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Development of regioselective deacylation of peracetylated β-D-monosaccharides using lipase from *Pseudomonas stutzeri* under sustainable conditions†

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The lipase-catalyzed regioselective deacylation of peracetylated pyranosides has been evaluated in biosolvents. Among the biocatalysts tested, lipase from *Pseudomonas stutzeri* showed the highest activity, displaying regiospecific activity towards the anomeric position. This lipase was also employed in the regioselective alcoholysis of peracetylated sugars in green solvents, contributing to improve the sustainability of the process. Yields up to 97% of the desired product with different biosolvents were found. These reactions took place without noticeable activity and with total regioselectivity, representing a considerable improvement over the use of an aqueous buffer or conventional organic solvents. Furthermore, scaled up reactions are feasible without losing catalytic action. In order to understand the role of these biosolvents in the enzyme's synthetic behaviour, molecular modelling and docking studies were performed in the presence of some selected biosolvents to conclude that the presence of biosolvents in the reaction media modifies the access of the alcohols to the enzymatic active site allowing the presence of small alcohols and not i-propyl and *t*-butyl residues in the alcohol.

Introduction

Carbohydrates play an important role in many molecular recognition processes such as embryogenesis, neuronal proliferation and apoptosis phenomena.[1–5] As the understanding of these biological functions increases, the need to develop more efficient synthetic procedures for oligosaccharide production in large quantities has become a major subject. The main limitation in the synthesis of oligosaccharides is represented by the preparation of sugar building blocks bearing only one free hydroxyl group in the desired position. Traditionally, the synthesis of mono-deprotected carbohydrates has been developed through multistep processes, involving different protecting groups, yielding mixtures of products and, in some cases, low yields.[6–9] In this context, the use of regioselective enzymes for the hydrolysis of per-acetylated sugars has been recently proposed as a simple and efficient procedure for the preparation of sugar intermediates using only acetyl for protection of hydroxyl groups.[6] Selectively acetylated sugars containing only one free position can be in fact easily used as key intermediates in the preparation of different glycoderivatives of biological interest.[7–9]

In this respect, lipases have been shown to be very versatile enzymes displaying high regio-, chemo- and enantioselectivity under mild reaction conditions and exhibiting very high stability in organic medium.[10–13] These enzymes have been widespread employed in stereoselective hydrolysis of esters and *trans*-esterification reactions.[11,14–16]

In particular, the regioselective hydrolysis of peracetylated saccharides has been reported using different lipases, such as those from *Candida rugosa* (CRL), lipase B from *Candida antarctica* (CAL-B), *Pseudomonas fluorescens* (PFL), *Thermomyces lanuginose* (TLL) and *Rhizomucor miehei* (RML), leading to diverse results depending on the enzyme, the immobilization procedure used for the enzyme and substrate employed.[8,14,17,18]

Thus, the search for a bio-catalyst with high activity, regio- and stereoselectivity towards a large number of substrates is an important challenge. For example, CRL is active towards many monosaccharides, allowing selective hydrolysis only in C-6 position,[6,8] while immobilized Lecitase® (phospholipase A_1) is moderately selective for the anomeric position, C-6 or both depending on the conducted immobilization.[19]

Two approaches are mainly used for the asymmetric deacylation of peracetylated sugars: hydrolysis, in reaction media

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normally containing some organic solvents to ensure substrate solubility, and alcoholysis reaction, where the solvent is also the reagent.[12,17,20]

The use of more sustainable solvents is a challenge in order to develop environmentally friendly processes. In this sense, green solvents must assemble the basic principles of green chemistry,[21–23] such as: less hazardous chemical synthesis, use of safe solvents, use of renewable feedstock and design to ensure a suitable degradation in the environment. Accordingly, solvents derived from biomass (bio-solvents),[24] such, as glycerol or dimethyl amide derivatives may be considered as green solvents.[25–27] Besides, fluorinated solvents are also considered "green solvents" due to their particular properties (temperature dependence solubility in organic–water media, low toxicity, and low volatility).[28–30] In addition, the employ of a biocatalytic strategy in combination with green solvents, actively contributes to greatly enhance the sustainability of a process allowing the development of preparative methods with high substrate concentration.[30–32]

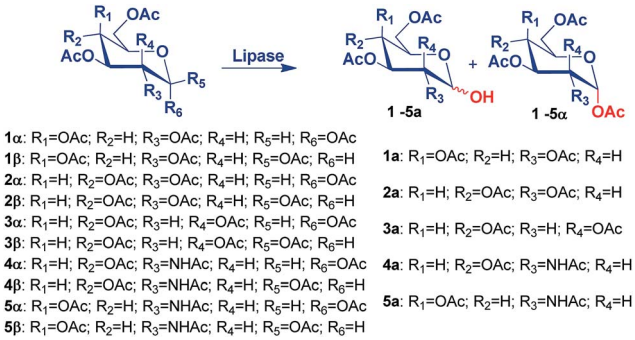
In this work, we have evaluated the activity of different lipases in the selective hydrolysis of peracetylated pyranosides in order to obtain different monosaccharides with a free hydroxyl group at the anomeric position, useful as intermediates to obtain, previous chemical activation and glycosylation, oligosaccharides of biological relevance. For developing an efficient preparative green bio-process, suitable to replace the toxic reagents (such as hydrazine acetate) and solvents currently used in chemical deprotection of the anomeric position[33] the search of a selective bio-catalysts has been combined with the use of green solvents. Thus, various "green" solvents have been studied to achieve high substrate concentration, selectivity and yields. In particular, the *Pseudomonas stutzeri* (PSL) was efficiently employed in hydrolysis and alcoholysis of several substrates and considered as catalyst for developing preparative bio-processes in green solvents. The good results obtained with this enzyme, improved compared with other lipases currently used in the hydrolysis of peracetylated pyranosides, have been explained by molecular modelling and docking studies.

Results and discussion

Screening of solvents and lipase activity

Lipases QLC, PSL and CAL-B were evaluated in order to determine their potential activity in the regioselective deacylation of peracetylated sugar (Scheme 1). 1,2,3,4,6-Penta-*O*-acetyl-β-D-galactopyranose (**1β**, see Scheme 1) was used as standard substrate with several wet-solvents (10% v/v water). The complete listing of the solvents used in this work is given in Fig. 1.

Some physico-chemical parameters of the solvents used in this work are gathered in Table S2 (ESI†). As can be seen, the structural diversity of the glycerol derivatives results in a wide range of hydrophobicities. Some of the glycerol-derived solvents used, namely those bearing fluorinated chains, display simultaneously both high hydrophobicity and hydrogen-bond donor ability, a rather unusual combination in conventional organic solvents.



Scheme 1 Lipase-catalyzed regioselective hydrolysis of pyranoses.

Concerning toxicity issues, there are some experimental data pointing to glycerol-derived solvents as being low hazardous, For instance, toxicities of glycerol based solvents like **S5** and the closely related 1,3-diethoxy-2-propanol (LD50 > 3000 mg kg^{-1}, oral in mice)[34] and 1,2,3-triethoxypropane (LD50 = 660 mg kg^{-1}, intraperitoneal in mice)[34] are very low, in some case even lower than that of butanol (LD50 = 2680 mg kg^{-1}, oral in mice). Fluorinated solvents will be probably more toxic than aliphatic ones, but lacking in experimental data for them it would be reasonable to take the toxicities of compounds produced in their probable decomposition, like trifluoroethanol (LD50 = 366 mg kg^{-1}, oral in mice)[35] trifluoroacetic acid (LDlo = 150 mg kg^{-1}, intraperitoneal in mice)[35] or 2,2,2-trifluoroethyl ethyl ether (LD_{50} = 5100 mg kg^{-1}, intraperitoneal in rats)[36] as referents of the toxicity of these kinds of solvents.

Moreover using these glycerol derivatives allow to reduce some other hazardous aspects. For instance, they have low volatility, so the concentration in the air using them is lower than using other conventional solvents. Albeit toxicities of these solvents are not well known at this moment it is clear they are not extremely toxic, meeting one of the key requirements for green applications.

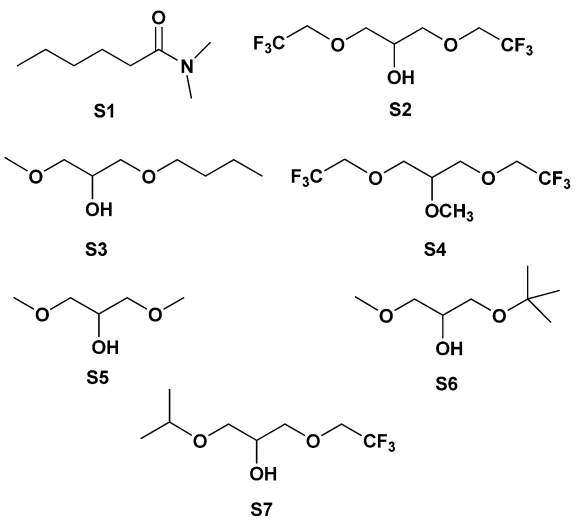


Fig. 1 Molecular structure of the solvents S1 to S7.

Table 1 Deacylation at the anomeric position of 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose (**1β**) catalyzed by several lipases under different solvents containing 10% v/v of water

Entry	Enzyme	Solvent	Time (h)	**1a** conv.[a] (%)
1	CAL-B[b]	**S1**	24	3
2	CAL-B[b]	**S2**	24	3
3	QLC[c]	**S1**	24	1
4	QLC[c]	**S2**	24	1
5	PSL[d]	**S1**	8	92
6	PSL[d]	**S2**	24	76
7	PSL[d]	**S3**	24	62
8	PSL[d]	**S4**	24	2
9	PSL[d]	THF	24	15
10	PSL[d]	2-MeTHF	24	25

[a] Determined by HPLC: Asahipak column NH_2P50-4E (4.6 mm × 250 mm); ELSD detector; mobile phase CH_3CN–H_2O, 80 : 20 (v/v), flow rate 0.8 mL min^{-1}. [b] 14.9 IU mg^{-1} protein. [c] 3.91 IU mg^{-1} protein. [d] 36.2 IU mg^{-1} protein.

Results from this experiment are summarized in Table 1. According to Table 1, PSL was the best enzyme to catalyze the deacylation of **1β** in wet-**S1** as reaction medium, obtaining 92% yield in 8 h (entry 5). The product was purified and the spectroscopic elucidation by NMR showed a regiospecific deacylation towards the anomeric position of the peracetylated substrate. Complete conversion of substrate **1β** into product **1a** (as a mixture of anomers α and β) was achieved without formation of other products as detected by HPLC analysis.

In fact, as reported in Fig. 2, maximum conversion (96%) was achieved after 24 h and the reaction proceeded with a complete regioselectivity, because all the consumed substrate was converted in product **1a**. After complete hydrolysis of **1β**, the concentration of the product remained constant (no further hydrolysis was observed). Good yields (76%) were also observed when **S2** was employed (entry 6). With PSL different other solvents were tested, although worst results were obtained (Table 1). CAL-B and QLC did not display good activity in **S1** and **S2** (Table 1) as well as in any other solvents tested (see Table S1 in ESI†). In particular CAL-B provided much poorer results compared with the process previously reported and performed using this immobilized enzyme.[18]

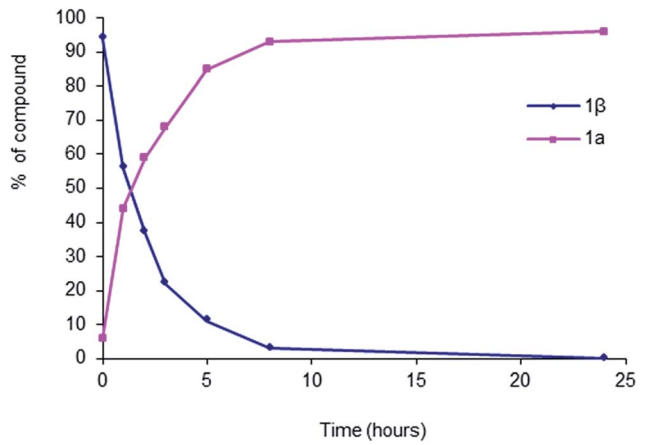


Fig. 2 Kinetic of the regioselective deacylation of **1β** catalyzed by PSL in **S1**.

It is worth of mention the good result obtained with PSL in the regioselective deacylation of the anomeric carbon in substrate **1β**, as not many hydrolases have been described to present high selectivity towards the hydrolysis of this position. *Thermomyces lanuginose* (TLL), *Aspergillus niger* (ANL) and *C. antarctica* lipases have been previously reported to provide hydrolysis in the anomeric position of **1β** but the type of immobilization used for the enzymes was crucial to achieve good yields in acceptable reaction times.[18] All those enzymes were almost inactive in the hydrolysis of **1α**, showing poor selectivity towards primary acetoxy group (C6 position) when used in the hydrolysis of peracetylated *gluco*pyranosides.[8,18]

The C-1 regioselective deacylation of *gluco*- and galacto pyranosides has also been achieved by using PFL as biocatalyst,[14] that belongs to *Pseudomonas* species, although the selectivity of the reaction and, consequently, the yields achieved, were strongly dependent on the substrate. Hydrolysis of the anomeric position of mannopyranoses, was instead reported with moderate yields by using the immobilized phospholipase "Lecitase", but this enzyme preferentially hydrolyzed the primary acetoxy group of *gluco*- and galactopyranoses.[19]

Furthermore, it should be considered that almost all the processes previously reported for the hydrolysis of pyranoses, were performed in aqueous homogenous reaction media (mainly using 20% v/v of acetonitrile)[6] and this reaction media strongly limited the operational range of substrate and product concentrations. In this context, the use of an enzyme active towards a wide range of peracetylated pyranosides, highly selective for the anomeric position and stable and in organic green solvents, could be considered extremely useful for developing preparative chemo-enzymatic process for the synthesis of oligosaccharides. Based on these assumptions PSL was selected for further studies.

Hydrolysis of other peracetylated substrates using *P. stutzeri* lipase

Accordingly, hydrolytic assays on some different peracetylated substrates were carried out with PSL (Scheme 1), in the presence of the solvents **S1** and **S2**. In order to study the potential selectivity of this biocatalyst towards α or β anomers, peracetylated α-glycopyranoses were also included in the study. Thus, the following peracetylated monosaccharides were employed as substrates: 1,2,3,4,6-penta-*O*-acetyl-α-D-galactopyranose (**1α**), 1,2,3,4,6-penta-*O*-acetyl-β-D-*gluco*pyranose (**2β**), 1,2,3,4,6-penta-*O*-acetyl-α-D-*gluco*pyranose (**2α**), 1,2,3,4,6-penta-*O*-acetyl-β-D-mannopyranose (**3β**), 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranose (**3α**), 2-acetamido-2-deoxy-1,3,4,6,-tetra-*O*-acetyl-β-D-*gluco*pyranose (**4β**), 2-acetamido-2-deoxy-1,3,4,6,-tetra-*O*-acetyl-α-D-*gluco*pyranose (**4α**) 2-acetamido-2-deoxy-1,3,4,6,-tetra-*O*-acetyl-β-galactopyranose (**5β**) and 2-acetamido-2-deoxy-1,3,4,6,-tetra-*O*-acetyl-α-D-galactopyranose (**5α**). Results are summarized in Table 2.

Table 2 Deacylation of peracetylated pyranoses catalyzed by PSL in different solvents containing 10% v/v of water

Entry	Substrate	Solvent	Time (h)	Product[a] (conv. %)
1	**1α**	**S1**	24	**1a** (ND)[b]
2	**2β**	**S1**	24	**2a** (96)
3	**2α**	**S1**	24	**2a** (ND)
4	**3β**	**S1**	24	**3a** (ND)
5	**3α**	**S1**	24	**3a** (ND)
6	**4β**	**S1**	24	**4a** (71)
7	**4α**	**S1**	24	**4a** (ND)
8	**5β**	**S1**	24	**5a** (15)
9	**5α**	**S1**	24	**5a** (ND)
10	**1α**	**S2**	24	**1a** (ND)
11	**2β**	**S2**	24	**2a** (40)
12	**2α**	**S2**	24	**2a** (ND)
13	**4β**	**S2**	24	**4a** (39)
14	**4α**	**S2**	24	**4a** (ND)

[a] Determined by HPLC: Asahipak column NH_2P50-4E (4.6 mm × 250 mm); ELSD detector; mobile phase CH_3CN–H_2O, 80 : 20 (v/v), flow rate 0.8 mL min^{-1}. [b] ND: not detected (<1%).

As can be seen in Table 2 very high conversions, similar to those achieved for substrate **1β**, were obtained in the case of compound **2β** when **S1** was employed as solvent (entry 2). The lipase displayed very high selectivity and it exclusively hydrolyzed the anomeric position. However, although this enzyme displayed also activity working in **S2**, the employ of this solvent led to much lower yields and in some cases no activity was observed. No conversion was detected if peracetylated mannoses **3α** and **3β** were used as substrates. In fact, formation of product **3a** in moderate yields was previously described using immobilized Lecitase,[19] but this product was not obtained with PSL. In contrast, good results were obtained with PSL when the β anomer of *N*-acetylglucosamine (**4β**) was considered (entry 6). However, in this last case, the lipase displayed a substantial lower performance compared with the homologous **2β** (71% of yield was obtained in 24 h), attributed to the presence of the acetamide group in the position C-2, according to the results previously obtained with other enzymes.[8,14,19] The activity of this enzyme was further reduced in the hydrolysis of peracetylated galactosamine **5β** (entry 8) and resulted completely inactive in the hydrolysis of both anomers of the peracetylated lactose (result not shown).

On the other hand, it is worth of mention that no production of monodeacylated product was detected when peracetylated α-saccharides were employed as substrates. Thus, for the substrates recognized by PSL (substrates **1**, **2** and **4**), this lipase is able to discriminate between the α and the β isomer, being possible to resolve a mixture of both isomers by a PSL-catalyzed regio- and stereoselective deacylation reaction.

Based on these results, we suspected that the PSL presented an active site suitable to accept only small molecules such as monosaccharides (β-D-lactose was not recognized), with a regio- and stereo-specificity of the enzyme towards the anomeric position. Accordingly, any variation at C-2 position strongly influenced the activity of this enzyme. In fact, both anomers of mannose were not recognized, probably because the acetoxy group at carbon 2 is inverted with respect to *gluco*- and galacto-pyranoses, which can be accepted as substrate by this enzyme. Also variation at the C-4 position of pyranoses somehow affected the activity of PSL, although with a minor influence compared with changes at C-2. Accordingly, *N*-acetylated pyranosides were less or poorly recognized by PSL compared with the corresponding substrates bearing in C-2 an acetoxy group and peracetylated galactosamine **5β** provided much worst results compared with glucosamine **4β**.

These results confirmed how the use of this green commercial solvent can be considered a new and promising approach for developing sustainable bio-processes for substrates characterized by a very poor water solubility.[37]

Alcoholysis of peracetylated substrates using *P. stutzeri* lipase

In order to study the alcoholysis of peracetylated substrates catalyzed by PSL, **1β** and **2β**, which provided the best results in the hydrolytic reaction, were employed as substrates. Not only common alcohols such propanol and 1-butanol were used, but also solvents **S2**, **S3**, **S5** and **S7**, bearing a free OH group (Fig. 1), were included in the study.

Results reported in Table 3 show that most of the alcohols tested may be successfully employed as nucleophiles for the alcoholysis of peracetylated **1β** and **2β**, except **S6** and **S7**. The yields afforded with most of alcohols range from 82 to 99%, which means that the lipase can efficiently use them as acceptors for the transfer of the anomeric acetyl group. Due to the broad variety of alcohols used PSL resistance to different polarities of solvents was proven. In these sense, solvents employed in these assays presented log *P* values ranging from −0.60 of **S5** up to 1.42 of **S2** (Table S2 in ESI†). Thus, the null activity exhibited by the enzyme in the presence of **S6** and **S7** may be related to an structural phenomena, where the access of these alcohols to the active site of the enzyme may be limited by the branched structure of the two groups (*tert*-butyl and i-propyl) present in these compounds.

Table 3 Alcoholysis of peracetylated monosaccharides catalyzed by *P. stutzeri* lipase in presence of anhydrous alcohols

Entry	Substrate	Alcohol	Time (h)	Product[a] (conv. %)
1	**1β**	2-Propanol	24	**1a** (82)
2	**1β**	1-Butanol	24	**1a** (87)
3	**1β**	**S2**	48	**1a** (99)
4	**1β**	**S3**	48	**1a** (71)
5	**1β**	**S5**	48	**1a** (84)
6	**1β**	**S6**	48	ND[b]
7	**1β**	**S7**	48	ND[b]
8	**2β**	1-Butanol	24	**2a** (84)
9	**2β**	**S2**	48	**2a** (97)
10	**2β**	**S3**	48	**2a** (88)
11	**2β**	**S5**	48	**2a** (97)

[a] Determined by HPLC: Asahipak column NH_2P50-4E (4.6 mm × 250 mm); ELSD detector; mobile phase CH_3CN–H_2O, 80 : 20 (v/v), flow rate 0.8 mL min^{-1}. [b] ND: not detected (<1%).

It is important to note that the results obtained in the study of different solvents showed that the best performances can be achieved using the solvent **S2**. This solvent provided the best results in the alcoholysis of substrate **1β**. In particular, PSL displayed a high preference to catalyze the reaction in the presence of solvent **S2**, compared with other solvents derived from glycerol. The behaviour of the lipase may be attributed to some specific interaction between the enzyme and the solvent as it has been previously described for other enzymes.[38–40] Consequently, **S2** can be considered a promising new green solvent suitable to be used for developing enzymatic bioprocesses.

Scale up process

An important point that must be evaluated after the synthesis of an interesting building block is the feasibility of scaling up the process (hydrolysis and alcoholysis). In this respect, an advantage of solvents **S1** and **S2** is that under stirring conditions an emulsion is created between biosolvent and aqueous buffer, then after reaction these biosolvents can be separated from reaction media by centrifugation. Moreover, carbohydrate compounds in the reaction media are not soluble in the biosolvent phase and remains in the aqueous phase. Centrifugation becomes a very useful tool for the isolation of the biosolvent from the reaction media, allowing its reuse in further reactions.

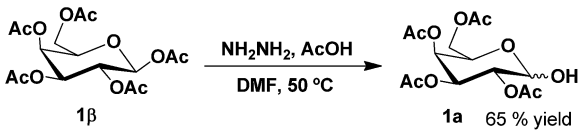
For substrate **1β** preparative processes were performed increasing the reaction volume and using solvent **S1** for hydrolysis and **S2** for alcoholysis. In both cases a complete conversion of the substrate into product was obtained (95%).

With this optimized separation procedure in hand, at the end of the reaction, the biosolvent (**S1** or **S2**) were recycled and reused and the product was easily purified by column chromatography using dichloromethane–methanol (95 : 5) as eluent (90% yield). Being the reaction almost complete (97–98% conversion at 24 h), the product recovered was pure at 95%. This process was repeated 3 times without losing catalytic action.

The comparison of the enzymatic process with the chemical reaction reported for the synthesis of product **1a**[33] showed that the enzymatic approach gives the best performances in terms of yields and environmental sustainability. In fact, the chemical process was performed in DMF using hydrazine in acetic acid, and provided 65% of yield (Scheme 2). Furthermore, for product isolation, purification was required, while the enzymatic reaction provided a product with a good purity by a simple work-up.

Molecular modelling and docking studies for *P. stutzeri* lipase

As described in material and methods, a molecular modelling study was carried out by homology of PSL and the already



Scheme 2 Chemical deprotection of **1β**.[33]

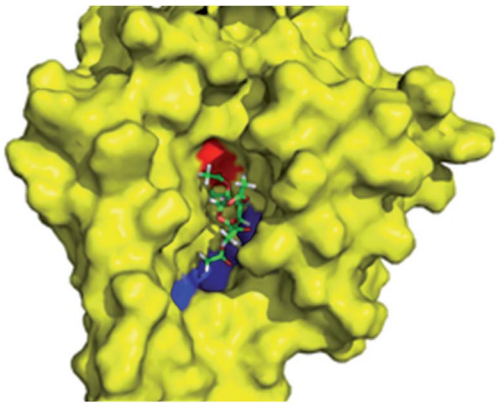


Fig. 3 Top view of the cavity of lipase from *P. stutzeri* with **1β** molecule, with the lids on the sides. In blue, large pocket; small pocket in red. Behind the oxianionic pocket is placed and the group of the anomeric carbon of **1β**.

available model of *Pseudomonas mendocina* (99% identity with PSL).[39,41] The model has been refined using the AMBER force field 03 and a steepest descent algorithm to minimize the energy function associated with the field for 2000 steps. Various parameters have been validated as Ramachandran angles and hydrophobicity profiles of residues, being all comparable to native structures crystallized. For the docking program was used Autodock 4.0.[42] In the proposed model, the enzyme has two pockets, small and large, the latter being clearly open to the solvent. The active site is protected by two lids, which are opened and in hydrophobic media tend to close in the presence of water (Fig. 3). In the case of the peracetylated derivatives, the substrate fits into the active site by size and shape, occupying the small pocket and exposing the rest of acetyl groups (large pocket), while the acetyl group at the anomeric position is arranged inferiorly to the oxoanionic pocket within striking distance of the catalytic serine (Ser-109). This arrangement is facilitated by a hydrogen bond, established between the Tyr-54 and the acetyl carbonyl of C-2 (Fig. 4). This residue has been shown to be crucial for enzyme activity.[41]

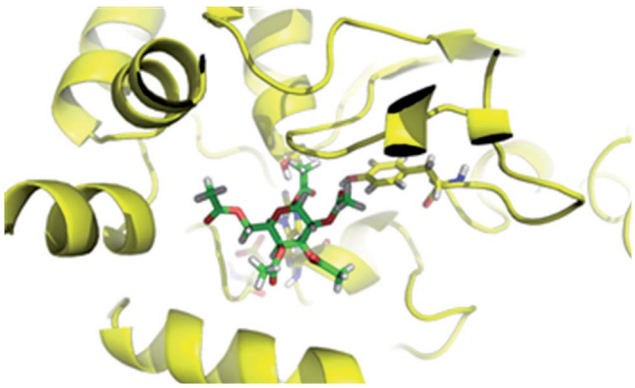


Fig. 4 Peracetylated galactose in the active site as proposed binding mode. Tyr-54 to the right and back Ser-109.

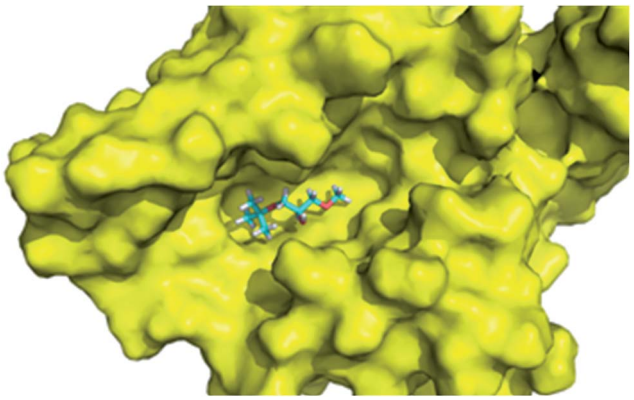


Fig. 5 Top view of the cavity of lipase from *P. stutzeri* with **S6** in the enzyme active center. The solvent has a *t*-butyl group that avoids the approach of the catalytic residue Ser-109 and therefore can not allow the nucleophilic attack of the alcoholysis reaction.

As we expected, this model could also explain why yields of *N*-acetylated derivatives (GalNAc or GlcNAc) and mannose (axial C-2) are so low. In the *N*-acetylated sugars, substrate binding could be hampered by the required planarity demanded by amide bond, while ethers may establish a H-bonding accepting the hydrogen from Tyr-54. In the case of mannose, the axial arrangement of the hydroxyl on C-2 prevents the substrate of being close enough to establish that interaction. The solvent in this model 3 interacts directly with acetyl groups, whereby their effect would be derived precisely from this fact. Depending on the characteristics of the solvent, these groups would be stabilized by exposure, or otherwise be forced to nonproductive binding modes to try repelling contact. For the alcoholysis, the proposed hypothesis was confirmed. The alcohols which are recognized by the enzyme in these reactions are: ethanol, 1-butanol, **S2**, **S3** and **S5**; they are able to enter the active site of the enzyme to react with the Ser-109, while the two alcohols not recognized by the enzyme (**S6** and **S7**) have a steric effect caused by the presence of chains of type i-propyl (**S7**) and *t*-butyl (**S6**), which avoids them from going into the pockets of the enzyme (Fig. 5).

Conclusions

In this work we describe an efficient enzymatic approach for preparing acetylated pyranosides (commercially available or easily obtained by chemical acetylation) bearing a free hydroxyl group only at the anomeric position. These intermediates are largely used for preparing the sugar donors currently used in chemical glycosylation and are currently prepared by using hazardous reagents and toxic solvents.

The results reported in the present work provide two major advantages: first, we found a lipase that cleaves with high regioselectivity the acetyl group at the anomeric position of different peracetylated β-monosaccharides and, secondly, we performed this regioselective deacylation under environmental friendly conditions, by using green solvents suitable to achieve high substrate concentration (30 g L^{-1}) and avoiding the use of any toxic reagent and solvents required in the classical chemical process.

Accordingly, PSL can be proposed as a useful biocatalyst for the preparation of acetylated monosaccharides with free hydroxyl group at the C-1 position. The bio-process can be performed either by hydrolysis or alcoholysis, using green solvents as reaction medium; besides, the process of deacylation may be scaled up with high efficiency and under green conditions respect to traditional chemical deprotection of the anomeric position.

The PSL catalyzed also alcoholysis in presence of several alcohols. In this case the best results were obtained using solvent **S2** and reactions can be affected by the presence of voluminous groups such *t*-butyl or iso-propyl residues as part of the side chains of secondary alcohol. By using the 3D structure of this lipase we have demonstrated that this enzyme is highly selective for β-peracetylated monosaccharides without activity towards the α-anomers. Thus, the regioselective hydrolysis of peracetylated monosaccharides catalyzed by PSL can be potentially employed as a facile procedure for the bio-separation of the two anomers too. The 3D structure of the active site also explains the reduced or the lack of activity observed in the case of peracetylated mannopyranoses and 2-acetaminopyranoses. In fact, any change in C-2 strongly influenced the enzyme activity at the close anomeric position, as consequence of a change of the interaction of the group in C-2 with the Tyr-54.

Also this molecular modelling and docking studies explained the alcoholysis results obtained with **S2** solvent. Small molecules of alcohols are able to enter to the active site of the enzyme to react with the Ser-109, while the presence of iso-propyl and *t*-butyl residues as part of the side chains of the alcohol avoids them from going into the pockets of the enzyme.

Thus, we could demonstrate that the lipase PSL efficiently catalyzes the hydrolysis or alcoholysis of peracetylated of β-D-galactose, β-D-glucosamine and β-D-glucose in the presence of green solvents, allowing for almost quantitative conversion in **S1** and **S2**. These solvents are considered safe and should be appropriate for the synthesis of building blocks that are very important in the synthesis of oligosaccharides of biological relevance. Furthermore, reaction scaling up is feasible without losing catalytic action.

Experimental

General

Peracetylated monosaccharides and disaccharides (*e.g.* β-D-*gluco*pyranose, β-D-galactopyranose, β-D-lactose, *etc*, Scheme 1), solvents (ethanol, tetrahydrofuran THF and 2-methyltetrahydrofuran 2-MeTHF) and *p*-nitrophenyl palmitate (*p*NPP) were purchased from Sigma Aldrich. Solvent **S1**, (Fig. 1) was a gift from COGNIS IP Management GmbH now part of BASF. Solvents **S2**, **S3**, **S4**, **S5**, **S6** and **S7** (Fig. 1) were a generous gift from Prof. Ignacio García,[26] (Universidad de Navarra, Spain; solvents features are shown in Table S2 see ESI†) Commercially available lipases from *Pseudomonas stutzeri* (PSL) and *Alcaligenes* sp. (QLC) were purchased from Meyto Sangyo (Japan). CAL-B was purchased from Sigma Aldrich. HPLC Jasco with

evaporative light scattering detector using NH2P50-4E amino column (Asahipak, Japan) eluted at 0.8 mL min^{-1}, 80% acetonitrile and 20% water was used. NMR spectra were recorded in $CDCl_3$ (δ = ppm) on a Bruker AV. 250 MHz and a Bruker AV III. 400 MHz.

Enzyme activity assay

The activities of the lipases were analyzed spectrophotometrically measuring the increment in the absorption at 410 nm produced by the released of *p*-nitrophenol in the hydrolysis of 1 mM *p*-nitrophenyl palmitate (*p*NPP) in 50 mM sodium phosphate buffer at pH 7 and 37 °C. To initiate the reaction 10 μL of the lipase solution (1.4 mg mL^{-1} in 50 mM sodium phosphate buffer pH 7) were added to 2.5 mL of substrate solution. The variation of the absorbance was monitored for 3 minutes. Each experimental assay was run at least three times with standard deviation under 5% of the samples average. An activity unit was defined as the amount of enzyme necessary to hydrolyze 1 μmol of *p*NPP per minute under the conditions described above.

The protein concentration was determined using Bradford method.[43] The protein calibration curve was obtained using BSA.

Hydrolysis of peracetylated substrates using *P. stutzeri* lipase

Standard assay was performed as follows: 15.0 mg of the peracetylated sugar were solved in 500 μL of solvent containing 50 μL of distilled water. The reaction was started by the addition of 10.0 mg of PSL[17] and the reaction progress was monitorized by HPLC. For identification of products, after complete reaction, the product was purified by column chromatography using dichloromethane–methanol (95 : 5) as eluent. Solvent was dried and purified products were analysed by ^{1}H-NMR and ^{13}C-NMR. Structures assignment was performed by means of 2D-COSY, HSQC experiments and HMBC. The NMR analytical data were corresponding to those reported in literature.[6,8,44]

2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranose (1a). Colorless oil, 90% yield (mixture of anomers). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 5.52 (bd, J = 3.4 Hz, 1H, H-1 α-anomer), 5.48 (dd, 1H, J = 1.3 Hz), 5.41 (dd, 1H, J = 3.5 Hz), 5.18 (dd, 1H, J = 3.4 Hz), 4.48 (t, 1H, J = 6.7 Hz), 4.11–4.09 (m, 2H), 2.10 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 2.00 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 171.5, 171.3, 171.2, 171.0, 96.9 (C-1 β-anomer), 91.0 (C-1 α-anomer), 71.9, 71.3, 69.2, 68.1, 62.7, 21.9, 21.7, 21.6, 21.5. Anal. calcd for $C_{14}H_{20}O_{10}$: C, 48.28%; H, 5.79%; found: C, 48.09; H, 5.80%. $[\alpha]_D^{20}$: +54.6 (c 1.98, $CHCl_3$).

2,3,4,6-Tetra-*O*-acetyl-α-D-*gluco*pyranose (2a). Colorless oil, 93% yield (mixture of anomers). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 5.54 (t, J = 9.7 Hz, 1H), 5.46 (d, 1H, J = 3.5 Hz, H-1 α-anomer), 5.09 (t, 1H, J = 9.8 Hz), 4.91 (q, 1H, J = 3.0 Hz), 4.74 (d, 1H, J = 8.5 Hz, β-anomer), 4.29–4.12 (m, 3H), 2.10 (s, 3H), 2.09 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 171.8, 171.7, 171.1, 170.6, 96.4 (C-1 β-anomer), 91.0 (C-1 α-anomer), 72.0, 70.8, 69.4, 68.1, 62.9, 21.6, 21.5. Anal. calcd for $C_{14}H_{20}O_{10}$: C, 48.28%; H, 5.79%; found: C, 48.12; H, 5.81%. $[\alpha]_D^{20}$: +46.9 (c 2.49, $CHCl_3$).

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-*gluco*pyranose (4a). Colorless oil, 65% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 6.00 (d, J = 9.5 Hz, 1H, NH), 5.33–5.26 (m, 2H), 5.14 (t, 1H, J = 9.4 Hz), 4.65 (d, 1H, J = 8.5 Hz, H-1 β-anomer), 4.32–4.27 (m, 1H), 4.23–4.20 (m, 2H), 4.16–4.10 (m, 1H), 2.11 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 172.42, 171.93, 171.47, 170.39, 92.48, 71.89, 69.18, 68.46, 63.04, 53.26, 24.09, 21.07, 21.58. Anal. calcd for $C_{14}H_{21}NO_9$: C, 48.41%; H, 6.09%; N, 4.03%; found: C, 48.29; H, 6.10; N, 4.04%.

Alcoholysis of peracetylated substrates using *P. stutzeri* lipase

Taking into account the catalytic behaviour of PSL toward peracetylated monosaccharides, **1β** and **2β** were selected for alcoholysis reactions. 15.0 mg of each substrate were dissolved in 500 μL of dry alcohol (1-butanol, **S2**, **S3**, **S5**, **S6** and **S7**). 10.0 mg of PSL[17] and molecular sieves (50.0 mg) were added to the mixture and the reactions were shaked at 30 °C during 48 h. Alcoholysis reactions were monitorized by HPLC. After complete reaction, products were purified by column chromatography using dichloromethane–methanol (95 : 5) as eluent. Solvent was evaporated and purified products were analysed by ^{1}H-NMR and ^{13}C-NMR. Structures assignment was performed by means of 2D-COSY, HSQC experiments and HMBC. NMR spectra were similar to those reported above and were concordant with literature.[6,8]

Preparative hydrolysis and alcoholysis

Substrate **1β** (2.4 g, 6.1 mmol) was dissolved in 80 mL of **S1** with 10% of distilled water (for hydrolysis) and in 80 mL of anhydrous **S2** containing 10% m/v of molecular sieves (for alcoholysis) by stirring during 24 hour at 30 °C. The biocatalyst (20 mg mL^{-1}) was then added to the reaction mixture under stirring. Aliquots (100 μL) were withdrawn at different intervals, ultra-filtered, and the progress of the reaction was checked by HPLC analysis. After complete reaction the biosolvent (**S1** or **S2**) was recycled and reused three times by centrifugation and the product **1a** was purified by column chromatography using dichloromethane–methanol (95 : 5) as eluent. Solvent was dried and purified products were analyzed by ^{1}H-NMR and ^{13}C-NMR. Structures assignment was performed by means of 2D-COSY, HSQC experiments and HMBC. NMR spectra were similar to those data previously reported and were concordant with literature.[6,8,44] This process was repeated 3 times without losing catalytic action.

Molecular modelling and docking

In order to explain results obtained by the hydrolysis and alcoholysis of peracetylated sugars with PSL, we decided to develop a study of molecular modelling and docking. The model was constructed by homology using as input the three-dimensional structure of the lipase from *Pseudomonas mendocina* (99% homology).[41] The model has been refined using the AMBER force field 03 and a steepest descent algorithm to minimize the energy function associated with the field for 2000 steps. Various parameters have been validated as

Ramachandran angles and hydrophobicity profiles of residues, being all comparable to crystallized native structures. For the docking program was used Autodock 4.0.[42] In the proposed model, the enzyme has two pockets, small and large, the latter being clearly open to the solvent. The active site is protected by two lids, which are opened and in hydrophobic media tend to close in the presence of water.

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