

Steric vs. electronic effects in the *Lactobacillus brevis* ADH-catalyzed bioreduction of ketones†

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Lactobacillus brevis ADH (LBADH) is an alcohol dehydrogenase that is commonly employed to reduce alkyl or aryl ketones usually bearing a methyl, an ethyl or a chloromethyl as a small ketone substituent to the corresponding (*R*)-alcohols. Herein we have tested a series of 24 acetophenone derivatives differing in their size and electronic properties for their reduction employing LBADH. After plotting the relative activity against the measured substrate volumes we observed that apart from the substrate size other effects must be responsible for the activity obtained. Compared to acetophenone (100% relative activity), other small substrates such as propiophenone, α,α,α -trifluoroacetophenone, α -hydroxyacetophenone, and benzoylacetone had relative activities lower than 30%, while medium-sized ketones such as α -bromo-, α,α -dichloro-, and α,α -dibromoacetophenone presented relative activities between 70% and 550%. Moreover, the comparison between the enzymatic activity and the obtained final conversions using an excess or just 2.5 equiv. of the hydrogen donor 2-propanol, denoted again deviations between them. These data supported that these hydrogen transfer (HT) transformations are mainly thermodynamically controlled. For instance, bulky α -halogenated derivatives could be quantitatively reduced by LBADH even employing 2.5 equiv. of 2-propanol independently of their kinetic values. Finally, we found good correlations between the IR absorption band of the carbonyl groups and the degrees of conversion obtained in these HT processes, making this simple method a convenient tool to predict the success of these transformations.

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Introduction

In the last few years, the use of alcohol dehydrogenases [ADHs, EC 1.1.1.x., also called ketoreductases (KREDs) or carbonyl reductases (CRs)] applied for synthetic purposes has largely increased, mainly due to the usually high catalytic efficiency and selectivity displayed to achieve carbonyl reductions or enantioselective alcohol oxidations under very mild reaction conditions.^{1–7} These enzymes are probably the most employed oxidoreductases to date, and several applications to obtain fine chemicals have already been found due to the use of very efficient systems to recycle the expensive

nicotinamide cofactors [NAD(P)H or NAD(P)⁺], needed to transfer electrons into and from the target substrate.^{8–11}

Lactobacillus brevis ADH (LBADH)¹² is a versatile short-chain alcohol dehydrogenase discovered by Hummel's group in 1997,¹³ which catalyzes the stereoselective reduction of ketones into the corresponding (*R*)-configured alcohols,¹⁴ or the oxidative kinetic resolution of *sec*-alcohols to obtain the remaining enantioenriched (*S*)-hydroxy derivatives,¹⁴ therefore displaying the so-called 'anti-Prelog' selectivity.¹⁵ Furthermore, it requires NADP(H) as a cofactor and usually works under the 'coupled-substrate' approach,¹¹ thus using 2-propanol (2-PrOH) or acetone to recycle the cofactor. This enzyme appears as a highly valuable tool since only a limited number of available ADHs show such good stereopreference and can be used with 2-PrOH.^{16–19}

Its amino acid sequence and three-dimensional structure have been determined,^{20,21} and several docking and modeling studies have been performed to explain the stereospecificity of this biocatalyst.^{20–23} The stereoselectivity emerges from an active site that possess two hydrophobic pockets, a small flexible one and a large open area, which host the small and large substituents of the substrate, respectively. Concerning the substrate spectra, this ADH is able to accept a more variable (often

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aromatic) bulky moiety such as a large ketone substituent,^{12,24} however, it has demonstrated a narrower acceptance for the small ketone substituent, accommodating preferentially methyl, ethyl, or chloromethyl groups.^{12,25}

The reduction of ketones coupled with the oxidation of alcohols in a hydrogen transfer (HT) fashion is a typical example where the redox potential between the hydrogen donor and the acceptor is not usually large enough to achieve quantitative conversions (conv), unless a huge excess of a sacrificial alcohol/ketone such as 2-propanol/acetone is used,¹¹ or activated ketones with an electron-withdrawing group at α -position such as chlorine,^{26,27} bromine,²⁸ or a carbonyl moiety at α -²³ or β -position^{23,29} are employed. In a recent contribution,²² we observed that for some substrates LBADH activity did not correlate with the conversions obtained, appearing some remarkable exceptions, and suggesting that mainly thermodynamic effects were involved in these processes. Furthermore, a method based on the infrared (IR) absorption band of the carbonyl group to predict the degree of conversion achieved in a HT-reduction of a ketone, was also proposed.

Herein, we have extended this study to a series of 24 α -substituted acetophenone derivatives (Fig. 1), differing in their steric and electronic properties, in order to provide a rationale for the final conversion of their HT reductions, as well as the influence of kinetics and thermodynamics using a huge excess of 2-propanol as a hydrogen donor (*standard conditions*), or just 2.5 equivalents (*minimal conditions*). Consequently the relative activities of LBADH with each substrate were measured compared with the calculated volume of the substrate. We

have also measured the IR absorption band of the carbonyl groups to check this predictive method with the enzymatic conversions. As a result, it will be shown that sterically demanding substrates can also be reduced by this enzyme.

Results and discussion

Enzyme activity of LBADH in the bioreduction of ketones

1–24a

A series of α - or β -substituted acetophenone derivatives (**1–24a**, Fig. 1) and the corresponding alcohols (**1–24b**) were selected based on their different steric and electronic properties in order to see the possible influence of the small substituent in their LBADH-catalyzed bioreductions. Thus, alkylated (**1–3a**), halogenated (**4–13a**), oxygenated (**14–16a**), nitrogenated (**17–20a**), and esterified (**21–24a**) derivatives were used as substrates in this study (see the ESI† for synthetic protocols).

Accordingly, NADPH depletion was used to determine the catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) of the LBADH-catalyzed reduction of ketones **1–24a** to subsequently calculate the relative activity with regard to acetophenone (**1a**, 100%). Ketone **1a** has been used in other studies as a model substrate for this enzyme due to its high reactivity.^{20–22} As shown in Table 1, compounds bearing one halogen atom at α -position (**4–6a**), α,α -difluoro- (**7a**), α,α -dichloro- (**8a**), and α -bromo- α -chloroacetophenone (**9a**) were the best substrates, being reduced approx. between 1.6 and 32-times more effectively than **1a**. Another halogenated compound such as α,α -dibromoacetophenone (**10a**)

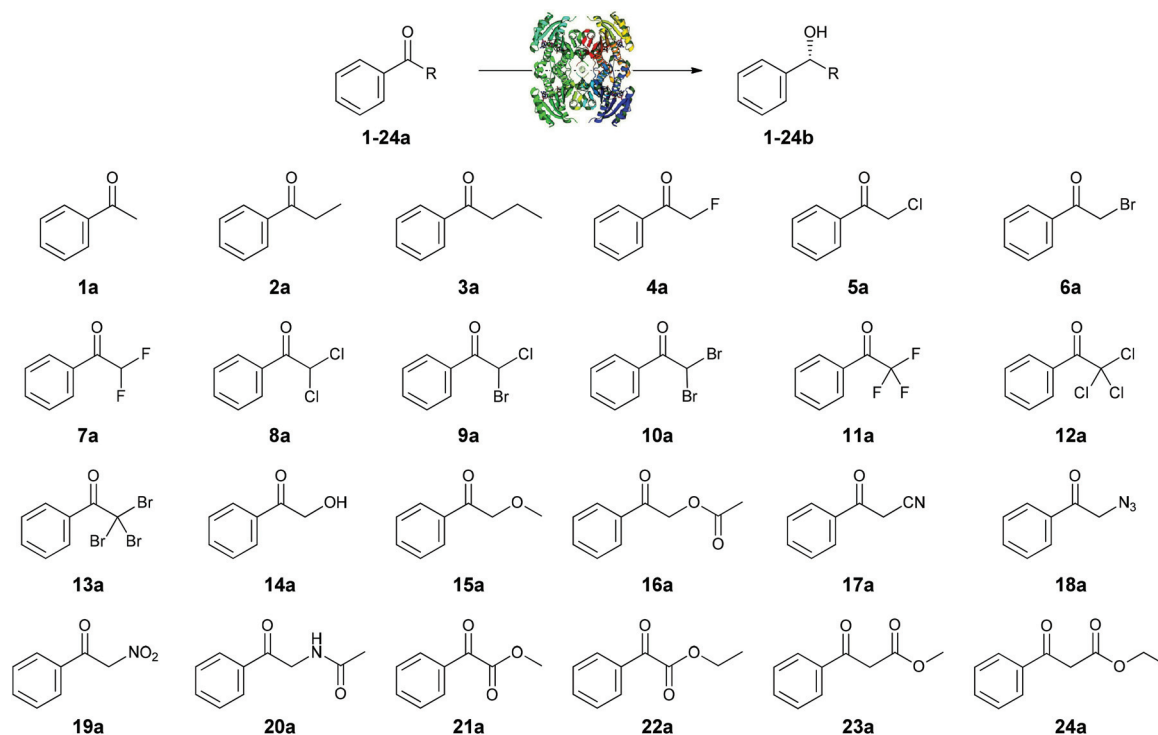


Fig. 1 Substrates employed to study the LBADH activity.



Table 1 Comparison of the relative activities for LBADH-catalyzed reduction of ketones **1–24a**

| Entry | Compound | Relative activity ^a (%) |
|-------|------------|------------------------------------|
| 1 | 1a | 100 |
| 2 | 2a | 29 |
| 3 | 3a | <1 |
| 4 | 4a | 3187 |
| 5 | 5a | 2358 |
| 6 | 6a | 547 |
| 7 | 7a | 780 |
| 8 | 8a | 472 |
| 9 | 9a | 159 |
| 10 | 10a | 73 |
| 11 | 11a | 21 |
| 12 | 12a | 26 |
| 13 | 13a | n.d. ^b |
| 14 | 14a | 5 |
| 15 | 15a | <1 |
| 16 | 16a | n.d. ^c |
| 17 | 17a | 3 |
| 18 | 18a | 4 |
| 19 | 19a | n.d. ^c |
| 20 | 20a | 1 |
| 21 | 21a | 27 |
| 22 | 22a | 27 |
| 23 | 23a | <1 |
| 24 | 24a | <1 |

^a 100% value corresponds to $k_{\text{cat}}/K_{\text{M}} = 16\,100\text{ M}^{-1}\text{ s}^{-1}$ (see ESI).

^b Solubility problems. ^c Absorbance interference. n.d. not determined.

was also a good substrate while α,α,α -trifluoro (**11a**) and α,α,α -trichloro (**12a**) derivatives could be reduced at levels around 0.2 times compared to **1a**. Ketone **2a** and α -keto esters **21a** and **22a** showed relative activities around 30%, while ketones **3a**, **14a**, **15a**, **17a**, **18a**, **20a**, **23a**, and **24a** can be considered as poor substrates from a kinetic point of view.

It was noticeable that going from acetophenone to propiophenone (**2a**) and butyrophenone (**3a**), the activity considerably dropped down (entries 1–3), supporting previous results obtained with this enzyme showing that a bulkier group than ethyl can hardly be accepted.²⁵ As mentioned before, when a family of halogenated derivatives was employed, it was observed that, on the one hand, monohalogenated compounds (entries 4–6) showed higher activities than dihalogenated ketones (entries 7–10), and these, in turn, exhibited higher velocities than the trihalogenated ones (entries 11 and 12). On the other hand, fluorinated compounds demonstrated to be better substrates than chlorinated and these ones better than brominated. These results can be explained based on steric reasons (see below) and also due to the higher electrophilicity of the carbonyl group when fluorine is present at α -position with regard to chlorine or bromine atoms.³⁰ For the oxygenated compounds (entries 14 and 15), it seemed that a polar hydroxyl group at α -location (**14a**) did not avoid completely the enzymatic activity, while a neutral methoxy moiety (**15a**) negatively affected it. The presence of a nitrile (**17a**), an azide (**18a**) or an acetamide (**20a**), caused a dramatic drop in LBADH reactivities (entries 17–20). The catalytic efficiencies of these nitrogenated derivatives mainly decreased due to the high K_{M}

values (see ESI†), leading to very low relative activities. Finally, we observed differences between keto esters (entries 21–24) depending on the carbonylic position, since α -substituted derivatives **21a** and **22a** showed a moderate enzymatic activity, while the β -substituted ones (**23a** and **24a**) seemed not to be appropriate substrates for LBADH.

Comparison of enzymatic activities and molecular volumes

Steric hindrance is usually taken as the most prominent reason to explain the low reactivity of a substrate with a (bio)-catalyst. For enzymes it is well known that (de)stabilizing interactions between the substrate and the enzyme can determine the activity and selectivity of the biocatalysts. For instance, based on these molecular interactions successful empiric rules have appeared for lipases (Kazlauskas' rule)³¹ or alcohol dehydrogenases (Prelog's rule).¹⁵ In an ADH, a ternary complex between an enzyme, ketone or alcohol substrate, and a cofactor in the reduced or oxidized form, must be produced to correctly transfer the electrons. Taking into account a model where two hydrophobic pockets differing in size would accommodate the acetophenone substrates **1–24a**, we decided to explore the correlation between the enzymatic activities with LBADH and the calculated substrate sizes. Moreover, the large group would be maintained (a phenyl moiety), while the small one would change in terms of steric demand.

Subsequently, the volume of all acetophenone compounds were estimated using the generalized AMBER force field (GAFF).³² For each derivative, a conformational search was performed using the Multiconf-Dock tool generating conformers by rotating all single, non-terminal, acyclic bonds.³³ The MSMS program³⁴ was used to carry out the numerical computation of the molecular volume. Both the solvent-excluded molecular volume (V_{SES}) for a probe sphere of 1.4 Å radius and the van der Waals molecular volume (V_{moi}) were computed for each compound and the results for different conformers were averaged (see the Experimental section and ESI† for more details). Thus, the molecular volume ranged from 117.49 Å³ (**1a**, R = CH₃) to 177.87 Å³ (**24a**, R = CH₂CO₂Et), meaning that the biggest substrate had a volume difference of approximately 1.5 times with regard to the smallest one. With these data in hand, a graphic correlation between the molecular volumes and the enzymatic activities for all ketones tested could be done as shown in Fig. 2.

Although there was a general trend correlating higher velocities with the smaller substrates, some noticeable deviations were observed. For instance, small ketones such as **2a** (R = CH₂CH₃), **11a** (R = CF₃), **14a** (R = CH₂OH), and **17a** (R = CH₂CN) had relative activities lower than 30%, while medium-sized ketones such as **6a** (R = CH₂Br), **8a** (R = CHCl₂), or **9a** (R = CHBr₂) presented relative activities between 150% and 550%. Even one of the biggest substrates, **22a** (R = CO₂CH₂CH₃, $V = 162.50\text{ Å}^3$) was moderately well accepted by this enzyme. This study shows that although undoubtedly the steric demand is a very important factor, other effects, *e.g.* electronic, must also have an influence and therefore should also be taken into account.³⁵



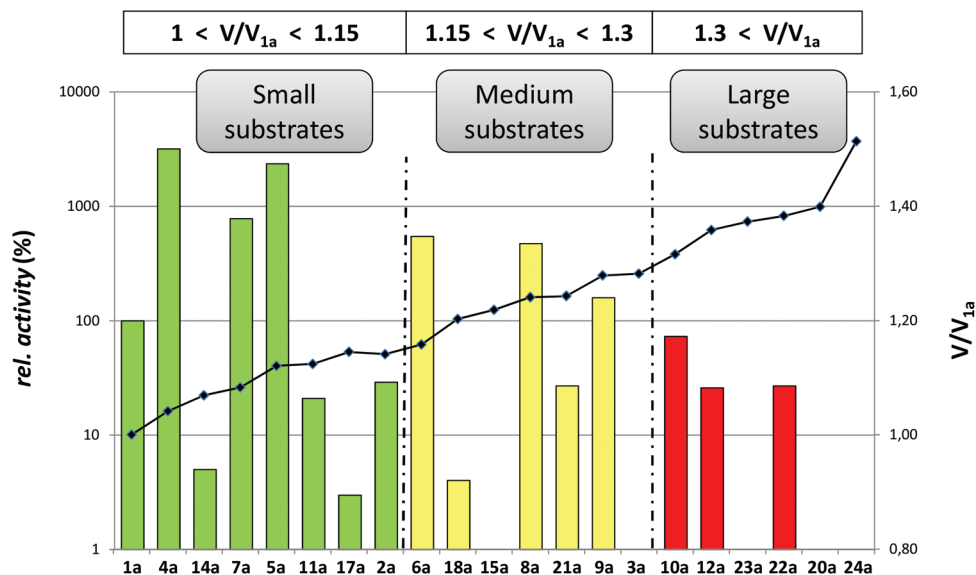


Fig. 2 Comparison of ketone volumes (black line) with relative activities (bars, logarithm scale). Ketone substrates are divided as small-sized ($1 < V/V_{1a} < 1.15$), medium-sized ($1.15 < V/V_{1a} < 1.3$), and large-sized ($1.3 < V/V_{1a}$). Substrates are ordered from left to right by an increasing value of calculated molecular volume.

Comparison of enzymatic activities and conversions: electronic effects and thermodynamic implications

Trying to answer whether a good substrate leads to complete conversion while a poor substrate affords low conversion, we analyzed the correlation between the kinetic results with the conversions obtained in the LBADH-catalyzed bioreductions. In a previous study²² it was demonstrated with a small set of ketones that high conversions were obtained with substrates bearing a chlorine atom at α -position. In fact, there was no clear correlation between the enzymatic conversions and the initial rates of the reactions. Although a trend was observed in some cases, there were some remarkable exceptions such as methoxyacetone, suggesting that the bioreduction of ketones was not under kinetic, but mainly under thermodynamic control.

Herein we have reduced the ketones listed in Fig. 1 with LBADH adding a catalytic amount of NADPH (1 mM) and employing a huge excess of 2-PrOH (5% v/v, standard conditions) or just using 2.5 equiv. of 2-PrOH (minimal conditions).²⁷ The variety of substituents enabled the drawing of a structure–activity relationship, so in a subsequent step, conversions obtained were plotted against the relative activities shown in Table 1 to easily detect possible deviations (Fig. 3).

Although acetophenone (**1a**) was reduced 4 times faster than propiophenone (**2a**), LBADH produced both alcohols with a conversion higher than 90% after 48 h using an excess of 2-propanol and approximately 50% when using just 2.5 equiv. Since in these cases the alcohol formation is not highly favored with regard to the 2-PrOH–acetone counterpart ($<0.5 \text{ kcal mol}^{-1}$),²² the thermodynamic equilibrium does not allow a very high conversion unless a huge excess of the hydrogen donor is applied. To ensure that the equilibrium was

reached using 2.5 equiv. of 2-PrOH, a time study of the bioreduction with **1a** was performed (Fig. 4), observing always a conversion lower than 50% within the time. Butyrophenone (**3a**) afforded very low formation of the corresponding alcohol (conv $<5\%$), showing that a propyl group was not suitable for this enzyme. The introduction of electronegative groups at the small substituent generally led to excellent conversions. Thus, α -halogenated derivatives **4–11a** could be reduced nicely (conv $>90\%$) under these mild conditions even just employing 2.5 equiv. of 2-PrOH (Fig. 3 and 4), independently of their relative activity (*i.e.*, the catalytic efficiency of **4a** was around 40 times higher than that of **10a**). This is due to the fact that the thermodynamic equilibrium in these HT reactions favors the hydrolyrin formation (between 4 and 6 kcal mol^{-1}),²² being probably involved in an intramolecular H-bond between the hydroxyl group and the halogen atom(s).³⁶ Just as in the case of very bulky substrates such as **12a** ($R = \text{CCl}_3$) and **13a** ($R = \text{CBr}_3$), the conversions were not high.

Concerning the oxygenated derivatives, only α -hydroxyacetophenone **14a** appeared as a suitable substrate for LBADH achieving quantitative conversion to **14b** under standard conditions, while 90% for the minimal setup, even at 8 h (Fig. 4). This suggested that in this case the intramolecular H-bond was not as effective as in the case of the halogenated compounds, although the equilibrium was clearly more shifted towards the alcohol product than for alkylated derivatives **1a** and **2a**.

Although nitrogenated ketones **19a** and **20a** were not appropriate substrates for this biocatalyst affording low conversions ($<15\%$), keto nitrile **17a** led to a formation of **17b** of approx. 70% with an excess of 2-PrOH and 55% employing 2.5 equiv. of the hydrogen donor. Remarkably, α -azidoacetophenone **18a**, despite its low relative activity (4%), could be reduced by



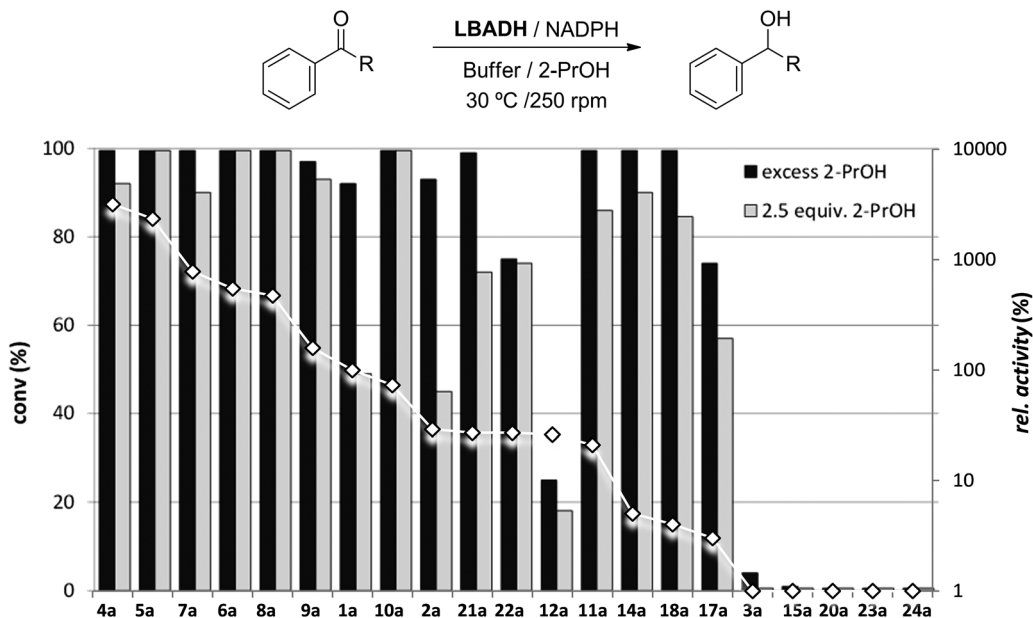


Fig. 3 Comparison of the initial rates (white line, logarithm scale) with LBADH-catalyzed bioreduction conversions ($t = 48$ h) under standard (5% v/v of 2-PrOH, black bars) and minimal conditions (2.5 equiv. of 2-PrOH, grey bars). Substrates (30 mM) are ordered from left to right by a decreasing value of enzymatic activity. In all cases, ee measured for the alcohol obtained was >99%.

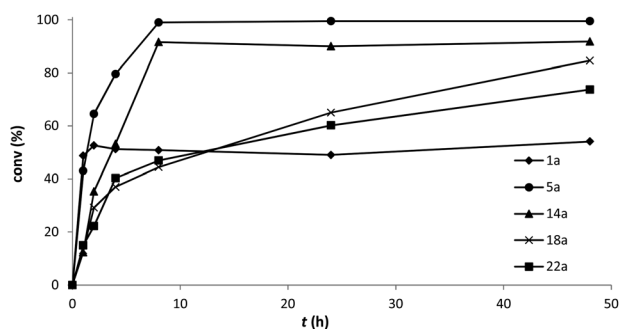


Fig. 4 Conversions of selected substrates (30 mM) within the time using 2.5 equiv. of 2-PrOH.

LBADH in a quantitative manner under the standard HT conditions. While a conversion around 60% was achieved after 24 h with a small excess of 2-PrOH, when the reaction was left for 48 h (Fig. 4), LBADH already led to 85% of alcohol **18b**.

For keto esters, while α -substituted **21a** and **22a** led to high conversions using both reaction settings (>70%), β -keto esters **23a** and **24a** were not accepted by the ADH. In these cases, the existence of a carbonyl group at α -position seems to highly favor the bioreduction, possibly due to an internal H-bond interaction between the alcohol and the carbonyl ester moiety.^{23,29} Again, since these ketones were moderately good substrates for LBADH, we performed the experiments with **22a** at different reaction times to confirm that when using an excess of the hydrogen donor we obtained a final conversion of around 75% at 24 and 48 h, while employing 2.5 equiv. (Fig. 4), the conversion still increased from 60% to 74%.

Substrate solubility is another important issue that can significantly affect these bioreductions.²⁴ Due to the fact that enzymatic reactions catalyzed by ADHs are generally carried out in an aqueous buffer solution to keep the enzyme stability, and since the majority of ketones of interest are highly hydrophobic, the use of organic cosolvents in biphasic^{37–39} and monophasic systems^{40–42} has been proposed. In the standard HT conditions, 5% v/v of 2-PrOH was used as a hydrogen donor and also helped to solubilize the substrates, but under the minimal setup 2% v/v of dimethylsulfoxide (DMSO) was added for solid ketone substrates that did not afford high conversions, to check whether their water-solubility could be an issue that would hamper higher degrees of alcohol formation. Thus, several bioreductions were repeated, observing a remarkable effect in some cases. Ketone **8a** was reduced quantitatively in the presence of DMSO while only affording 63% of **8b** with just 2.5 equiv. of 2-PrOH. Conversion with ketone **9a** was enhanced from 75% to >99% employing DMSO, and a similar effect (from 11% to 57%) was observed for keto nitrile **17a**. It must also be mentioned that in all cases stereoselectivities were higher than 99% for the formation of the anti-Prelog alcohols, thus demonstrating that the binding of these substrates was not altered (phenyl linked to the big pocket, and the smaller ketone derivative to the small pocket).

Enzyme stability should also be taken into account when measuring enzymatic conversions. Therefore, LBADH was incubated for 24 h at 30 °C in a buffer in the presence or in the absence of 2-PrOH before addition of **1a**, in order to measure the enzymatic conversion after 24 h. In both cases, transformations around 90% were achieved, confirming the excellent enzyme stability during the experimental time-frames studied.



IR carbonyl stretching bands to predict biocatalyzed HT conversions

We previously measured the IR carbonyl absorption bands of a small set of ketones and plotted them against the degrees of conversion at equilibrium using a slight excess of the hydrogen donor, obtaining a good correlation between them.²² The presence of electron-withdrawing groups led to higher IR frequencies, since the dipolar form of these ketones (Fig. 5) was disfavored, therefore destabilizing their ground resonance form and making them more reactive.⁴³ It was concluded that IR could be employed as a reliable methodology to qualitatively predict if a ketone could be a good hydrogen acceptor under HT conditions.

Due to the fact that we had a broad set of structurally related ketones, we decided to further explore the reliability of this methodology. IR carbonyl stretching bands were measured for all reactive acetophenones and then these values were plotted against the normalized enzymatic conversions, that is, the LBADH-catalyzed conversion obtained with 2.5 equiv. of 2-PrOH divided by the one achieved using an excess of the hydrogen donor (Fig. 5).⁴⁴

From our previous study,²² ketones having a C=O value lower than 1690 cm⁻¹ did not show quantitative conversions under the minimal conditions while those presenting values higher than 1690 cm⁻¹ were highly reduced even with a small excess of the hydrogen donor. As can be seen, from the 15 ketones studied, just **6a** did not fit with this criterion. α -Bromoacetophenone showed a lower value (1683.9 cm⁻¹) compared to other halogenated derivatives. This is because α -brominated and α -iodinated carbonylic derivatives present lower C=O shifts in comparison with fluorinated or chlorinated ketones due to the dihedral angle (φ) between C=O and C-Br or C-I bonds.⁴⁵

In contrast, a correlation to the ¹³C-NMR signal of the carbonyl moiety did not lead to any conclusive data.

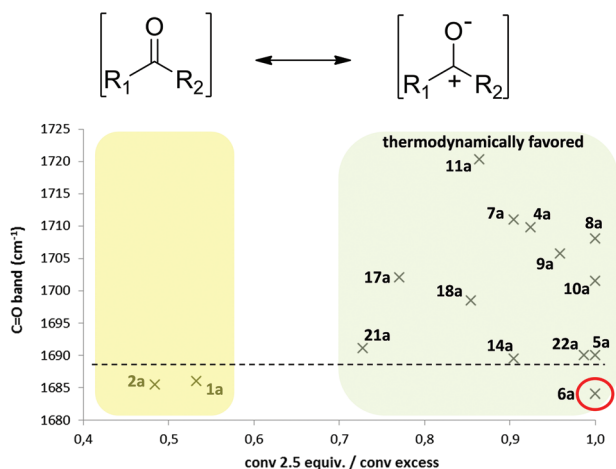


Fig. 5 Ketone resonance structures and plot of IR carbonyl stretching bands of acetophenone derivatives against the normalized degrees of conversion for LBADH-catalyzed HT reactions. The exception found is circled in red.

Conclusions

Lactobacillus brevis ADH is a very robust and versatile oxidoreductase which is often employed since it can work under the highly convenient ‘coupled-substrate’ approach displaying ‘anti-Prelog’ selectivity. Until now it has been described as a very active biocatalyst for alkyl or aryl ketones possessing methyl, ethyl, or chloromethyl as a small substituent in the ketone failing for other bulkier substrates. Herein we have shown that not only these moieties, but also other groups such as α,α -dibromomethyl, α,α,α -trichloromethyl or α -ethoxycarbonyl can be accepted by this enzyme achieving good to excellent conversions and stereoselectivities to the corresponding alcohols. When the molecular volumes of the substrates were measured, a trend was observed regarding the enzymatic activity, although there was a clear deviation for some ketones. Hence, steric effects are important in these biocatalyzed reductions, but other influences, *i.e.* electronic, cannot be ignored.

These hydrogen transfer reactions are a good example where ΔE^0 between the hydrogen donor and the acceptor is usually not large enough to achieve quantitative conversions necessitating the use of a large excess of a co-substrate to drive the equilibrium in the desired direction. In this sense, the use of α -halo- and α - or β -carbonyl ketones as hydrogen acceptors in nearly stoichiometric amounts has been utilized for synthetic purposes.^{26–30} This behavior has been studied for LBADH obtaining excellent conversions with substrates bearing electron-withdrawing groups such halogen(s), azido, cyano, hydroxyl, and carbonyl. Although some of these derivatives appeared as poor substrates for this enzyme from a kinetic point of view, they could be converted into the alcohol products at high conversion, since these hydrogen transfer processes are mainly driven by thermodynamics. While for some of the formed alcohols a stabilizing intramolecular H-bond is assumed, for others an inductive effect can be ascribed for the low alcohol oxidation rates.

Finally, IR frequencies of the carbonyl band correlated well with the normalized conversions, and except for the special case of α -brominated ketone **6a**, this method appears as an easy and straightforward predictive methodology for the conversion obtained by a ketone under HT conditions.

Our data demonstrate that the frequently employed statement “*due to steric reasons*” to explain the low reactivity or conversion of a substrate with an (enzyme) catalyst must be carefully assessed since other important effects contribute as well. Furthermore, a low (enzyme) activity does not necessarily imply low conversion, especially in the case of HT transformations catalyzed by ADHs.

Experimental

General methods

NADPH was acquired from Codexis. Chemical reactions were monitored by analytical TLC, performed on Merck silica gel 60



F254 plates and visualized by UV irradiation. Flash chromatography was performed using silica gel 60 (230–400 mesh). IR spectra were recorded on a Perkin-Elmer 1720-X infrared Fourier transform spectrophotometer on NaCl pellets dissolving the compounds with a drop of CH_2Cl_2 . ^1H -, ^{13}C -NMR, and DEPT were obtained using a Bruker DPX-300 and NAV-400 (^1H , 300.13 MHz and ^{13}C , 75.5 MHz) spectrometer for routine experiments. The chemical shifts (δ) are given in ppm and the coupling constants (J) in hertz (Hz). ESI⁺ mode was used to record mass spectra (MS) and ESI-TOF for HRMS. Gas chromatography (GC) analyses were performed on a Hewlett Packard 6890 Series II chromatograph. Optical rotations were measured using a Perkin-Elmer 241 polarimeter and are quoted in units of $10^{-1} \text{ deg cm}^2 \text{ g}^{-1}$. Steady-state kinetic parameters were determined using a Varian Cary50Bio UV/Vis spectrophotometer.

Protein expression and purification

E. coli/LBADH was grown overnight at 37 °C in 250 mL of Luria–Bertani medium supplemented with 100 $\mu\text{g mL}^{-1}$ ampicillin. The protein expression was induced by the addition of anhydrotetracycline hydrochloride (0.2 mg L^{-1} , 0.4 μM final concentration). The cultivation was performed overnight at 20 °C and 130 rpm to avoid the formation of inclusion bodies. The cells were harvested by centrifugation (5600 rpm, 20 min, r.t., Heraeus SEPATECH, Megafuge 1.0) and washed with phosphate buffer 50 mM, pH 7.5. Cells were disrupted by ultrasonication (Branson, S250D CE, 200 W, 5 mm spike, 50 mL tubes, 1 s impulse, 4 s pause, amplitude 40%, 2 min and 30 s, 4 °C) and subsequently subjected to centrifugation (5600 rpm, 20 min, r.t.). The supernatant was used for protein purification.

The sample was filtered off and LBADH-Strep was purified *via* Strep-Tag chromatography following the procedure described by the company (GE Healthcare, StrepTrap HP 5 mL column) and LBADH-Strep was obtained at a concentration of 145 μM . The purity of protein samples was assessed by SDS polyacrylamide gel electrophoresis.

Steady-state kinetic analysis of LBADH

The enzymatic activities of LBADH in the reduction of ketones **1a–24a** were determined spectrophotometrically by monitoring the NADPH consumption at 340 nm ($\epsilon_{\text{NADPH},340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). In some cases, high concentrations of the substrate or the proper compound resulted in absorption values exceeding 1 unit. In these experiments, the coenzyme consumption was measured at 370 nm ($\epsilon_{\text{NADPH},370} = 2.7 \text{ mM}^{-1} \text{ cm}^{-1}$).

Stock solutions of substrates (1.0 M) were made in DMSO since the presence of 1% DMSO resulted in a slight decrease in LBADH activity, while higher solubility of certain compounds could be achieved. A reaction mixture (1.0 mL) usually contained the corresponding ketone in 50 mM Tris-HCl, pH 7.5, 100 μM NADPH, 1% v/v DMSO, and 0.02–0.15 μM of LBADH.

Molecular volume calculations

The generalized AMBER force field (GAFF) was employed for carrying out the molecular modeling of the selected PhCOR compounds.³² The GAFF parameters including AM1-BCC atomic charges were generated automatically using the antechamber module included in the Amber11 package.⁴⁶ For each compound, a conformational search using the Multiconf-Dock tool³³ was performed generating conformers by rotating all single, non-terminal, acyclic bonds. Subsequently, the SANDER module in Amber11 was employed to minimize and score all the non-redundant conformers in terms of their relative GAFF energies.

The MSMS program³⁴ was used to carry out the numerical computation of the molecular volume. To improve the accuracy of the results, a high value (10 vertex points per Å^2) for the triangulation density was employed. We computed both the solvent-excluded molecular volume for a probe sphere of 1.4 Å radius and the van der Waals molecular volume. The Bondi atomic radii were used in all the volume calculations and the results for different conformers were averaged (see ESI†).

LBADH-catalyzed reduction of ketones

Protocol using an excess of 2-propanol. In a 1.5 mL Eppendorf vial, LBADH (3U) was added in 50 mM Tris-HCl buffer, pH 7.5 (600 μL , 1 mM NADPH, 1 mM MgCl_2) and mixed with 2-propanol (32 μL , 5% v/v) and the corresponding ketone (30 mM). Reactions were shaken at 250 rpm and 30 °C for 48 h and stopped by extraction with ethyl acetate ($2 \times 0.5 \text{ mL}$). The organic layer was separated by centrifugation (13 000 rpm, 2 min) and dried over Na_2SO_4 . Conversions and *ee* of the corresponding alcohols were determined by GC (see ESI†). For α -brominated ketones, 50 mM Tris- H_2SO_4 buffer, pH 7.5 (600 μL , 1 mM NADPH, 1 mM MgBr_2) was used to avoid undesired $\text{S}_{\text{N}}2$ reactions.²⁸

Protocol using 2.5 equiv. of 2-propanol. In a 1.5 mL Eppendorf vial, LBADH (3U) was added into 50 mM Tris-HCl buffer, pH 7.5 (600 μL , 1 mM NADPH, 1 mM MgCl_2) and mixed with 2-propanol (3 μL , 2.5 equiv.) and the corresponding ketone (30 mM, 1 equiv.). Reactions were shaken at 250 rpm and 30 °C for 48 h and stopped by extraction with ethyl acetate ($2 \times 0.5 \text{ mL}$). The organic layer was separated by centrifugation (13 000 rpm, 2 min) and dried over Na_2SO_4 . Conversions and *ee* of the corresponding alcohols were determined by GC (see ESI†). For α -brominated ketones 50 mM Tris- H_2SO_4 buffer, pH 7.5 (600 μL , 1 mM NADPH, 1 mM MgBr_2) was used.²⁸ In some cases, 12 μL of DMSO (2% v/v) were added to improve substrate solubilization.

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