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Covalent Immobilization of *Candida rugosa* Lipase at Alkaline pH and Their Application in the Regioselective Deprotection of Per-*O*-acetylated Thymidine

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Abstract: Lipase from *Candida rugosa* (CRL) was stabilized at alkaline pH to overcome the inactivation problem and was immobilized for the first time by multipoint covalent attachment on different aldehyde-activated matrices. PEG was used as a stabilizing agent on the activity of CRL. At these conditions, CRL maintained 50% activity at pH 10 after 17 h incubation in the presence of 40% (w/v) of PEG, whereas the enzyme without additive was instantaneously inactive after incubation at pH 10. Thus, this enzyme was covalently immobilized at alkaline pH on three aldehyde-activated supports: aldehyde-activated Sepharose, aldehyde-activated Lewatit105 and heterofunctional aldehyde-activated EDA-Sepharose in high overall yields. Heterogeneous stable CRL catalysts at high temperature and solvent were obtained. The aldehyde-activated Sepharose-CRL preparation maintained 70% activity at 50 °C or 30% (v/v) acetonitrile after 22 h and exhibited high regioselectivity in the deprotection process of per-*O*-acetylated thymidine, producing the 3′-OH-5′-OAc-thymidine in 91% yield at pH 5.

Keywords: *Candida rugosa* lipase; stabilization; covalent immobilization; PEG; alkaline pH; regioselectivity; nucleosides

1. Introduction

Enzymes are natural catalysts working at mild conditions with high specificity by substrate and excellent selectivity which could constitute a green solution for the industry. However, enzymes are unstable out of their natural environments, and parameters such as pH and temperature (T) are relevant to their stability. Therefore, the stabilization mechanism represents an important issue in enzyme development for possible industrial implementation. Low enzyme stability has been overcome using several strategies: genetic engineering [1], chemical modifications [2], the addition of stabilizing agents [3–6], or the use of different immobilization mechanisms [7,8].

Particularly, multipoint covalent attachment on macroporous supports has been described as a very interesting approach to stabilize enzymes [9–11].

The functionalization of the support materials by molecules as short spacer arms assures the attachment of an enzyme molecule to the matrix through various covalent linkages. The amino-acidic residues of the protein involved in the covalent immobilization should be rigid, conserving the relative positions against changes in the protein conformation, e.g., with the effect of distorting agents, such as heat and organic solvents [12–14].

In this way the use of supports functionalized by aldehyde groups under alkaline conditions promotes enzyme immobilization by a specific orientation, through the richest area containing the highest number of lysines, generating a multipoint covalent interaction [15]. The advantages of the aldehyde groups are: (i) they are reactive toward unprotonated primary amines; (ii) they are stable under alkaline conditions; (iii) they do not exhibit steric hindrances for intramolecular reactions [16]. In fact, several industrial enzymes have been stabilized by multipoint covalent attachment on these aldehyde-activated supports [15].

However, enzymes sensitive to alkaline pH are not able to be immobilized through this methodology. *Candida rugosa* lipase (CRL), a very useful enzyme in biotransformations [17–19], is one of these enzymes with very limited conditions for proper use, especially in regards to pH [20]. The immobilization methods for the covalent attachment of this lipase described in the literature use the application of functionalized supports, e.g., with glutaraldehyde or cyanogen bromide groups, for covalent immobilization at neutral pH, which mainly correspond to the reaction to the terminal amino group, with a low, intense covalent immobilization [20,21]. Also, the adsorption on hydrophobic supports at neutral pH has been used for improving its stability, although this is a reversible immobilization and the enzyme can be leached from the support in the presence of some concentration of solvent or detergent [20]. Therefore, the multipoint covalent immobilization of this enzyme on aldehyde supports would be an excellent strategy, considering the advantages described, for obtaining an irreversible, immobilized biocatalyst with high stability.

Improvements of the enzyme's stability with the presence of some additives such as polyols, solvents or sugars have been reported [22,23].

The use of these additives has the advantage of providing enzyme activity at high temperatures and alkaline pH [24]. A study of the protective effect of PEG, trehalose and glycerol revealed an increase with the reagent concentration and length of the carbon chain [25,26]. These compounds are known to have a more or less pronounced effect on water activity and on the degree of water molecule association [25]. On the other hand, aqueous solutions of polyols, polymers and sugars, as additives, were used to study the thermostabilization of enzymes [27].

Herein, we propose a methodology to stabilize *Candida rugosa* lipase (CRL) at alkaline pH using PEG as an additive, permitting for the first time its immobilization by multipoint covalent attachment on aldehyde-activated (Ald) derivative supports. Two different covalent immobilization strategies involving different orientations of the protein (Figure 1) in the immobilization were used (Scheme 1). These new immobilized CRL biocatalysts were used to catalyze the regioselective monodeprotection of per-*O*-acetylated thymidine, an interesting intermediate in the synthesis of different fungicidal, antitumor, and especially antiviral agents [28].

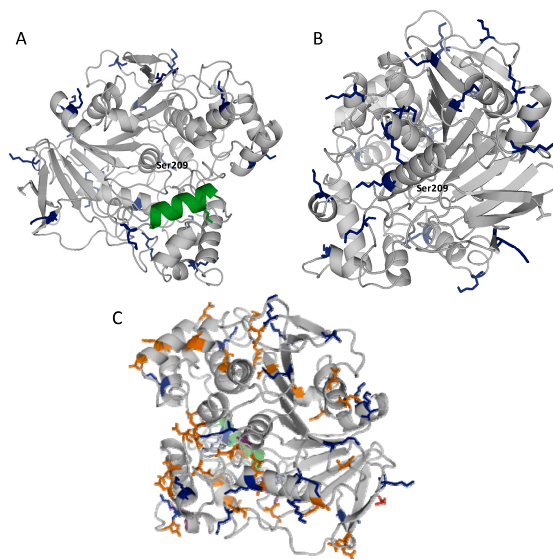
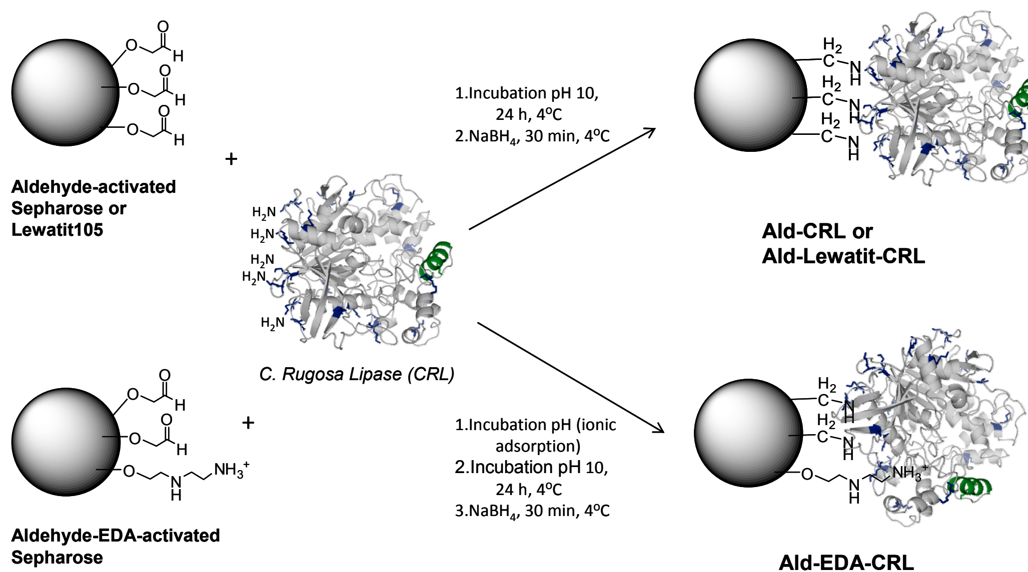


Figure 1. Structure of mature CRL. (**A**) Lid side. Oligopeptide lid (green), Lys (blue); (**B**) Lid opposite side marked lysines; (**C**) Lid opposite side marked lysines and aspartic and alutamic acids (orange). The structure of CRL was obtained from the Protein Data Bank (pdb code: CRL) and the picture was created using Pymol v. 0.99.



Scheme 1. Covalent immobilization of CRL by different methodologies.

2. Results and Discussions

2.1. Stabilization of CRL at Alkaline pH

The stability of soluble pure CRL by incubation at different pHs was first studied (Figure 2). This lipase has been described to be a very sensitive enzyme [20]. After purification, the enzyme incubated at pH 7 maintained around 92% activity during 25 h at 4 °C. When the pH was increased up to 9, 60% activity was found after 25 h. However, the activity of the enzyme was totally lost immediately after incubation at pH 10, exhibiting the extremely low stability at alkaline pH, which is mandatory to perform the multipoint covalent immobilization on an aldehyde-activated support.

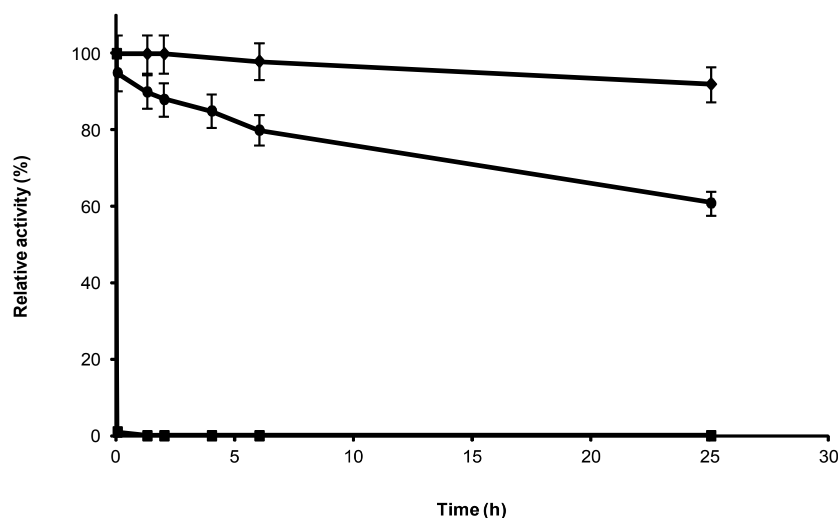


Figure 2. Stability of purified CRL at 25 °C and different pHs: pH 7 (rhombus), pH 9 (circles), pH 10 (squares); 0.27 mg purified free lipase was used in each experiment.

Thus, different additives—at 20% (*w*/*v*) concentration—were added to the enzyme solution to study their effect on the enzyme stability (Figure 3). The best result was achieved when PEG was previously added to the lipase alkaline solution (pH 10), where the enzyme retained 40% activity after incubation for 4.5 h, whereas the soluble enzyme without additives maintained only 20% activity. Using polyols such as glycerol, dextran or trehalose, a slightly improved activity was observed.

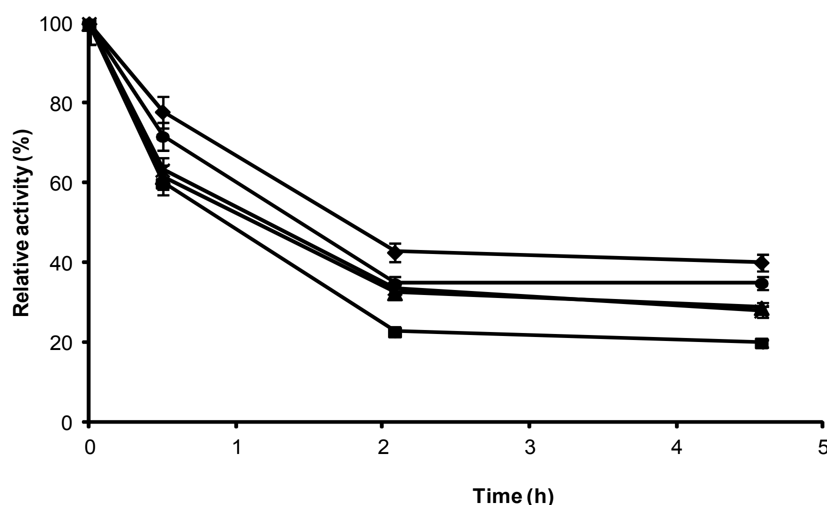


Figure 3. Stabilization of CRL in the presence of different additives. The additives were added at 20% (*w*/*v*) concentration and the experiments were performed at pH 10 and 4 °C. Without additive (squares), PEG (rhombus), glycerol (circles), dextran (triangles), threalose (×).

PEG was selected as a stabilizing agent and the effect of the additive concentration and the molecular size on the lipase stability was studied (Figure 4). PEG1500 stabilized the enzyme slightly better than PEG6000 at a 15% concentration (*w*/*v*), although the best results were found using 40% (*w*/*v*) PEG1500, where CRL conserved 50% activity after 17 h incubation (Figure 4).

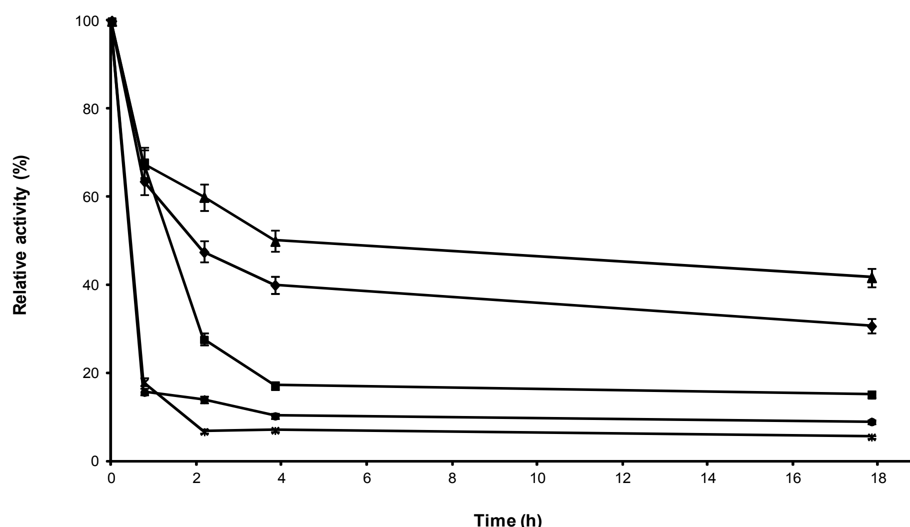


Figure 4. Effect of PEG concentration on the stability of CRL at 4 °C and pH 10. Without additive (×), PEG-1500 15% (squares), PEG-6000 15% (circles), PEG-1500 20% (rhombus), PEG-1500 40% (triangles).

The possible mechanism for the high stabilization of lipase achieved by the addition of PEG could be explained by two different effects. The first is (1) a strong physical adsorption of the PEG molecules to the hydrophobic area of the protein, as previously has been reported [29]. Especially here, in lipases the most hydrophobic area is concentrated on the lid and the surrounding active site; therefore, the PEG could generate protection of the active site, significantly improving the stability in an extreme condition such as alkaline pHs. The second effect is (2) due to the fact that the use of higher PEG concentrations causes a high viscosity which also may prevent undesired changes in the enzyme structure promoted by the alkaline pH [30].

2.2. Multipoint Covalent Immobilization of CRL

The enzyme was immobilized at optimal conditions (with 40% PEG in solution) using two different strategies to get multipoint covalent immobilization of the lipase throughout different orientations (Scheme 1). CRL was immobilized on Ald-Sepharose at 89% yield (loading of 4.9 mg lipase/g support) at pH 10.2 over 24 h retaining 53% initial activity, whereas a 69% yield of lipase immobilized maintaining 39% of initial activity was obtained using Ald-Lew105 (Table 1).

Table 1. Covalent immobilization of *Candida rugosa* lipase on different supports at 4 °C.

Support	Immobilization yield (%) [a]	Retained Activity (%)
Ald-Sepharose	89	53
Ald-Lew105	69	39
Ald-EDA-Sepharose	95	37

[a] Immobilization for 24 h; 5.5 mg pure lipase was offered per gram of support.

The immobilization on heterofunctional Ald-EDA-Sepharose was performed at pH 8 and around 90% yield was achieved after 3 h incubation. After that, the immobilized preparation was incubated at pH 10 for 24 h to promote a possible multipoint covalent attachment. The immobilized preparation retained 37% overall initial activity (Table 1).

2.3. Stability of Different Covalent Immobilized Preparations of CRL

To evaluate the effect of the immobilization method on the stabilization of the enzyme, inactivation experiments at different conditions were studied (Figures 5 and 6).

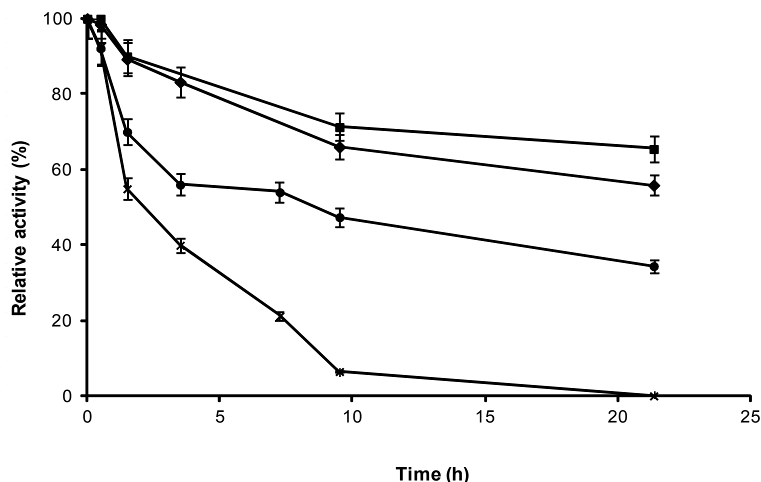


Figure 5. Thermal inactivation course of different CRL immobilized preparations. Experiments were performed at 50 °C and pH 5. Free CRL (×), Ald-EDA-CRL (rhombus), Ald-CRL (squares), Ald-Lew105-CRL (circles).

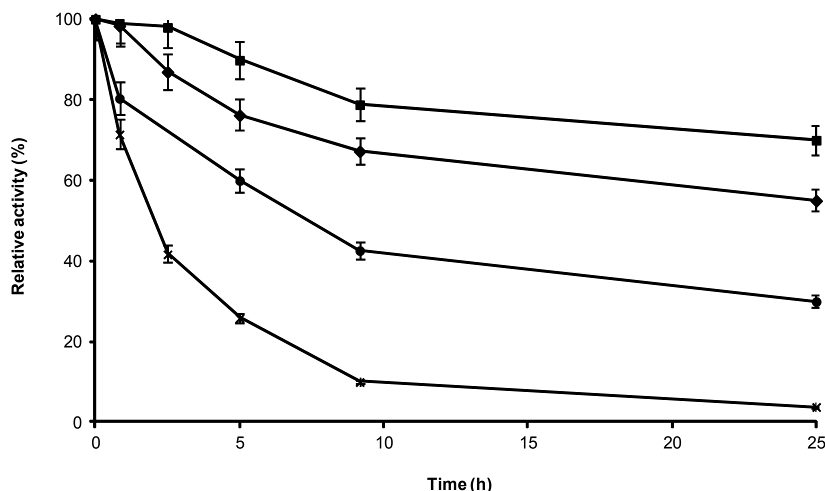


Figure 6. Inactivation profile of different CRL immobilized preparations in the presence of co-solvent. Experiments were carried out at 25 °C, pH 5 and 30% (*v*/*v*) acetonitrile. Free CRL (×), Ald-EDA-CRL (rhombus), Ald-CRL (squares), Ald-Lew105-CRL (circles).

At 50 °C, the best stabilization of CRL was achieved after immobilization on Ald-Sepharose. The Ald-CRL, Ald-lew105-CRL and Ald-EDA-CRL immobilized preparations conserved more than 50% activity after 9 h incubation at 50 °C, whereas the free lipase only retained 6% activity (Figure 5). Indeed, the Ald-CRL preparation still conserved 70% of the initial activity after 22 h at 50 °C whereas the Ald-Lew105-CRL preparation only maintained 38% activity at this time. This demonstrates the effect of the matrix on the lipase stabilization.

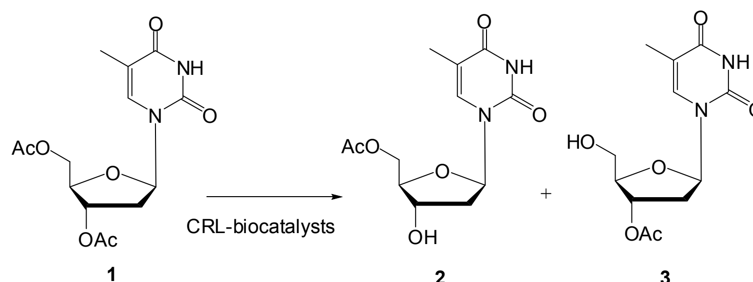
Also, of the differently oriented catalysts, Ald-EDA-CRL showed good activity, maintaining around 60% activity after 22 h incubation (Figure 5).

When the CRL preparations were incubated at 30% (*v*/*v*) acetonitrile at 25 °C, the effect was even clearer (Figure 6). The Ald-CRL preparation was the most stable catalyst also in the presence of a co-solvent, retaining 70% activity after 25 h incubation, conditions where the soluble enzyme was completely inactive. The enzyme immobilized on the Ald-EDA support conserved 55% activity whereas the Ald-Lew105-CRL preparation was again less stable than the Sepharose one (Figure 6).

2.4. Regioselective Deprotection of Per-O-acetylated Thymidine by Immobilized CRL Biocatalysts

The different covalent immobilized preparations of CRL were used as catalysts in the hydrolytic deacetylation of per-*O*-acetylated thymidine (**1**) at different pHs (Table 2).

Table 2. Regioselective deprotection of 3,5-*O*-diacetylated thymidine **1** with different aldehyde-activated CRL biocatalysts at 25 °C in aqueous media.



Biocatalyst	pH	Initial Rate [a]	Reaction Time (h)	Yield [b] (%)	2 (%)	3 (%)	Thymidine
free CRL	5.0	0.08	104	99	81	9	10
Ald-CRL	5.0	0.24	71	100	91	3	6
Ald-EDA-CRL	5.0	0.09	144	100	70	10	20
Lew105-CRL	5.0	0.06	144	100	17	15	67
free CRL	7.0	0.08	104	99	90	8	2
Ald-CRL	7.0	0.26	51	100	90	4	6
Ald-EDA	7.0	0.09	152	100	88	10	3
Lew105	7.0	0.04	150	62	28	32	2
free CRL	8.0	0.08	120	100	75	11	13
Ald-CRL	8.0	0.11	73	100	88	8	4

[a] the initial rate in μmol × mgprot^{-1} × h^{-1}. It was calculated at 10%–30% conversion. [b] yield of the monohydroxy acetylated product at 100% conversion.

The Ald-CRL preparation showed the highest activity (three times higher than the soluble enzyme) and regioselectivity in the monoacetylation of **1** at pH 5 and 7, producing the C-3 hydroxy monoacetylated thymidine **2** in around 90% yield. The other CRL preparations showed lower regioselectivity, and no differences in activity compared with the soluble enzyme (Table 2). In particular, CRL immobilized on Lew105 lost the specificity and the regioselectivity. Also, the results using the Ald-CRL preparation at pH 8 were better than using the soluble enzyme (Table 2). The role of the immobilization method on the modulation of the activity and regioselectivity has been shown. In the case of CRL, immobilization by this strategy generates a particular orientation of the enzyme (from the protein lid's opposite side (Figure 1C)) and a strong rigidification of its structure. This phenomenon alters the open-closed movement of the oligopeptide lid during catalysis and the exact shape of the open structure which is translated in a significant modulation of the lipase properties. We have already observed these alterations in lipase enantioselectivity [31], and also in the regioselective deprotection of different glycoderivatives [18], and it has been recently observed in the production of 2-glyceryl derivatives [32].

3. Experimental Section

3.1. Materials

Lipase from *Candida rugosa* (CRL), ethylendiamine (EDA), p-nitrophenyl butyrate (pNPB), dithiothreitol (DTT), polyethyleneglycol (PEG) (Mr 1500, 6000), glycerol, dextran (Mw 1500) and trehalose were from Sigma Chem. Co (St. Louis, MO, USA). Sepharose 10BCL, octyl-Sepharose and cyanogen bromide (CNBr) activated Sepharose beads were from GE-Healthcare (Uppsala, Sweden).

Aldehyde-activated Sepharose or Lewatit VP OC105 (Ald or Ald-Lew105) were prepared as previously described [33]. 3,5-*O*-diacetylated thymidine **1** was prepared as previously described [34].

3.2. Lipase Activity Assay

The activities of the soluble lipase (without additives or in the presence of different concentrations of PEG, glycerol, DTT, trehalose or dextran), supernatant and enzyme suspension were analyzed spectrophotometrically measuring the increment in absorbance at 348 nm produced by the release of p-nitrophenol (pNP) (ϵ= 5.150 $M^{-1} \cdot cm^{-1}$) in the hydrolysis of 0.4 mM pNPB in 25 mM sodium phosphate at pH 7 and 25 °C. To initialize the reaction, 0.05–0.2 mL of lipase solution or suspension was added to 2.5 mL of substrate solution in magnetic stirring. Enzymatic activity is given as one µmol of p-nitrophenol released per minute per mg of enzyme (IU) under the conditions described above.

3.3. C. rugosa *Lipase Purification*

The enzyme was purified from commercial crude extract by interfacial adsorption as previously described [35]. Lipase commercial extract was dissolved in 25 mM sodium phosphate buffer at pH 7 to give 200 mg extract/mL, and submitted to gentle stirring during 1 h at 4 °C, and centrifuged at 12,000 rpm during 15 min. The supernatant was separated from the pellet and the protein amount was calculated by Bradford method [36] (5.5 mg prot/mL). Then 1 mL of this supernatant was diluted in 9 mL of 25 mM phosphate buffer pH 7 and the solution was added to one gram of octyl-Sepharose. The reaction was performed at 4 °C for 1 h. After that, the suspension was filtered by vacuum and the solid was washed several times with distilled water. More than 95% of the enzyme was immobilized.

For the preparation of the covalent immobilized catalysts, the lipase was desorbed from the support (one gram of octyl-CRL) adding 20 mL of a solution of 25 mM phosphate buffer pH 7 with 0.4% Triton X-100 (*v*/*v*) and incubated it for 1 h. A final solution of 0.27 mg purified lipase/mL was obtained.

3.4. Preparation of EDA-Aldehyde–Activated Sepharose Support (Ald-EDA)

Sepharose 10 BCL (10 g) was suspended in a mixture solution of 44 mL water, 16 mL acetone, 3.28 g NaOH, 0.2 g NaBH4 and 11 mL epichlorhydrine. The suspension was stirred mildly for 16 h and washed with an excess of water. One gram of epoxy-Sepharose support was suspended in 10 mL of ethylenediamine (0.1 M) solution at pH 8 for 6 h. Finally the support was oxidized adding a solution of 10 mL of water with 140 µmol of sodium periodate per gram of support during 90 minutes and washed abundantly with distilled water and store at 4 °C.

3.5. Multipoint Covalent Immobilization of CRL on Different Aldehyde-Activated Sepharose Supports (Ald)

3.5.1. Immobilization on Aldehyde-Activated Sepharose (Ald) or Aldehyde-Activated Lewatit-105 (Ald-Lew105) at Alkaline pH

First 20 mL of lipase solution (0.27 mg_{lipase}/mL) was dissolved in 20 mL solution of 100 mM sodium bicarbonate pH 8.2 containing 40% PEG1500 (*w*/*v*) and the pH was adjusted at pH 10.15. After that, one gram of Ald-Sepharose or Ald-Lew-105 was added and the reaction was maintained during 16 h at 4 °C. Finally the enzyme-support multi-interaction was ended by adding 1 mg of sodium borohydride per mL of suspension during 30 min [33] (Scheme 1). The immobilization yields are shown in Table 1.

3.5.2. Immobilization on Aldehyde-Activated EDA-Sepharose (Ald-EDA) at pH 8 and Incubation at pH 10

One gram of Ald-EDA-Sepharose was added to 10 mL of purified CRL solution (0.27 mg lip/mL) containing 40% PEG1500 (*w*/*v*). Then the suspension was stirred for 2 h at pH 8 and 4 °C. Periodically, samples of the supernatants and suspensions were withdrawn, and the enzyme activity was measured

as described above. After the preparation was filtrated by vacuum and the solid was incubated in 10 mL sodium bicarbonate buffer at pH 10 for 24 h. Finally, the preparation was reduced by addition of 10 mg sodium borohydride for 30 minutes and then washed with water (Scheme 1). The immobilization yields are shown in Table 1.

3.6. Inactivation of CRL Immobilized Preparations against T and Co-Solvent

First 0.5 g of biocatalyst were dissolved in 5 mL of 25 mM sodium phosphate buffer (with 30% (v/v) acetonitrile) at 25 °C or incubated at 50 °C in acetate buffer at pH 5. The remaining activity at different times was measured by the assay described above using pNPB as substrate.

*3.7. Enzymatic Hydrolysis of 3′,5′-Di-O-Acetylthymidine (**1**)*

Substrate **1** (5 mM) was dissolved in a mixture of acetonitrile (5%, v/v) in 10 mM sodium phosphate at pH 7.0 or 10 mM sodium acetate at pH 5.0. 0.2 g of biocatalyst was added to 2 mL of this solution at 25 °C. During the reaction, the temperature and the pH value was maintained constant using a pH-stat Mettler Toledo DL50 graphic (Mettler-Toledo, LLC 1900 Polaris Parkway, Columbus, OH, USA). The degree of hydrolysis was analyzed by reverse phase HPLC (Spectra Physic Thermo SP 100 coupled with an UV detector Spectra Physic SP 8450 (Thermo Fisher-Scientific, Waltham, MA, USA). For these assays a Kromasil C18 5 µm φ (25 cm×0.4 cm) column was used and the following gradient program (A: mixture of acetonitrile (10%, v/v) in 10mM ammonium phosphate at pH 4.2; B: mixture of miliQ water (10%, v/v) in acetonitrile; method: 0–6 min 100% A, 6–14 min 85% A to 15%B, 14–22 min 100% A, flow: 1.0 mL·min^{-1}). UV detection was performed at 260 nm. The unit of enzymatic activity was defined as micromoles of substrate hydrolyzed per minute per mg of immobilized protein. The monodeprotected 3-OH (**2**) and 5-OH (**3**) were used as pure standards. The retention time was 2.4 min for Thymidine, 9.4 min for 2 and 10.2 min 5 and 19 min for **1**.

4. Conclusions

Lipase from *C. rugosa* has been stabilized at alkaline pH to overcome the inactivation problem by the addition of PEG1500 as a stabilizing agent. Therefore, this has permitted its immobilization for the first time by multipoint covalent attachment on different aldehyde-activated supports in high overall yields. Very stable CRL biocatalysts have been prepared; in particular CRL immobilized on Ald-Sepharose was much more stable than the soluble enzyme. This stable biocatalyst showed an excellent regioselectivity in the monodeprotection of per-*O*-acetylated thymidine, producing the 3-OH-5′-OAc-thymidine in 91% yield at pH 5. Therefore, this new biocatalyst represents an interesting alternative to the octyl-CRL preparation, which has been described as an excellent catalyst in nucleosides and especially in monosaccharides deprotection [18]. However, Ald-CRL presents the advantage of being an irreversible catalyst with high stability in the presence of solvent. This strategy can be also extended to other pH-sensitive enzymes to generate highly stable and active biocatalysts.

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Author Contributions: J.M.P. conceived and designed the experiments; C.W.R. performed the experiments; J.M.P. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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