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Authors: Ciara Callaghan, Martin Redmond, rOBSON c. Alnoch, Cesar Mateo, Marco Filice, and Jose M Palomo

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Biocatalytic process optimization for production of high-added value 6-*O*-hydroxy and 3-*O*-hydroxy glycosyl building blocks

Ciara Callaghan[a], Martin Redmond[a], Robson Carlos Alnoch[a], Cesar Mateo[a], Marco Filice*[a,b] and Jose M. Palomo*[a]

[a] C. Callaghan, M. Redmond, R. C. Alnoch, Dr. C. Mateo, Dr. M. Filice, Prof. Jose M. Palomo

Department of Biocatalysis. Institute of Catalysis (CSIC). Marie Curie 2. Cantoblanco.

Campus UAM, 28049 Madrid, Spain. Fax: +34-91-585-4760.

E-mail: josempalomo@icp.csic.es / marco.filice1@gmail.com

[b] Dr. M. Filice

Current address: Advanced Imaging Unit (CNIC), CIBERES, Melchor Fernandez Almagro, 3, 28029, Madrid. Spain.

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Abstract

A biocatalytic optimization process to synthesize regioselective monohydroxy glycosyl building blocks has been performed. Lipases immobilized on commercial supports were treated with water soluble carbodiimide (EDC) at different concentrations. In the presence of co-solvents, the stability of lipases adsorbed on octyl-Sepharose improved after the EDC modification. The new *Candida rugosa lipase* (CRL) modified heterogeneous biocatalysts were tested in the production of 6-OH hydroxyl-tetraacetyl glucose by a regioselective monodeacetylation in aqueous media. Improvements in activity and excellent regioselectivity were obtained for octyl-CRL-EDC10mM preparation, with 95% isolated yield of product in multimilligrams scale. Also excellent recyclability was observed. The C-6 alcohol was transformed in C-3 by chemical migration and both compounds were successfully transformed in the corresponding novel trichloroacetimidyl glucoderivatives. Modified CRL biocatalysts were also tested in the selective deprotection of peracetylated thymidine, and octyl-CRL-EDC10mM showed excellent specificity and improved regioselectivity, producing 3-hydroxy-5-acetyl-thymidine, precursor of azidethymidine (AZT), in 95% yield. The new RML modified heterogeneous biocatalysts showed excellent regioselectivity and recyclability in the 3-OH monodeprotection of peracetylated lactal.

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Introduction

Glycoderivatives are a versatile class of molecules, useful as key intermediates in the synthesis of novel bio-active compounds such as drugs and vaccines, for example, which will allow human beings to overcome diseases, improving the quality of life of patients, or even in the development of early diagnosis and more effective and targeted therapeutic modalities.[1-5] Therefore, the development of more effective and economic strategies for preparing these molecules is mandatory.

In particular, one of the key steps in glycochemistry is the efficient access to new partially protected monosaccharides containing a strategically positioned free hydroxyl group (a nucleophilic acceptor) as building blocks for a regio- and stereochemical controlled synthesis of oligosaccharides and glycoderivatives. Different chemical methods have been described in literature based on regioselective orthogonal protection strategies and different combinations, there for to give access to these specific monosaccharides.[6-7] However, the ideal strategy would be one where only a single protecting group is used, ensuring a high conversion and good final yields. An extremely high control of selectivity during the synthesis is mandatory in order to reduce the synthetic steps and to obtain pure homogeneous compounds with the highest economic efficiency.

In this way, regioselective deprotection of fully protected (with the same protecting group) monosaccharides represents one of the best choices. However, the preparation of these molecules by chemical methods is tedious and hard work (several steps, tedious work-up, time-consuming purification, moderate yields),[8] because of the difficulty to discriminate between groups with a very similar reactivity which does not completely fulfill the requirement of a very regioselective methodology.

In recent years our group has developed a straightforward strategy to synthesize diversely monodeprotected mono- and disaccharide building blocks from fully acetylated carbohydrates catalyzed by immobilized lipases at very mild conditions.[9]

The strategy is based on the complex catalytic mechanism of lipases which implies dramatic conformational changes of the enzyme molecule between a closed and an open form.[10]

Indeed, in most cases the lipase adsorbed on a hybrophobic surface, fixing the open conformation, resulting in the best catalyst in terms of reaction yields and regioselectivity providing valuable intermediates, such as the *C-6* or *C-3* monodeacetylated molecule (key positions in the synthesis of biological relevant oligosaccharides).[9a,11] High activity and regioselectivity have been obtained using lipase from *C. rugosa* (CRL) adsorbed on commercial octyl-Sepharose as catalyst for regioselective one-pot preparation of different *C-6* monodeprotected monosaccharides[9a] and the lipase from *R. miehei* (RML) adsorbed on commercial octyl-Sepharose for regioselective deprotection *C-3* in peracetylated lactal.[12]

However, despite the extremely high catalytic parameters shown by these immobilized lipases in these processes, a reversible immobilization was promoted in both cases. As consequence, the enzyme catalyst can be desorbed from the heterogeneous support in precise conditions (e.g. in the presence of hydrophobic or amphiphilic agents).[13]
Therefore, issues such as stability of the catalyst, reutilization, activity and control of the excellent regioselectivity require to be optimized before a possible implementation at industrial scale of this biocatalytic regioselective process.
The free-hydroxyl acetylated molecules can be easily convertible in substituted mono- and polysaccharides useful in chemical, biological and medicinal studies. This process is

performed by a previous activation of the hydroxyl group. Many strategies have been described in literature although in most cases by activation of the anomeric position of the sugar. [14-15] Here, we produce selectively 6-*O* and 3-*O* hydroxy glycosyl building blocks which can be a new alternative position for activation making a great relevance in biomedical studies. The biological activity of natural products in many cases derives from the sugar moieties and changes in a simple single position in the sugar structure could be dramatically important such as occur in the different ABO blood groups.[16] The chemical activation of anomeric hydroxyl group in sugar is usually performed by using tricloroacetonitrile at base conditions generating the corresponding tricloroacetamidate (TCA) derivative.[15c] This modification in other positions of the sugar will permit to create a new class of neo-glycoconjugates and glycoderivatives.

Herein, the chemo-enzymatic regioselective monodeprotection process of different peracetylated glycoderivatives catalyzed by CRL and RML lipases immobilized on commercial octyl-Sepharose has been improved by a post-immobilization treatment with water-soluble carbodiimide, enhancing the catalyst stability while conserving its excellent regioselectivity. By this way, the production of different monodeprotected building-blocks in multimilligram scale has been successfully achieved. Subsequently to this biocatalytic optimization, the chemical activation of the produced free hydroxyl group producing the novel trichloroacetimidyl glucoderivatives (6-OTCA and 3-OTCA) was satisfactorily carried out.

Results and Discussion

Lipase from *C. rugosa* adsorbed on commercial octyl-Sepharose (octyl-CRL) or Lewatit1600 (Lew-CRL) and lipase from *R. miehei* adsorbed on commercial octyl-Sepharose (octyl-RML) were incubated with EDC solution (at different concentrations) by a solid-phase reaction strategy in order to avoid the leakage of the enzyme from the catalysts. The effect of this treatment in the enzyme activity of the immobilized preparations was evaluated in the hydrolysis of *p*-nitrophenylbutyrate. The activity was almost conserved in all cases after treatment with 10 mM of EDC (Lew-CRL suffered a decrease of 20% value), whereas loss of almost 30-40% in activity was observed using higher EDC concentrations (Table 1).

In order to choose the optimal modification for the particular biotransformation, the stability of the different modified-EDC lipase preparations by incubation at different concentrations of co-solvents was studied.

First, the stability of the CRL preparations was evaluated in the presence of 20% or 50% (v/v) acetonitrile (Figure 1 and 2). CRL is a very sensitive enzyme compared with other lipases [13a,17] and 50% co-solvent represents a very harsh condition. After incubation in an aqueous solution of 50% acetonitrile, an improvement on stability for the modified octyl-CRL preparations was observed (Figure 1) whereas for Lew-CRL a dramatic loss in stability was observed after 1 h incubation (less than 20% of remaining activity) (Figure 1A). In the case of octyl-CRL, the non-modified preparation suffered a strong activity lost caused by the release of the enzyme from the support at these conditions (soluble enzyme is instantly inactive at these conditions).[13a] In fact in 1 h less than 50% activity was recovered, whereas the EDC modified catalysts conserved 75% activity at the same time. For longer incubation time, also non-modified Lew-CRL and octyl-CRL showed less than 10% activity after 5 h incubation. On the other hand, at

the same time, the EDC modified octyl-CRL catalysts conserved more than 25% activity whereas the corresponding modified Lew-CRL catalysts were completely inactive (Figure 1).

Considering this profile in very drastic conditions, the biocatalysts stability was studied using 20% (v/v) co-solvent at 25ºC (Figure 2). At these conditions the variations between the non-modified, 10mM EDC or 50 mM EDC were more apparent.

CRL immobilized on Lewatit was less stable than immobilized on octyl-Sepharose, and again the EDC modification resulted negative for the enzyme stability in the former (Figure 2A). The enzyme was completely inactive after 2 h in the 50 mM EDC preparation and conserving 25% activity in the case of 10 mM EDC preparation, whereas the 75% activity of Lew-CRL was conserved at this time.

The chemical modification of the octyl-CRL with 10 mM EDC generated once again the best catalyst, maintaining almost complete activity after 50h incubation, whereas the CRL preparation modified with 50 mM EDC was slightly better than the non-modified which conserved 50% activity after 50 h (Figure 2B).

The stability of octyl-RML was also evaluated in the presence of 25% and 50% acetone (v/v) at 25ºC (Figure 3). The non-modified catalyst showed a decrease of 40% activity after incubation in 25% acetone after 25 h. The different EDC modified octyl-RML catalysts showed an excellent stability conserving 100% activity at these conditions in all cases (Figure 3A). When the RML biocatalysts were incubated at 50% acetone (v/v), octyl-RML resulted inactive after 1h, whereas the EDC modified catalyst conversed more than 50% activity at that time. The 25mM and 50mM EDC modified catalysts were the most stable maintaining around 60% activity after 25 h incubation (Figure 3A). Therefore, in the case of CRL, the chemical modification was positive for octyl-CRL

and negative for Lew-CRL and the best results were obtained using 10 mM EDC modification conditions whereas for octyl-RML in all cases, the EDC modification strongly improved the stability. Considering the stability and the remaining activity, 25 mM EDC modification was selected as optimal conditions.

RML and CRL adsorbed on commercial octyl-Sepharose can be completely desorbed using 0.5% triton X-100.[13] The covalent attachment effect of this modification strategy was evaluated. These modified biocatalysts were incubated in this detergent solution and no releasing of the enzyme from the support was observed.

Therefore to reveal the reason of the covalent interaction between the enzyme and the solid support, a FT-IR analysis of the dry octyl-Sepharose was performed (Figure 4). The spectrum showed a broad peak around 3380 cm^{-1}, which is due to the stretching vibration of O-H bonds, while peaks around 2890 cm^{-1} are due to the C-H stretch. In the range 1070–1140 cm^{-1}, peaks related to C-O-C stretch vibration that represents the glycosidic bond between monosaccharide units (typical of agarose structure) and ester groups.[18] A peak of 933 cm^{-1} represents the C-O-C bridge of 3,6-anhydrogalactose unit. Finally, a peak around 1635 cm^{-1} is clearly observed, which corresponds to the CO_2 stretch vibration of carbonyl group at carboxylic acid (Figure 4). [18] Thus this result directly demonstrates the presence of carboxylic groups in the agarose beads which are the responsible of the covalent attachment of the enzyme in the EDC treatment. Furthermore, analyzing the enzyme structure (Figure 5) it has been observed how different aminoacids such as lysines can be involved in the reaction with the activated carboxylic groups of the support, especially in the open form fixed in the immobilized enzyme on the octyl-Sepharose (Figure 5B).

In this way, in order to evaluate the applicability of this technique in terms of specificity and regioselectivity, the different modified enzyme preparations were used as catalysts in the monodeprotection of different peracetylated molecules (**1**, **6** and **9**) at 25ºC and pH 5.

In the course of different projects, we have determined that octyl-CRL is an optimal catalyst for the regioselective hydrolysis of peracetylated glucose (**1**). These catalysts selectively produced the 6-OH hydroxyl-tetraacetyl glucose (**2**) at very high excellent yield with a simple work-up[9a] (Scheme1).

Consequently, the effect of the modification with EDC of this catalyst was tested in this reaction (Table 1). Also Lew-CRL was evaluated in this reaction. The preparation of immobilized lipase on this support have been described as very promising in some cases[19-22] and indeed the widely used commercial biocatalyst Novozym435 is the immobilized form of *Candida antarctica* B lipase on Lewatit1600.[23]

The octyl-CRL-EDC10mM preparation showed excellent specificity and regioselectivity as the non-modified enzyme in the hydrolysis of **1** at pH 5, 25ºC in the presence of 20% acetonitrile, being slightly more active than the former obtaining in both cases >99% yield of **2** (Table 1, Scheme 1).

Surprisingly, the Lew-CRL preparation was around 3-fold more active than the octyl-CRL in the deprotection although the enzyme in this form showed less specificity, producing 85% yield of the desired monodeprotected product, and 15% of other products (other regioisomers and hydrolyses in other positions) (Table 1). The modification of the enzyme in this case did not alter any property of the enzyme.

At this point the recyclability of the regioselective octyl-CRL preparations was studied (Figure 6). The results after 4 cycles of reaction were very different. The octyl-CRL

suffered a decrease in the activity with the cycles, conserving only 29% initial value after 4 cycles. However, the octyl-CRL-EDC10mM preparation almost conserved total activity after the second cycle and more than 70% after the fourth cycles (Figure 6). In all cycles the regioselectivity of the enzyme was conserved.

Therefore, these results demonstrate that the modification permits to conserve the excellent properties of the catalyst adding the higher stability to the enzyme, being the last a relevant characteristic for a possible industrial application.

The final monodeprotected product **2** was obtained in each case with a 95% isolated yield. This is a very interesting building block in carbohydrate chemistry. One of the most useful activated sugars for oligosaccharides synthesis is the trichloroacetamidyl ester (TCA) product. Therefore, the free OH of **2** was activated by using trichloroacetonitrile and DBU as nucleophilic base at 0 ºC to produce the corresponding 6-OTCA-tetraacetyl-glucose **3** in excellent yields (>90%) (Scheme 1).

In order to widen the activation possibilities and the overall scope of our proposed strategy, we decided to apply on product **1** an optimized chemo-enzymatic strategy developed by ourselves permitting to achieve in one-pot glucoderivatives regioselectively deprotected in other positions then 6-OH. [9a] In more details, the 3-OH glucose **4** was obtained in 25% overall yields after incubation of **1** solution at alkaline pH and chromatography purification.[9a] Also this building-block was used in the activation synthesis with TCA. By this way, the novel 3-OTCA compound **5** was obtained in excellent yields (>90%) (Scheme 1).

To assess the applicability of the proposed strategy, another interesting biotransformation was tested: the selective deprotection of per-acetylated thymidine **6**. Indeed, the 3-hydroxy-5-acetyl-thymidine (**7**) is a key direct intermediate in the

synthesis of azidethymidine (AZT) (Scheme 2) also called zidovudine, the first antiretroviral drug. The catalytic performance of the different CRL biocatalysts was assessed in the hydrolysis of **6** at 25ºC and pH 5 (Table 3). Comparing octyl-CRL and Lew-CRL, the latter was more active although slightly worst in term of specificity producing less amount of **7** but similar in regioselectivity, producing in both cases 8% of free hydroxyl in 5´(**8**) (Table 3). The EDC modification caused different effects depending on the immobilized CRL preparation. The octyl-CRL-EDC10mM showed excellent specificity and improved regioselectivity compared to the non modified catalyst, producing 95% of **7** at full conversion (Table 3). However, Lew-CRL-EDC10mM exhibited a loss in specificity in comparison with Lew-CRL, only 48% yield of **7**, although slight improvement of regioselectivity was observed (Table 3).

Therefore these examples have demonstrated that the EDC modification of CRL immobilized on commercial octyl-Sepharose greatly improves the catalyst performance.

Furthermore, in order to broad the scope of the method, this treatment performed in octyl-RML was also evaluated in term of specificity and regioselectivity of the enzyme in the hydrolysis of peracetylated-lactal (**9**) (a lactose-derivative compound very interesting in oligosaccharide synthesis) at pH 5 and 25ºC in the presence of 20% acetone as co-solvent (Scheme 3). In this time the three modified RML catalysts with different EDC modification (10, 25 and 50 mM) were tested.

In all cases the enzyme activity of the EDC modified catalyst was 3 times lower compared to octyl-RML. However, the high regioselectivity of the octyl-RML was conserved after the different EDC modifications which even slightly improved the specificity of the enzyme producing more than 90% of *C*-3 monodeprotected product **10**. Therefore the octyl-RML-EDC25mM was chosen as the best one considering

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selectivity and stability, it was recycled in this reaction up to seven times and complete activity of the enzyme was conserved (data not shown).

Conclusions

The solid-phase chemical modification by water-soluble carbodiimide of lipases previously immobilized on octyl-Sepharose have permitted the obtainment of a more stable and in some cases more active and more regioselective enzyme. This new catalyst overcomes the disadvantage of reversibility of this immobilization technology, generating heterogeneous biocatalysts, which couple the numerous advantages of the octyl-Sepharose support to covalent immobilization, with an excellent recyclability of the catalyst. Also this strategy is a suitable optimization because in all cases an excellent regioselectivity in the monodeprotection of peracetylated molecules was conserved, obtaining different glycosyl and nucleosidic building blocks in multimiligram scale. Therefore, this strategy represents a simple and effective methodology for interesting industrial application of the preparation of these molecules. This process could be scale at grams and especially for the 6-OH monodeprotected tetraacetylated glucose it could be possible to multigram scale.

Experimental

General

Lipase from *Candida rugosa* (CRL), per-*O*-acetylated α-glucose (**1**), per-*O*-acetylated lactal (**9**), thymidine, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), trichloroacetonitrile (TCA), Boron trifluoride ethyl etherate ($BF_3.OEt_2$), 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) were from Sigma Chem. Co (St. Louis, USA). Lipase from *Rhizomucor miehei* (RML) was kindly donated by Novozymes (Denmark). Lewatit® VP OC 1600, a macroporous DVB-crosslinked polymer in spherical bead form based on methacrylic esters, was from Lanxess AG (Germany). 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 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 flow of 1 mL/min and UV detector L-7400.

Synthesis of per-*O*-acetylated thymidine (6)

Thymidine (1g, 4.12 mmol) was dissolved in 6 mL acetonitrile and then triethylamine (TEA, 4 equiv.), acetic anhydride (4 equiv.) and a catalytic amount of 4-(dimethylamino) pyridine (DMAP) were added. The reaction was stirred at room temperature until the reaction was complete, followed by TLC analysis (ethyl acetate/hexane (7/3) as eluent (R_f= 0.32) and visualized by UV light (254 nm)). Then the solution was diluted with chloroform and water (1:1) (6 mL). The organic phase was separated, washed with water (4x 20mL) and dried (Na_2SO_4), filtered, and concentrated *in vacuo*. NMR data are in agreement with the reported values.[24]

Standard enzymatic activity 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 (pNP) in the hydrolysis of 0.4 mM *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 pNPB per minute per mg of enzyme (IU) under the conditions described above.

Preparation of biocatalysts

Four grams of CRL commercial powder was dissolved in 130 mL of 50 mM sodium phosphate pH 6 (solution of 0.5 mg lipase per mL) and slow stirring for 30 min at 4ºC. Then, solution was centrifuged at 12,000 rpm during 30 min and the supernant was recovered.

Six milliliters of liquid solution of RML (containing aprox. 6 mg of lipase)[12] was dissolved in 114 mL of 25 mM sodium phosphate buffer at 25ºC.

Octyl-Sepharose support was added to the enzyme solution; 2 g in the 65 mL CRL solution and 1g in 120 mL solution of RML. The other 65 mL of CRL solution was added to 2 g of Lewatit1600. The reactions were incubated at 4ºC for CRL and 25ºC for RML for 4 h. After this time, enzymatic activity of the suspension and supernatant were determined using the pNPB 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 on the fridge. In all cases more than 95% of enzymatic activity

was immobilized on the support and the immobilized enzyme conserved the initial activity.

Finally for the modified biocatalysts, one gram of the immobilized CRL biocatalyst (octyl-CRL, Lewatit-CRL) and 0.5 g of octyl-RML was incubated in 10 mL of distilled water pH 4.8 containing EDC at different concentrations (10, 20, 25, 50 mM) for 2 h and then the suspension were filtrated, washed with distilled water and stored in the fridge.

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

The different unmodified and EDC-modified biocatalysts were incubated in phosphate buffer solution containing 25% or 50% (v/v) of co-solvent (acetone or acetonitrile). The activity of the immobilized enzyme was evaluated during the time by using the pNPB assay.

FT-IR Spectra

Octyl-Sepharose support, after dissolved in distilled water, was freeze-dried. Infrared spectra were then recorded using a Fourier transform infrared (FT-IR) spectra were obtained on a PerkinElmer Spectrum 400 Series spectrometer (PerkinElmer, USA); each spectrum was obtained by averaging 32 interferograms with a resolution of 1 cm^{-1}.

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

Per-*O*-acetylated α-glucose (**1**) (144 mg, 0.37 mmol) was dissolved in 4 mL of acetonitrile. This solution was slowly mixed with 16 mL of 50 mM sodium phosphate buffer at pH 4.8 and then 1 g of CRL preparations was added. The reaction was followed by TLC and HPLC analysis, maintaining the hydrolysis reaction under

mechanical stirring at 25°C and the pH value constant by automatic titration in order to avoid the chemical acyl migration. TLC conditions were Hexane:AcOEt 5:5 v/v, $R_{f\,2}$: 0.25, R_{f1}:0.56. HPLC conditions were: eluent water: ACN 7:3 v/v, pH 4.3 adjusted by adding TFA). R_{t1} =20 min, R_{t2} =7.3 min at 225 nm

When complete monodeprotection was reached, the reaction mixture was filtered by vacuum on a sintered glass filter. The catalyst was washed with distilled water (x 3 times) and reused in a second reaction. This procedure was repeated two more times (4 cycles).

To obtain the monodeprotected product, the 20 mL solution containing the 6-OH product was saturated with NaCl (6 g) and extracted with ethyl acetate (3 x 20 mL). The organic layers were combined, wash with saturated aqueous sodium hydrogen carbonate (2 x 20 mL) and dried it by adding anhydrous sodium sulfate. Solvent was evaporated in a rotary evaporator and crystallized with cold diethyl ether to obtain the product as a white solid (**2**, 136 mg, 95% yield). ^{1}H-NMR data are in agreement with the reported values.[9a]

Synthesis of 6-trichloroacetamidyl-tetra-*O*-acetyl-glucopyranose (3)

100 mg of **2** (0.26 mmol) was dissolved in 10 mL of CH_2Cl_2 in an ice bath and 2.6 mmol of trichloroacetonitrile was added. Then 0.54 mmol of DBU was added to the mixture drop by drop and incubated for 1 h or until disappearance of **2**. The reaction procedure was followed by TLC (Hexane:AcOEt 5:5 v/v). The product was purified by 'Cal Bridge/ Mini-chromatography column (90% of **3**, 0.23 mmol). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.52 (s, 1H-NH), 6.35 (d, J = 3.7 Hz, 1H-1), 5.48 (t, J = 9.8 Hz, 1H-3), 5.19 (t, J = 9.8 Hz, 1H-4), 5.09 (dd, J = 10.3, 3.7 Hz, 1H-2), 4.39 (d, J = 11.9, 3.3 Hz, 2H-6a,b), 4.16 (m, 1H-5), 4.22 (m, 1H-5), 2.16-2.01 (4s, 12H-Ac).

Synthesis of 3-trichloroacetamidyl-tetra-*O*-acetyl-glucopyranose (5)

The 3-OH precursor **4** was synthesized as described elsewhere.[9a] 100 mg of **4** (0.26 mmol) were dissolved in 10 mL of CH_2Cl_2 in an ice bath and 2.6 mmol of trichloroacetonitrile was added. Then 0.54 mmol of DBU was added to the mixture drop by drop and incubated for 1 h or until disappearance of **4**. The reaction procedure was followed by TLC (Hexane:AcOEt 5:5 v/v). The product was purified by 'Cal Bridge/ Mini-chromatography column (90% of **5**, 0.23 mmol).^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.52 (s, 1H-NH), 6.35 (d, *J* = 3.57 Hz, 1H-1), 5.78 (t, *J* = 10.0 Hz, 1H-3), 5.35 (t, *J* = 9.85 Hz, 1H-4), 5.28 (dd, *J* = 10.26, 3.79 Hz, 1H-2), 4.26 (dd, *J* = 12.3, 4.0 Hz, 1H-6a), 4.16 (m, 1H-5), 4.10 (dd, *J* = 12.27, 4.0 Hz, 1H-6b), 2.19-2.00 (4s, 12H-Ac).

Biocatalytic deprotection of per-*O*-acetylated thymidine (4)

Substrate **4** (40 mg, 0.12 mmol) was dissolved in a mixture of acetonitrile (5%, *v*/*v*) in 10 mM sodium acetate at pH 5.0. 0.2 g of CRL preparations was added to 2 mL of this solution at 25°C. The degree of hydrolysis was analyzed by TLC and reverse phase HPLC. TLC conditions were: dichloromethane/methanol, 92.5/7.5 v/v, R_{f4}: 0.25, R_{f5}:0.12, R_{f6}:0.10. HPLC conditions were: eluent was obtained by 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 monodeprotected 3-OH (**5**) and 5-OH (**6**) were used as pure standards. The retention time was 2.4 min for Thymidine, 9.4 min for **5** and 10.2 min **6** and 19 min for **4**.

The reaction was then scale-up, starting with 200 mg (0.61 mmol) of thymidine peracetylated in 25 mL volume (containing 5% ACN) and 1.5 g of octyl-CRL-

EDC10mM. The full conversion was achieved after 162 h with the same regioselectivity. The product was purified and characterized in agreement with the previous report.[25]

Biocatalytic deprotection of per-*O*-acetylated lactal (7)

7 (0.01 mmol) was added to 2 mL solution of phosphate buffer (25 mM) with acetonitrile (20%) at pH 5, 25ºC 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. Hydrolysis reactions were followed by HPLC, where the eluent was an isocratic mixture of 40% acetonitrile in 10 mM ammonium phosphate buffer at pH 3.8 and UV detection at 215 nm. The retention time of the product **7** and **8** was 18.50 min and 11.10 min respectively in these conditions.
When complete monodeprotection was reached, the reaction mixture was filtrated by vacuum on a sintered glass filter. The catalyst was washed with distilled water (x 3 times) and reused in a second reaction. This procedure was repeated six times (7 cycles).

The reaction was scale-up using 5 g of octyl-RML-EDC catalyst in the hydrolysis of 0.53 mmoles (300 mg) of peracetylated lactal (**6**) in 40 mL volume (containing 20% ACN) at pH 5. After 24 h, full conversion was achieved and the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (3x40mL). 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 (7, 270 mg, 90%). NMR data are in agreement with previously reported.[12]

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Captions

Scheme 1. Chemobiocatalytic synthesis of glucose building blocks. (a) Biocatalyst, 20% acetonitrile/80% buffer at pH 4.8, 25ºC. (b) Biocatalyst, 20% acetonitrile/80% buffer at pH 4.8, 25ºC and after complete conversion pH changed from 4.8 to 9. (c) tricloroacetonitrile, DBU, 0ºC, CH_2Cl_2.

Scheme 2. Biocatalytic regioselective preparation of monodeprotected thymidine. (a) Biocatalyst, 5% acetonitrile/95% acetate buffer at pH 5, 25ºC.

Scheme 3. **Biocatalytic production of monodeprotected lactal**. (a) Biocatalyst, 20% acetonitrile/80% buffer at pH 5, 25ºC.

Figure 1. **Stability of different CRL preparations in the presence of acetonitrile 50% (v/v) as co-solvent**. A. Lew-CRL (triangles), Lew-CRL-EDC10mM (squares), Lew-CRL-EDC50mM (rhombus). B. Octyl-CRL (triangles), octyl-CRL-EDC10mM (squares), octyl-CRL-EDC50mM (rhombus).

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Figure 3. Stability of different RML preparations in the presence of acetone as co-solvent. A. 25% ; B. 50% (v/v). Octyl-RML (rhombus), octyl-RML-EDC10mM (squeares), octyl-RML-EDC25mM (triangles), octyl-RML-EDC50mM (asterisk).

Figure 4. FT-IR spectra of octyl-Sepharose.

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Figure 5. A. Structure of CRL. B. Adsorption of the open form of CRL on the octyl-Sepharose matrix. Lid oligopeptide (light blue), ϵ-NH_2 Lys (red), hydrophobic residues (orange), serine active site (green). The structure of CRL was obtained from the Protein Data Bank (pdb code: 1CRL) and the picture were created using Pymol v. 0.99

Figure 6. Comparison in reuse of octyl-CRL and octyl-CRL-EDC10mM in the hydrolysis of 1. Octyl-CRL (blue), octyl-CRL-EDC10mM (red).

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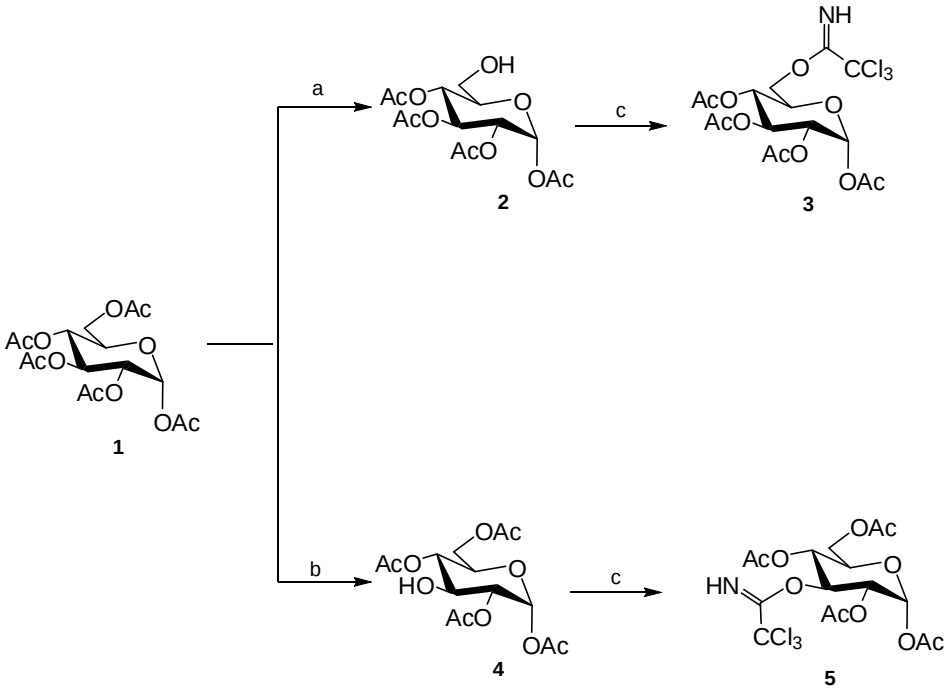

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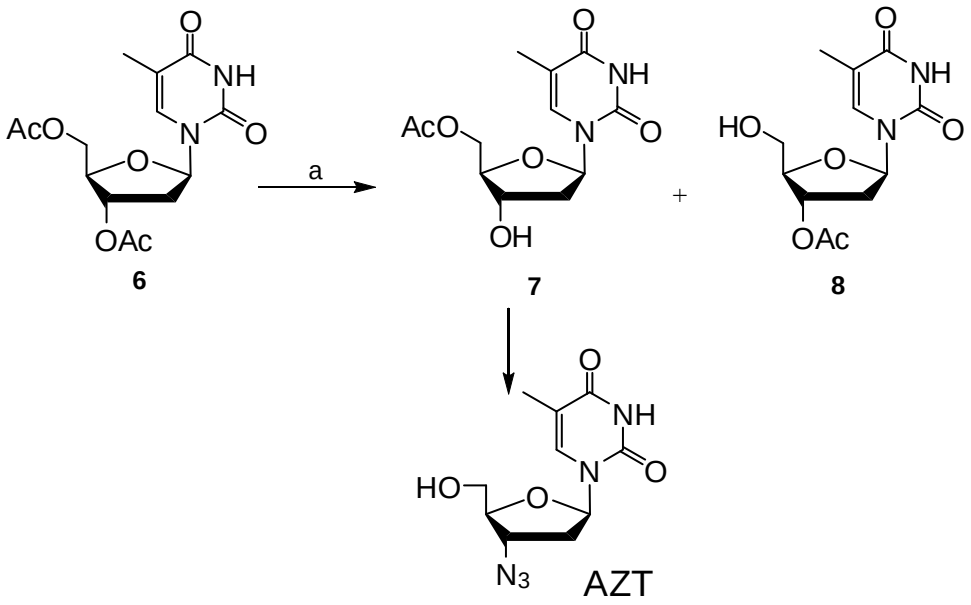

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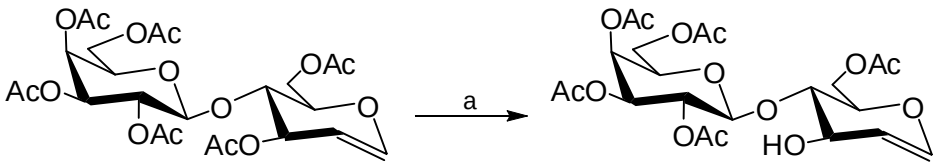

9 **10**

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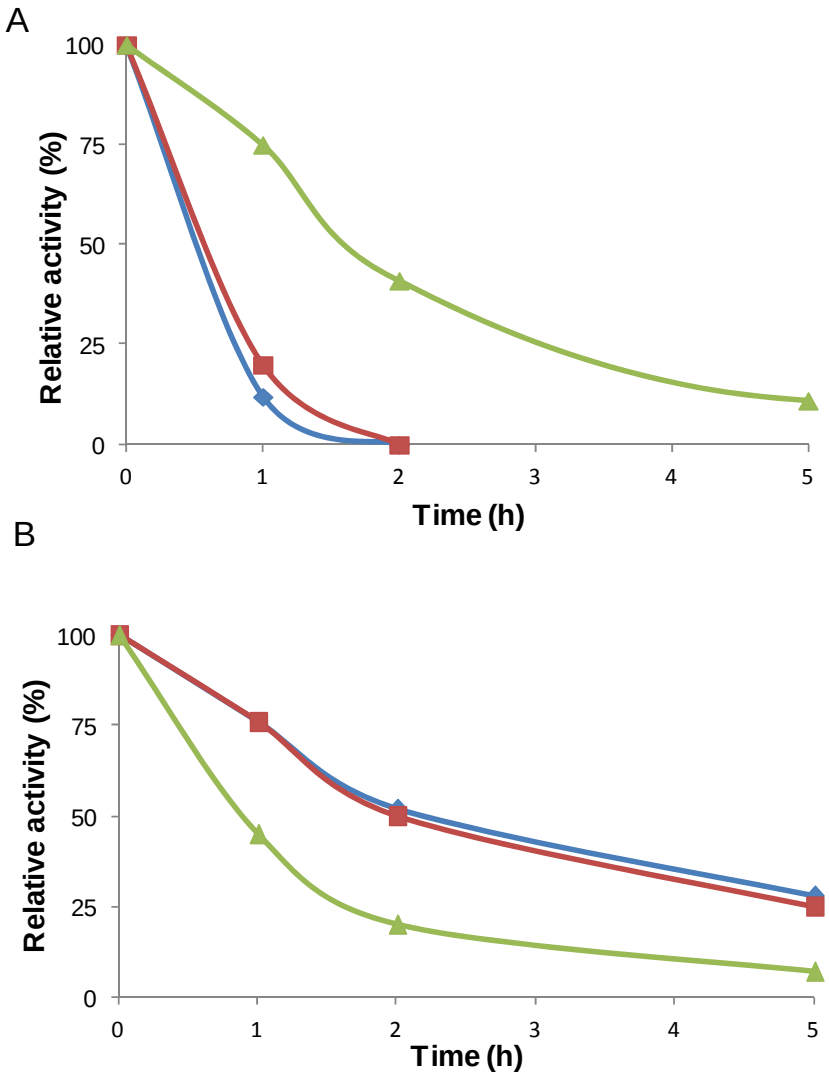

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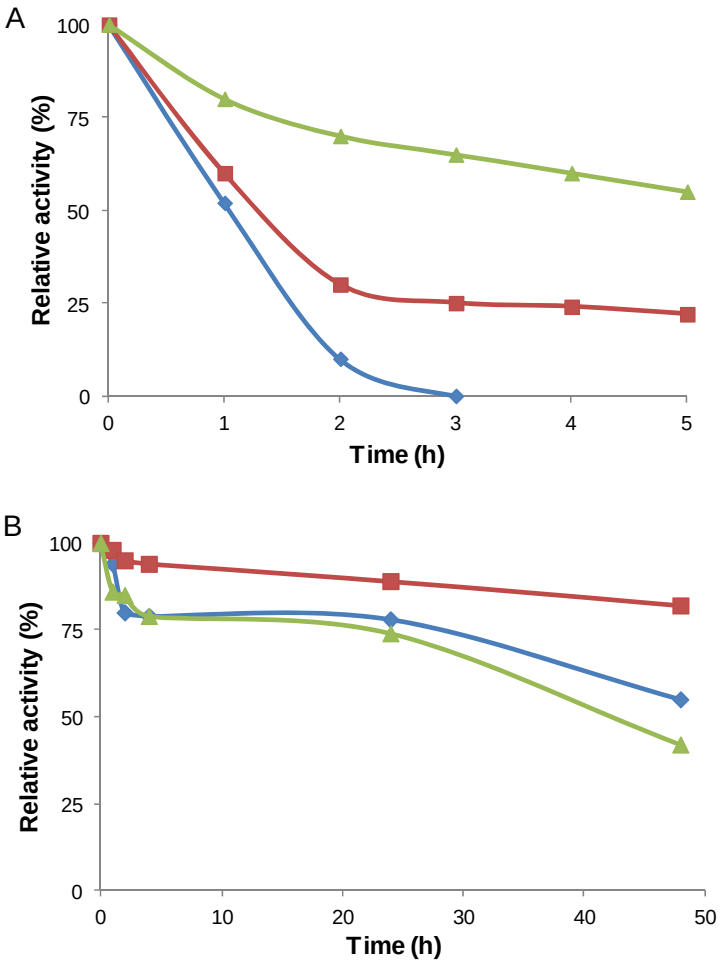

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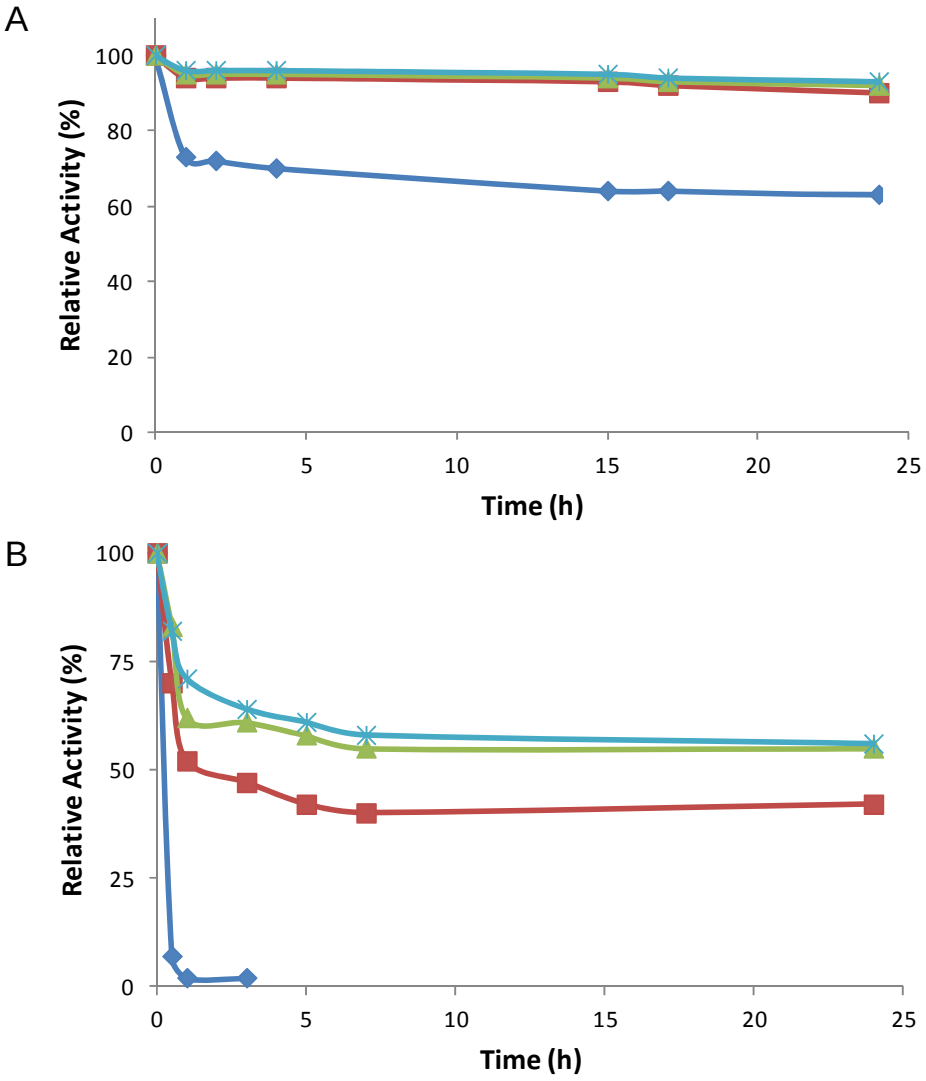

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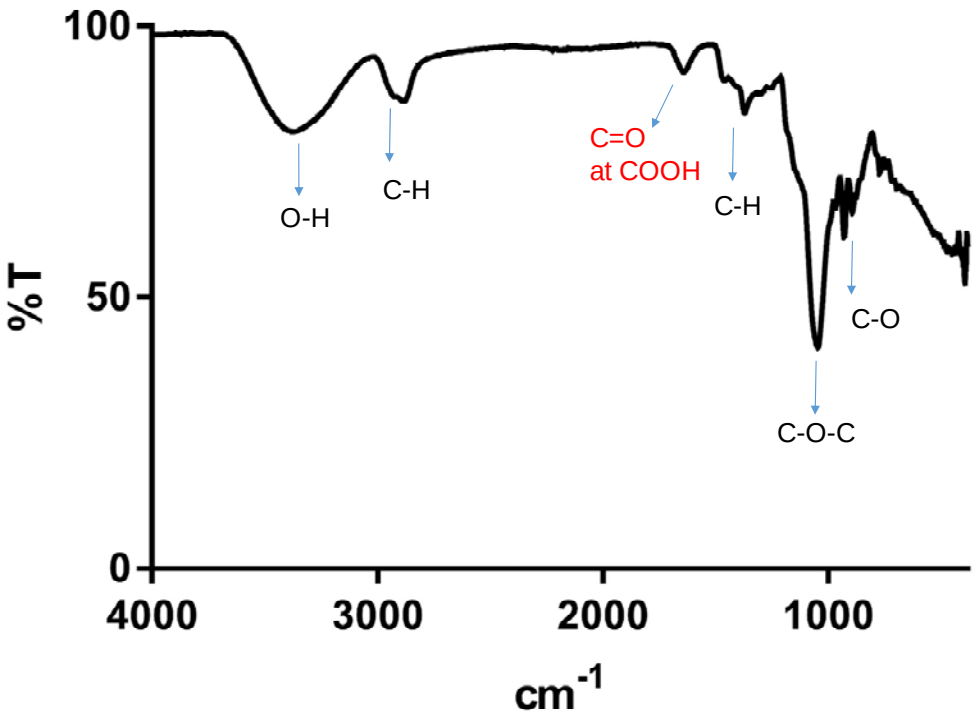

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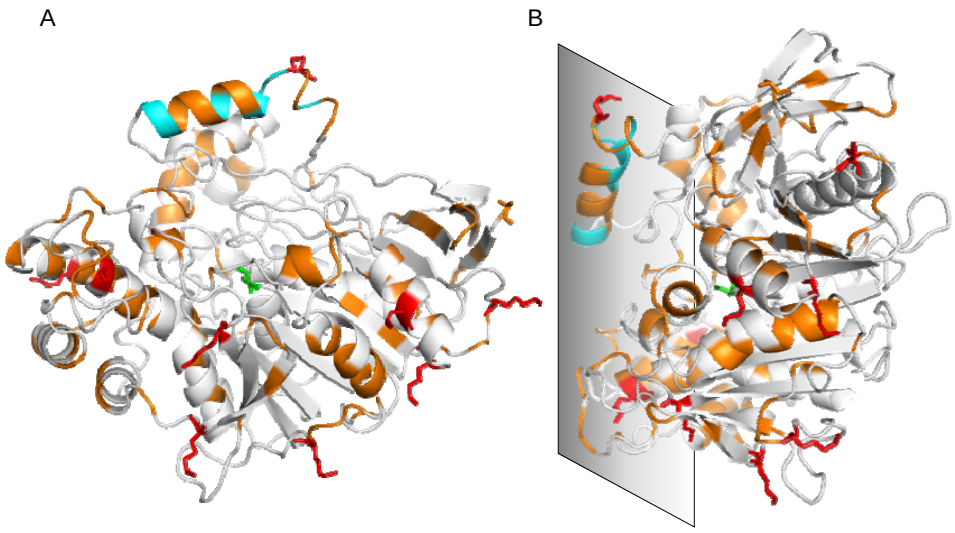

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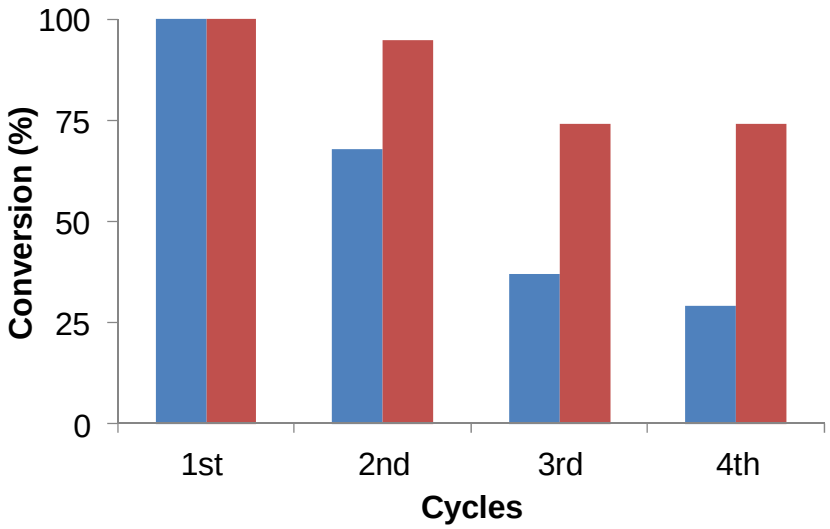

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Table 1. Enzymatic activity values of immobilized enzymes after EDC treatment

Biocatalyst	Enzymatic activity (%)[a]			
	EDC [mM]			
	0	10	25	50
Octyl-CRL	100	99	nd	60
Lew-CRL	100	80	nd	47
Octyl-RML	100	90	78	68

[a] the values correspond to the percentage considering the initial activity of the biocatalyst in pNPB assay as 100%. Nd: not determined

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Table 2. Biocatalytic hydrolysis of **1**

Biocatalyst	Specific activity[a]	Reaction time (h)	Conversion (%)	**2** (%)	Others (%)
Octyl-CRL	1.0	30	100	>99	0
Octyl-CRL-EDC10mM	1.3	30	100	>99	0
Lew-CRL	3.5	16	100	95	5
Lew-CRL EDC10mM	3.0	16	100	90	10

[a] the initial rate in μmol x mg_{prot}^{-1} x h^{-1}. It was calculated at 10-30% conversion.

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Table 3. Biocatalytic hydrolysis of **6**

Support	Specific activity[a]	Reaction time (h)	Conversion (%)	**7** (%)	**8** (%)	thymidine
Octyl-CRL	1.0	24	99	74	8	18
Octyl-CRL-EDC10mM	1.3	50	99	95	5	0
Lew-CRL	3.5	6	99	69	8	22
Lew-CRL-EDC10mM	3.0	50	99	48	4	48

[a] the initial rate in μmol x mg_{prot}^{-1} x h^{-1}. It was calculated at 10-20% conversion

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Table 4. Biocatalytic hydrolysis of **9**

Biocatalyst	Specific activity[a]	Reaction time (h)	Conversion (%)	**10** (%)	Others (%)
Octyl-RML	0.7	24	100	90	10
Octyl-RML-EDC10mM	0.2	64	100	92	7
Octyl-RML-EDC25mM	0.2	64	100	92	7
Octyl-RML-EDC50mM	0.2	64	100	92	7

[a] the initial rate in μmol x mg_{prot}^{-1} x h^{-1}. It was calculated at 10-20% conversion.