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Influence of Chitosan Derivatization on Its Physicochemical Characteristics and Its Use as Enzyme Support

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ABSTRACT: Chitosan was derivatized by two methodologies for analyzing their effect on chitosan physicochemical characteristics and its applicability as carrier for *Bacillus circulans* β-galactosidase immobilization. Glutaraldehyde (GA) and epichlorohydrin (EPI) were used for crosslinking and activation of chitosan, producing the corresponding supports (C-GA and C-EPI-EPI) after a one-step and a two-step process, respectively. The spherical shape and mean diameter of chitosan particles was not significantly affected by polymer derivatization, while Fourier transform infrared analysis showed that in both cases, chitosan polymer was chemically modified. TGA analysis indicated that C-EPI-EPI was the most thermally stable. The high degree of activation of C-EPI-EPI (586 μmol of aldehydes/g) resulted in the highest loss of activity during immobilization; hence a support with 100 μmol of aldehydes/g was produced (C-EPI-EPI$_{100}$). The highest expressed activity (89.3 IU/g) was obtained with the enzyme immobilized in C-GA, while the biocatalyst with highest thermal stability at 60°C was obtained with C-EPI-EPI$_{100}$ (half-life was 84-fold higher than the one of the soluble enzyme). The best compromise between biocatalyst expressed activity and thermal stability corresponded to β-galactosidase immobilized in C-EPI-EPI$_{100}$. According to this study, chitosan derivatized with EPI is a thermally stable carrier appropriate for producing highly stable immobilized *B. circulans* β-galactosidase. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40171.

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INTRODUCTION

Enzymes are powerful catalysts due to their high selectivity, high activity at mild reaction conditions, high turnover number and biodegradability, advantages that provoke an increasing interest for applying them in the food, pharmaceutical and agrochemical industry.[1] Enzymes are intrinsically labile catalysts, being one of the main drawbacks for their utilization as process catalysts; therefore, different strategies have been developed to improve their performance including protein engineering, directed evolution, environmental engineering, and enzyme immobilization. This latter strategy offers the additional advantage of producing an insoluble biocatalyst, which is an asset from an economical and technical point of view.[2]

Chitosan is a deacetylated derivative of chitin, which is a polymer found in the exoskeletons of crustaceans, the cuticles of insects, and the cell walls of fungi. Chemically, chitosan is a linear polysaccharide composed of β-(1-4)-linked D-glucosamine and *N*-acetyl-D-glucosamine.[3] The presence of amine and hydroxyl groups in the chitosan molecule makes it an attractive support for enzyme immobilization and in fact several immobilization methods using chitosan as support have been developed, including encapsulation,[4] entrapment,[5,6] physical adsorption,[7,8] and covalent attachment.[9,10] Additionally, the nontoxicity and biocompatibility of chitosan make it an appropriate support for producing immobilized biocatalysts, especially for food and pharmaceutical processes.[3]

Among immobilization methods, covalent bonding of the enzyme to the support normally offers the highest degree of stabilization.[11,12] Chitosan may be derivatized in order to introduce additional functional groups that can react with the functional groups of the amino acid residues in the protein surface. Glutaraldehyde (GA) is a bifunctional reagent frequently utilized for chitosan activation, since the aldehyde groups of GA may form Schiff bases with native amino groups of chitosan, introducing aldehyde groups to the support.[13–15] Epichlorohydrin has been also utilized in chitosan activation;[16,17] it reacts with the amino and/or hydroxyl groups of chitosan depending on the reactions conditions, introducing epoxy and diol groups to the support. Hydrolysis of epoxy groups and further oxidation of diols to aldehydes

results in a support with a more chemically homogeneous surface for the immobilization through the ε-amino groups of the lysine residues of the enzyme.

The application of chitosan for enzyme immobilization also requires the crosslinking of the chitosan chains in order to produce a mechanically resistant support and to avoid the dissolution of chitosan under acidic conditions. The crosslinking process is carried out using bifunctional reagents that can also activate the support for the immobilization of enzymes.[3]

β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) is a widely utilized enzyme in the dairy industries mainly due to their ability to hydrolyze lactose from milk.[18] During the last years, the ability of β-galactosidases to catalyze transgalactosylation reactions has gained importance because of the potential of producing high value oligosaccharides. In the reaction of synthesis, a galactose moiety is transferred to a nucleophilic acceptor different than water, potentially any sugar present in the reaction medium, forming oligosaccharides with different polymerization degree. If lactose is the initial substrate, transgalactosylation results in the production of galactooligosaccharides, a product well recognized for its prebiotic capacity.[19]

This work highlights the effect of the type of derivatization on the physicochemical properties of chitosan and on its applicability as support for β-galactosidase immobilization. The aim is the generation of a cheap, ecofriendly and stable support, which allows the production of an immobilized biocatalyst well balanced in terms of expressed activity and thermal stability. The β-galactosidase from *Bacillus circulans* was used as case of study, considering its high synthetic capacity.[20] In order to achieve such objective, GA and epichlorohydrin (EPI) were evaluated as crosslinking and activating molecules of chitosan to produce a suitable matrix for β-galactosidase immobilization.

MATERIALS AND METHODS

Materials

A commercial preparation of β-galactosidase from *Bacillus circulans* (Biolactasa-NTL CONC X2) was purchased from BIOCON (Barcelona, Spain), presenting 31.3 ± 0.5 mg of protein per mL and 1353 ± 11 IU per mL (conditions of activity measurement are in Section Enzyme Activity Assay). *o*-nitrophenyl-β-D-galactopyranoside (*o*-NPG) was from Carbosynth Limited (Bershire, UK). Sodium metaperiodate and glycerol were purchased from Merck (Darmstadt, Germany). EPI, GA, glycidol, sodium borohydride, *o*-nitrophenol (*o*-NP), and chitosan (≥75% deacetylated; molecular weight 190,000–375,000 daltons; batch 061M0046V) were from Sigma-Aldrich (St. Louis, MO). Sepharose CL-6B bead (6% agarose, cross-linked with EPI) was purchased from GE Healthcare (Uppsala, Sweden). All other reagents were of the highest available purity and used as purchased.

Support Preparation

Preparation of Chitosan Particles. Chitosan particles were produced by the neutralization method previously reported,[21] varying only the equipment utilized for particle formation. Chitosan was dissolved to a final concentration of 1.5% (w/v) in 3% (v/v) acetic acid solution and stirred for 12 h to ensure that all chitosan flakes were dissolved. The chitosan solution was fed by gravity into a 1.3-mm nozzle and sprayed using compressed air into 1*M* NaOH in 40% (v/v) ethanol solution under stirring. The particles were recovered and thoroughly washed with distilled water and 0.05*M* phosphate buffer pH 7.0, and stored at 4°C.

Derivatization of Chitosan Particles. ***Glutaraldehyde.*** Chitosan particles were derivatized with GA as previously reported,[21] designating this carrier as C-GA (see Figure 1). One typical derivatization process was performed as follows: 1 g of chitosan particles were contacted with 10 mL of 5% (w/w) GA solution and kept under stirring for 30 min at 4°C. Particles were recovered by filtration and thoroughly washed with distilled water and 50-m*M* phosphate buffer pH 7.0, and stored at 4°C.

Epichlorohydrin. Chitosan particles were derivatized with EPI in a two-step process with the aim of favoring chitosan crosslinking and activation separately (see Figure 2). Chitosan particles were first crosslinked as previously reported[22]; however, in order to favor the reaction between chitosan chains, a ratio of 1 mole of EPI per 4 moles of primary hydroxyl groups on chitosan was utilized (this ratio was calculated assuming 75% of deacetylation). Considering this condition, 1 g of chitosan particles were contacted with 2 mL of an aqueous solution containing 4.8 g/L of EPI and 2.7 g/L of NaOH at 45°C for 2 h under stirring. The crosslinked polymer carrier was designated as C-EPI. After washing, C-EPI was activated with EPI through a second reaction with EPI solution in basic media. This carrier was designated as C-EPI-EPI. One typical activation process was performed as follows: 1 g of C-EPI was suspended in 6 mL of an aqueous solution containing 40 g/L of NaOH, 37% (v/v) acetone, and 3.8 g/L of $NaBH_4$ at 4°C. Then, EPI was added to achieve a final concentration of 185 g/L. The excess of EPI ensures the activation of all the primary hydroxyl groups in crosslinked chitosan. After adding EPI, the mixture was stirred at 25°C for 18 h. C-EPI-EPI was carefully washed with 20% (v/v) aqueous acetone solution, then with distilled water and finally with 50-m*M* phosphate buffer pH 7.0. C-EPI-EPI was finally contacted with 0.5*M* H_2SO_4 solution during 1 h in order to hydrolyze all epoxy groups and then the diol groups were oxidized during 2 h with an aqueous solution containing 17.1 g/L of $NaIO_4$ (in both steps the ratio of 1 g of support per 10 mL of solution was kept).

Preparation of Glyoxyl Agarose. The support (Gx-A) was prepared by activation of the agarose matrix with glycidol and subsequent oxidation with periodate, as previously reported.[23]

Chitosan Characterization. The morphology of chitosan particles was studied by optical microscopy (Nikon Eclipse 50i Microscope) and scanning electron microscope (Evo MA 10, Carl Zeiss). The particles analyzed by scanning electron microscopy (SEM) were previously freeze-dried (Alfa 1-2/LD Plus, Martin Christ Lyophilizer). Particle size distributions were determined by the analysis of optical images using the software Image J2x.

Fourier transform infrared (FTIR) spectroscopy (Perkin Elmer Spectrum One model with DTGS detector) was used for

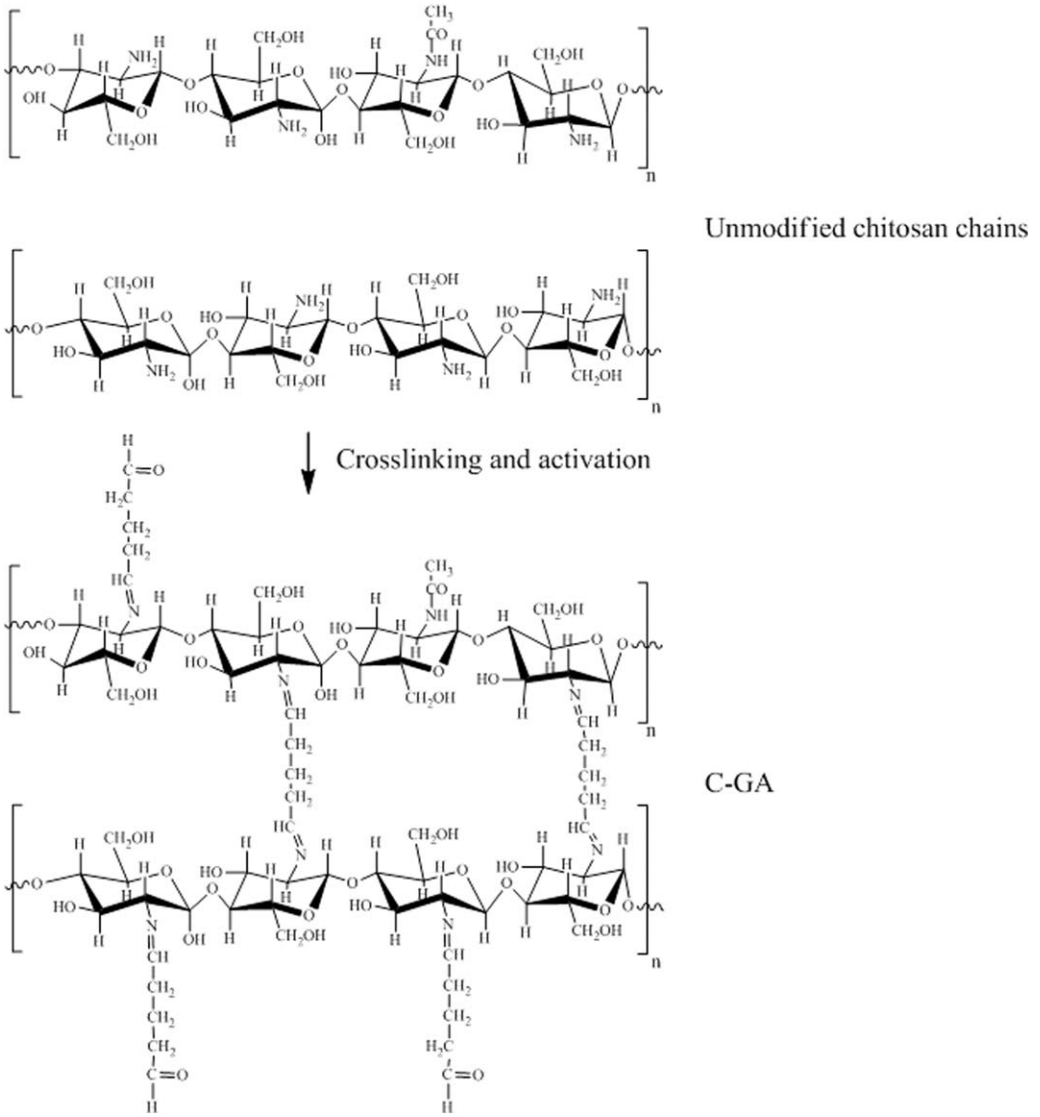

Figure 1. Schematic representation of chitosan derivatized with GA.

determining changes in chitosan chemistry before and after derivatization. Samples previously dried at 30°C under vacuum were pelletized with KBr. The spectra were obtained at room temperature with 32 scans and 4 cm^{-1} of resolution.

The quantification of aldehydes groups in C-EPI and C-EPI-EPI was carried out by back-titration of $NaIO_4$.[23]

Thermogravimetric analysis (TGA) was carried out in a model Q500 equipment from TA Instruments, in order to evaluate the thermal stability of the unmodified and derivatized chitosan particles. The samples were heated at 10°C min^{-1} from room temperature up to 600°C, under nitrogen atmosphere.

Enzyme Activity Assay

The enzymatic activity was assayed using *o*-NPG as substrate and measuring *o*-NP release spectrophotometrically at 420 nm using a temperature-controlled cell with constant magnetic stirring. To initiate the reaction, 50 μL of β-galactosidase solution or suspension was added to 2 mL of substrate solution. One international unit of β-galactosidase activity (IU) was defined as the amount of enzyme producing 1 μmole of *o*-NP per minute from a 45 m*M* *o*-NPG solution in 0.1*M* citrate-phosphate buffer pH 6 at 25°C. The extinction molar coefficient of *o*-NP under assay conditions was 560 M^{-1} cm^{-1}.

Enzyme Immobilization

Immobilization of β-galactosidase was carried out contacting 10 mL of enzyme solution with 1 g of support at 25°C, until the activity in the supernatant varied by less than 10%. The enzyme solution (1.3 mg of protein/mL) was prepared in 0.1*M* bicarbonate buffer pH 10, with 20% (v/v) glycerol to avoid enzyme inactivation at the immobilization conditions. A final step of reduction by 30 min using 1 mg/mL of $NaBH_4$ was required to obtain stable bonds between the enzyme and the support.

Immobilization was monitored by measuring the enzyme activity in the suspension and in the supernatant. Immobilization yields were determined in terms of expressed activity (IY_A) and bound protein (IY_P):

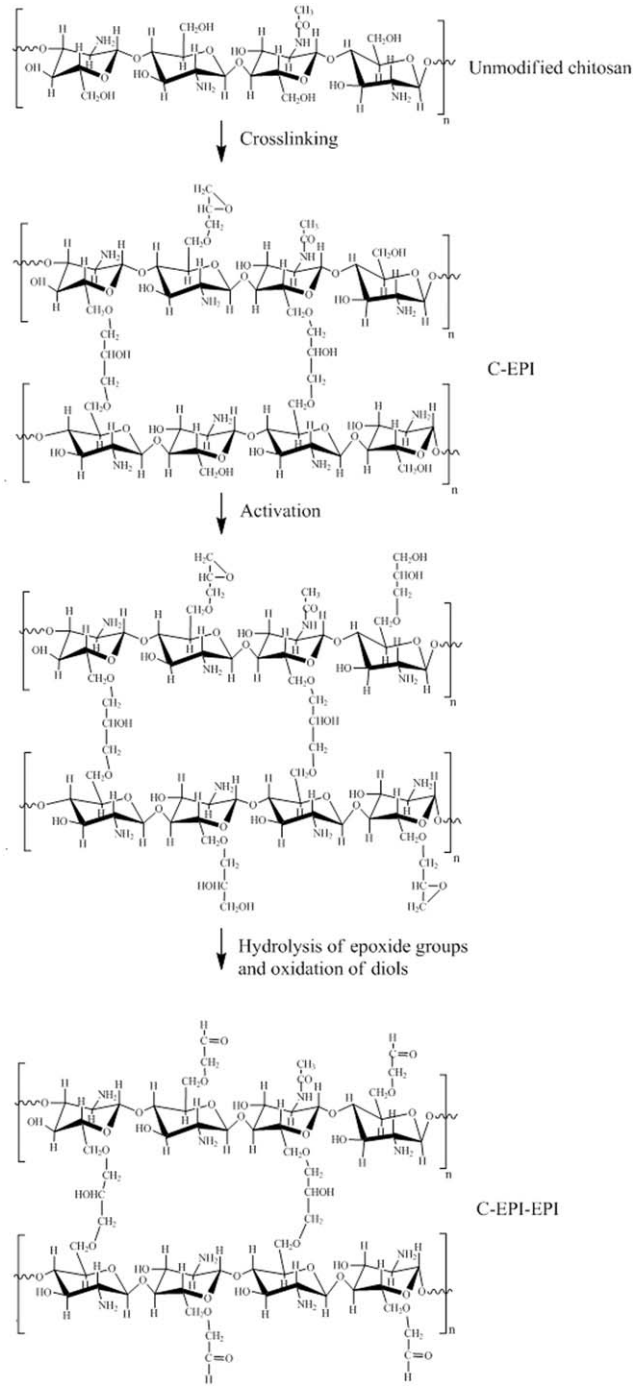

Figure 2. Schematic representation of chitosan derivatized with EPI in a two-step process.

$$IY_A = \frac{A_I}{A_C} \cdot 100 \quad (1)$$

$$IY_P = \frac{P_I}{P_C} \cdot 100 \quad (2)$$

where A_I is the activity expressed by the immobilized biocatalyst, A_C is the contacted enzyme activity, P_I is the immobilized protein (difference between contacted protein and unbound protein in the supernatant) and P_C is the contacted protein. Protein concentrations were determined by Bradford methodology.[24]

Biocatalysts Thermal Inactivation

Immobilized and soluble β-galactosidases were incubated in 0.1*M* citrate-phosphate buffer pH 6 at 60°C. Aliquots were withdrawn periodically under stirring in order to have a homogeneous biocatalyst suspension. Stability of immobilized biocatalysts were compared in terms of the stability factor (SF), defined as the ratio of the half-life of immobilized and soluble biocatalyst, and the lumped parameter maximum catalytic potential (MCP) that considers both the activity and thermal stability of the immobilized biocatalyst (Eq. 3).

$$MCP = \int_{t_0}^{t_f} A_I \, dt \quad (3)$$

MCP was determined by evaluating the variation of expressed activity along a period of biocatalyst inactivation. Even though, final time of biocatalyst utilization (t_f) should be established from an economic analysis of its reuse, considering that it is usual practice to replace the enzyme catalyst when residual activity reaches values in the range from 50 to 10% (~1 to 3 half-lives),[25–27] a value of t_f, corresponding to 35% of residual activity, was taken as catalyst replacement criterion for the evaluation of MCP.

RESULTS AND DISCUSSION

The effect of the procedure utilized for chitosan derivatization (crosslinking and activation) on its physicochemical characteristics was evaluated in order to select a carrier for the immobilization of *B.circulans* β-galactosidase.

Characterization of Chitosan Support

Morphology. C-GA and C-EPI-EPI were analyzed by optical microscopy (Figure 3) showing that, even though both supports present an irregular shape, most particles are spherical. The supports were also analyzed by SEM. The images of each one at two different magnifications are presented in Figure 4. Both supports showed a rough surface and the presence of macropores, without a clear difference between them. In Figure 4 (a.2), it is possible to observe the internal microstructure of the freeze-dried hydrogel. This area was magnified in Figure 5, showing that it is formed by a network of chitosan fibers.

Particle-Size Distribution. The size of chitosan particles will influence the applicability of the immobilized enzyme in industrial processes because of their impact in the filtration time if it is used in stirred tank reactors in repeated batch mode or in the back pressure and flow rates if a column reactor is utilized. The size distribution of C-GA and C-EPI-EPI showed an asymmetric behavior (positively skewed) and the particle diameter varied from 3 to 64 μm, with more than 75% of the particles being in the range from 3 to 32 μm (Figure 6). The mean and median particle size for C-GA were 14 and 12 μm, respectively, while for C-EPI-EPI were 13 and 10 μm, respectively.

Chemical Composition. The chemical modification of chitosan polymer was assessed by FTIR analysis. The characteristic bands for unmodified chitosan polymer are shown in Figure 7. The saccharide structure exhibits bands at 1155 cm^{-1} for

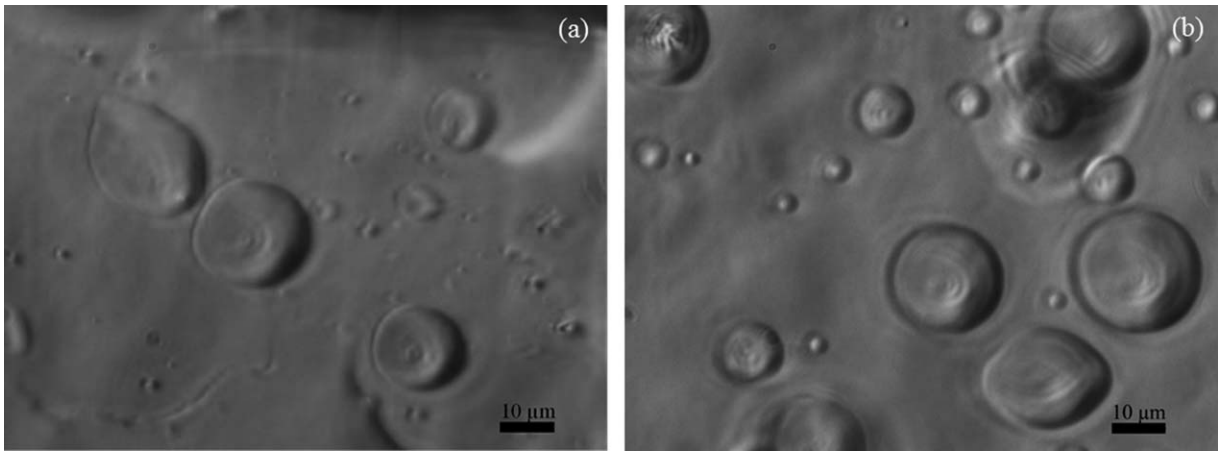

Figure 3. Optical microscope photography of derivatized chitosan particles: (a) C-GA and (b) C-EPI-EPI.

asymmetrical stretching vibrations of the C—O—C glycosidic linkages, 1070 cm^{-1} for C—O stretching vibration of C—OH, and weak bands at 1322 and 1257 cm^{-1} assigned for CH_2 vibrations of the pyranose ring.[28] The band at 1652 cm^{-1} can be attributed to the C—O stretching vibration of amide groups of the acetylated fraction of chitosan, while band at 1561 cm^{-1} can be assigned to the bending vibration of primary amino groups.[29]

After crosslinking and activation, the chemical surface changed according to the derivatizing reagent utilized (Figure 8). The use of GA reduced the ratio between primary amino and hydroxyl groups of chitosan, which was reflected in a neat separation of the amino and hydroxyl bands of its FTIR spectra, 3620 and 3188 cm^{-1}, respectively. Likewise, the best resolution among bands at 1650 and 1570 cm^{-1} indicates that the relative quantity of carbonyl and amine groups changed due to the presence of

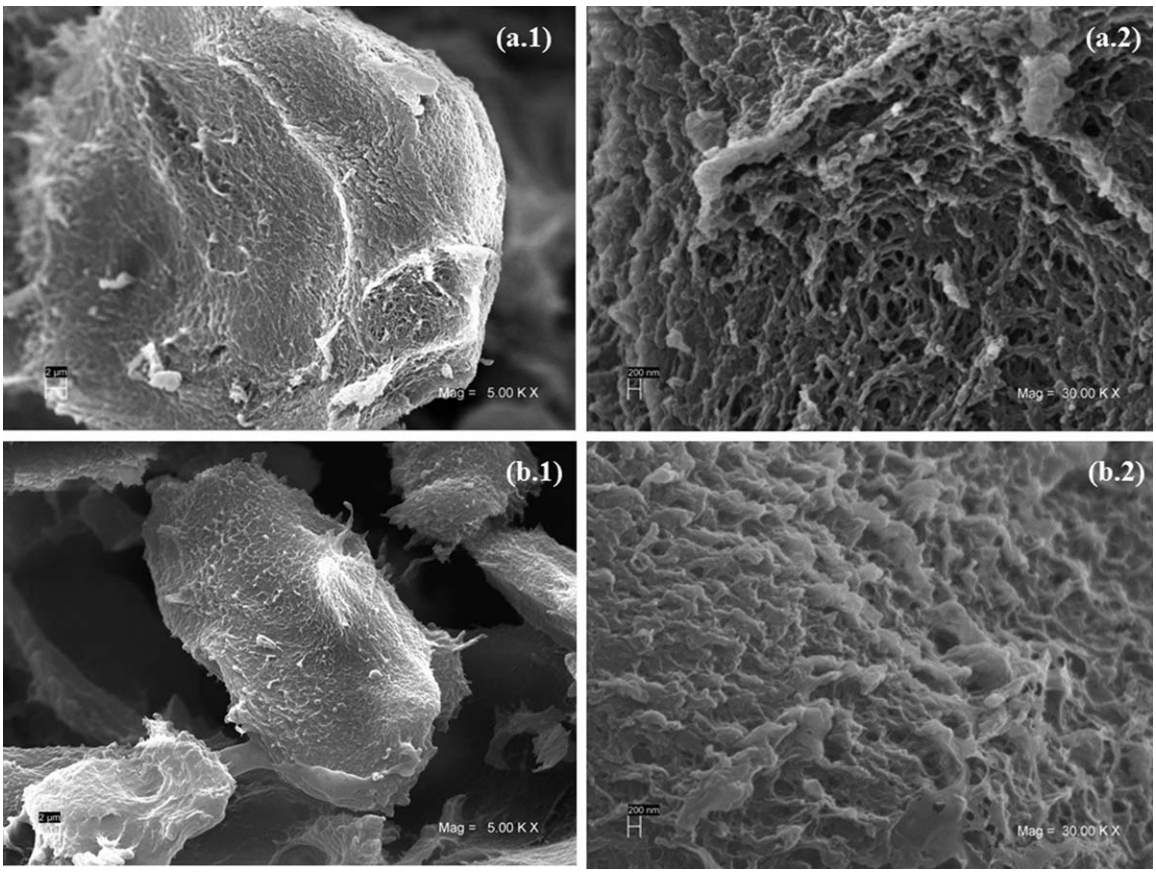

Figure 4. SEM images of chitosan supports with 5000 X (1) and 30000 X (2) magnifications. C-GA (a) and C-EPI-EPI (b).

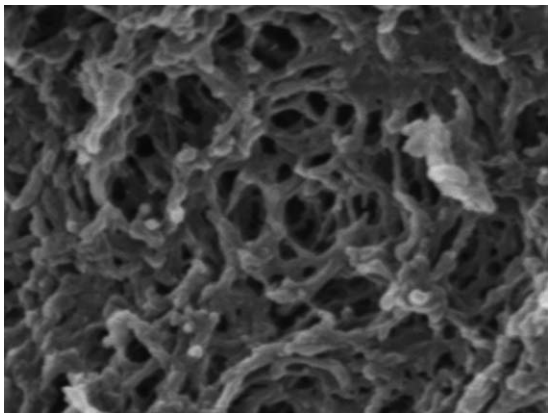

Figure 5. Magnification of C-GA inner structure.

new imine groups in the polymer as a consequence of the formation of bonds between chitosan and GA (Figure 8, spectra b).[30] In the case of C-EPI-EPI, the steps of crosslinking and activation were analyzed separately. The first step of chitosan crosslinking did not produce a significant change in the bands present in the unmodified chitosan spectra; however, the relative intensity of 3400 cm^{-1} and 1652 cm^{-1} bands changed indicating that part of the primary amino and/or hydroxyl groups have reacted during the crosslinking process [Figure 8(c)]. The further activation of C-EPI led to a major modification of the support [Figure 8(d)], reflected in the resolution of amino and hydroxyl bands (3620 and 3188 cm^{-1}); unlike C-GA, FTIR spectra of C-EPI-EPI shows that EPI mainly reacted with hydroxyl groups leaving a higher proportion of amino to hydroxyl groups than when GA was utilized, as was expected.

Aldehydes groups of C-EPI-EPI were quantified by back-titration of $NaIO_4$, corresponding to 586.4 ± 2.8 μmol per gram of wet support. This aldehyde concentration was almost three times the value reported by other authors that have derivatized the support in a one-step process under conditions similar to the ones utilized for the activation of C-EPI.[15] In the case of C-GA, maximum aldehyde concentration may be estimated by stoichiometry considering that 75% of chitosan is deacetylated and that one molecule of GA reacts with only one amino group of chitosan, resulting in an aldehyde concentration of 328 μmol per wet gram of support. It can be expected this value to be actually lower since GA also crosslinks chitosan chains, reacting with two amino groups of the polymer. Rodrigues et al.[15] had reported that chitosan activated with GA under similar conditions than in this study, presents an aldehyde concentration of 212 μmol per wet gram of support.

Thermal Degradation. The thermal degradation of chitosan supports was evaluated by TGA and differential thermogravimetric analysis (DTG). Variations of peak areas and position after support derivatization evidenced physical and molecular changes of chitosan polymer. It can be expected that the support with the highest thermal stability will exhibit the highest life-span as a consequence of the decrease of polymer chains freedom for their crosslinking.[31] The TGA and DTG curves for unmodified chitosan, C-GA, C-EPI, and C-EPI-EPI are illustrated in Figure 9. The first stage corresponds to the loss of water adsorbed on the surface of the materials, while the second stage corresponds to the thermal decomposition of chitosan through vaporization and elimination of volatile products. It is known that pyrolysis of polysaccharides starts by a random split of the glycosidic bonds, followed by further decomposition to acetic and butyric acids and lower fatty acids, where C2, C3, and C6 species predominate.[32] The thermal degradation of the assessed polymers occurred between 170 and 480°C depending on the derivatization process [Figure 9(b)]. Unmodified chitosan polymer exhibited the highest mass loss at 279°C, which is the same result reported in other studies.[33,34] This temperature was slightly increased when derivatization consisted in just one step, reaching 281°C for C-GA and 291°C for C-EPI [Figure 9(b)]. Further activation of C-EPI resulted in the most thermally stable material with the highest mass loss at 322°C, which is in agreement with other studies where chitosan was derivatized in several steps.[33] The determination of vitreous transition (Tg) was not tested by DSC because, according with TGA experiment (Figure 9) and some

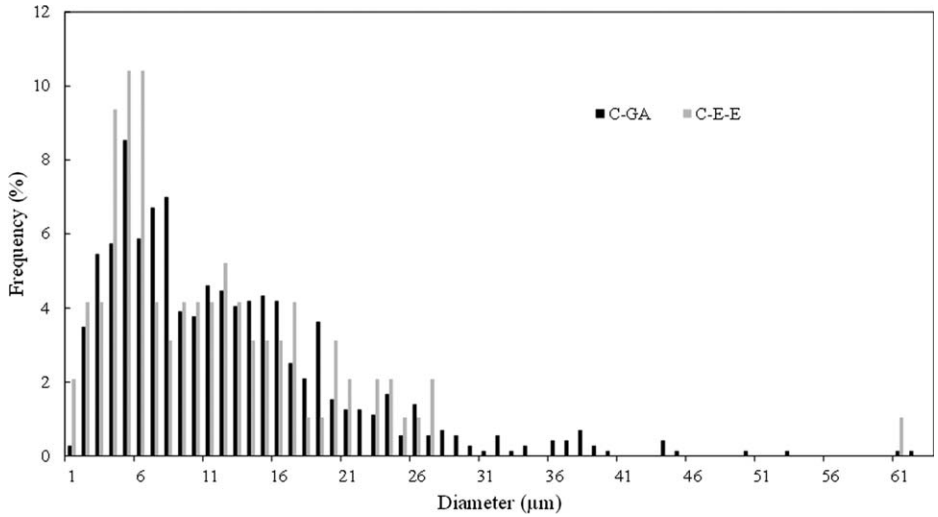

Figure 6. Particle size distribution of chitosan particles according to the derivatization process.

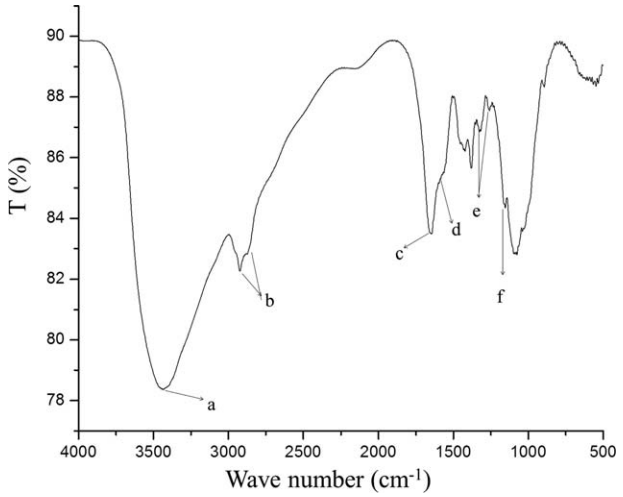

Figure 7. FTIR spectra of unmodified chitosan polymer. (a) amino and hydroxyl groups vibrations, (b) C—H vibrations, (c) C—O stretching vibration of amide groups in the acetylated fraction, (d) bending vibration of primary amino groups, (e) CH2 of the pyranose ring, (f) glycosidic linkages.

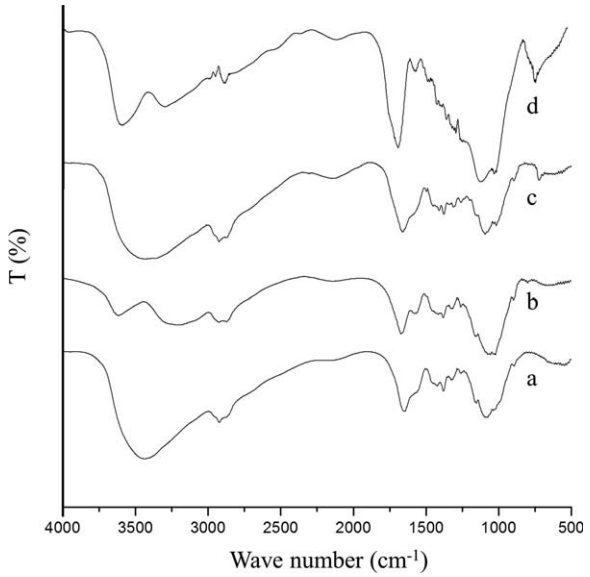

Figure 8. Comparison between chitosan polymers: FTIR spectra of (a): unmodified chitosan, (b): C-GA, (c): C-EPI, and (d): C-EPI-EPI.

previous reports,[35,36] the chitosan decomposition is very close to Tg process, making its determination very difficult. However, these results indicate that in all cases the chitosan was chemically functionalized (C-GA, C-EPI, and C-EPI-EPI) and the resulting structure modified. In all cases, the higher weight loss presented after chitosan derivatization can be explained by the extra organic content.

Immobilization of *B. circulans* β-Galactosidase

β-galactosidase from *B. circulans* was immobilized in chitosan supports in order to evaluate the effect of the derivatization methodology on enzyme immobilization. For a higher chemical homogeneity of the support, epoxy groups were hydrolyzed and oxidized to aldehydes.

The covalent bonding of the biocatalyst to the support was carried out at pH 10 to favor the formation of Schiff bases between aldehyde groups of the support and ε-amino groups of lysine residues in the protein surface.[12] The supports were compared with the widely utilized carrier Gx-A, known to produce high degrees of stabilization to different enzymes.[37] The kinetics of the immobilization process varied according to the utilized support (Figure 10). According to the activity of the supernatant, immobilization kinetics in Gx-A and C-EPI-EPI were similar during the first hour of contact and the immobilization in these supports was faster than when C-GA was utilized. However, the activity of the suspension dropped drastically as soon as the enzyme was immobilized in the C-EPI-EPI, unlike the immobilization in Gx-A where only 20% of the initial suspension activity was lost during the first hour of immobilization. In the case of C-GA, there is also a loss of activity of the suspension; however, the effect of support-enzyme interaction was not as detrimental as for C-EPI-EPI. Since the blank did not lose activity during immobilization, its detrimental effect may be explained by the reaction of lysine residues near to the catalytic

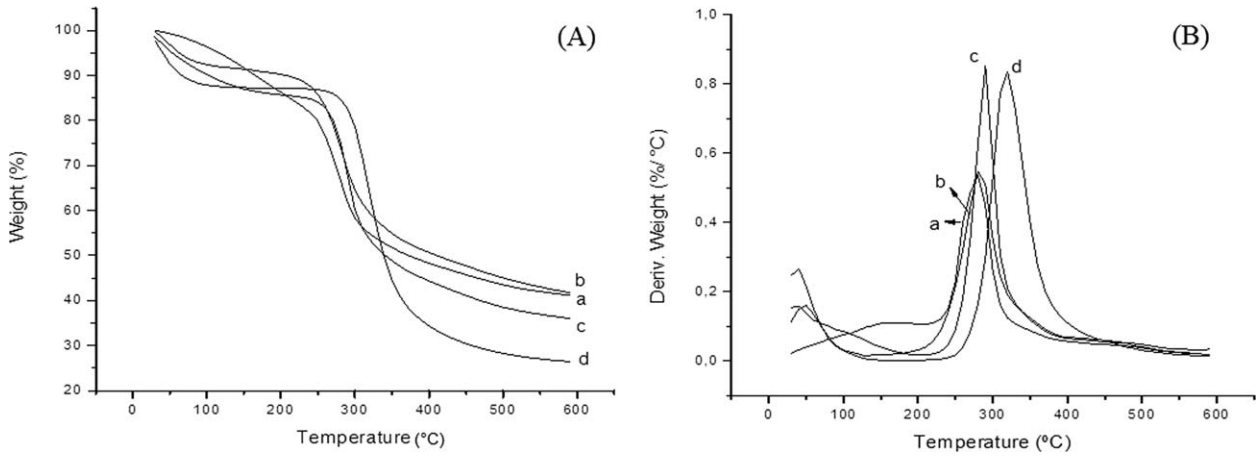

Figure 9. (A) Thermogravimetric and (B) differential thermogravimetric curves for chitosan particles. (a) unmodified chitosan, (b) C-GA, (c) C-EPI, and (d) C-EPI-EPI.

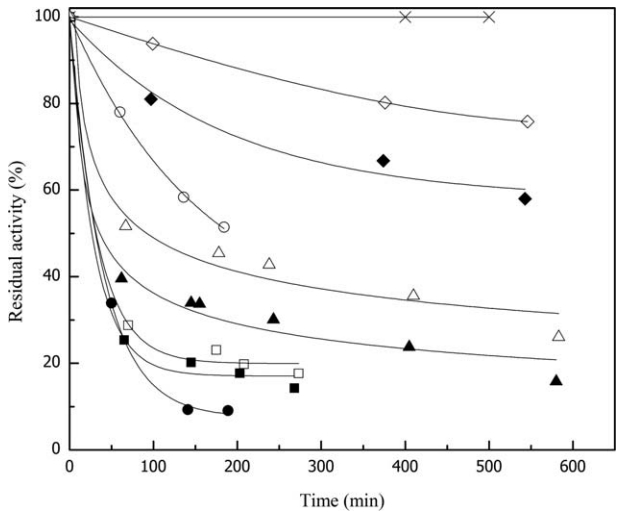

Figure 10. Immobilization of *B.circulans* β-galactosidase (at 25°C in bicarbonate buffer 0.1*M* pH 10, 20% of glycerol) in different supports. Squares: C-EPI-EPI586; Rhombuses: C-EPI21 EPI100; Triangles: C-GA; Circles: Gx-A; Crosses: Blank. Closed and empty symbols represent the activity in the supernatant and in the suspension respectively.

site of the enzyme and/or by excessive enzyme stiffening as a consequence of an intense multipoint interaction between the support and the catalyst. The last argument may be supported by the aldehyde concentration of the carriers, since Gx-A presented 100 μmol per gram (17% of the aldehyde concentration of C-EPI-EPI), as quantified by back-titration of $NaIO_4$. In this analysis C-GA was not considered as its degree of deacetylation was not experimentally determined, being hard to control the ratio between GA and amine groups in order to adequately modulate the aldehyde content. In order to overcome the strongly negative effect of the high degree of activation of C-EPI-EPI, its aldehyde concentration was controlled in the step of support oxidation in order to obtain a carrier with only 100 μmol of aldehydes per gram (C-EPI-EPI_{100}). The lower aldehyde concentration of this support was reflected in the kinetics of immobilization (Figure 10), a higher time of contact being required to obtain the same degree of immobilization than when the C-EPI-EPI with 586 μmol per gram was utilized. Even though the aldehyde concentration of the C-EPI-EPI_{100} was the same than in Gx-A, the slower immobilization may be explained by the differences in the support structure. The expressed activity of C-EPI-EPI after the reduction of its activation degree was increased 5.3-fold, but lower than obtained

Table I. Parameters of β-Galactosidase Immobilization on Chitosan Supports and Glyoxyl Agarose

Support	Expressed activity (IU/g)	IY_A (%)	IY_P (%)
C-EPI-EPI_{564}	10.0 ± 1.5	2.0 ± 0.2	47.3 ± 2.4
C-EPI-EPI_{100}	53.1 ± 1.9	10.6 ± 0.3	44.9 ± 2.0
C-GA	89.3 ± 11.3	17.8 ±1.5	85.7 ± 0.4
Gx-A	199.0 ± 6.0	39.8 ± 0.8	93.5 ± 0.6

IY_P: immobilization yield in terms of protein, IY_A: immobilization yield in terms of activity.

with C-GA and Gx-A biocatalyst. Values of immobilization yield and expressed activity of the biocatalysts obtained with each support are summarized in Table I.

Thermal Stability of Immobilized *B. circulans* β-Galactosidase

Thermal stability of each immobilized biocatalyst was evaluated under nonreactive conditions at 60°C without considering Q-EPI-EPI with 586 μmol of aldehyde per gram due to its low activity. Inactivation kinetics of each biocatalyst was modeled according to a biphasic deactivation mechanism[38]:

$$\frac{e}{e_0} = \left[1+\alpha\cdot\frac{k_1}{k_2-k_1}\right]\cdot\exp(-k_1\cdot t) - \left[\alpha\cdot\frac{k_1}{k_2-k_1}\right]\cdot\exp(-k_2\cdot t) \quad (4)$$

where e/e_0 represents the residual activity at time t, α is the specific activity ratio of the intermediate species with respect to the native enzyme, and k_1 and k_2 are the transition rate constants from the native to the intermediate and the intermediate to the final enzyme species respectively. Values of the model constants are in Table II.

As illustrated in Figure 11, C-EPI-EPI_{100} offered the highest thermal stabilization of the enzyme, followed by Gx-A and C-GA biocatalysts. It has been reported that immobilization on supports containing short-chain aliphatic aldehydes, as in the case of C-EPI-EPI and Gx-A supports, exhibit a chemistry quite different from the one in the immobilization on supports activated with GA reactant (C-GA), and may lead to the immobilization at a different area of the enzyme and/or to a lower multipoint interaction between the enzyme and the support.[39,40] These facts may explain the low stabilization obtained after immobilization on C-GA. Conversely, the different degree of stabilization that was obtained after the immobilization on

Table II. Inactivation Parameters for *B. circulans* β-Galactosidase Immobilized in Different Supports, Determined According to a Series Biphasic Model [eq. 4]

Biocatalyst	k_1 (h^{-1})	k_2 (h^{-1})	α	R^2	Half-life (min)	SF	MCP (μmole/$mg_{catalyst}$)
Soluble enzyme	75.49	3.05	0.51	0.99	2.4	1.0	-
β-gal/C-EPI-EPI_{100}	1.23	0.03	0.53	0.99	201.8	84.1	21.7
β-gal/C-GA	14.78	1.61	0.72	0.99	17.8	7.4	1.6
β-gal/Gx-A	1.69	0.03	0.36	0.99	53.0	22.1	14.8

$R_2$32: Coefficient of determination, SF: stability factor, MCP: maximum catalytic potential.

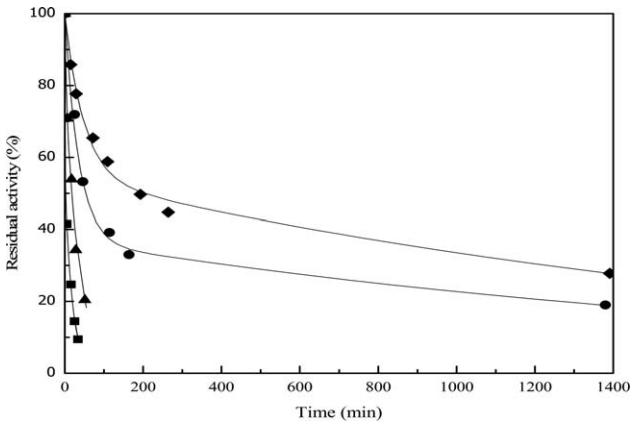

Figure 11. Thermal inactivation of soluble and immobilized *β*-galactosidase at 60°C in 0.1*M* citrate-phosphate buffer pH 6. Squares: soluble enzyme, Rhombuses: C-EPI-EPI100 biocatalyst, Triangles: C-GA biocatalyst, Circles: Gx-A biocatalyst. Line represents the inactivation model.

C-EPI-EPI and Gx-A may be explained by a different support structure.

To compare quantitatively the stability of each biocatalyst, the widely used SF was calculated (Table II). Even though C-EPI-EPI_{100} and Gx-A have the same concentration of aldehydes groups, the SF obtained with C-EPI-EPI_{100} was 3.8-fold higher than the one achieved with its agarose counterpart. SF of C-EPI-EPI_{100} biocatalyst was higher than values reported for the immobilization of *B. circulans* *β*-galactosidase on acrylic resine[41] and meso-macroporous silica,[42] both activated with short-chain aliphatic aldehydes. These results highlight that chitosan activated with EPI in a two-step process is a suitable carrier for obtaining a highly stable biocatalyst of immobilized *B. circulans* *β*-galactosidase.

Considering that the expressed activity of Gx-A was 3.8-fold higher than the one obtained with Q-EPI-EPI_{100}, a sound comparison between these supports should consider both the expressed activity and thermal stability of the catalysts, since both parameters will influence biocatalyst performance in a given process. MCP represents a meaningful lumped parameter for the comparison of different immobilized biocatalysts, because it adequately balances the expressed activity and thermal stability. The high MCP of Q-EPI-EPI_{100} biocatalyst indicates that the high thermal stabilization offered by Q-EPI-EPI compensates the lower expressed activity obtained in comparison with the Gx-A catalyst (Table II), resulting in an immobilized enzyme that along the period of thermal inactivation presents a better catalytic performance.

CONCLUSIONS

A complete study about the effect of the derivatization methodology on the chitosan physicochemical characteristics was carried out, evaluating EPI and GA as derivatizating agents. Spherical shape of chitosan particles was not modified by the different derivatization methodologies and in both cases more than 75% of the particles were between 3 and 32 μm in diameter. FTIR analysis showed that the supports were chemically modified reacting part of their original amino and hydroxyl groups and so making the carbonyl groups more available for the covalent bonding of the enzyme. Conversely, the chitosan derivatization with EPI through a two-step process allowed improving significantly the thermal stability of the chitosan polymer. Immobilization of *B. circulans* *β*-galactosidase in chitosan supports was strongly affected by the derivatization methodology. Chitosan derivatized with EPI in a two-step process and activated with 100 μmole of aldehyde/g offered the highest degree of *β*-galactosidase stabilization, with a SF higher than the ones reported by other authors. Additionally, in comparison with the commonly used glyoxyl-agarose, chitosan derivatized with EPI offered as a support a better compromise of expressed activity and thermal stability to *B. circulans* *β*-galactosidase.

The results obtained in this study indicate that chitosan is a polymer that properly derivatized may result in a high quality support for the generation of robust biocatalysts.

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