C=N bands are very similar. This observation indicates that varying the two parameters (concentration of glutaraldehyde and the time of crosslinking reaction) did not lead to significant differences in terms of the biopolymer interaction with the crosslinking agent.

Therefore, two conclusions can be drawn from the FTIR analysis. Firstly, the chitosan beads interacted with the glutaraldehyde, since the imine group band was observed in the crosslinked chitosan beads, and secondly, the interaction of chitosan with glutaraldehyde was not significantly affected by the amount of glutaraldehyde or the time of crosslinking. Thus, the conditions of 1.25% (v/v) of glutaraldehyde and 6 h of crosslinking reaction were selected for the immobilization of the lipase (BCL) and for further studies on the application of the immobilized lipase.

In addition, when the support prepared with higher concentration of glutaraldehyde (5% v/v) was used for the immobilization of BCL, no improvements on the immobilization efficiency (amount of BCL immobilized per mg of support used) was observed, mainly because aldehydes (such as glutaraldehyde) have inhibitory effects on the enzymatic activity when used in high concentrations. This crosslinker not only can cause damage to the active sites of the enzyme but also has adverse health effects to humans. The toxicity of aldehydes to enzymes is well known and described in literature (Cavello et al., 2014; Karnchanajindanun et al., 2011). This reason reinforced the choice for using smaller concentration of glutaraldehyde for the preparation of the beads.

A ninhydrin assay was conducted in order to quantify the amount of free amino groups remaining in the chitosan chain after crosslinking. Thus, the percentage of free amino groups in the crosslinked beads was calculated based on the amino groups remaining after the crosslinking reaction, applying the different glutaraldehyde concentrations (1.25 and 5.0% v/v) and reaction times (6 and 12 h), compared with the uncrosslinked chitosan beads, in which the free amino group content was assumed to be equal to its deacetylation degree (81%).

The reaction of ninhydrin with a primary amino group forms a colored reaction product, diketohydrindylidene-diketohydrindamine, also called Rüehmann's purple (Prochazkova et al., 1999). The reaction of the crosslinked chitosan microspheres with ninhydrin was accurate, reproducible and sensitive. The results are shown in Fig. 3.

As can be observed in Fig. 3, with the same glutaraldehyde concentration, the free amino group content decreased with increasing crosslinking time, as expected. Also, the content of free amino groups decreased with increasing glutaraldehyde concentration for both

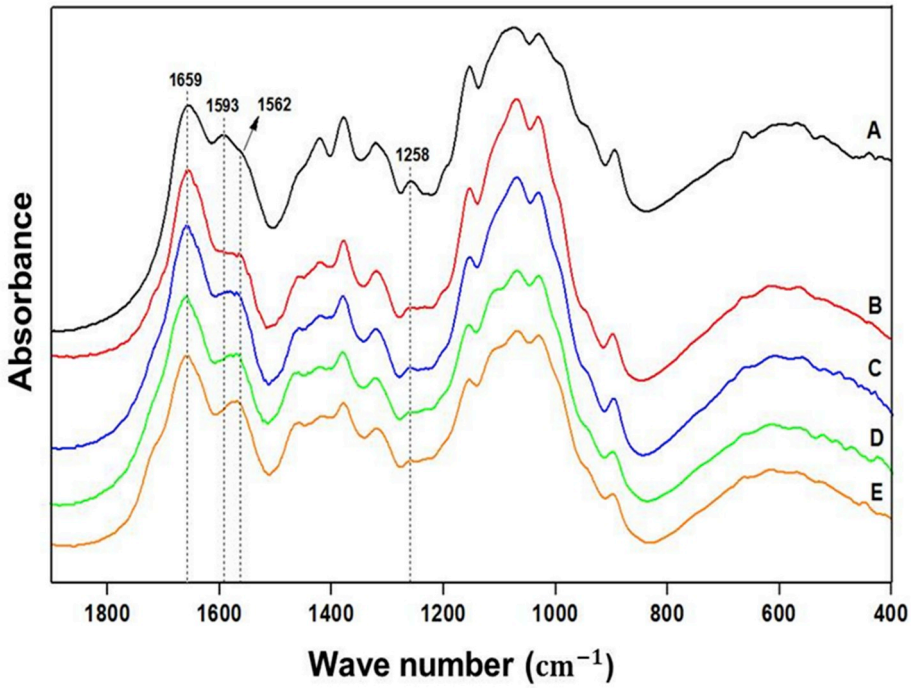

Fig. 2. FTIR spectra for the chitosan beads: **(A)** uncrosslinked chitosan and chitosan crosslinked with 1.25% (v/v) glutaraldehyde for **(B)** 6 h and **(C)** 12 h, and chitosan crosslinked with 5.0% (v/v) glutaraldehyde for **(D)** 6 h and **(E)** 12 h (KBr pellets).

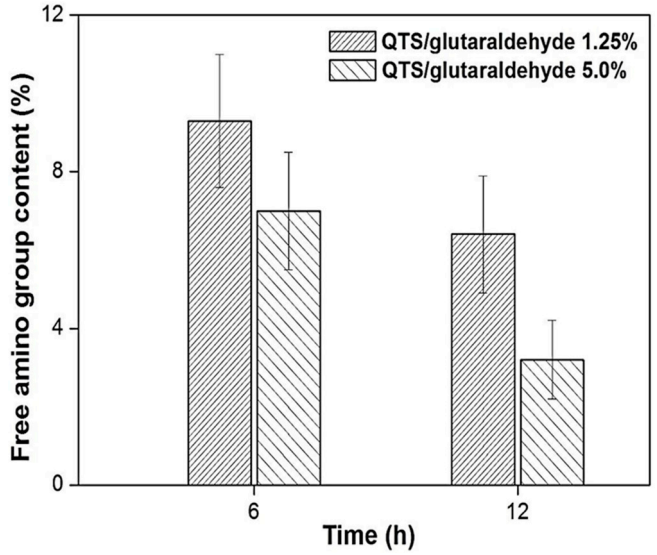

Fig. 3. The residual free amino group content (%) on the crosslinked chitosan beads.

reaction times. In addition, the chitosan-based supports all showed a high degree of crosslinking, since the free amino content did not exceed 10%. These data demonstrate that the chitosan microspheres were successfully crosslinked by glutaraldehyde and that the degree of crosslinking increased on increasing the concentration of the crosslinking agent or the reaction time.

3.2. Preparation of geranyl acetate

Early experiments for this study were carried out employing the commercially available lipase from *Rhizopus oryzae* (Amano, lipase F-AP15). This lipase was first chosen based on previous works done in our group, which used it either in its free form or immobilized in a solid supports. For instance, F-AP15 lipase was used as biocatalyst for the (chemo)-enzymatic epoxidation of cyclohexene (Moreira et al., 2005) and in the epoxidation of β-caryophyllene (Da Silva and Nascimento, 2014), showing good catalytic activity on both studies. Thus, the goal here was to check whether this lipase would be also suitable or not, to catalyze the intended reaction (transesterification of geraniol into its corresponding acetate compound). However, the conversions into the ester **3** were very low, either using free or immobilized lipase on the crosslinked chitosan beads, under the same reaction conditions (*n*-hexane as organic solvent, 35 °C, up to 72 h of reaction).

Due to this, BCL was taken as second option and the results obtained herein were much more promising. Thus, from this point and on, all the following experiments were carried out employing BCL (free or immobilized) as biocatalyst. Regarding the superiority of BCL to produce geranyl acetate, BCL has been described as a very robust and versatile biocatalyst and has been used to obtain compounds with high optical purity (high enantioselectivity). This lipase is also stable under temperature and pH changes, showing higher enzymatic activity at 50°C and the pH range of 7–8, as reported elsewhere (Sasso et al., 2016). Besides, BCL has been reported as highly stable when interacting with several organic solvents, such as methanol, which is a promising feature for organic synthesis with the aid of enzymes (Pan et al., 2010). Thus, for all these reasons, BCL immobilized on the chitosan support has shown to be a good choice to produce geranyl acetate.

3.2.1. Lipase loading

In a first approach, the loading of lipase (BCL) was evaluated using different amounts of this lipase to catalyze the reaction applied to obtain geranyl acetate. In this study, increasing amounts (20–100 mg) of BCL were dissolved in 1.0 mL of a 0.1 mol/L sodium phosphate buffer solution (pH 7.2) and added to 300 mg of chitosan beads crosslinked with 1.25% (v/v) glutaraldehyde (6 h of crosslinking). No enzyme was used in the control reaction. Fig. 4 shows the degrees of conversion for the preparation of the terpenic ester **3** (Scheme 1).

As shown in Fig. 4, the degree of conversion of geraniol **1** to geranyl acetate **3** increased as the initial amount of BCL increased from 0 to 40 mg/mL, at which the maximum percentage of conversion was observed (40%). However, the conversion degree decreased (from 40 to

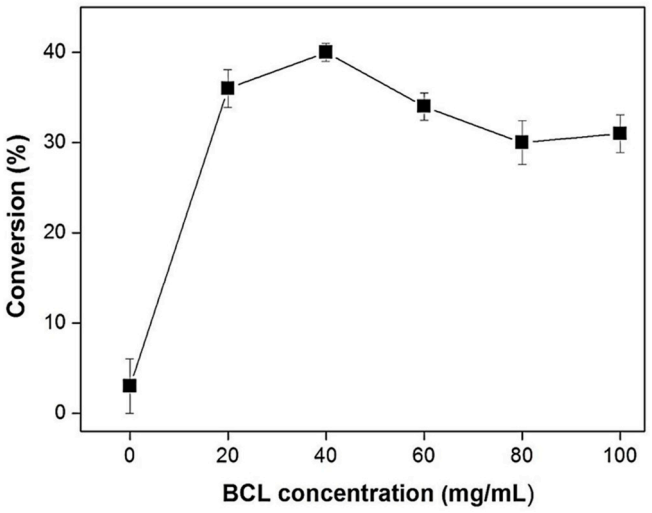

Fig. 4. Influence of the concentration of BCL used for immobilization on the glutaraldehyde-crosslinked chitosan support (QTS/glutaraldehyde 1.25% (v/v), 6 h). [Reaction conditions: geraniol (10 mmol), vinyl acetate (10 mmol), *n*-hexane (25 mL), 48 h, 35 °C, 300 mg of support, 150 rpm.].

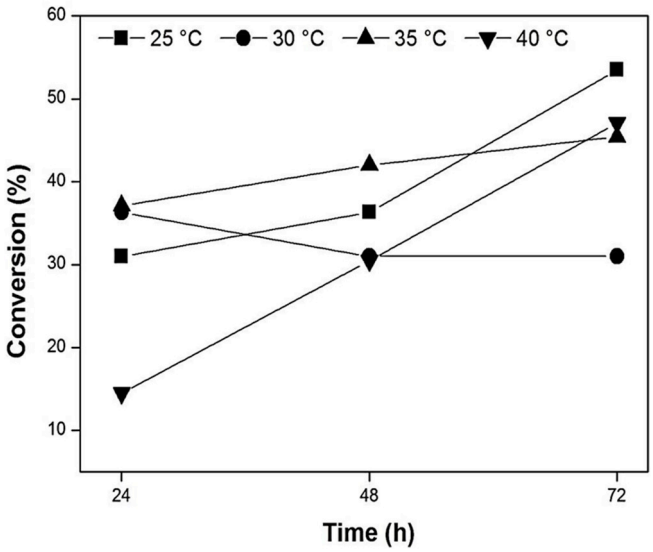

Fig. 5. Influence of time and temperature on the performance of lipase (BCL) immobilized on the chitosan-based support (QTS/glutaraldehyde 1.25% (v/v)/ 6 h) as a catalyst in the preparation of geranyl acetate. [Reaction conditions: geraniol (10 mmol), vinyl acetate (10 mmol), 40 mg/mL of BCL loaded in 300 mg of crosslinked-chitosan beads, *n*-hexane (25 mL), 150 rpm.].

30%) as the lipase loading was further increased from 40 to 100 mg/mL. Thus, the amount of 40 mg/mL was chosen for the subsequent studies on the immobilized lipase.

The reactions employing the free lipase (BCL) showed higher degrees of conversion to the terpenic ester **3**, varying from 61 to 76% (results not shown).

These observations can be explained by the fact that there are no specific trends that help to predict whether an enzyme will be more stable and active after immobilization or not. In other words, each immobilization method may have inherent advantageous and disadvantageous (Faber, K., 2010; Liese, A. and Hilterhaus, L., 2013).

However, it is well known that an enzyme is usually more resistant to pH and temperature variations, as well as the influence of the organic medium, when immobilized on a support rather than present in its free form. This observation is attributed to the fact that immobilized enzymes are less susceptible to inactivation because their structure is more rigid (Faber, K., 2010).

Immobilization methods have another important advantage which is that the enzyme can be reused in successive reaction cycles. Thus, although the free BCL showed greater activity during the preparation of ester **3**, its immobilization on the chitosan-based support enabled its recovery and reuse, validating the method.

The protein content determined for the BCL immobilized on this chitosan-based support was 0.91 ± 0.13 mg/g of support.

3.2.2. Influence of the organic solvent

In this study, BCL was immobilized on the chitosan-based support crosslinked with 1.25% (v/v) glutaraldehyde (6 h of crosslinking) and the influence of the organic solvent was evaluated. Twenty organic solvents with different log *P* values (Laane et al., 1987) were evaluated in the transesterification reaction of geraniol with vinyl acetate: *n*-heptane (log *P* 4.00) *n*-hexane (log *P* 3.50), cyclohexane (log *P* 3.20), toluene (log *P* 2.50), chloroform (log *P* 2.00), diisopropyl ether (log *P* 1.70), *tert*-butanol (log *P* 1.45), methyl *tert*-butyl ether (MTBE, log *P* 1.43), dichloromethane (log *P* 0.93), ethyl ether (log *P* 0.85), ethyl acetate (log *P* 0.68), tetrahydrofuran (THF, log *P* 0.49), propyl alcohol (log *P* 0.25), isopropyl alcohol (log *P* 0.05), acetone (log *P* −0.23), ethanol (log *P* −0.24), acetonitrile (log *P* −0.33), methanol (log *P* −0.76), 1,4-dioxane (log *P* −1.1), and petroleum ether (no log *P* available).

The influence of organic solvents in biotransformations and enzyme-catalyzed processes is well established in the literature (Laane et al., 1987; Silva and Nascimento, 2016; Nascimento et al., 2015). Several studies have shown that the reactions carried out using solvents with log $P > 4.0$ tend to be more efficient, since these are able to trap the water around the enzyme, creating a micro aqueous layer, which helps to maintain the active conformation of the enzyme, preserving its catalytic activity. On the other hand, polar solvents (log $P < 3.0$) tend to alter the amount of water needed for the enzyme to maintain its catalytic activity, compromising their stability and the capacity to conduct reactions (Silva and Nascimento, 2016; Nascimento et al., 2015).

As can be observed in Fig. 5, the degree of conversion of geraniol to geranyl acetate was dependent on the organic solvent used to carry out the transesterification reaction. When *n*-hexane was used as the solvent, the degree of conversion to the terpenic ester **3** was 40%. On using other non-polar solvents (log $P > 3$), such as *n*-heptane and cyclohexane, the conversion degrees were good to moderate (36 and 23%, respectively). Good conversion degrees were also observed using solvents with moderate polarity. With the use of diisopropyl ether, toluene, and methyl *tert*-butyl ether, for instance, the conversion degrees were 29, 17, and 13%, respectively.

When the more polar solvents (log $P < 1.43$), such as dichloromethane, chloroform, ethyl ether, tetrahydrofuran, acetonitrile and isopropyl alcohol, were used, the conversion degrees were low (< 5%). In fact, when polar solvents, such as methanol, ethanol and propyl alcohol, were used, the formation of the product **3** was not detected, indicating that these solvents in some way influenced the BCL conformation, causing its inactivation, or they were not effective in solubilizing the reagents, thus hindering the reaction (Andriani et al., 2015).

However, it is interesting to note that when acetone and 1,4-dioxane (log $P < 0.0$) were used, the conversion degrees to **3** were 13 and 12%, respectively, showing that some aprotic polar solvents do not affect the catalytic activity of immobilized BCL. In general, no direct relation between the conversion degrees and log *P* values was observed.

The selection of an organic solvent for synthetic processes is also dependent on other factors, such as the health criterion. According to this criterion, each solvent is scored between 1 and 10, in which the highest value is related to solvents that result in little or no damage to human health (e.g. water), while the lowest value relates to potentially

Table 1
Influence of organic solvents on the preparation of geranyl acetate **3** using BCL immobilized on chitosan-based support crosslinked with 1.25% (v/v) glutaraldehyde, 6 h[a].

Entry	Solvent	Log *P* [b]	Health criterion [c]	Conversion (%)[d]
1	*n*-heptane	4.00	8	36
2	*n*-hexane	3.50	4	40
3	petroleum ether	–	5	18
4	cyclohexane	3.20	7	23
5	toluene	2.50	4	17
6	chloroform	2.00	3	< 5
7	diisopropyl ether	1.70	6	29
8	*tert*-butanol	1.45	6	10
9	MTBE	1.43	5	15
10	dichloromethane	0.93	4	5
11	ethyl ether	0.85	5	8
12	ethyl acetate	0.68	8	5
13	THF	0.49	6	< 5
14	propyl alcohol	0.25	5	< 5
15	isopropyl alcohol	0.05	8	< 5
16	acetone	−0.23	8	13
17	ethanol	−0.24	8	< 5
18	acetonitrile	−0.33	6	< 5
19	methanol	−0.76	5	< 5
20	1,4-dioxane	−1.10	4	12

[a] Reaction conditions: geraniol (10 mmol), vinyl acetate (10 mmol), 40 mg/mL of BCL loaded in 300 mg of crosslinked-chitosan beads, organic solvent (25 mL), 48 h, 35 °C, 150 rpm.
[b] Laane et al., 1987.
[c] Curzons et al. (1999); Prat et al. (2014).
[d] Determined by ^{1}H NMR.

hazardous solvents that can, in some circumstances, cause injury through mishandling and/or following inhalation or accidents (Prat et al., 2014; Curzons et al., 1999).

In Table 1, it can be observed that for each solvent the health criterion is listed. It should be noted that this criterion has no direct relationship with the log *P* parameter, which reflects the relationship between the organic solvent and the lipase activity. Thus, a solvent that may be good at preserving the lipase activity may be hazardous for human health, for instance, *n*-hexane. This solvent has a log *P* value of 3.50 and thus it is ideal for use in biotransformations because it tends to preserve the stability of the biocatalyst. However, its health criterion is 4, which means its use is not recommended, since it is potentially hazardous to health.

It is therefore clear that it is important to handle these substances very carefully and make use of protective equipment and clothing, besides having the necessary knowledge and training to work safely in the laboratory.

3.2.3. Influence of reaction time and temperature

Biotransformations are generally dependent on the time and, more notably, on the temperature. The temperature can alter the reaction rate besides affecting the conformation, activity, stability, and selectivity of biocatalysts (Faber, 2010).

Thus, in the next part of this study, the lipase (BCL) immobilized on the chitosan-based supports, crosslinked with 1.25% (v/v) glutaraldehyde for 6 h, was used in the reaction of geraniol with vinyl acetate in *n*-hexane applying different times (24, 48, and 72 h) and temperatures (25, 30, 35, and 40 °C). For comparison, control reactions were carried out in the absence of lipase under the same experimental conditions, and the terpenic ester (3) was detected in conversion degrees of < 5%.

Fig. 5 shows the plot obtained in the study on the time and temperature applied for the preparation of **3**.

As shown in Fig. 5, both the reaction time and temperature influenced the activity of the immobilized lipase in the catalysis of the transesterification reaction studied.

The reactions studied showed interesting results regarding the influence of time and temperature on the activity of the immobilized lipase. Regardless of the temperature, in the majority of the reactions performed the conversion degree increased as the time of reaction increased, except for the reactions conducted at 30 °C, for which the opposite effect or no variation in the conversion degree was observed. For the reactions carried out at lower temperatures (25 and 35 °C), the degree of conversion increased only slightly with the reaction time, while at 40 °C the increase was more evident.

According to Faber, higher temperatures may have a negative effect on the enzyme activity by lowering the deactivation energy more than the activation energy of the enzymatic reaction, resulting in the deactivation process becoming more rapid (Faber, 2010).

However, for the reactions performed in this study the highest conversion was obtained after 72 h of reaction at 40 °C (~47%). This observation provides evidence that the immobilization process enabled the lipase to catalyze the reaction at high temperatures without compromising its catalytic activity.

Due to practical purposes, the reaction time of 48 h was chosen to conduct the study regarding the reusability of the biocatalyst. Therefore, the best temperature to perform transesterification reactions of 1 under this condition was 35 °C since the higher conversion degrees for the reactions conducted for 48 h were obtained employing this temperature (42%).

3.2.4. Recycling of the immobilized lipase

One of the main objectives of enzyme immobilization is to recuperate the biocatalyst at the end of the reaction, allowing its reuse and making the process less onerous and more sustainable (Faber, 2010; Liese and Hilterhaus, 2013; Hanefeld et al., 2009).

Thus, to evaluate the efficiency of using BCL immobilized on a chitosan-based support, the transesterification reaction of geraniol with

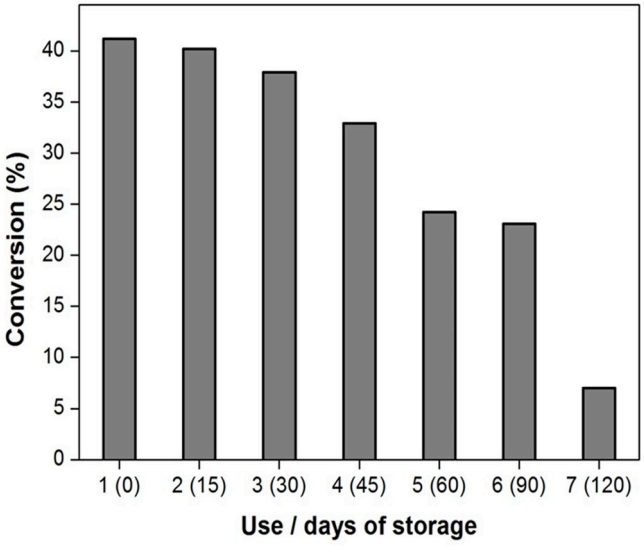

Fig. 6. Effect of number of uses and storage time on the lipase (BCL) immobilized on chitosan-based support crosslinked with 1.25% (v/v) of glutaraldehyde for 6 h [Reaction conditions: geraniol (10 mmol), vinyl acetate (10 mmol), 40 mg/mL of BCL loaded in 300 mg of crosslinked-chitosan beads, *n*-hexane (25 mL), 150 rpm, 48 h, 35 °C].

vinyl acetate was performed applying seven successive cycles over a period of 120 days. The results are reported in Fig. 6.

As can be observed, the conversion degree decreased slightly (from 42 to 33%) after 45 days of storage and 4 reaction cycles. In the fifth and sixth uses (after 60 and 90 days of storage), the conversion degrees were 24 and 23%, respectively. After 120 days of storage, the conversion degree decreased considerably (to 7%), indicating the loss of catalytic activity of the immobilized lipase.

These data demonstrate that the immobilization of BCL on the chitosan-based support is a satisfactory and attractive option, since this process both protected the lipase from organic solvent deactivation and maintained it relatively stable for over 90 days of storage.

Lastly, it should be noted that no macroscopic changes in the support were observed, which represents an additional advantage of this immobilization method.

4. Conclusions

Chitosan beads crosslinked with varying concentrations of glutaraldehyde were prepared and characterized. The lipase originating from *B. cepacia* PS-SD (BCL) was successfully immobilized on the chitosan-based support (QTS/glutaraldehyde 1.25% v/v, 6 h) and used to carry out the transesterification reaction of geraniol with vinyl acetate in organic medium under mild conditions. The best degrees of conversion to 3 were obtained for a BCL loading of 40 mg/mL (40%) on the glutaraldehyde-crosslinked chitosan beads. The reactions were best conducted in non-polar solvents, such as *n*-hexane (40%), *n*-heptane (36%), cyclohexane (23%) and diisopropyl ether (29%), and in some polar solvents, such as MTBE (15%), acetone (13%) and 1,4-dioxane (12%). The reaction time and temperature considerably affected the catalytic activity of the immobilized lipase and the highest conversion degree was obtained with 72 h of reaction at 40 °C (47%). The efficiency of the immobilization and the stability of the lipase after immobilization were evaluated by investigating the enzyme reuse (7 cycles) and storage (120 days). The immobilized lipase (BCL) showed considerable stability for over 90 days of storage and six reaction cycles (23% of 3). These results demonstrate that this immobilization process is efficient and of low cost.

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