

Article

Synthesis of Benzyl Acetate Catalyzed by Lipase Immobilized in Nontoxic Chitosan-Polyphosphate Beads

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Abstract: Enzymes serve as biocatalysts for innumerable important reactions; however, their application has limitations, which could be overcome by using appropriate immobilization strategies. Here, a new support for immobilizing enzymes is proposed. This hybrid organic-inorganic support is composed of chitosan—a natural, nontoxic, biodegradable, and edible biopolymer—and sodium polyphosphate, which was the inorganic component. Lipase B from *Candida antarctica* (CALB) was immobilized in microspheres by encapsulation using these polymers. The characterization of the composites (by infrared spectroscopy, thermogravimetric analysis, and confocal Raman microscopy) confirmed the hybrid nature of the support, whose external part consisted of polyphosphate and core was composed of chitosan. The immobilized enzyme had the following advantages: possibility of enzyme reuse, easy biocatalyst recovery, increased resistance to variations in temperature (activity declined from 60°C and the enzyme was inactivated at 80°C), and increased catalytic activity in the transesterification reactions. The encapsulated enzymes were utilized as biocatalysts for transesterification reactions to produce the compound responsible for the aroma of jasmine.

Keywords: chitosan; polyphosphate; microspheres; immobilization; lipase, CALB.

1. Introduction

Esters are chemicals of great economic importance in the food, pharmaceutical, and cosmetic industries [1]. Among the esters of short-chain carboxylic acids, benzyl acetate is of vital interest, with applications in different fields, and the annual production of this compound is up to 10,000 tons [2]. This ester can be obtained from natural sources, since it is present in plants, such as jasmine and gardenia; however, its direct extraction and purification are highly complex and expensive [2]. Although the synthesis of this ester by conventional chemical methods is possible, such production methods have many disadvantages, such as high temperature, toxicity of reagents, use of corrosive catalysts, and low selectivity, and thus, increasing the possibility of the generation of by-products that compromise the purity or hinder the purification of the target product [3]. Moreover, it cannot be labeled as a green product.

In this regard, the use of lipases as biocatalysts has been gaining prominence in the recent years, since they differ from conventional catalysts, because they often function at room temperature in different types of solvents (ionic liquids, supercritical fluids, organic solvents, and less contaminant, aqueous media), and are completely biodegradable [4–6]. The use of lipases as catalysts results in selective synthesis, with low or no production of undesirable products and being environmentally safe [7].

Among the enzymes with good performance in organic syntheses, lipases occupy a central position [8]. Lipases are an important class of enzymes in biological systems [9,10]. However, the use of enzymes for industrial biocatalysis has certain disadvantages; for instance, the production cost is high because they are synthesized in small concentrations by cells, and their extraction and purification are expensive. This drawback has been overcome by the development of immobilization techniques, which maintain enzyme activity and allow easy recovery of the catalyst from the reaction medium through filtration as well as its cyclical use, thus reducing operational costs [11,12]. Considering that enzyme immobilization is virtually mandatory in the design of industrial biocatalysts, many efforts have been made to combine immobilization with the attempts to overcome other limitations of enzyme use. Thus, an appropriate immobilization technique can be used for improving the stability, activity, selectivity, and specificity of the enzyme as well as its resistance to inhibitors or denaturing chemicals during enzyme purification [13–18].

One of the preferred techniques for enzyme immobilization is immobilization on pre-existing supports [19]; however, the production of *de novo* solid supports may also be of great interest. These *de novo* solid supports may be synthesized from a mixture of polymers with opposite ionic charges. Anionic polysaccharides, such as alginate [20–22], form coacervates maintained via strong interactions or microspheres with cations (e.g., Ca^{2+}) or cationic polysaccharides, such as chitosan [23–25]. Chitosan is a natural polymer, which is produced from chitin [26]. Chitin is normally isolated from the exoskeletons of many species of insects and crustaceans [27]. It is non-toxic and biodegradable. Partial deacetylation of chitin yields chitosan, a cationic polymer that is used extensively in the field of biotechnology for the immobilization of enzymes and cells because of its properties, such as the ability to stabilize proteins or enzymes while preserving their biological activities [28,29]. The immobilization of lipase on polymeric beads, such as those of chitosan or cellulose, has been reported in the literature [30–33].

It should be considered that the structure of the final microsphere that is used for enzyme immobilization may determine its final performance in the target applications, such as the separation and adsorption of biomacromolecules, or in the design of biosensors [26,34].

Sodium polyphosphate is an inorganic, polymeric, poly anionic electrolyte with high cation sequestration ability [35]. Initially, it was named sodium hexametaphosphate, and later, the nomenclature was modified because the widely used method for the production of this polymeric salt produces a mixture of linear polymers with different lengths [36]. Sodium polyphosphate is still prominent as the only water-soluble phosphate polymer, and because it consists of anionic groups that are distributed throughout the polymeric chain, cations can interact with its anionic chain [37].

There are many techniques for enzyme immobilization, of which most have advantages and disadvantages [38]. There are two types of approaches to enzyme immobilization, namely, irreversible and reversible. The most common irreversible methods are covalent binding [39],

entrapment in supports [40], cross-linking of the enzymes that were previously adsorbed physically on the supports [41,42,43], and microencapsulation. The most common reversible method of immobilization is physical adsorption [44]. The objective of this study was to analyze the effects of the encapsulation of a model enzyme on a novel support formed of chitosan and polyphosphate. As the model enzyme, we had selected the lipase B from *Candida antarctica* (CALB), which is one of the most widely reported enzymes in the literature [29,45]. This biocatalyst was utilized to produce benzyl acetate via a transesterification reaction. In the kinetically controlled reactions, the final yields are transient (the product could be hydrolyzed), and the maximum yields are determined by the properties of the biocatalysts [46].

2. Results and Discussion

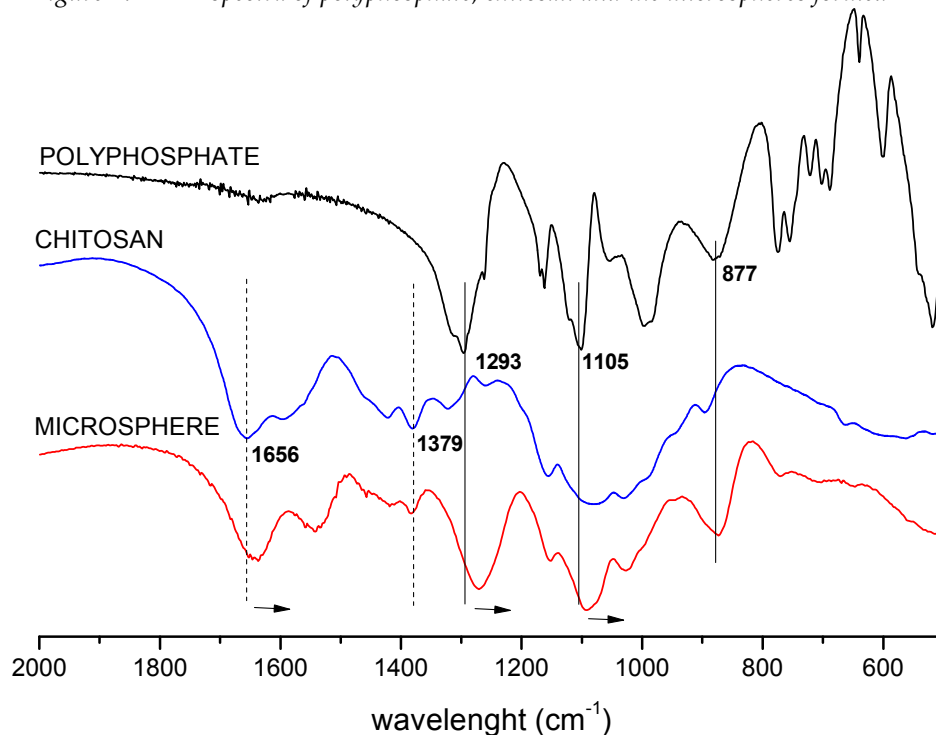
2.1. Characterization of microspheres in the LPCS (lipase immobilized on polyphosphate and chitosan support)

2.1.1. Infrared Spectroscopy

The degree of chitosan deacetylation was directly related to the increase in the number of cationic sites present in the polymer. This could be followed by monitoring the axial deformation band of the C = O amide bond at approximately 1656 cm^{-1} .

The positions of the bands that characterized the center of the polyphosphate chain, the chain and the terminal atoms were displaced (they were ν_s (P-O-P), ν_s (terminal PO_3), and ν_{as} (PO_2^- middle of the chain) at 877, 1105, and 1293 cm^{-1} , respectively). The displacement of these bands suggested that the center of the anionic chain and terminal sites of phosphate are involved in ion exchange with the cationic chain of chitosan. Figure 1 shows that the identity bands of each precursor polymer appeared in the spectral profile of the produced microspheres. This confirmed the hybrid (organic-inorganic) nature of the microspheres.

Figure 1. FT-IR spectra of polyphosphate, chitosan and the microspheres formed



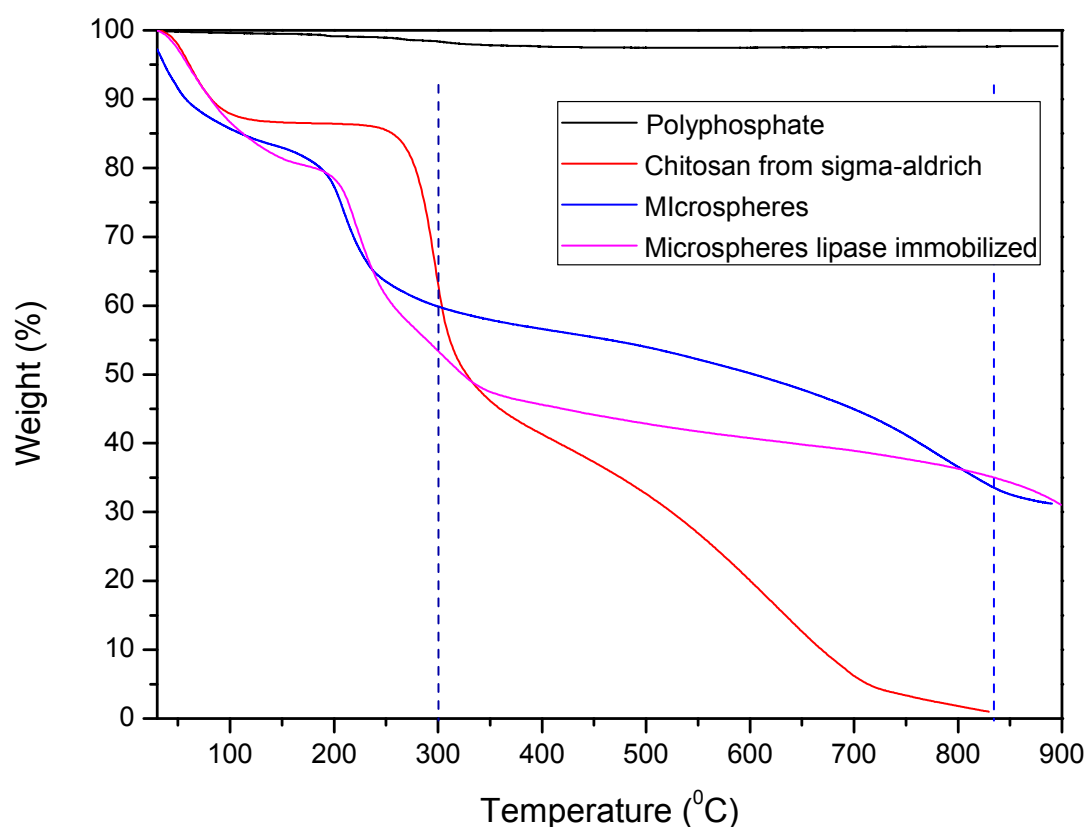
2.1.2. Thermogravimetric analysis

In the thermal decomposition curve of chitosan, two peaks were observed: the first one, which denoted an endothermic reaction, corresponded to the process of dehydration, and it was observed at approximately 263°C; the second one, which denoted an exothermic reaction, corresponded to the process of chitosan decomposition, which continued beyond the temperature limit, and resulted in the formation of a residual solid mass of 1.8%.

Using polyphosphate, there was almost no decomposition even at 900°C, as it was a vitreous precursor. The thermal resistance of the microspheres was higher than that of the precursors because of strong interactions between sodium polyphosphate and chitosan. Figure 2 shows that the first decomposition event occurred between 150°C and 250°C, depending on the sample of microspheres, and the other decomposition event extended over a wide peak, during which, for the sample without lipase, for the non-lipase sample, the thermal decomposition of the sample occurred between 299°C and 834°C.

The residual mass of chitosan was 1.8%, whereas the sample of sodium polyphosphate did not exhibit any loss in mass. On the other hand, the residual masses were 36.8 and 35% when the mixed microspheres were used with and without lipase, respectively. This allowed us to conclude that virtually all of the residual mass was sodium polyphosphate.

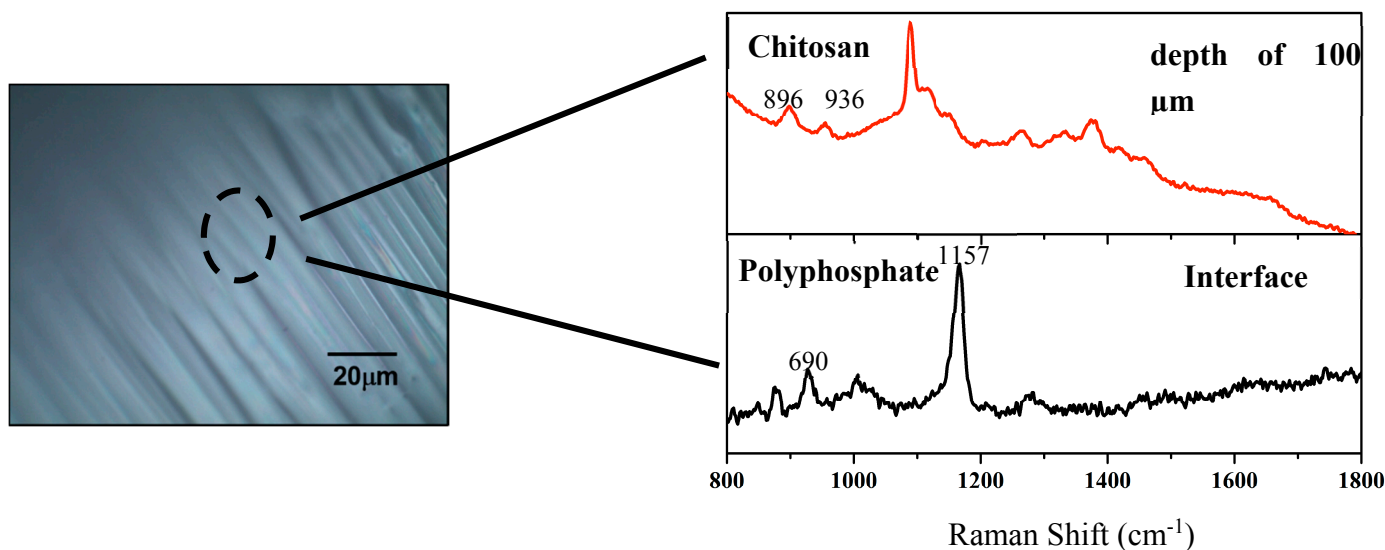
Figure 2. TGA curves of polyphosphate, chitosan, and microspheres.



2.1.3. Confocal Raman microscopy

Confocal Raman microscopy suggested that the shells of the microspheres were composed of sodium polyphosphate, and the cores were composed of mainly chitosan, as illustrated in Figure 3.

Figure 3. Confocal Raman spectra of two points located on the outside and inside of the microspheres, at a depth of 100 μm .



The peaks for polyphosphate, which were used as signature peaks, were of P-O-P vibrations and PO_2^- stretches, which were observed at approximately 690 and 1157 cm^{-1} , respectively [47]. Although the spectra of chitosan were similar to that of polyphosphate, it was possible to differentiate between them. The spectral profiles exhibited by the cores of the microspheres suggested that chitosan was the main component, because the peaks were observed at 896 and 936 cm^{-1} , which were characteristic of C-H bending vibrations and C-N stretching [48].

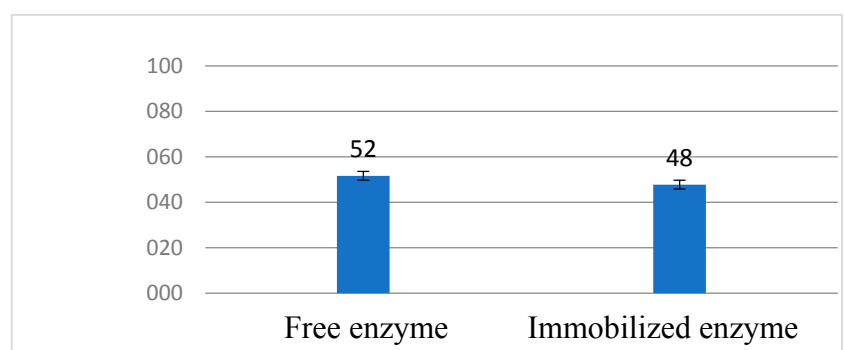
The extent of encapsulation through the process of immobilization of the biocatalyst in the LPCS support was approximately 100%, and no protein was detected in the supernatant by the Bradford's method, which suggested the complete encapsulation of the enzyme in the microspheres.

2.2. Characterization of LPCS

2.2.1. Preservation of the catalytic activity of the biocatalyst by LPCS

The ability of the biocatalyst to catalyze hydrolytic reactions was preserved upon its encapsulation by the polyphosphate-chitosan support (Figure 4), which maintained 92% of the hydrolytic activity of the free enzyme. This proved that the support that was developed in this study did not compromise the catalytic activity of the enzyme.

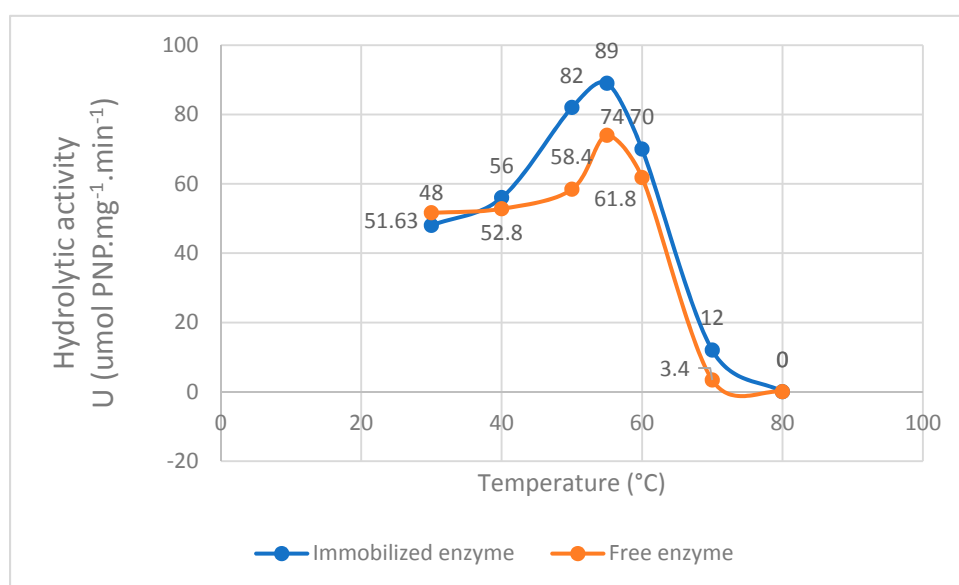
Figure 4. Comparative bar graph of the hydrolytic activities of the free and immobilized enzyme.



2.2.2. Effect of temperature on the activity of LPCS

The hydrolytic activity of the immobilized enzyme was evaluated at different temperatures. Figure 5 shows that the activity of the immobilized enzyme increased up to 55°C and decreased at temperatures higher than 60°C; the enzyme became fully inactive at 80°C while the free enzyme is fully inactive at 70°C.

Figure 5. Activity profile of LPCS at different temperatures.



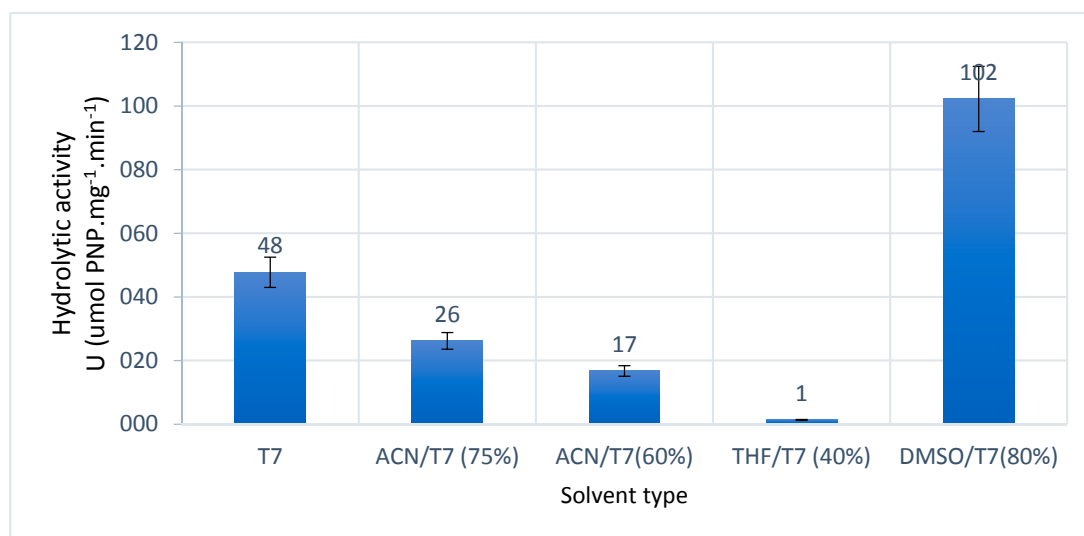
2.2.3. Catalytic ability of the enzyme immobilized using different solvents.

The hydrolytic activity of the enzyme immobilized in the LPCS support was studied using different mixtures of solvents and aqueous buffers (Figure 6). LPCS exhibited better performance in the medium composed of dimethyl sulfoxide/phosphate buffer 7.0 mixture; the enzyme activity in this medium was more than double that in pure aqueous medium. The lowest activity was obtained when tetrahydrofuran/buffer 7.0 was used as the solvent for the reaction, as shown in figure 6.

Acetonitrile had a greater negative effect at 60% than at 75%, which suggested the possibility of positive effects at lower concentrations. It is important to note that although the hydrolytic activity of the enzyme in the different solvents was different, the immobilized enzyme was active in all of them. It was also possible to highlight that the use of binary solvent mixtures with higher dielectric constant (water, acetonitrile, and DMSO) resulted in higher hydrolytic activity of the enzyme. The high resistance of the enzyme to organic solvents, together with the prevention of enzyme aggregation could be due to the partition of the organic solvents away from the highly hydrophilic

environment of LPCS, and this phenomenon of solvent partition has been described in other cases using other ionic polymers [49,50].

Figure 6. Hydrolytic activity of the enzyme in different media.

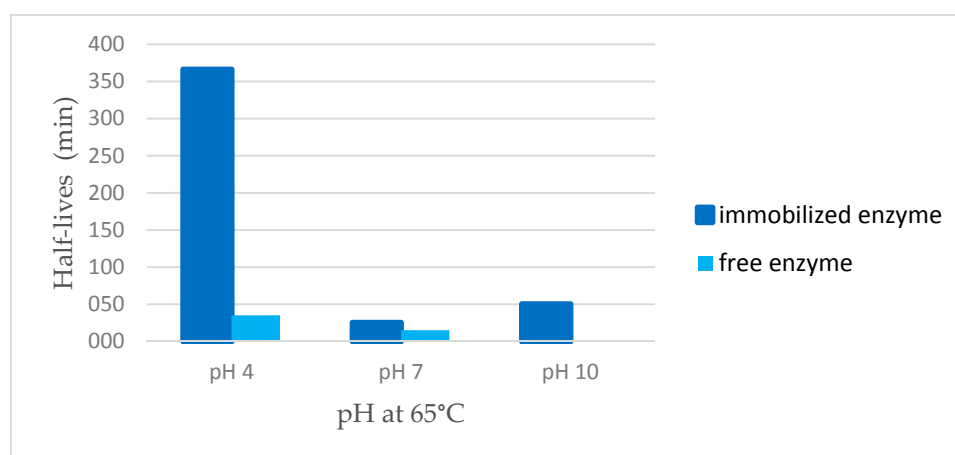


2.2.4 Stability of the enzyme immobilized in the LPCS support at different values of pH

The analysis of the stability of LPCS at different values of pH at 65°C is represented in Figure 7. The enzyme was highly stable at pH 4.0, with a half-life of 366 minutes. When the medium was basic (pH 10.0), the biocatalyst half-life was reduced to 50 min, and even lower, to 25 min at pH 7.0. At pH 7, the enzyme could interact with both cationic and anionic groups in the support, which was the driving force for the enzyme inactivation being faster at neutral pH value than at extreme pHs.

These results suggested greater thermal stability of the enzyme immobilized in the LPCS support at different values of pH than those obtained when the enzyme was immobilized in a pre-formed chitosan/polyphosphate support via ion exchange. Using LPCS with immobilization method and support, the enzyme activity was reduced 45 times, while by the novel method, more than 90% of the enzyme activity was maintained. The strong ion exchange between the enzyme and the *ex novo* support, which involved the entire enzyme structure and reduced enzyme mobility, may be the reasons for the excellent results in terms of activity and stability [51].

Figure 7. Half-lives (expressed in minutes) of the LPCS at different pH values at 65°C.



*. Other specifications are provided in the Materials and Methods section.

The enzyme stability of the immobilized in the acidic media agreed with the results of the confocal Raman microscopy that polyphosphate constituted the shell of the microspheres, because although it could undergo hydrolysis, this biopolymer was more resistant in acid media than chitosan, which was highly soluble in acidic media. In this way, at acidic pH, the microsphere appeared to be completely stable, and this prevented enzyme inactivation.

The temperature of 65°C for the pH test was selected in order to render the system less stable, since preliminary results had already suggested a link between the high resistance of the immobilized enzyme and variations in pH.

2.2.5. Enzymatic synthesis of the compound responsible for the aroma of jasmine catalyzed by LPCS

Another potential application of LPCS, which was evaluated in this study, was its ability to artificially synthesize the compound responsible for the aroma of jasmine (Figure 8). This compound has wide applications in the cosmetic and perfume industries [52]. The immobilized enzyme was able to catalyze the acetylation of benzyl alcohol with different acyl donors. The activated acyl donor that permitted the highest enzyme activity in the acetylation reaction was vinyl acetate, which gave rise to the highest maximum yield. When acetic acid (this was a direct esterification reaction), ethyl acetate, or butyl acetate (these were transesterification reactions) were used, the conversion levels were below 40% (Figure 9). The yields of the direct esterification reaction were higher than those of the transesterification reactions using esters, because in the latter, the released alcohols might have competed with benzyl alcohol. In any case, in the reaction involving vinyl acetate, during which no such competitive alcohol was released, an extremely high yield was observed within a short time (74% conversion after 12 h, and 98 after 24 h, Figure 10). These results were better than those of previous reports on the synthesis of esters used in perfumes, where the reported conversion levels were usually lower. For example, conversion levels of less than 90% were reported after 24 hours of reaction when the commercial enzyme Lipozyme RM IM was used [54].

Figure 8. Enzymatic production of the compound responsible for the aroma of jasmine.

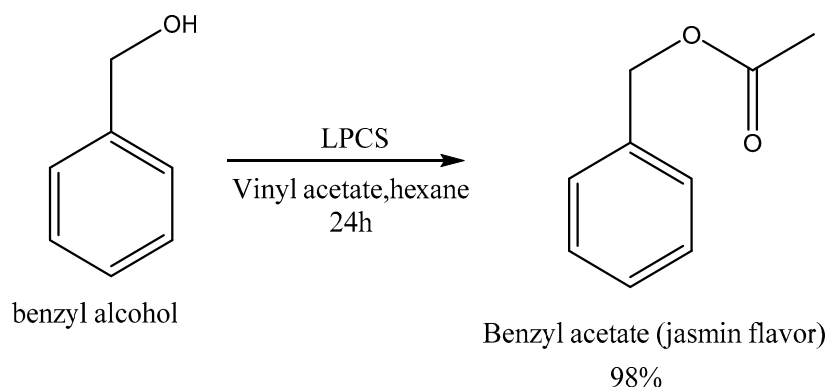
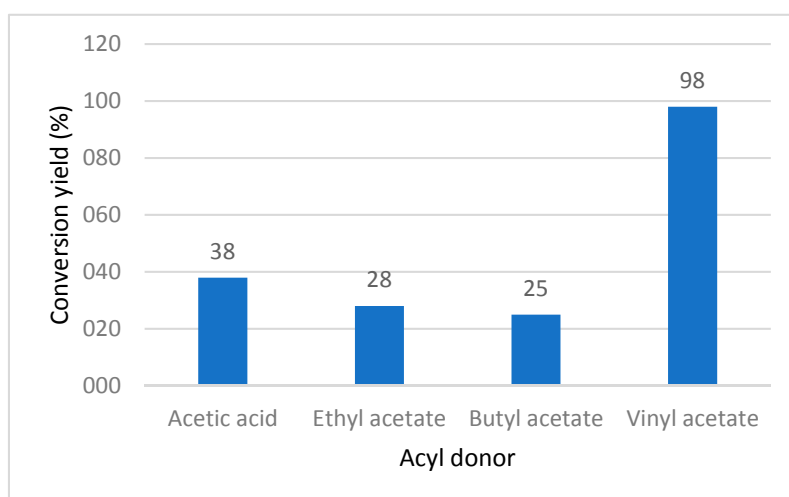


Figure 9. Effect of acyl donors on the acetylation of benzyl alcohol, catalyzed by LPCS.



.This enzymatic process has differential great interest, both from environmental and operational perspectives, because in typical chemical acetylation reactions, toxic catalysts (pyridine) are often used with onerous recovery methods, and in general, they are not practicable [53,54].

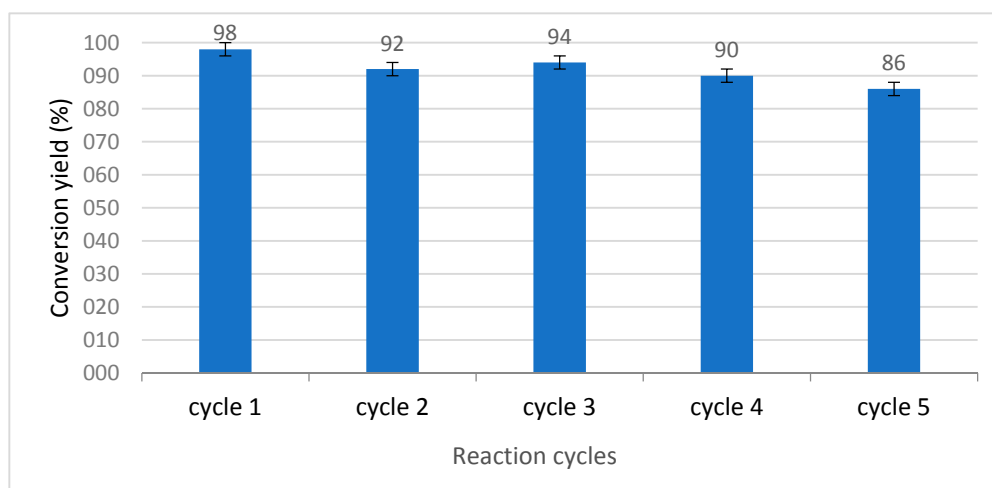
2.2.6. LPCS recycling and storage stability

In order to evaluate the possibility of the reutilization of LPCS in this reaction, the same LPCS biocatalyst was utilized for five consecutive cycles. It was possible to observe that the reaction yields that were obtained using LPCS exhibited a progressive but relatively small decrease over successive cycles, from 98% to 86% after the fifth cycle (Figure 10). Thus, the biocatalyst could be reused for several cycles in this reaction.

2.2.7. LPCS recycling

In order to evaluate the possibility of the reutilization of LPCS in this reaction, the same LPCS support was utilized for five cycles. It was possible to observe that the reaction yields that were obtained using LPCS exhibited a progressive but relatively small decrease over successive cycles, from 98% to 86% after the fifth cycle (Figure 10).

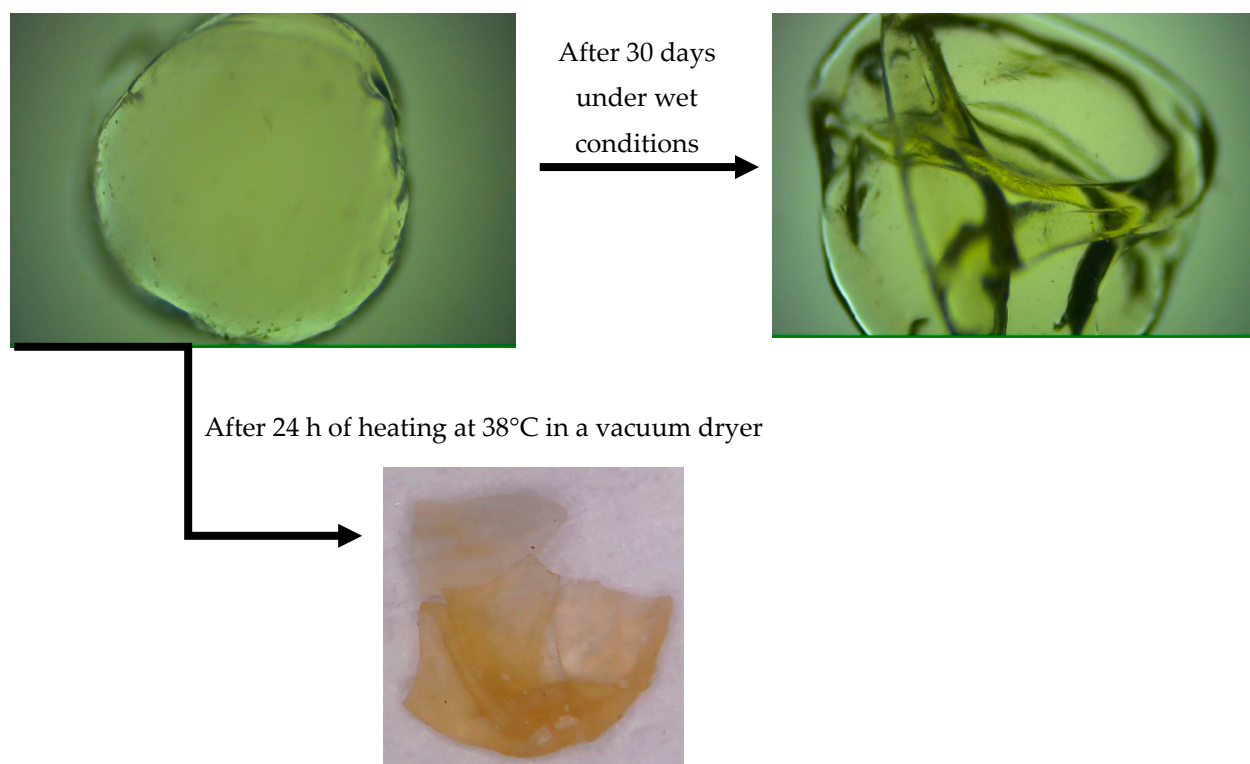
Figure 10. Reuse of LPCS in production of the compound responsible for jasmine aroma.



Note: The reactions consumed 20 μL of benzyl alcohol, 5 mg of the immobilized enzyme, 50 μL of acyl donor, and 2 mL of hexane as the solvent in a reaction time of 24 h

The microspheres lost their structures when they were stored under wet conditions at room temperature after 30 days. This could be clearly visualized by the release of the liquid that was trapped inside the LPCS support into the medium (as shown in the figure 1). However, when dried, the spheres retained their shape, the particles became harder, and the enzyme remained immobilized and active within them during after 30 days. This implied that the use of LPCS in an anhydrous medium would be of great interest.

Figure 11. Photomicrographs of the microspheres at 50X magnification.



3. Materials and Methods

3.1. Materials

The enzyme that was used in this study was lipase from *Candida antarctica* (CALB), which was supplied by Novozymes (Spain). Chitosan was obtained from Sigma-Aldrich (USA); sodium polyphosphate was supplied by Merck (USA), and used without further refinement. deionized water was used in this study.

3.2. Equipment used for the characterization of the biocatalyst

- Fourier transform infrared (FTIR) spectra from 4000 to 400 cm^{-1} were recorded using an FTLA 2000-102 FTIR spectrophotometer (ABB – Bomem) using the KBr pellet technique at room temperature.
- Thermogravimetric (TG) analysis was performed using a thermogravimetric analyzer (model TGA/SDTA851e; (Mettler-Toledo) by heating the samples from 30°C to 900°C at 10°C.min⁻¹ in a synthetic air atmosphere.
- The Raman spectra were obtained using a Renishaw's Raman equipment coupled to a Leica DM2500 M microscope, a Renishaw MS 20 motor stage with axial and lateral resolution of 0.10 mm,

which was controlled using the wire 3.6 software, excited with radiation at 632.8 nm (He-Ne, Renishaw) and at 785 nm (diode, Renishaw). The scattered radiation was dispersed using a diffraction grating of 1200 lines·mm⁻¹, and recorded using a peltier-cooled CCD camera. The samples were observed using a LEICA objective lens with a numerical aperture of 0.9 and magnification of 50X.

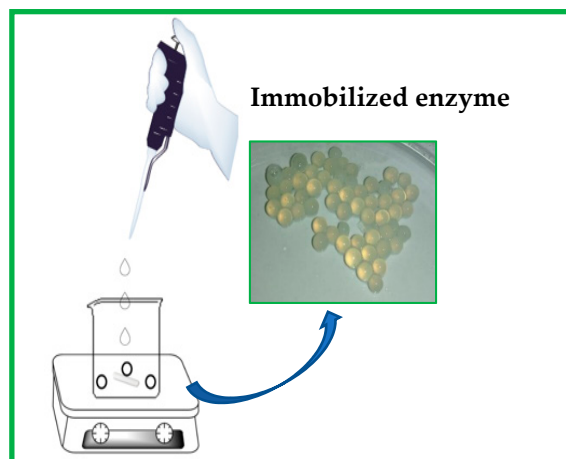
- A QP2010SE Plus GC / MS apparatus (Shimadzu) using a 30-m Rtx®-5MS (95% dimethylpolysiloxane and 5% diphenyl) capillary column, with an internal diameter of 0.25 mm and film thickness of 0.1 µm of the fixed phase was used; The temperatures of the injector and detector were 240°C and 280°C, respectively. The column conditions were: 60–80°C at 5°C·min⁻¹, held for 3 minutes, then, from 80°C·min⁻¹ to 280°C at 30°C·min⁻¹, remaining at this temperature for 10 minutes using He as the gas for entrainment with a flow rate of 1.0 mL·min⁻¹. Analysis was performed using the mass spectrometer in scan mode with an analysis time in 23.67 min; The mass spectra were recorded in the range of 35 to 500 daltons per electron impact (EMIE) with an ionization energy of 70 eV (voltage of 1.5 KV) using a quadrupole mass analyzer and an ion source at 240°C;
- The enzyme activities of free lipase and immobilized lipase were monitored by UV-Vis spectroscopy using a FEMTO 600 S spectrophotometer at a specific wavelength.
- Images of the microspheres were observed using a LABOMED/ANALITICA LX 400 optical microscope, with Siedentopf Binocular and Trinocular heads inclined at 30°, and 360°, respectively rotation an interpupillary distance of 48–75 mm, with 10X/20 mm wide-field eye-pieces of optical Infinite RP Series, and Achromatic Flat DIN Objectives of 4X (WD 30.0 mm), 10X (WD 7.0 mm), 40X (WD 0.65 mm; retractable), and 100X (WD 0.23 mm).

3.3. Synthesis of the microspheres from chitosan and polyphosphate

Microspheres were synthesized by adding a 75–85% deacylated solution of chitosan (CH, 2% m/v) drop wise to a solution of sodium polyphosphate (PP), (Merck ≥ 68%, 0.2 mol L⁻¹), with agitation at room temperature. Microsphere samples with a molar ratio (PP:CH) ranging from 10:1 to 1000:1 were synthesized.

The chitosan solution was added drop wise to an aqueous solution of sodium polyphosphate at a concentration of 0.2 mol·L⁻¹. Ten milliliters of each of the polymer solutions was used; then, these spheres were recovered by decantation, and washed with distilled water (figure 12). The standard molar ratio of the two polyelectrolytes (polyphosphate: chitosan) used was 1000: 1, since this ratio resulted in the synthesis of microspheres with higher mechanical resistance than those generated at the ratios of 100:1 and 10:1. The supernatants were collected for further analysis by UV-Vis spectroscopy.

Figure 12. Schematic representation of the procedure for the formation of microspheres.



3.4. Immobilization of CALB

CALB solution (0.38 mL; 1 mg/mL) was mixed with 2.5 mL of 1% (w/v) chitosan solution, and the system was subjected to magnetic stirring for 30 minutes; then, all the mixture of chitosan and CALB was added drop wise to 10 mL of sodium polyphosphate solution, as described in the previous section, and subjected to 30 minutes of mild magnetic stirring. The formation of microspheres was instantaneous for each added drop. The new biocatalyst-polyphosphate-chitosan support that encapsulated the lipase was referred to as LPCS.

3.5. Determination of the concentration of the immobilized enzyme

The concentration of the immobilized enzyme in the support was determined according to equation 1.

Equation 1. Concentration of the immobilized enzyme

$$I\% = 100 \left[\frac{1 - \text{Protein in supernatant}}{\text{Free Protein Content}} \right]$$

3.6. Hydrolytic Activity

The evaluation of the hydrolytic activity of CALB was based on the hydrolysis of *p*-nitrophenyl acetate (PNPA). An aliquot of 300 μ L of PNPA dissolved in acetone at 20 mmol was added to 3 mL of phosphate buffer solution pH 7.0 (0.05 mol.L⁻¹). Then, 5 mg of the immobilized enzyme was added to the system with stirring (175 rpm) for 10 min. After this, the solution was filtered, and the concentration of PNP that was released was quantified using a spectrophotometer at 410 nm. One unit of hydrolytic activity (A) was defined as the amount of enzyme required to release 1 μ mol of PNP per minute (μ mol.min⁻¹). At certain instances, the temperature was varied between 30°C and 80°C, and different solvents were added. The hydrolytic activity of the enzyme was calculated according to equation 2:

Equation 2. Hydrolytic activity

$$A = \frac{\text{molar concentration (in } \mu\text{mol, PNPA)}}{\text{Mass of immobilized enzyme (mg) . time (min)}}$$

3.7. Activity in organic solvents

The activity of the enzyme that was encapsulated in the microspheres was tested in different mixtures of organic solvents (acetonitrile:CAN; Tetrahydrofuran (THF) and dimethyl sulfoxide) and phosphate buffer (pH 7.0), according to the following methodology: 3.0 mL of the evaluated solvent was added to 300 μ L of *p* nitrophenyl acetate in acetone at 20 mmol.L⁻¹ with 5 mg of dried immobilized enzyme.

3.8. Effect of pH

For the analysis of the thermal inactivation of the biocatalyst under stress, 5 mg of biocatalyst was suspended in 1 mL of sodium acetate at 10 mmol.L⁻¹ and pH 4, sodium phosphate at pH 7, or sodium carbonate at pH 9 at 65°C (± 2). Periodically, samples were withdrawn, and the activity was measured using PNPA. The deactivation constant and half-life of each immobilized derivative were calculated according to the model of Sadana and Henley [55] using Microcal Origin version 8.1 [29].

3.9. Enzymatic synthesis of esters— Effect of the acyl donor groups and enzymatic synthesis of the compound responsible for the aroma of jasmine

A quantity of 5 mg of the immobilized enzyme was added to 20 μ L of benzyl alcohol and 50 μ L of acyl donor (vinyl acetate in acetic acid, ethyl acetate, and butyl acetate) in 2 mL of hexane solution. Then, aliquots were withdrawn after 12 and 24 h, and analyzed by gas chromatography coupled with mass spectrometry using chiral columns.

3.10. Reuse of the catalyst

For analyzing the reusability of the immobilized enzyme, a reaction with 20 μ L of benzyl alcohol was performed. The quantities of (5 mg) the immobilized enzyme and vinyl acetate (50 μ L) were maintained with hexane (2 mL) as the solvent. The reaction products were analyzed at different reaction times (12 and 24 h). After 24 h, the reaction medium was filtered, and the immobilized enzyme was washed twice with 5 mL of hexane, dried at room temperature for 30 minutes, and then, subjected to a new reaction cycle (five times).

4. Conclusions

The new support that was formed by the mixture of polyphosphate and chitosan is highly suitable for the physical entrapment of enzymes. The highly ionic support allowed for the partition of the enzyme into the hydrophobic compounds, such as organic solvents, and formed interactions with the immobilized enzyme. It is important to emphasize that the use of microencapsulation for immobilization using these polymers and this enzyme, as well as its use for the synthesis of molecules of industrial importance is unprecedented in the literature.

The proposed biocatalyst, which is LPCS, exhibited higher thermal stability and operational stability than the free lipase, as well as the retention of high activity in most of the organic solvents that were tested, and facilitated the recovery of high activity. The biocatalyst exhibited good performance during the production of the compound responsible for the aroma of jasmine, which surpassed results, which were published previously in the literature.

The mechanism of enzyme molecules protection can be understood equilibrium thermodynamics of electrolytes because the existence of attractive forces for all components LPCS. The mixture of polyphosphate and lipase to produce a turbid system, a clear evidence of the electrostatic attraction between like charged polyphosphate and cationic enzyme sites.

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Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Sample Availability: Samples of the compounds are available from the authors.

Graphical abstract

