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# One-Way Biohydrogen Transfer for Oxidation of *sec*-Alcohols

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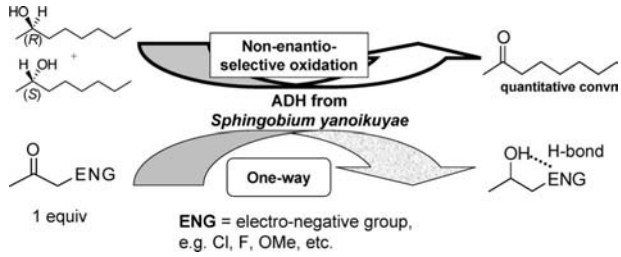
## ABSTRACT




**Quasi-irreversible oxidation of *sec*-alcohols was achieved via biocatalytic hydrogen transfer reactions using alcohol dehydrogenases employing selected ketones as hydrogen acceptors, which can only be reduced but not oxidized. Thus, only 1 equiv of oxidant was required instead of a large excess. For the oxidation of both isomers of methylcarbinols a single nonstereoselective short-chain dehydrogenase/reductase from *Sphingobium yanoikuyae* was identified and overexpressed in *E. coli*.**

Oxidation of alcohols to the corresponding carbonyl compounds belongs to the most fundamental processes in organic chemistry and many recent efforts are devoted to develop selective, "green", and catalytic oxidation methods.[1] Chemocatalytic oxidations of secondary alcohols based on asymmetric hydrogen-transfer reactions (with Ru, Rh, and Ir)[2,3] or Oppenauer oxidations (with Al, Zr, and lanthanides)[4] employing acetone as hydrogen acceptor gained considerable attention. For these reactions, it is still difficult to shift the equilibrium toward completion and to suppress undesired side reactions.[3c,5] Biocatalytic oxidation methods are less employed for secondary alcohols[6,7] via hydrogen transfer with a single biocatalyst.[8,9] This approach would represent a very simple, "green", and elegant method since only a single enzyme—an alcohol dehydrogenase (ADH)—is required to perform the whole cycle (Scheme 1).

However, as for the chemical variant, the huge excess of cosubstrate (ketone, aldehyde) required driving the equilibrium to the product side represents a serious drawback. Furthermore, for an efficient oxidation process both enantiomers of *sec*-alcohols have to be oxidized to access the

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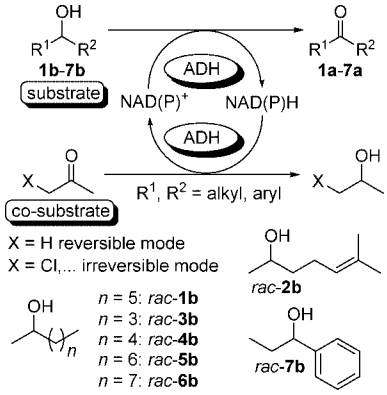
**Scheme 1.** Different Modes of Biocatalytic Hydrogen Transfer




corresponding ketone in high yield. Due to the intrinsic chirality of enzymes and therefore their high enantioselectivity a kinetic resolution will occur in general, delaying or even disabling the oxidation of the second isomer.[10] Therefore, we faced two challenges: (i) finding a hydrogen transfer oxidation method favoring product formation and (ii) a single nonstereospecific enzyme, which oxidizes the substrate and also recycles the cofactor, thus working in a coupled substrate approach.

Our first aim was to identify an alcohol dehydrogenase which can be used for oxidation via hydrogen transfer showing diminished enantiopreference. This is in sharp contrast to all other studies performed for the reduction of ketones to access optical pure alcohols.[11] As a first approach, we thought to search for wild-type micro-organisms that reduce ketones in a nonstereoselective fashion. However, a possible hit does not mean that we found a micro-organism containing the desired right enzyme: due to the large number of alcohol dehydrogenases in organisms it is very likely that two enzymes with opposite stereopreference mimic a microbial catalyst with low stereoselectivity. Subsequently, we hypothesized that an ADH which can reduce ketones with two bulky substituents has such a large open active site that ketones with one smaller group will be reduced with low stereopreference and consequently also the enantioselectivity for the reverse reaction—the oxidation—will be low. Testing our culture collection, we identified *Sphingobium yanoikuyae* DSM 6900[12] as a possible hit. Thus, employing lyophilized cells of *S. yanoikuyae*, hexanophenone (*n*-pentyl phenyl ketone) was reduced to the corresponding (*S*)-alcohol employing either ethanol or 2-propanol as hydrogen donor with good ee [97 (*S*) and 89 (*S*), respectively]. To identify the involved enzyme, a genomic library of this organism in *E. coli* was screened for the oxidation of 2-propanol employing a fluorescence assay for NAD(P)H formation.[13] A short chain dehydrogenase (annotated arbitrary SyADH) with 262 amino acids and with a preference for NADPH was identified, cloned, and overexpressed in *E. coli*. The overexpressed SyADH showed blue fluorescence, as it was recently described for an ADH from *Vibrio vulnificus*.[14] As a confirmation to have identified the "right" enzyme, hexanophenone was reduced employing the overexpressed catalyst with good ee (96%) comparable with the wild-type micro-organism. Testing less hindered substrates like 2-octanone **1a** (see the Supporting Information), the enzyme showed good activity and to our delight the desired low stereoselectivity for the reduction, leading to alcohols in almost racemic form confirming our hypothesis.

According to these results, this enzyme appeared to be a perfect candidate to allow complete oxidation of carbinols with at least one small alkyl-group. Indeed, employing acetone as hydrogen acceptor in large excess (10 equiv), *rac*-2-octanol **1b** could be oxidized efficiently with 99% conversion within 24 h. In a following step, alternative hydrogen acceptors (oxidants) were tested. In addition to acetone, 4-methyl-2-pentanone (methyl isobutyl ketone, MIBK), acetaldehyde, and even pyruvate were accepted, emphasizing the high flexibility of this enzyme to accept different types of cosubstrates in the active site. To our delight, the stability of the catalyst allowed the substrate concentration to be increased up to 400 g $L^{-1}$ (Figure 1). Over a broad substrate concentration range (40 g $L^{-1}$ to 400 g $L^{-1}$) the space time yield did not show significant deviations with acetone;

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(4) For a review, see: de Graauw, C. F.; Peters, J. A.; van Bekkum, H. *Synthesis* **1994**, 1007.

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(9) For employing two enzymes and acetone as hydrogen acceptor see: Fossati, E.; Polentini, F.; Carrea, G.; Riva, S. *Biotechnol. Bioeng.* **2006**, *93*, 1216.

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(12) DSM number refers to strains commercially available from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, German Collection of Microorganisms and Cell Cultures, http://www.dsmz.de/).

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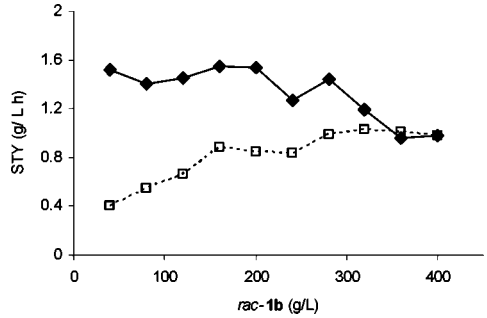


**Figure 1.** Dependence of space time yield (STY) on varied substrate concentration (*rac*-2-octanol, **1b**) using as hydrogen acceptor (a) acetone (15% v $v^{-1}$, ◆); (b) MIBK (10% v $v^{-1}$, □).

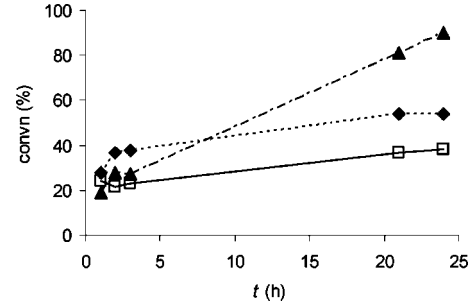


**Figure 2.** Time course of *E. coli*/SyADH-catalyzed oxidation of *rac*-2-octanol **1b** (30 g $L^{-1}$) employing 1.5 equiv of oxidant [acetone (◆); MIBK (□); chloroacetone (▲)].

additionally, MIBK proved to be rather efficient in a two-phase system at higher substrate concentration as well.

When investigating the scope of substrates which can be oxidized by SyADH, we experienced a surprise. Halohydrins like 1-chloro-2-octanol or 2-chloro-1-phenylethanol were not oxidized at all. First, we expected that this was a curiosity of this special enzyme, therefore we tested approximately 60 commercial alcohol dehydrogenases (e.g., ADHs from *Lactobacillus kefir* and *brevis*, ADH-'A' from *Rhodococcus ruber*, or screening kits from Biocatalytics/Codexis) for the oxidation of halohydrins with an excess of the corresponding cofactor. Again for all 60 commercial ADHs no oxidation-products were detectable via GC, thus obviously chlorohydrins cannot be oxidized by ADHs. As a consequence, we concluded that although α-chloro ketones would be reduced to 1-chloro-2-alcohols, the latter would not be oxidized backward anymore. Therefore, if chloroacetone[15] is used as an oxidant instead of acetone the system should become quasi-irreversible (Scheme 1). Comparing the oxidation of *rac*-2-octanol **1b** with 1.5 equiv of various oxidants (chloroacetone, MIBK, acetone), it became clear that although the oxidation with chloroacetone was slower at the beginning compared to acetone, the equilibrium was shifted toward the product side employing chloroacetone, while the equilibrium for acetone was at approximately 50% conversion after 24 h (Figure 2).

To show the applicability of this methodology, several racemic aliphatic and aromatic secondary alcohols (**2b**−**7b**) were oxidized to the corresponding ketones at elevated substrate concentrations (30 g $L^{-1}$) using just 1.5 equivalents of chloroacetone (Table 1) as hydrogen acceptor. In case of more bulky alcohols like **7b** lower conversions were obtained since in this case a kinetic resolution occurs due to the higher enantioselectivity of the enzyme for such type of substrates.

Performing a preparative oxidation of *rac*-2-octanol (0.2 g) with just 1.1 equiv of chloroacetone and lyophilized cells of *E. coli*/SyADH (0.2 g) at 30 °C, complete conversion (>99%) was achieved within 24 h.

To show the scope of the biocatalytic one-way concept with another ADH, the enantioselective ADH-'A' from *Rhodococcus ruber*[16] was employed in a kinetic resolution of *rac*-**1b**. Varying the substrate concentration up to 100 g $L^{-1}$ just 0.6 equiv of chloroacetone as oxidant was required to reach full conversion for the prefered (*S*)-enatiomer (Figure 3) within 24 h, thus leaving enantiopure (*R*)-**1b** behind.

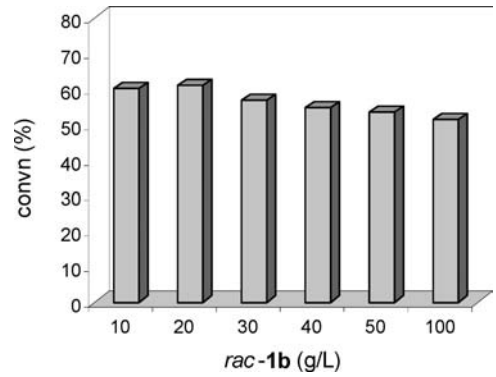


**Figure 3.** Kinetic resolution of *rac*-2-octanol **1b** at varied substrate concentrations via quasi-irreversible oxidative hydrogen transfer employing *E. coli*/ADH-'A' and 0.6 equiv of chloro acetone ($t$ = 24 h).

Consequently, the question was raised as to which other oxidants might be used and what is the reason for this quasi-irreversibility. As a first approach, the time course of the oxidation of various 2-propanol derivatives bearing different substituents (1-fluoro-2-propanol, 1,3-difluoro-2-propanol, 1,1,1,3,3,3-hexafluoro-2-propanol, 1,3-dichloro-2-propanol, and 1-bromo-2-propanol) was followed by a UV assay for the formation of NADPH from $NADP^+$ employing SyADH and compared with 2-propanol and 1-chloro-2-propanol.[17] From all these compounds only 2-propanol led to the

(15) 100 g of chloroacetone costs 14 euros at Sigma-Aldrich (Austria).

(16) This (*S*)-selective enzyme has a strong preference for NADH/$NAD^+$. See: Edegger, K.; Gruber, C. C.; Poessl, T. M.; Wallner, S. R.; Lavandera, I.; Faber, K.; Niehaus, F.; Eck, J.; Oehrlein, R.; Hafner, A.; Kroutil, W. *Chem. Commun.* **2006**, 2402.

(17) Pure compounds had to be synthesized, since the commercial samples had only technical grade.

**Table 1.** Quasi-irreversible Oxidation ($t = 24$ h) of *rac-sec*-Alcohols (30 g $L^{-1}$) Catalyzed by Lyophilized *E. coli*/SyADH Cells Employing Chloroacetone as Hydrogen Acceptor (1.5 equiv)

| entry | substrate | convn (%)[a] |
|---|---|---|
| 1 | *rac*-**1b** | >99[b] |
| 2 | *rac*-**2b** | 90 |
| 3 | *rac*-**3b** | >99 |
| 4 | *rac*-**4b** | 95[b] |
| 5 | *rac*-**5b** | 97 |
| 6 | *rac*-**6b** | 70[b] |
| 7 | *rac*-**7b** | 55[b] |

[a] Measured by GC. [b] Using double quantity of catalyst.

formation of NADPH; thus, for all other alcohols no ketone formation could be detected within the observation time (see the Supporting Information). It can therefore be assumed, that all the corresponding ketones represent one-way oxidants employing alcohol dehydrogenases with $NAD(P)^+$. This was confirmed experimentally employing SyADH with 1,1- and 1,3-dichloroacetone, 1,1,1-trichloroacetone, and ethyl acetoacetate as stoichiometric oxidants leading to complete conversion. As a first simplified explanation for the one-way oxidation, the formation of an additional intramolecular hydrogen bond[18] between the new obtained alcohol moiety and the electronegative group (F, Cl, Br, C=O[19]) can be assumed gaining $\sim$3−4 kcal $mol^{-1}$.

Thus, calculating the Gibbs free energies for the hydrogenation of the ketones 1-fluoro-, 1-chloro-, and 1-bromoacetone to the corresponding alcohols and comparing them with the values for acetone, a clear result was obtained: hydrogenation of all halo ketones led to products which are about 4−6 kcal $mol^{-1}$ more favored[20] than 2-propanol, which is a clear explanation for the quasi-irreversibility of the observed reactions.[21]

In summary, we have identified an alcohol dehydrogenase accepting all common oxidants (acetone, acetaldehyde, MIBK, pyruvate). Due to its low stereoselectivity for methylcarbinols, both enantiomers could be oxidized at reasonable rate. Furthermore, we could show that oxidation via hydrogen transfer does not necessarily need a huge excess of a hydrogen acceptor: By employing activated ketones, like chloroacetone,[14] 1,1- and 1,3-dichloroacetone, 1,1,1-trichloroacetone, or ethyl acetoacetate, to name just a few possibilities, only stoichiometric amounts of oxidant were required. This leads to a dramatically reduced amount of organic reagents needed. Due to the increasing importance of biocatalytic transformations in organic synthesis,[22] it is expected that oxidation methods employing enzymes will gain increasing significance especially, since biocatalysts are easily accessible, cheap, biodegradable, and show perfect chemoselectivity.

**Acknowledgment.** Financial support by DSM, FFG, and the Province of Styria is gratefully acknowledged. B.F.-S. acknowledges the FAPSEP (Fundação de Amparo a Pesquisa do Estado de São Paulo) and the CAPES (Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

**Supporting Information Available:** Gene library construction, cloning and overexpression of SyADH, measurement of kinetic constants, computational details, and analytics are detailed. This material is available free of charge via the Internet at http://pubs.acs.org.

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