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Solid-surface activated recombinant *Rhizopous oryzae* lipase expressed in *Pichia pastoris* and chemically modified variants as efficient catalysts in the synthesis of hydroxy monodeprotected glycals

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Lipase of *Rhizopus oryze* expressed in *Pichia pastoris* (ROL_{pp}) was selectively immobilized on octyl-Sepharose, fixing the open and active conformations. This enzyme was compared to the commercial one (ROL_{sigma}) and a unique enzyme of 32 kDa was selectively adsorbed in both cases. Small differences in the N-terminal peptide sequence of both lipases seem to be involved in the enzyme fixing on the solid support, affecting the active site structure. This phenomenon resulted in a strong difference in catalytic properties between immobilized enzymes, with ROL_{pp} being the most active, specific and regioselective heterogeneous biocatalyst in the hydrolysis of lactal hexaacetate. Immobilized ROL_{pp} was 8 times more active, and more specific than immobilized ROL_{sigma}, with excellent regioselectivity (monodeprotection in 3-OH, >99% yield). Solid-phase chemical modification of the N-terminus of immobilized ROL_{pp} was attempted because of the moderate results obtained in the hydrolysis of glucal triacetate. Different biomolecules were introduced and the enzyme catalytic properties in this reaction were assessed. The modification of ROL_{pp} with a polycarboxylated peptide (pA) improved the activity, specificity and regioselectivity of the enzyme, producing mainly the 3-OH monodeprotected glucal. The presence of acetonitrile 3% (v/v) in the reaction medium negatively affected ROL_{pp}, being completely unspecific, whereas ROL_{pp} modified with p1 conserved the specificity and regioselectivity shown in fully aqueous medium. The presence of dioxane improved the specificity and varied the regiopreference of the immobilized lipase (from C-3 to C-6 and C-4 monohydrolyzed products). The posterior modification with pA improved the specificity and the regiopreference of ROL_{pp} towards C-6 monohydrolyzed product.

Introduction

Glycals, pyranose derivatives containing an unsaturated bond, are quite useful in organic chemistry and in particular in carbohydrate chemistry, with tremendous versatility in the preparation of different biologically relevant molecules such as carbohydrate-based vaccines.[1–3]

In the application of these molecules to the architecture of a final active structure it is necessary to consider adequate strategies to obtain a regioselectively free unique hydroxyl group in the molecules without affecting the stability of the double bond. Indeed, many chemical deprotection–protection approaches modify this double bond.[4] In this way the use of biocatalytic strategies has been recognized as one of the most successful methods to obtain this kind of building block without affecting the double bond. This green process permits the achievement of these molecules in a highly specific and regioselective way.[5] However, the stability and the production of the enzyme must be taken into account in the final process for practical industrial implementation.

In this way, the use of the methylotrophic yeast, *Pichia pastoris*, as a cell factory for the expression of recombinant

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proteins has become increasingly popular in recent times and a substantial workhorse for biotechnology.[6]

In particular, *P. pastoris* used as a host for the production of *Rhizopus oryzae* lipase (ROL) offers advantages compared to other hosts such as high productivity, the capacity to grow in a minimal medium at high cell densities, low levels of endogenous protein secretion and the ability to efficiently secrete heterologous protein.[7,8]

ROL is widely used in industrial applications[10] and recently this heterologous ROL version from *P. pastoris* has been successfully described as an interesting biocatalytic tool in the preparation of asymmetric compounds, cholesterol derivatives and biofuels.[9–13]

Nevertheless, the production of this lipase in *P. pastoris* (ROL_{pp}) generates small differences in the N-terminal sequences[14] compared with the same lipase expressed in *E. coli* (ROL_{sigma}).[15] The structure of closed conformation of the lipase from *R. niveus* (RNL)[16] – which presents an identity with ROL of over 95% – is shown in Fig. 1. In general the N-terminal sequences of RNL, ROL_{pp} and ROL_{sigma} show a highly hydrophobic character (more than 10 of the last 28 aminoacids present hydrophobic residues)[16] and part of these sequences is stabilized by a near β-sheet (Fig. 1).

The N-terminal analysis showed some differences between the recombinant and native lipases, especially in a tetrapeptide sequence; the Glu-Ala-Glu-Phe sequence in ROL_{pp} (where two aminoacids correspond to the final sequence of the alpha-factor of *Saccaharomyces*, and the next two correspond to the restriction site where ROL_{pp} was cloned)[14] *versus* the Thr-Asn-Ser-Ala sequence in ROL_{sigma} (aminoacids from the prosequence of ROL) (Fig. 1). Indeed, the

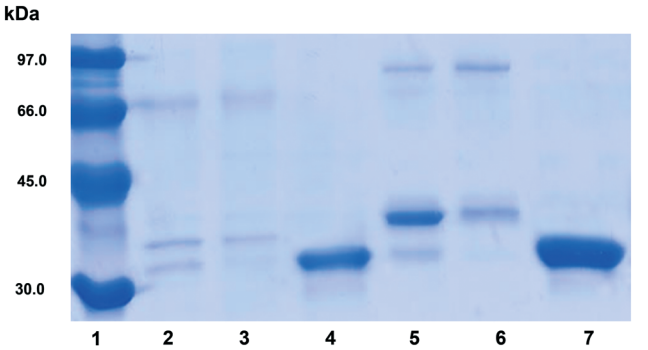


Fig. 2 SDS-PAGE of the immobilized form of ROL in octyl-Sepharose. Lane 1. LMW marker. Lane 2. Soluble recombinant ROL_{pp}. Lane 3. Supernatant after immobilization of ROL_{pp} on octyl-Sepharose. Lane 4. Octyl-Sepharose-ROL_{pp} immobilized preparation. Lane 5. Soluble commercial ROL_{sigma}. Lane 6. Supernatant after immobilization of ROL_{sigma} on octyl-Sepharose. Lane 7. Octyl-Sepharose-ROL_{sigma} immobilized preparation.

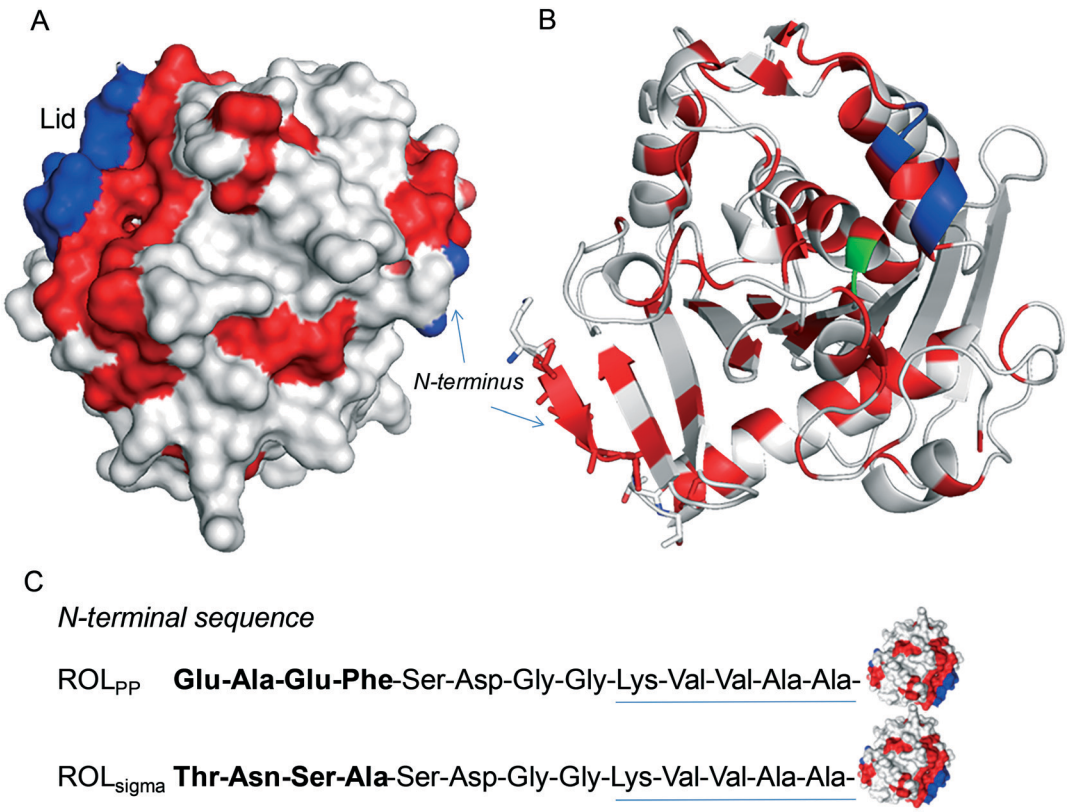


Fig. 1 A. Surface lipase structure in closed conformation. B. Cartoon structure of *Rhizopus* lipase. The protein structure corresponds to *Rhizopus niveus* lipase (>95% homology with ROL, identical N-terminus sequence with ROL_{sigma}) obtained from the Protein Data Bank (pdb code: 1LGY) and the pictures were created using Pymol v. 0.99. Lid oligopeptide (blue), hydrophobic residues (red), serine active site (green). C. N-terminal sequences of ROL_{pp} and ROL_{sigma}. The underlined aminoacids correspond to the N-terminus sequence in the protein structure in A and B.

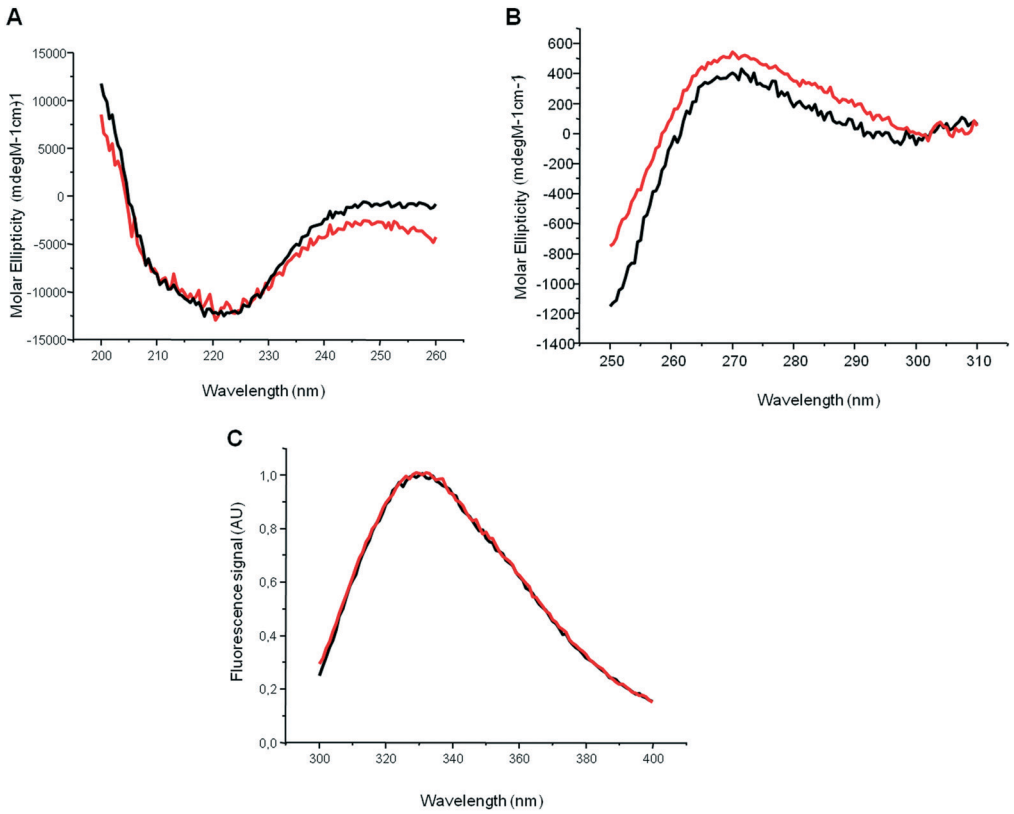


Fig. 3 Characterization of ROL lipases. A) Far-UV CD spectra; B) near-UV CD; C) fluorescent spectra. ROL_{pp} (black line), ROL_{sigma} (red line).

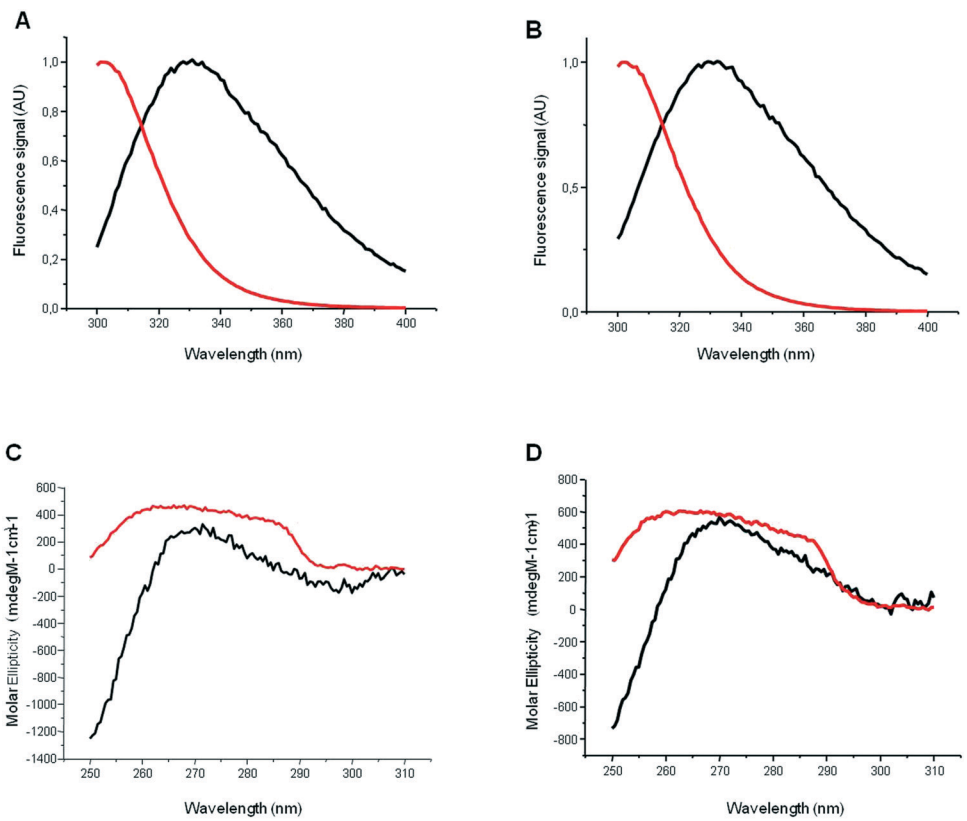


Fig. 4 Characterization of ROL lipases in the presence of Triton X-100. A) Fluorescent spectra of ROL_{pp}. B) Fluorescent spectra of ROL_{sigma}. C) Near-UV CD spectra of ROL_{pp}. D) Near-UV CD of ROL_{sigma}. Without Triton (black line), with Triton (red line).

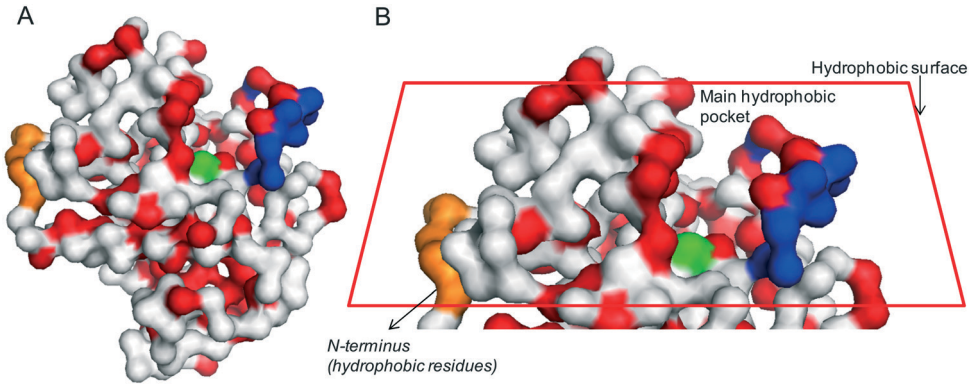
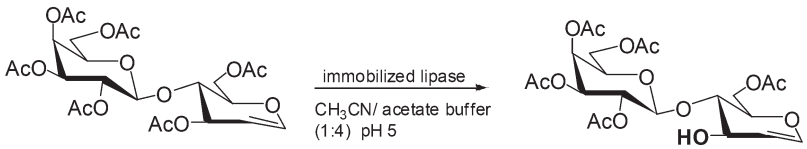


Fig. 5 A. Surface lipase structure in open conformation. B. Lipase in open conformation adsorbed onto a hydrophobic surface. The protein structure corresponds to *Rhizopus delemar* lipase (>95% homology with ROL, identical N-terminus sequence with ROL_{sigma}) obtained from the Protein Data Bank (pdb code: 1TIC) and the pictures were created using Pymol v. 0.99. Lid oligopeptide (blue), hydrophobic residues (red), serine active site (green), N-terminus sequence (orange).

Table 1 Biocatalytic hydrolysis of **1**

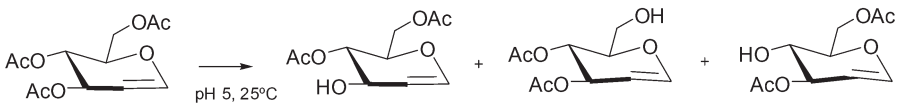


1 **2**

Biocatalyst	Specific activity[a]	Time (h)	Conversion (%)	Yield of **2** (%)	Others
Octyl-Sepharose-ROL_{sigma}	1.83 ± 0.1	72	100	48	52
Octyl-Sepharose-ROL_{pp}	13.6 ± 0.6	23	100	>99	—

[a] The initial rate in μmol × mg_{prot}^{-1} × min^{-1} × 10^{-3}. It was calculated at 10–30% conversion.

Table 2 Hydrolysis of **3** catalyzed by different butyl-Sepabeads-ROL_{pp} preparations at pH 5 and 25 °C



3 **4** **5** **6**

Biocatalyst	Specific activity[a]	Time (h)	Conversion (%)	Yield of **4** (%)	Yield of **5** (%)	Yield of **6** (%)	Others
Butyl-Sepabeads-ROL_{sigma}	10.83 ± 0.86	28	95	29	8	2	56
Butyl-Sepabeads-ROL_{pp}	11.12 ± 0.55	28	95	23	5	6	61
Butyl-Sepabeads-ROL_{pp}-pA	16.99 ± 0.85	24	73	46	5	2	20
Butyl-Sepabeads-ROL_{pp}-pB	13.33 ± 0.52	24	93	6	5	7	75
Butyl-Sepabeads-ROL_{pp}-pC	13.01 ± 0.60	24	84	24	7	7	46
Butyl-Sepabeads-ROL_{pp}-pD	17.49 ± 0.80	24	91	11	8	8	64

[a] The initial rate in μmol × mg_{prot}^{-1} × min^{-1} × 10^{-3}. It was calculated at 10–20% conversion. Ac-Cys-Asp-Asp-Asp-Asp-Asp-COOH (pA), Hippuryl-Arg-OH (pB), dipeptide Boc-Ala-Gly-OH (pC) and polygalacturonic acid (pD).

N-terminus in ROL_{pp} is slightly more hydrophobic than that in ROL_{sigma} (Fig. 1C).

Some reports have demonstrated differences between these two ROL enzymes in terms of specific activity with long-chain esters, although all these studies were performed with the lipase in soluble form.[14] However, lipases in solution are in a certain equilibrium between a closed and an inactive conformation, where the active site is secluded by an oligopeptide chain (lid), (main) and an open and active conformation, where the lid is shifted, making the active site accessible, (minor). This heterogeneity of lipase solution means that enzyme concentration strongly affects the final activity

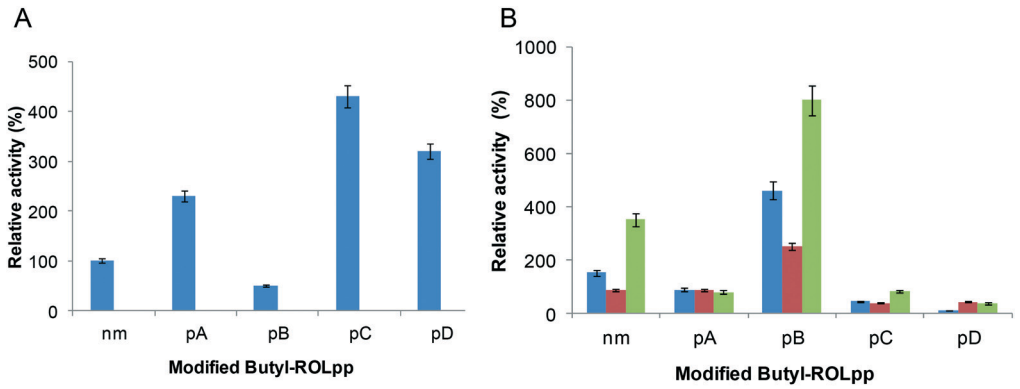


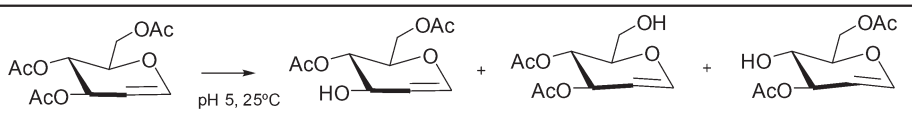
Fig. 6 Activity remaining of the different butyl-Sepabeads-ROL preparations in the presence of solvent. Relative activity values were calculated considering the activity of the non-modified biocatalyst (nm) 100%. A) Activity of the butyl-Sepabeads-ROL after modification. B) Activity of the different immobilized ROL catalysts in the presence of co-solvent (v/v). Acetonitrile (10%) (blue), acetonitrile (20%) (red), dioxane (20%) (green). Activity was determined using the *p*NPB activity assay. Ac-Cys-Asp-Asp-Asp-Asp-Asp-COOH (pA), Hippuryl-Arg-OH (pB), dipeptide Boc-Ala-Gly-OH (pC) and polygalacturonic acid (pD).

Table 3 Stability of the different butyl-Sepabeads-ROL_{pp} preparations in the presence of co-solvent (v/v). The biocatalysts were incubated in the solvent solution and the activity was measured after 3 h

	Relative activity[a] (%)		
Biocatalyst	10% (v/v) acetonitrile	20% (v/v) acetonitrile	20% (v/v) dioxane
Butyl-Sepabeads-ROL_{pp}	56 ± 2.8	100 ± 6.0	60 ± 2.4
Butyl-Sepabeads-ROL_{pp}-pA	42 ± 2.5	20 ± 1.3	100 ± 4.0
Butyl-Sepabeads-ROL_{pp}-pB	41 ± 2.0	83 ± 4.1	40 ± 1.6
Butyl-Sepabeads-ROL_{pp}-pC	56 ± 2.6	60 ± 3.0	60 ± 2.9
Butyl-Sepabeads-ROL_{pp}-pD	25 ± 1.6	30 ± 1.8	40 ± 1.5

[a] The relative activity is determined considering 100% the activity of the biocatalyst dissolved in phosphate buffer 25 mM pH 7. Ac-Cys-Asp-Asp-Asp-Asp-Asp-COOH (pA), Hippuryl-Arg-OH (pB), dipeptide Boc-Ala-Gly-OH (pC) and polygalacturonic acid (pD).

Table 4 Hydrolysis of **3** catalyzed by different butyl-Sepabeads-ROL_{pp} preparations at pH 5 and 25 °C in the presence of 3% (v/v) acetonitrile



3 **4** **5** **6**

Biocatalyst	Specific activity[a]	Time (h)	Conversion (%)	Yield of **4** (%)	Yield of **5** (%)	Yield of **6** (%)	Others
Butyl-Sepabeads-ROL_{pp}	14.66 ± 0.88	48	99	1	1	1	96
Butyl-Sepabeads-ROL_{pp}-pA	1.66 ± 0.1	72	85	48	0	3	34
Butyl-Sepabeads-ROL_{pp}-pB	4.10 ± 0.23	48	100	0	0	0	100
Butyl-Sepabeads-ROL_{pp}-pC	4.16 ± 0.20	48	100	0	1	0	99
Butyl-Sepabeads-ROL_{pp}-pD	3.83 ± 0.20	48	100	1	2	0	97

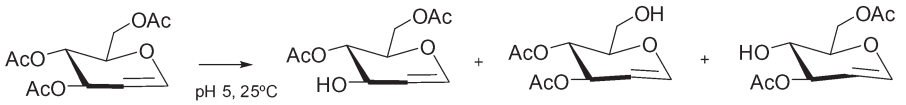
[a] The initial rate in $\mu mol \times mg_{prot}^{-1} \times min^{-1} \times 10^{-3}$. It was calculated at 10–20% conversion. Ac-Cys-Asp-Asp-Asp-Asp-Asp-COOH (pA), Hippuryl-Arg-OH (pB), dipeptide Boc-Ala-Gly-OH (pC) and polygalacturonic acid (pD).

values.[17] Therefore the application of immobilization strategies where a unique conformation of lipase can be present on a solid-phase is quite important for a real comparison.

For the regioselective deprotection of carbohydrates using lipases, the application of enzymes in open conformation has been demonstrated to be mandatory for successful results.[5] Many different examples demonstrate that lipases adsorbed by hydrophobic interactions result in a fixed open and more stable conformation.[18–21] Indeed, in the case of carbohydrate reactions, the best catalysts using lipases have been an immobilized form where the lipase is adsorbed onto a hydrophobic support material.[5]

In this work we analyzed the effect of fixing the open conformation of both ROL lipases, by selective adsorption on a hydrophobic matrix. The effect on the catalytic properties and especially their application in the regioselective hydrolysis of different peracetylated glycals have been tested. Also site-specific modifications of the ROL_{pp} on the solid-phase

Table 5 Hydrolysis of **3** catalyzed by different butyl-Sepabeads-ROL_{pp} preparations at pH 5 and 25 °C in the presence of 3% (v/v) dioxane



3 **4** **5** **6**

Biocatalyst	Specific activity[a]	Time (h)	Conversion (%)	Yield of **4** (%)	Yield of **5** (%)	Yield of **6** (%)	Others
Butyl-Sepabeads-ROL_{pp}	13 ± 0.66	24	83	8	14	14	47
Butyl-Sepabeads-ROL_{pp}-pA	2.50 ± 0.12	72	89	9	29	13	38
Butyl-Sepabeads-ROL_{pp}-pB	10 ± 0.5	24	62	0	2	2	58
Butyl-Sepabeads-ROL_{pp}-pC	15 ± 0.74	24	95	4	5	5	81
Butyl-Sepabeads-ROL_{pp}-pD	14 ± 0.70	24	90	10	11	14	55

[a] The initial rate in μmol × mg_{prot}^{-1} × min^{-1} × 10^{-3}. It was calculated at 10–20% conversion. Ac-Cys-Asp-Asp-Asp-Asp-Asp-COOH (pA), Hippuryl-Arg-OH (pB), dipeptide Boc-Ala-Gly-OH (pC) and polygalacturonic acid (pD).

were performed by introducing different peptide sequences in the N-terminal and their effect on catalytic properties was studied.

Experimental section

General

Ac-Cys-Asp-Asp-Asp-Asp-Asp-COOH (pA) was purchased from Isogen (Netherlands). Lipase from *Rhizopus oryzae* (ROL_{sigma}), per-*O*-acetylated lactal (**1**), per-*O*-acetylated glucal (**3**), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), Hippuryl-Arg-OH (pB), Boc-Ala-Gly-OH (pC), polygalacturonic acid (25–50 kDa) (pD) and *p*-nitrophenyl butyrate (*p*NPB) were from Sigma Chem. Co (St. Louis, USA). Octyl-Sepharose 4BCL fast flow was from GE heathcare (Uppsala, Sweden). TLC analyses were run on silica plates (Merck 60 F_{254}). HPLC analyses were performed using an HPLC-spectra P400 (Thermo Separation products). The column was a Kromasil-C18 (250–ϕ4.6 mm and 5 mm) from Analisis Vinicos (Tomelloso, Spain). Analyses were run at 25 °C using an L-7300 column oven at a flow of 1 mL min^{-1} and UV detector L-7400. NMR spectra were recorded using a Varian Mercury 500 MHz spectrometer and calibrated in accordance with solvent standard peaks.

Production of *Rhizopus oryzae* lipase in *Pichia pastoris*

Rhizopus oryzae lipase (ROL) was produced by a mixed substrate fed-batch cultivation of a recombinant *P. pastoris* strain using methanol as an inductor.[22] The culture broth was centrifuged and microfiltered to remove the biomass. The supernatant was concentrated by ultrafiltration with a Centrasette® Pall Filtron system (New York, USA) equipped with an Omega membrane with a 10 kDa cut-off, and subsequently dialyzed against 10 mM Tris-HCl buffer pH 7.5 and thereafter lyophilized.[23]

Enzymatic activity assay (*p*NPB assay)

In order to follow the immobilization process, the activities of the soluble lipases and their immobilized preparations were analyzed spectrophotometrically, measuring the increment in absorbance at 348 nm ($\in$ = 5.150 M^{-1} cm^{-1}) produced by the release of *p*-nitrophenol (*p*NP) in the hydrolysis of 0.4 mM of *p*-nitrophenol butyrate (*p*NPB) in 25 mM sodium phosphate buffer at pH 7 and 25 °C. To initialize the reaction, 0.05–0.2 mL of lipase solution (blank or supernatant) or suspension was added to 2.5 mL of substrate solution. Enzymatic activity was determined as μmol of hydrolyzed *p*NPB per minute per mg of enzyme (IU) under the conditions described above.

Immobilization of lipases

Two grams of ROL powder (containing 5 mg total protein per gram) was dissolved in 60 mL of sodium phosphate buffer pH 7.

Octyl-Sepharose support was added to the enzyme solution; 1 g in 30 mL of ROL solution. The other 30 mL of ROL solution was added to 1 g of butyl-Sepabeads. The reactions were incubated at 25 °C for 4 h. After this time, enzymatic activity of the suspension and supernatant were determined using the *p*NPB enzymatic assay to determine the immobilization yield and remaining enzymatic activity in the immobilized preparation. After immobilization, the enzyme derivative was recovered by filtration under vacuum, washed with abundant distilled water and conserved dry in the fridge. In all cases more than 95% of enzymatic activity was immobilized on the support and the immobilized enzyme conserved the initial activity.

N-terminal chemical modification of ROL immobilized on butyl-Sepabeads

Compounds pA–pD (5 equiv.) were respectively dissolved in 0.5 mL of acetonitrile and added to 10 mL of distilled water pH 4.8 containing EDC (10 mM). After 30 min, one gram of the ROL immobilized on butyl-Sepabeads (butyl-Sepabeads-ROL) was added to the solution and the mixture was incubated for 18 h. Then, the suspension was filtrated, washed with abundant distilled water and stored in the fridge.

Stability of the different biocatalysts in the presence of co-solvent

The different unmodified and modified butyl-Sepabeads-ROL biocatalysts were incubated in phosphate buffer solution with different amounts of co-solvent (v/v) (dioxane or acetonitrile). The activity of the immobilized enzyme was evaluated over time using the *p*NPP assay. At least triplicates of each assay were made.

Biocatalytic deprotection of per-*O*-acetylated lactal (1)

1 (0.01 mmol) was added to a 2 mL solution of phosphate buffer (25 mM) with acetonitrile (20%) at pH 5, 25 °C and the reaction was initialized by adding 0.4 g of biocatalyst. The hydrolytic reaction was carried out under mechanical stirring, and the pH value was controlled by automatic titration. At least triplicates of each assay were made. Hydrolysis reactions were followed by TLC. TLC conditions were hexane : AcOEt 5 : 5 v/v, R_{f2}: 0.25, R_{f1}: 0.56. The reaction was also followed by HPLC, where the eluent was an isocratic mixture of 40% acetonitrile in 10 mM ammonium phosphate buffer at pH 3.8, UV detection at 220 nm and a flow rate of 1 mL min^{-1}. Under these conditions, the retention times were: **1**: 18.50 min, **2**: 11.01 min.[24]

The reaction was scaled up using 2 g of biocatalyst in the hydrolysis of 0.05 mmol (24 mg) of **1** in 15 mL volume (containing 20% ACN) at pH 5. When full conversion was achieved, the reaction mixture was filtrated by vacuum on a sintered glass filter. The catalyst was washed with distilled water (3 times) and stored at 4 °C. The aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (3 × 15 mL). The collected organic layers were dried over anhydrous Na_2SO_4 which was then removed by filtration and concentrated under vacuum, producing a white solid (**2**, 21 mg, 90%) which was identified by ^{1}H-, ^{13}C-NMR and 2D-COSY. ^{1}H-NMR (500 MHz, $CDCl_3$) of **2**. δ (ppm): 6.33 (d, 1H, J = 5.90 Hz, H-1), 5.38 (d, 1H, J = 3.39 Hz, H-4′), 5.25 (t, 1H, J = 8.37 Hz, H-2′), 5.00 (dd, 1H, H-3′), 4.76 (dd, 1H, H-2), 4.59 (d, 1H, J = 8.0 Hz, H-1′), 4.43 (m, 1H, H-3), 4.20–4.10 (m, 2H, H-6a, 6b), 4.13–4.07 (m, 2H, H-6′a, 6′b), 4.08 (m, 1H, H-5′), 3.98 (m, 1H, H-5), 3.63 (m, 1H, H-4), 2.15–2.09 (5s, 15H, 5 × CH_3). ^{13}C-NMR (300 MHz,$CDCl_3$). δ (ppm): 170.58, 170.03, 169.60, 143.75, 102.66, 102.19, 81.96, 73.77, 71.51, 70.82, 68.70, 68.26, 66.98, 62.58, 62.10, 20.79, 20.59, 20.55, 20.45.

Biocatalytic deprotection of per-*O*-acetylated glucal (3)

Per-*O*-acetylated glucal (**3**) (1.5 mM) was dissolved in 1 mL of 25 mM sodium phosphate buffer at pH 4.8, containing in some cases 3% of co-solvent (v/v). 0.1 g of butyl-Sepabeads-ROL preparation was added. The reaction was followed by HPLC analysis. At least triplicates of each assay were made. Analyses were run at 25 °C using an L-7300 column oven and UV detector L-7400 at 220 nm. The mobile phase was an isocratic mixture of 20% acetonitrile–80% water (containing 0.01% TFA); flow rate 1.0 mL min^{-1}. For the product characterization the reaction was scaled up 5 times. When the reaction was complete, the reaction mixture was filtered by vacuum on a sintered glass filter and the solution was saturated with NaCl and extracted with ethyl acetate. After evaporation of the solvent under reduced pressure, the crude product was purified by flash chromatography and determined by ^{1}H-NMR.

^{1}H-NMR (500 MHz, $CDCl_3$) of **4**. δ (ppm): 6.42 (dd, J = 6.1 Hz, 1H, H-1), 4.95 (dd, J = 6.2, 2. Hz, 1H, H-4), 4.84 (dd, J = 6.2, 3.2, 2.7 Hz, 1H, H-2), 4.43 (ddd, J = 6.7, 5.3 Hz, 1H, H-5), 4.22–4.38 (m, 2H, H-6A, H-6B), 4.20–4.11 (dd, J = 6.2, 2.2 Hz, 1H, H-3), 2.55 (bs, 1H, OH), 2.16 (s, 3H, CH_3), 2.11 (s, 3H, CH_3).

^{1}H-NMR (500 MHz, $CDCl_3$) of **5**. δ (ppm): 6.49 (dd, J = 6.1 Hz, 1H, H-1), 5.41–5.50 (m, 1H, H-3), 5.22 (dd, J = 9.0, 6.5 Hz, 1H, H-4), 4.81 (dd, J = 5.9, 2.8 Hz, 1H, H-2), 3.98–4.09 (m, 1H, H-5), 3.66–3.86 (m, 2H, H-6A, H-6B), 2.07–2.13 (2s, 6H, 2CH_3).

^{1}H-NMR (500 MHz, $CDCl_3$) of **6**. δ (ppm): 6.45 (dd, J = 6.2, 1.5 Hz, 1H, H-1), 5.31 (ddd, J = 7, 2.4 Hz, 1H, H-3), 4.76 (dd, J = 3.6, 2.6 Hz, 1H, H-2), 4.38–4.57 (dd, J = 14.7, 7 Hz, 2H, H-6A, H-6B), 4.01 (m, J = 2.6 Hz, 1H, H-5), 3.84 (bt, J = 9.6 Hz, 1H, H-4), 3.54 (bs, 1H, OH), 2.14–2.16 (s, 6H, 2CH_3).

HPLC determination: retention times of the different products in HPLC were: R_{t3} = 24.6 min, R_{t4} = 6.3 min, R_{t5} = 6.6 min, R_{t6} = 8.1 min.

Circular dichroism

Circular dichroism (CD) spectra of the different lipases were recorded in a Chirascan spectropolarimeter (Applied Photophysics) at 25 (±1) °C. Near-UV spectra were recorded at wavelengths between 250 and 310 nm in a 1 cm path-length cuvette and far-UV spectra were recorded at wavelengths between 200 and 260 nm in a 0.1 cm path-length cuvette. Protein concentrations were 20 and 10 μ, respectively, in phosphate buffered saline, pH 7.2 (PBS; bioMerieux).

Fluorescence spectroscopy

Fluorescence measurements were performed in a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) monitoring the intrinsic tryptophan fluorescence in 2 μM of different ROL variants and peptide conjugate solutions, using an excitation wavelength of 280 nm, with excitation and emission bandwidths of 5 nm, and recording fluorescence emission spectra between 300 and 400 nm. All spectroscopic measurements were made in water.

Results and discussion

Characterization and adsorption of *Rhizopus oryzae* lipase (ROL)

The two lipases, ROL_{pp} and ROL_{sigma}, were adsorbed on commercial octyl-Sepharose at 25 °C for 3 h. In both cases, the lipase immobilization yield was excellent (>95%). This immobilization method, using low ionic strength during the incubation, permits the specific immobilization of lipases in a crude mixture, *e.g.* containing esterases.[25] Thus, the SDS-PAGE analysis of the crude, supernatant and suspension of the different immobilized proteins revealed that ROL_{pp} presents three

bands, one around 34 kDa, another slightly more intense at 36 kDa and another around 70 kDa (Fig. 2, lane 2). In the case of ROL_{sigma}, 3 bands also appeared, the main one at 40 kDa and another two slighter bands at 34 kDa and around 97 kDa, respectively (Fig. 2, lane 5). These results showed different proteins contained in the crude extract of both ROL lipases. However, in both cases only a single strong band at 34 kDa was observed in the immobilized form (Fig. 2, lane 4 and 7). The other bands were not adsorbed on octyl-Sepharose under these conditions, which could indicate that those proteins are not lipases, (*e.g.* the 40 kDa which has been identified as esterase).[14] Thus, the two immobilized ROL preparations can be completely comparable for an evaluation of whether the variation in the N-terminal sequence has any effect on the catalytic properties of the enzyme.

In order to know if some structural effect can be observed, both enzymes were desorbed from the support after adding 1% Triton X-100 and the soluble pure enzymes were concentrated by ultracentrifugation using an Amicon Ultra filter 10 kDa.

Experiments of far circular dichroism (CD) to study possible modifications in the secondary structure and near CD and fluorescence assays to analyze the tertiary structure were performed (Fig. 3). According to the far CD spectrum, the content of α helix secondary structure in the enzymes was the same, which suggested that no significant differences in that were observed (Fig. 3A). Additionally, small differences in intensity were observed in the near CD spectrum but not in shape and the fluorescence spectra of both enzymes were identical (Fig. 3C). These results suggested that this small variation in the N-terminal seems not to be significant enough to structurally affect the whole enzyme.

CD and fluorescent analyses of ROL_{pp} and ROL_{sigma} in the presence of detergent were performed (Fig. 4).

In the fluorescence spectrum there was a displacement in the maximum of 28 nm (from 330 to 302 nm), identical for both lipases (Fig. 4A and B). This data showed the conformational change from mainly closed (without detergent) to open conformation (in the presence of detergent) of the enzyme, which is similar in both cases.

Near CD spectra also showed a change in the tertiary structure with the presence of detergent in both cases; some deviations were observed in the range of 310–285 nm in a comparison between both enzymes.

The N-terminus of the protein seems to be also involved in the interaction of the protein with a hydrophobic surface (28-mer peptide sequence with 50% hydrophobic groups),[16] considering the main hydrophobic pocket, the lid and surrounding areas in the open conformation (Fig. 5). Furthermore, the N-terminal peptide of ROL is also interacting with a loop (Asp232-Phe242) bearing the catalytic aspartic acid, which could be influencing the final catalytic properties when this lipase is fixed on a hydrophobic interface.[25,26]

Application of immobilized ROL preparations

The octyl-Sepharose immobilized forms of ROL_{pp} and ROL_{sigma} were used as catalysts in the regioselective hydrolysis of lactal hexaacetate **1** at pH 5 and 25 °C. Although the structural results did not show clear differences, a tremendous difference in catalytic properties was observed for this particular reaction. ROL_{pp} immobilized on octyl-Sepharose was 8-fold more active than the immobilized ROL_{sigma} (Table 1). Furthermore, octyl-Sepharose-ROL_{pp} showed excellent specificity and regioselectivity, producing exclusively the 3-OH monohydrolyzed **2** in >99% yield, whereas immobilized ROL_{sigma} showed moderate specificity (Table 1). Thus ROL showed a similar regio-preference in the monodeacetylation to other lipases,[5,27,28] although this result using immobilized ROL_{pp} is the best in terms of activity and excellent regioselectivity. This excellent result supports the theory by which a simple change in some aminoacids in the N-terminal sequence of the enzyme expressed by *Pichia pastoris* generates a more active and specific enzyme in this complex biocatalytic reaction.

In this way in order to expand the study, the ROL catalysts were evaluated in the hydrolysis of glucal triacetate (**3**). To this end, ROL enzymes were immobilized on butyl-Sepabeads, a specific resin which does not suffer swelling, which can thus be used both in aqueous and in organic media (where Sepharose collapsed[29]).

In the hydrolysis of **3**, ROL_{sigma} was slightly better in specificity and selectivity than ROL_{pp} (Table 2). The butyl-Sepabeads-ROL_{pp} preparation showed low specificity in monodeprotection and also low regioselectivity, producing 3-OH (**4**), 6-OH (**5**), and 4-OH (**6**) monohydrolyzed products, with moderate yields for **4** (23%) (Table 2).

Therefore, considering these moderate results for butyl-Sepabeads-ROL_{pp}, a new strategy for improving the specificity and regioselectivity of the enzyme in the hydrolysis of **3** was attempted.

After the clear differences in catalytic performance of both ROL enzymes in the hydrolysis of lactal due to the tetrapeptide difference in the protein sequence (higher anionic with hydrophobic degree residues in ROL_{pp}) as a key role, a site-specific chemical modification in the N-terminus of immobilized ROL_{pp} was attempted.

Different biomolecules were selected for incorporation into the protein, considering a balance of hydrophilic/hydrophobic. For that purpose, a polycarboxylated peptide Ac-Cys-Asp-Asp-Asp-Asp-Asp-COOH (pA), which was recently demonstrated to greatly alter the properties of a genetically modified lipase,[30] was used. Also Hippuryl-arginine (pB) which presents a hydrophobic *N*-protecting group with a positively charged character (opposite to pA), dipeptide Boc-Ala-Gly-OH (pC) (introducing hydrophobicity) and finally a polysaccharide, polygalacturonic acid (pD), a hydrophylic and very carboxylated structure.

The effect of the modification on the enzyme activity was evaluated (Fig. 6). The modification using molecules containing carboxylic groups (pA, pC and pD) improved the enzyme activity from 2 to 4 fold, whereas the introduction of N-terminal arginine protected by a hydrophobic group (pB), decreased by half the activity of the immobilized enzyme

(Fig. 6A). The activities analyzed in the presence of different solvents and concentration results are shown in Fig. 6B. The butyl-Sepabeads-ROL_{pp} underwent an increase in activity especially in the presence of dioxane. Concerning the modified butyl-Sepabeads-ROL_{pp} a high hyper-activation of enzyme activity is observed with pB modification in the presence of dioxane (around 8 fold) and also with acetonitrile (4 fold), whereas the other modified ROL_{pp} biocatalysts just conserved the activity, or suffered strongly decreasing values such as was the case of modification with pD.

These new biocatalysts were tested in the hydrolysis of **3** at pH 5 and 25 °C (Table 2).

Under these conditions, the butyl-Sepabeads-ROL_{pp}-pA showed better activity (2 fold) and much better specificity and regioselectivity than the non-modified catalyst. This biocatalyst permitted us to obtain mainly 3-OH product (46% yield from 73% conversion). The enzyme modification with the other carboxylated molecules (pC and pD) improved the activity but not significantly the specificity. Interestingly, the butyl-Sepabeads-ROL_{pp}-pB worsened the enzyme specificity, also decreasing the regioselectivity (Table 2).

Stability of the new butyl-Sepabeads-ROL_{pp} biocatalysts

The different modified ROL_{pp} biocatalysts were incubated for 3 h in the presence of different co-solvent concentrations and the remaining activity was calculated in the *p*NPB assay (Table 3). In the presence of 10% (v/v) acetonitrile, around half the activity value was conserved in the non-modified ROL preparation, whereas the chemically modified catalyst showed similar or less activity after this time. More inhibition was observed after incubation in the presence of 20% acetonitrile. In this case the butyl-Sepabeads-ROL-pB conserved higher activity than the non-modified one, whereas the other modifications did not improve the result. As we previously observed in Fig. 6 acetonitrile in general inhibited enzyme activity more than dioxane. Indeed, in the presence of 20% (v/v) dioxane, butyl-Sepabeads-ROL conserved up to 60% activity in 3 h of incubation. Surprisingly, the modification with pA maintained the full initial activity of the enzyme (Table 2). In the other biocatalysts the modification did not help the stability of the enzyme.

Thus, it was clear that the modification performed on the N terminus of ROL_{pp} showed an important effect on activity and stability.

Hydrolysis of **3** catalyzed by butyl-Sepabeads-ROL_{pp} biocatalysts in the presence of co-solvent

In order to alter the catalytic properties of the immobilized enzyme, the hydrolytic reaction of **3** catalyzed by butyl-Sepabeads-ROL_{pp} biocatalysts was performed in aqueous media containing 3% (v/v) of different co-solvents (Tables 4 and 5). This relatively low co-solvent concentration was demonstrated to be enough to alter the enzyme structure to improve the specificity and regioselectivity,[31] and considering the previous results of immobilized ROL stability at higher co-solvent concentration, a solvent percentage high enough to conserve the enzyme stability during the reaction times.

In the presence of 3% (v/v) of acetonitrile, most butyl-Sepabeads-ROL_{pp} preparations suffered a strong decrease in activity and specificity (Table 4). The butyl-Sepabeads-ROL_{pp} modified with pA was unique in that it conserved the specificity and regioselectivity from that without co-solvent.

When 3% (v/v) dioxane was in the reaction medium, an improvement in specificity for butyl-Sepabeads-ROL_{pp} and some modified preparations was observed (Table 5). However, the most significant change was the enantiopreference of the enzyme: now the three different monohydrolyzed products were formed, in higher amounts for **5** (6-OH) and **6** (4-OH) instead of **4** (3-OH). The best result was obtained with butyl-Sepabeads-ROL_{pp}-pA, which showed the best specificity and regioselectivity towards **5** (Table 5). The other modified catalysts showed the same regioselectivity but worse specificity than butyl-Sepabeads-ROL_{pp} (Table 5).

The specificity and regioselectivity achieved by the different immobilized ROL biocatalysts were maintained during all the reaction times.

Therefore, the site-specific chemical modification of ROL_{pp} with pA, a tetracarboxylated peptide, seems to be the best catalyst for this monodeprotection reaction. Without a solvent, this catalyst showed the best specificity, stability and regioselectivity in the production of **4**. However, acetonitrile addition did not improve the enzyme catalytic properties for this reaction, whereas the presence of dioxane had an interesting effect in modulating the regioselectivity of the enzyme, and in particular for this modified ROL, producing mainly the **5** (6-OH) in a ratio of 2.23 against **6** (4-OH).

Conclusions

The expression of *R. oryzae* lipase in *P. pastoris* generates small changes in the N-terminal sequence of the protein compared with the commercial version, which seem to be structurally not significant although strongly affecting the lipase catalytic properties. The adsorption onto a hydrophobic solid support permitted a real comparison of both enzymes, because a unique enzyme is fixed in an active form (open conformation) in both cases. The N-terminal sequence with a hydrophobic nature is involved in fixing the enzyme on the solid support which is also related to modifications in the active site structure. This resulted in a strong difference in activity and specificity between immobilized enzymes, with ROL from *P. pastoris* being the most active, specific and regioselective catalyst in the hydrolysis of fully acetylated disaccharide lactal.

In a second approach, chemical modification of the N-terminus of ROL_{pp} was attempted. Different biomolecules were introduced at this position on the immobilized enzyme, obtaining excellent results by modification with a polycarbxylated peptide, increasing the activity, specificity and regiseolectivity of the enzyme in the hydrolysis of glucal, especially important when a co-solvent was used. Therefore, the

directly immobilized enzyme or site-specific modified ones could be excellent heterogeneous biocatalysts for application in the improved preparation of glycosyl building blocks.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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