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E. coli/ADH-A: An all-inclusive catalyst for the selective biooxidation and deracemisation of secondary alcohols

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The NAD^+ regeneration system present in *E. coli* cells was exploited for the oxidation and deracemisation of secondary alcohols with the over-expressed alcohol dehydrogenase from *Rhodococcus ruber* DSM 44541 (*E. coli*/ADH-A). Thus, various racemic alcohols were selectively oxidised employing lyophilised or resting *E. coli*/ADH-A cells, without requiring external cofactor or co-substrate. Simple addition of these substrates to the *E. coli*/ADH-A cells in buffer afforded the corresponding ketones and the remaining enantioenriched (*R*)-alcohols. This methodology was applied for the desymmetrisation of a *meso*-diol, and for the synthesis of the highly valuable raspberry ketone. Moreover, a biocatalytic concurrent process was developed with resting cells of *E. coli*/ADH-A, ADH from *Lactobacillus brevis* (LBADH) and glucose dehydrogenase (GDH), for the deracemisation of various secondary alcohols, producing the desired enantiopure alcohols in up to >99% *ee* starting from the racemic mixture. The reaction time of deracemisation for 1-phenylethanol was estimated to be less than 30 min. The stereoinversion of (*S*)-1-phenylethanol to its pure (*R*)-enantiomer was also successfully achieved, thus providing a biocatalytic alternative to the chemical Mitsunobu inversion reaction.

Introduction

Biooxidation and deracemisation processes carried out by whole-cell microorganisms or isolated enzymes are nowadays recognized as valuable tools for the preparation of high added-value compounds such as chiral alcohols used as building blocks for active pharmaceutical ingredients (APIs). Oxidoreductases such as alcohol dehydrogenases depend on their (expensive) nicotinamide cofactor $NAD(P)^+$ for oxidation.[1] These enzymes therefore require an efficient recycling method, as shown with the various coupled systems developed in the past decades,[2] to perform cost-effective enzymatic redox processes at a higher scale.[3]

Although the oxidised nicotinamide cofactors $NAD(P)^+$ are more stable in solution than their corresponding reduced forms NAD(P)H, synthetic schemes for efficient regeneration of $NAD(P)^+$ are far less developed than for the reduced counterpart NAD(P)H.[2] Consequently, several types of enzymes have been used in coupled systems for $NAD(P)^+$ regeneration, such as L-lactate dehydrogenase employing pyruvic or glyoxylic acid as formal oxidant,[4] and glutamate dehydrogenase, used to regenerate NAD^+ or $NADP^+$ coupled with the reductive amination of α-ketoglutarate.[5] For alcohol dehydrogenases (ADHs), the use of acetone or acetaldehyde in a huge molar excess has been demonstrated in a "coupled-substrate" approach,[6] although in several cases these conditions are not compatible with the stability of the enzyme. Another category of enzymes, the NAD(P)H oxidases (NOXs), especially those affording water as by-product at the expense of molecular oxygen, have appeared as an interesting alternative to oxidise NAD(P)H for recycling purposes.[7] Recently, this cofactor regeneration system was also described with the simultaneous over-expression of a NAD^+ dependent enzyme and a NOX (H_2O producing NADH oxidase from *Lactobacillus brevis*) in a whole-cell biocatalyst.[8]

ADHs are stereocomplementary for oxidation and reduction processes (enzyme-based stereocontrol) and can therefore catalyse the enantioselective oxidative kinetic resolution of *sec*-alcohols to obtain enantioenriched products, although only with a maximum 50% yield (Scheme 1a). More attractive (and more challenging) methods include deracemisation protocols. Thus, a highly valuable optically pure product with a theoretical100% yield and 100% *ee* can be obtained from a cheap racemic substrate in a concurrent one-pot process combining multiple catalysts (Scheme 1b).[9] In this case, each step must be carefully balanced

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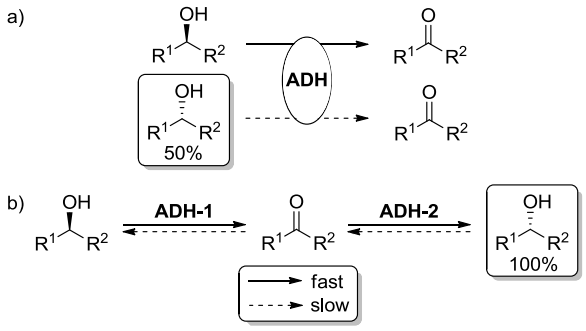
to ensure that the catalytic processes run at comparable rates and that the various catalytic reactions do not interfere with one another. Recently, efficient systems have appeared for the deracemisation (or stereoinversion) of secondary alcohols,[10] employing isolated enzymes solely[7e,11] or in combination with whole-cell biocatalysts.[12]



Scheme 1. a) ADH-catalysed oxidative kinetic resolution; and b) deracemisation or (stereoinversion) of a racemic *sec*-alcohol catalysed by two stereocomplementary ADHs.

While screening ADHs for oxidation reactions, we observed that *E. coli* cells recycle NAD^+ already on their own without the need to over-express an additional NADH oxidase. Herein, we describe the use of lyophilised or resting cells of *E. coli* over-expressing the solvent tolerant alcohol dehydrogenase from *Rhodococcus ruber* DSM 44541 (ADH-A),[13] as an efficient catalyst to achieve the biooxidative kinetic resolution of several *sec*-alcohols without the need for an external cofactor or recycling system. The application of this methodology to deracemise *sec*-alcohols by a concurrent process with NADP-dependent *Lactobacillus brevis* ADH (LBADH)[14] and a NADPH recycling system was also successfully achieved.

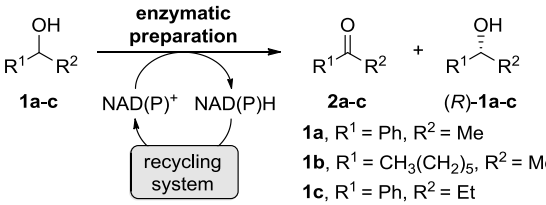
Results and Discussion

Biooxidative Kinetic Resolution of Secondary Alcohols

As a continuation of our work employing recombinant ADHs over-expressed in *E. coli*,[13a,15] the biooxidation of racemic alcohols was explored to perform oxidative kinetic resolutions (Table 1). Thus, racemic 1-phenylethanol (**1a**, 40 mM) was added to a mixture of *E. coli*/ADH-A, a catalytic amount of NAD^+ (1 mM) and a molar excess of acetone as co-substrate (entry 1). As expected, kinetic resolution was achieved, affording 50% of acetophenone (**2a**) and 50% of the remaining enantiopure (*R*)-1-phenylethanol (**1a**) after 24 h. As a control experiment, the same reaction was performed without acetone (entry 2), and surprisingly, the same results were observed, obtaining 50% of the enantiopure (*R*)-**1a**. In a subsequent experiment, no additional NAD^+ was added to the reaction medium, finding again excellent conversion and enantioselectivity (entry 3). These observations led to the hypothesis that a catalytic amount of NADH/NAD^+ present in the lyophilised *E. coli* cells was being internally oxidatively recycled and used by the over-expressed ADH-A. The membranes of *E. coli* are known to contain an electron transport chain that oxidises NADH with oxygen as an electron acceptor: various types of enzyme complexes or isolated enzymes oxidising NADH have been described in *E. coli*. Among them, the most studied are the NADH dehydrogenases, such as the NADH:ubiquinone oxidoreductase (analogue to the mitochondrial NADH:ubiquinone oxidoreductase called Complex I) involved in the aerobic respiratory chain,[16] and flavohemoglobins (e.g.Hmp),[17] which can act as "NADH oxidase-like" enzymes.[18] Moreover, the NADH:ubiquinone oxidoreductase that can account for the NADH oxidase activity of *E. coli* is highly dependent on NADH.[19]

To confirm the presence of an integrated NAD^+ regeneration system in *E. coli*, lyophilised *E. coli* cells (TOP10) as well as purified commercially available ADH-A were tested separately under the same reaction conditions leading to no detectable conversion (entries 4 and 5). When lyophilised *E. coli* cells and purified ADH-A were combined, 18% of ketone **2a** was obtained (entry 6). Other lyophilised *E. coli* preparations over-expressing NADP-dependent ADHs were also explored, such as *Lactobacillus brevis* (LBADH), [14] *Ralstonia* sp. (RasADH),[20] *Sphingobium yanoikuyae* ADH (SyADH),[21] *Thermoanaerobacter ethanolicus* (TeSADH)[22] or *Thermoanaerobium* sp. ADH (ADH-T),[23] but conversions with their respective model substrates were modest (entries 7 to 11). This observation may be due to the higher cytosolic concentration of NAD^+/NADH with respect to $NADP^+$/NADPH in *E. coli*.[24] Additionally, the aerobic respiratory chain is more efficient for NADH than for NADPH.[25] Therefore, since these ADHs are more selective towards NADPH, the overall system is less efficient. Nonetheless, when acetone (5% v/v) was added as co-substrate with *E. coli*/LBADH, 50% of acetophenone (**2a**) and 50% of the remaining enantiopure alcohol (*S*)-**1a** were obtained (entry 12).

Table 1. Study of several enzymatic systems for the biooxidation of substrates **1a-c**[a]



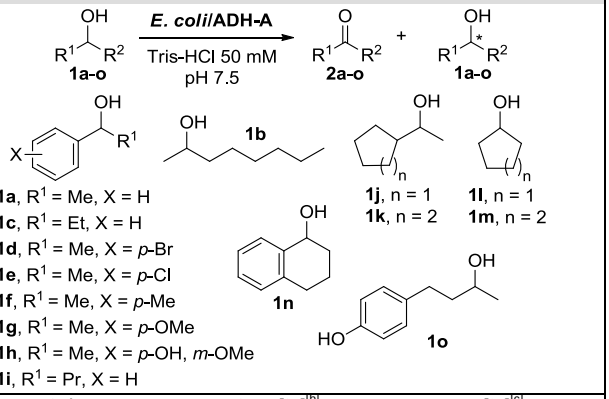
Entry	Biocatalyst	Substrate	**2a-c** [%][b]	*ee* **1a-c** [%][c]
1	lyo *E. coli*/ADH-A	**1a**[d,e]	50	>99 (*R*)
2	lyo *E. coli*/ADH-A	**1a**[e]	50	>99 (*R*)
3	lyo *E. coli*/ADH-A	**1a**	50	>99 (*R*)
4	lyo *E. coli*	**1a**	<1	n.d.
5	ADH-A	**1a**	<1	n.d.
6	ADH-A + lyo *E. coli*	**1a**	18	22 (*R*)
7	lyo *E. coli*/TeSADH	**1b**	30	31 (*R*)
8	lyo *E. coli*/ADH-T	**1b**	25	23 (*R*)
9	lyo *E. coli*/RasADH	**1c**	38	38 (*R*)
10	lyo *E. coli*/SyADH	**1c**	6	8 (*R*)
11	lyo *E. coli*/LBADH	**1a**	24	23 (*S*)
12	lyo *E. coli*/LBADH	**1a**[c]	50	>99 (*S*)

[a] Reaction conditions: substrate = [40 mM], lyophilised (lyo) cells of *E. coli* or *E. coli*/ADH (20 mg) or commercial ADH-A (3 U), Tris-HCl buffer (50 mM, pH 7.5, total end volume 0.6 mL), shaken at 250 rpm at 30 ºC for 24 h. [b] Conversion determined by GC. [c] Enantiomeric excess measured by chiral GC. [d] Acetone (5% v/v) was added as co-substrate. [e] NAD^+ was added (1 mM). n.d. = not determined.

Interestingly, the reverse reaction, i.e. the reduction of acetophenone (**2a**) to 1-phenylethanol (**1a**), with either *E. coli*/ADH-A or *E. coli*/LBADH, was also achieved by simply adding 2-propanol (5% v/v) without external addition of NADH, affording **1a** in 85 and 86% conversion, respectively (from a 40 mM substrate concentration), and >99% (*S*) *ee* with ADH-A and >99% (*R*) *ee* with LBADH. The presence of the nicotinamide cofactor in *E. coli* cells thus proves to be useful to reduce the cost for these redox reactions.

Once the lyophilised cells of *E. coli*/ADH-A were established as a suitable preparation to achieve the biooxidative kinetic resolution of a *sec*-alcohol under very simple conditions without additional co-substrate or NAD^+, different alcohols were tested to determine the substrate scope of this straightforward process (Table 2).

Table 2. Substrate screening with lyophilised cells of *E. coli*/ADH-A[a]



Entry	Substrate	**2a-o** [%][b]	*ee***1a-o**[%][c]
1	(*S*)-**1a**	92	>99 (*S*)
2	**1b**	48	4 (*R*)
3	**1c**	34	77 (*R*)
4	**1d**	15	21 (*R*)
5	**1e**	16	24 (*R*)
6	**1f**	42	91 (*R*)
7	**1g**	47	94 (*R*)
8	**1h**	42	92 (*R*)
9	(*S*)-**1i**	11	>99 (*S*)
10	**1j**	47	94 (*R*)
11	**1k**	49	97 (*R*)
12	**1l**	52	n.a.
13	**1m**	80	n.a.
14	**1n**	17	21 (*R*)
15	(*S*)-**1o**	91	>99 (*S*)

[a] Reaction conditions: [substrate] = 40 mM, lyophilised cells of *E. coli*/ADH-A (20 mg), Tris-HCl buffer (50 mM, pH 7.5, total end volume 0.6 mL), shaken at 250 rpm at 30 ºC for 24 h. [b] Conversion determined by GC. [c] Enantiomeric excess measured by chiral GC. n.a. = not applicable.

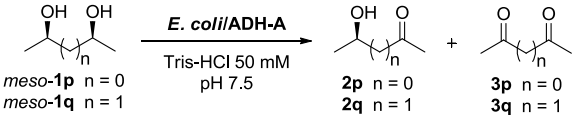
A first series of 1-phenylethanol derivatives (**1a,c**-**h**) were employed as substrates for this system, obtaining in general good results. (*S*)-1-Phenylethanol (**1a**) was almost fully oxidised under these conditions (92% conversion). 1-Phenyl-1-propanol (**1c**, entry 3) was also successfully oxidised to the corresponding propiophenone (**2c**, 34% conversion), yielding enantioenriched (*R*)-**1c**. Among a series of *para*-substituted derivatives, the ones bearing an electron-withdrawing group (EWG, entries 4 and 5) afforded lower conversions than when an electron-donating group (EDG) was present (entries 6 to 8). Previous experiments performed with LBADH and ADH-A did demonstrate that the oxidation of the alcohol was slowed down when the substrate bared an EWG.[26] Pleasingly, a bulky substrate such as (*S*)-1-phenyl-1-butanol (**1i**) was oxidised with this system to some extent (entry 9). 2-Octanol (**1b**, entry 2) was used as substrate but although conversion was close to 50%, almost racemic **1b** remained. Indeed, aliphatic substrates are known to be oxidised by ADH-A in a less selective manner than their corresponding bioreductions.[27] 1-Cycloalkylethanol compounds **1j** and **1k** were also oxidised successfully, obtaining the remaining enantioenriched alcohols with very high *ee* (entries 10 and 11). Cyclic alcohols **1l** and **1m** were also employed, obtaining good conversions into the corresponding ketones (entries 12 and 13), while for 1-tetralol (**1n**, entry 14) a lower conversion was observed.

The preparation of an important ketone, the raspberry ketone **2o**, was also envisaged using this mild biooxidation methodology.[28] Raspberry ketone is currently extensively used in industry, especially for flavouring and as a food additive. Thus, (*S*)-4-(4-hydroxyphenyl)-2-butanol (**1o**) was converted into **2o** with lyophilised cells of *E. coli*/ADH-A in buffer, affording 91% conversion (entry 15). An upscale of the reaction (60 mg) also achieved good conversion (88%) and isolated yield (73%).

Desymmetrisation of *meso*-Diols

Regio- and stereoselective reduction of diketones and oxidation of diols by biocatalytic hydrogen transfer have been previously performed using nicotinamide cofactor recycling systems to obtain chiral hydroxy ketones or diols.[29] These derivatives are important building blocks of many natural compounds such as pheromones or antitumor agents, and they can also be used as precursors for fine chemicals in the flavour and fragrance, agrochemical, and pharmaceutical industry. Particularly interesting is the mono-oxidation of *meso*-diols since, in the best case, they can afford an enantiopure hydroxy ketone in a theoretical yield of 100%. Therefore, *meso*-diols 2,3-butanediol (**1p**) and 2,4-pentanediol (**1q**) were investigated by simply employing lyophilised cells of *E. coli*/ADH-A, the (*S*)-configured secondary alcohols should be oxidised preferentially, affording the enantiopure (*R*)-hydroxy ketone. Table 3 shows the results of the reactions with lyophilised cells of *E. coli*/ADH-A after 24 h at 30 ºC.

Table 3. Desymmetrisation of *meso*-diols **1p-q** with lyophilised cells of *E. coli*/ADH-A[a]



Entry	Substrate	**1p-q** [%][b]	**2p-q** [%][b]	*ee***2p-q** [%][c]	**3p-q** [%][b]
1	*meso*-**1p**	<1	97	<1	3
2	*meso*-**1q**	<1	>99	99 (*R*)	<1

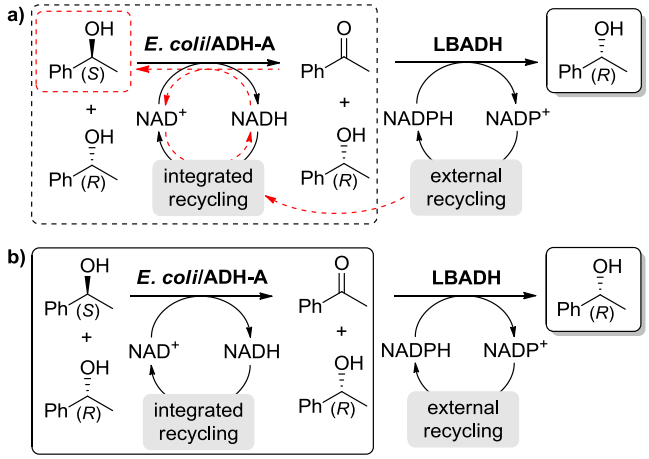
[a] Reaction conditions: [substrate] = 30 mM, lyophilised cells of *E. coli*/ADH-A (20 mg), Tris-HCl buffer (50 mM, pH 7.5, total end volume 0.6 mL), shaken at 250 rpm at 30 ºC for 24 h. [b] Percentage of products determined by GC. [c] Enantiomeric excess measured by chiral GC.

For *meso*-2,3-butanediol (**1p**), ADH-A displayed no stereospecificity, thus 3-hydroxy-2-butanone (**2p**) was obtained in racemic form, together with 3% of 2,3-butanedione (**3p**, entry 1). For *meso*-2,4-pentanediol (**1q**), excellent conversion (>99%) and *ee* (99%) were achieved, affording (*R*)-4-hydroxy-2-pentanone (**2q**) in the absence of 2,4-pentanedione (**3q**, entry 2). Therefore, the use of lyophilised cells of *E. coli*/ADH-A without adding any external cofactor and co-substrate proved to be an economical route for the desymmetrisation of a *meso*-diol, generating an enantiopure hydroxy ketone that in other chemical ways is tedious to synthesise.

Deracemisation of Secondary Alcohols

Considering the NAD^+ preference of the above system, a biocatalytic concurrent process was envisaged for the deracemisation of *sec*-alcohols (Scheme 2a). This process would be achieved by coupling *E. coli*/ADH-A with the stereocomplementary NADP-dependent LBADH[14] using an external recycling system that could independently regenerate

NADPH and be the driving force of the process, leading to the enantiopure alcohol product starting from the racemic mixture.[7e] First, either glucose dehydrogenase (GDH) or glucose-6-phosphate dehydrogenase (G6PDH) were tested with varying concentrations of glucose and glucose-6-phosphate (G6P), respectively, to determine the best external recycling system for NADPH (Table 4).



Scheme 2. Concurrent biooxidation and reduction processes for the deracemisation of *rac*-**1a** using: a) lyophilised *E. coli*/ADH-A cells; and b) resting *E. coli*/ADH-A cells; together with purified LBADH, external NADPH and a NADP cofactor recycling system. The undesired NADH-recycling catalysed by the external cofactor regeneration system in Scheme 2a is highlighted in red.

Table 4. Deracemisation process of **1a** using lyophilised cells of *E. coli*/ADH-A, LBADH and GDH or G6PDH[a]

Entry	[**1a**] [mM]	Cofactor recycling system	[G6P] or [Glu] [mM]	**2a** [%][b]	**1a** [%][b]	*ee* **1a** [%][c]
1	40	G6PDH	20	17	83	<1
2	40	G6PDH	100	4	96	40 (*R*)
3	40	GDH	100	<1	>99	35 (*R*)
4	40	GDH[d]	100	<1	>99	8 (*R*)
5	40	GDH	250	<1	>99	65 (*R*)
6	30	GDH	250	<1	>99	86 (*R*)
7	30	GDH[e]	250	<1	>99	65 (*R*)
8	30	GDH	500	<1	>99	86 (*R*)
9	30	GDH	750	<1	>99	92 (*R*)
10	30	GDH	1000	<1	>99	94 (*R*)

[a] Reaction conditions: substrate, lyophilised cells of *E. coli*/ADH-A (20 mg), LBADH (3 U), GDH or G6PDH (3 U), glucose or G6P, Tris-HCl buffer (50 mM, pH 7.5, 1 mM NADPH, total end volume 0.6 mL), shaken at 250 rpm at 30 ºC for 24 h. [b] Percentage of products determined by GC. [c] Enantiomeric excess measured by chiral GC. [d] Additional NAD^+ (0.5 mM) was added. [e] A total of 6 U were added.

In a first set of experiments, the influence of the nicotinamide cofactor regenerating enzyme (GDH or G6PDH) was studied (entries 1 to 3) using 40 mM of racemic 1-phenylethanol (**1a**) and 20 mM or 100 mM of glucose or G6P. When using G6PDH, the ketone **2a** was detected, whereas with GDH no traces were observed and (*R*)-**1a** was obtained with 35% *ee*.[30] Although an excess of G6P or glucose was employed, the deracemisation process was still incomplete, perhaps due to the partial disruption of the lyophilised cells, allowing G6PDH or GDH, which can accept both NADPH and NADH, to interfere and recycle NADH up-taken by ADH-A thus reducing ketone **2a** back to the alcohol (*S*)-**1a** (Scheme 2a). The addition of NAD^+ into the reaction mixture did not seem to improve the system (entry 4). In order to shift the equilibrium towards the alcohol (*R*)-**1a** and achieve a complete process, higher glucose concentrations (250 mM, entry 5) or lower substrate concentrations (30 mM, entry 6) were attempted and improved the *ee* to 86%. Doubling the amount of GDH led to lower *ee* (65%, entry 7), confirming the interference between both oxidation-reduction reactions, as mentioned above. Finally, increasing the molar excess of glucose up to 1 M (entries 8-10) led to the alcohol (*R*)-**1a** with 94% *ee* after 24 h, without any trace of acetophenone (entry 10).

Nevertheless, the deracemisation system required a high concentration of glucose (more than 30 equivalents with regards to the substrate) at a 30 mM substrate concentration to achieve over 90% *ee*. To further improve this methodology, we envisaged compartmentalising the oxidation reaction from the reduction process by using resting cells of *E. coli*/ADH-A (Table 5) with their cell wall intact. In this manner, the interference of the external recycling enzyme was minimised while still allowing the transport of the alcohol and the ketone (Scheme 2b). An amount of 35 mg of wet resting cells was subsequently used for all reactions based on the best results obtained. Under these conditions, only 100 mM of glucose (a 10-fold decrease) were required to obtain 96% *ee* (*R*)-**1a** with no trace of **2a** after 24 h (entry 1). A concentration of 200 mM of glucose was found to be the minimum needed to obtain enantiopure (*R*)-**1a**(entries 2 and 3), whereas an increase up to 500 mM did not have any detrimental effect (entries 4 and 5). Using these optimal conditions, the deracemisation of *rac*-1-phenylethanol (**1a**) was successfully upscaled (50 mg), affording the enantiopure (*R*)-1-phenylethanol with an isolated yield of 82% after purification. The concentration of **1a** could also be increased to 60 mM to obtain >99% (*R*) *ee*, while at 80 mM a lower *ee* of 90% (*R*) was observed.

Table 5. Deracemisation process of 1-phenylethanol (**1a**) with resting cells of *E. coli*/ADH-A, LBADH and GDH[a]

Entry	[Glu] [mM]	**2a** [%][b]	**1a** [%][b]	*ee***1a**[%][c]
1	100	<1	>99	96 (*R*)
2	150	<1	>99	98 (*R*)
3	200	<1	>99	>99 (*R*)
4	250	<1	>99	>99 (*R*)
5	500	<1	>99	>99 (*R*)

[a] Reaction conditions: [substrate] = 40 mM, wet resting cells of *E. coli*/ADH-A (35 mg), LBADH (3 U), GDH (3 U), glucose, Tris-HCl buffer (50 mM, pH 7.5, 1 mM NADPH, total end volume 0.6 mL), shaken at 250 rpm at 30 ºC for 24 h. [b] Percentage of products determined by GC. [c] Enantiomeric excess measured by chiral GC.

To show the broad applicability of this method, substrates which performed well in the biooxidative process (Table 2) were also deracemised (Figure 1). Thus, 1-(4-methoxyphenyl)ethanol (**1g**) and 1-cyclopentylethanol (**1j**) were obtained in their (*R*)-enantiopure form, while the other substrates (**1f,h,k**) afforded the enantioenriched (*R*)-enantiomer in modest to high enantiomeric excess (81-91%). The lower enantioenrichment observed with **1b**(59%) was due to the fact that ADH-A is not selective for its oxidation (Table 2, entry 2), therefore in this case both enantiomers were oxidised and then the ketone was subsequently reduced.

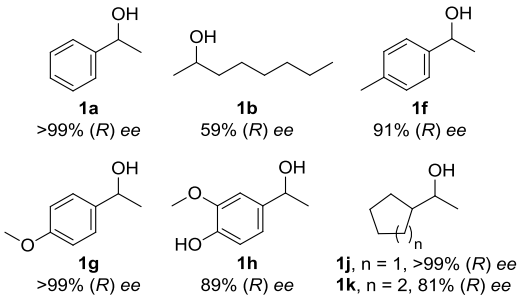


Figure 1. Deracemisation of secondary alcohols **1a-b**, **1f-h**, and **1j-k** at 40 mM concentration.

Finally, an estimation of the reaction time for the deracemisation of **1a** was determined using resting cells of *E. coli*/ADH-A (Figure 2). After only 5 min of reaction, 66% *ee* of (*R*)-**1a** was obtained. Within 10 min, 98% *ee* of (*R*)-**1a** was already achieved. To obtain >99% with no trace of acetophenone (**2a**) only 30 min were necessary, demonstrating the usefulness of this quick and cost-effective process for the deracemisation of secondary alcohols.

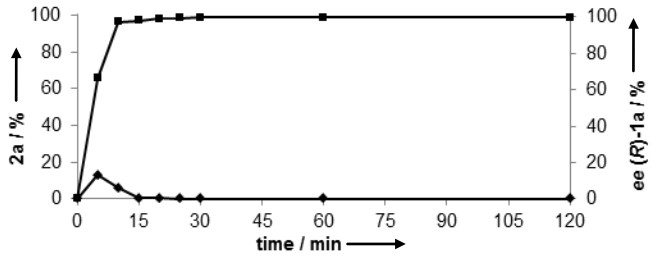
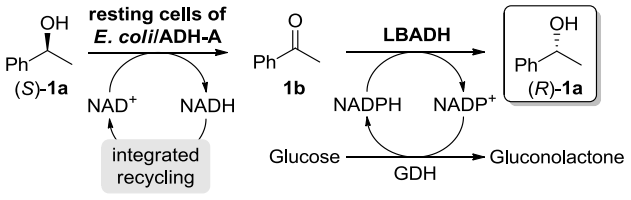


Figure 2. Time frame of the deracemisation of *rac*-1-phenylethanol (**1a**). Legend: **2a** (◆), *ee*(*R*)-**1a** (■).

Stereoinversion of a Secondary Alcohol

The deracemisation process developed above was also applied to perform the stereoinversion of a secondary alcohol, starting from (*S*)-1-phenylethanol to obtain the enantiopure (*R*)-1-phenylethanol (Scheme 3). Thus, with a concentration of 30 mM of (*S*)-**1a** in buffer and with the combined *E. coli*/ADH-A and LBADH system previously described, the (*R*)-enantiomer was exclusively obtained in >99% *ee* after 24 h.



Scheme 3. Concurrent biooxidation and reduction processes for the stereoinversion of (*S*)-**1a**.

Conclusion

More efficient, cheaper and simpler systems are increasingly required to face the growing demand of chiral intermediates needed for pharmaceuticals, fine chemicals or other applications. While previous methodologies described the use of whole cells applied to bioreductions by simply adding glucose in the absence of additional cofactors,[31] examples for biooxidative protocols were still missing. Herein we have demonstrated the use of a simple methodology to perform selective oxidative transformations only employing lyophilised or resting cells of *E. coli*/ADH-A in aqueous medium, without the need for an external cofactor regeneration system. Hence, a variety of racemic *sec*-alcohols were successfully oxidised leading to the corresponding enantioenriched (*R*)-alcohols, a *meso*-diol was desymmetrised to afford an enantiopure hydroxy ketone that can be used as a chiral building block, and a valuable derivative such as the raspberry ketone was obtained in high isolated yield. This system also allowed the deracemisation of racemic 1-phenylethanol through a biocatalytic concurrent process to form enantiopure (*R*)-1-phenylethanol in >99% *ee* under 30 min when coupled with the stereocomplementary LBADH and an external recycling system for NADPH (GDH with glucose). Several other aromatic and aliphatic secondary alcohols were also successfully enantioenriched. This deracemisation process avoids the time-consuming and yield-lowering protection-deprotection chemistry and can be used for stereoinversion processes as an equivalent to the Mitsunobu reaction. At this stage more studies are needed to determine the exact nature of this internal NAD^+ recycling system, which could lead to the application of this methodology to other NAD^+-dependent enzymes over-expressed in *E. coli* at higher substrate concentrations. This method offers the potential to reduce cost and increase efficiency due to shorter time and fewer steps for the production of high added-value (enantioenriched) compounds.

Experimental Section

General

Alcohols **1a**-**n**, *meso*-diol **1p**, ketones **2a**-**o**, hydroxy ketones **2p**-**q**, and diketones **3p**-**q** were commercially available. (*S*)-**1o** was obtained as previously described.[28] *meso*-2,3-Butanediol (**1p**) was obtained commercially. Racemic 2,4-pentanediol (**1q**) was purchased from commercial sources and the racemic and *meso*-isomers were separated by column chromatography (Et_2O) as previously described.[29] All other commercial reagents and solvents were purchased with the highest purity available and used as received. NMR spectra were recorded on a Bruker DPX-300 or Bruker AV-300 spectrometer at 300 (^1H) and 75 (^{13}C) MHz. GC analyses were carried out on a Varian 3900 gas chromatograph equipped with a FID. HPLC analyses were performed using a Hewlett-Packard 1100 with the chiral column AS with UV detection. Commercial enzymes D-glucose dehydrogenase 002 (GDH, 30 U/mg), D-glucose-6-phosphate dehydrogenase (G6PDH, 640 U/mg), ADH-A (20 U/mg) and LBADH (300 U/mL) were purchased from Codexis, as well as the sodium salts of the nicotinamide coenzymes $NAD(P)^+$ and NAD(P)H (purity >99%). The following ADHs over-expressed in *E. coli* were obtained as previously described:[13a,20,21] *Rhodococcus ruber* (ADH-A), *Lactobacillus brevis* (LBADH), *Ralstonia* sp. (RasADH), *Sphingobium yanoikuyae* ADH (SyADH), *Thermoanaerobacter ethanolicus* (TeSADH) or *Thermoanaerobium* sp. ADH (ADH-T). A 50 mM Tris-HCl buffer at pH 7.5 was used for all ADH-catalysed experiments.

Procedure for the biooxidative kinetic resolution of a *sec*-alcohol

For the biooxidation of alcohols **1a**-**o**, in an eppendorf tube, the lyophilised *E. coli*/ADH-A (20 mg) and Tris-HCl buffer (600 µL, 50 mM at pH 7.5) were added, and left to shake for 30 min at 30 ºC and 250 rpm. Then the substrate (40 mM) was added. The reaction was shaken at 30 ºC and 250 rpm for 24 h. The reaction mixture was then

extracted with EtOAc (2 × 0.5 mL) and centrifuged after each step (13,000 rpm, 90 seconds). The combined organic layers were dried with Na_2SO_4 and the resulting crude was analysed by GC (see Supporting Information for details).

Synthesis of raspberry ketone (2o)

For the biooxidation of (*S*)-rhododendrol (*S*)-**1o** to raspberry ketone **2o**, in an eppendorf tube, the lyophilised *E. coli*/ADH-A (20 mg) and Tris-HCl buffer (600 µL, 50 mM at pH 7.5) were added and left to shake for 30 min at 30 ºC and 250 rpm. Then, (*S*)-rhododendrol (40 mM) was added. The reaction was shaken at 30 ºC and 250 rpm for 24 h. The reaction mixture was filtered on celite and then extracted with EtOAc (2 × 0.5 mL). The combined organic layers were dried with Na_2SO_4 and the resulting crude was analysed by HPLC (see SI for details). The upscale (60 mg of substrate) involved the same reaction conditions, with the same equivalences in a 15 mL falcon tube. The resulting crude reaction product was dried and analysed by HPLC and NMR spectroscopy, leading to a good conversion (88%). Flash column chromatography (CH_2Cl_2/MeOH 9:1) afforded the pure isolated raspberry ketone **2o** (73%).

Procedure for the deracemisation of a *sec*-alcohol using resting *E. coli*/ADH-A cells

For the deracemisation of the racemic *sec*-alcohols, in an eppendorf tube, fresh resting cells of *E. coli*/ADH-A (35 mg) were suspended in Tris-HCl buffer (420 µL, 50 mM at pH 7.5), with LBADH (3 U, 10 µL), GDH (3 U, 10 µL), glucose (100 µL, 250 mM), NADPH (60 µL, 1 mM) and the racemic *sec*-alcohol (40 mM) was added. The reaction was shaken at 30 ºC and 250 rpm for 24 h. The reaction mixture was then extracted with EtOAc (2 × 0.5 mL) and centrifuged after each step (13,000 rpm, 90 seconds). The combined organic layers were dried with Na_2SO_4 and the resulting crude was analysed by GC (see Supporting Information for details). The stereoinversion of (*S*)-**1a** was performed in the same way but with a substrate concentration of 30 mM.

For the upscale deracemisation of 1-phenylethanol, in a falcon tube, fresh resting cells of *E. coli*/ADH-A (700 mg) were suspended in Tris-HCl buffer (8.4 mL, 50 mM at pH 7.5), with LBADH (60 U, 200 µL), GDH (100 U, 200 µL), glucose (2 mL, 250 mM), NADPH (1.2 mL, 1 mM) and racemic 1-phenylethanol (**1a**, 50 µL, 35 mM) was added. The reaction was stirred at 30 ºC in an orbital shaker for 24 h at 250 rpm, and after filtration on celite, it was extracted with EtOAc (3 × 5 mL). The solvent was evaporated and (*R*)-**1a** was isolated as a clear colourless oil (40.3 mg, 81% isolated yield) in >99% *ee* purity, >99% conversion by GC analysis (see Supporting Information for details).

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Keywords: biooxidation • deracemisation • desymmetrisation • secondary alcohols • stereoinversion

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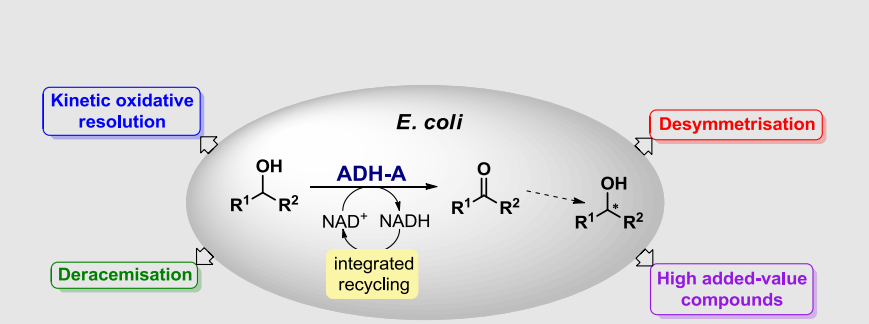

An integrated recycling system: A NAD^+ regeneration system present in *E. coli* cells was exploited for the oxidation of secondary alcohols with *E. coli*/ADH-A. Racemic alcohols were selectively oxidised without requiring external cofactor or co-substrate, affording the remaining enantioenriched (*R*)-alcohols. Deracemisation, desymmetrisation and stereoinversion processes were developed leading to optically pure high added-value compounds.

*C. E. Paul, I. Lavandera, V. Gotor-Fernández, W. Kroutil, V. Gotor**

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***E. coli*/ADH-A: An all-inclusive catalyst for the selective biooxidation and deracemisation of secondary alcohols**