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Biocatalytic oxidation of *sec*-alcohols via hydrogen transfer

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Abstract

The oxidations of alcohols via hydrogen transfer represents an environmental benign ‘green’ redox protocol. In this context biocatalytic hydrogen transfer employing the alcohol dehydrogenase ADH-‘A’ from *Rhodococcus ruber* DSM 44541 results in oxidation of *sec*-alcohols in aqueous medium at room temperature. High concentrations of the hydrogen acceptor (acetone, up to 50% v/v) are tolerated by the enzyme. Examples for the enantio-, and regioselective oxidation of *sec*-alcohols employing ADH-‘A’ in the wild type strain as well as over-expressed ADH-‘A’ in *E. coli* are given.
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Driven by the increased public awareness on hazards derived from chemical production, environmentally benign oxidation methods have gained increasing importance [1–4]. In this context, the oxidation of alcohols at the expense of a sacrificial ketone employing aluminium as metal (known since the 1920’s as the Oppenauer oxidation) constitutes a typical ‘green’ redox-reaction (Scheme 1) [5].

As a consequence, this method has been re-investigated recently [6–9] in order to replace the previously employed metal alkoxides by catalysts showing improved properties. In addition asymmetric variants have been pursued by using enantioselective hydride transfer protocols based on chiral transition metal complexes [7] or chiral hydride sources [6].

Along the same scheme, but following a different reaction mechanism, oxidation of *sec*-alcohols via hydrogen transfer employing transition metals (Ru, Rh, Ir) in combination with chiral organic ligands allows synthesis of non-racemic *sec*-alcohols via oxidative kinetic resolution [10–14]. Although satisfactory enantioselectivities were achieved, it is still difficult to shift the reaction towards completion and to suppress undesired racemization [11].

Methods for the oxidation of alcohols involving enzymes/micro-organisms gained increasing importance during the last few years [15–22]. Biocatalytic asymmetric hydrogen transfer is catalyzed by alcohol dehydrogenases requiring nicotinamide-cofactors. They show several advantages over the chemical methods, such as (i) their intrinsic asymmetry, (ii) absence of side reactions, such as aldol condensation, and (iii) they operate under essentially mild reaction conditions, typically at ambient temperature, under air and in aqueous solution. However, their large-scale application has predominantly been impeded by the requirement for cofactor-recycling (Scheme 2) [23,24].

The vast majority of dehydrogenases which oxidize *prim*- and *sec*-alcohols require $NAD(P)^+$ as cofactor, which are relatively unstable and too expensive when used in molar amounts. Therefore, a good deal of research has been devoted to the development of $NAD(P)^+$ regeneration-techniques to ensure that only catalytic amounts are required furnishing economic processes.

In order to render a simple process, the so called ’coupled substrate’ approach is favored, where a single enzyme simultaneously catalyzes the desired oxidation as well as the recycling of the cofactor. However, the fact that the sacrificial carbonyl compound acting as oxidant, e.g. acetone is required in excess to shift the reaction towards high conversion, represents a serious hurdle, due to the low stability of many enzymes [25,26]. Only recently, we reported on an exceptionally stable NAD^+-dependent alcohol dehydrogenase from *Rhodococcus ruber* DSM 44541 termed ADH-‘A’, which accepts acetone as co-substrate for NAD^+-regeneration (thereby producing 2-propanol as co-product) and at the same time performs the desired alcohol oxidation (Scheme 3) [27,28].

Most importantly from a preparative point of view, the enzyme is remarkably stable towards elevated co-substrate concentrations, allowing the use acetone for cofactor regeneration

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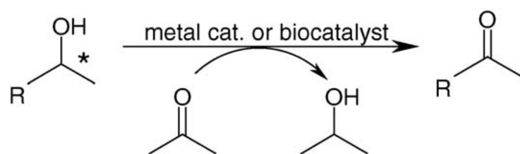
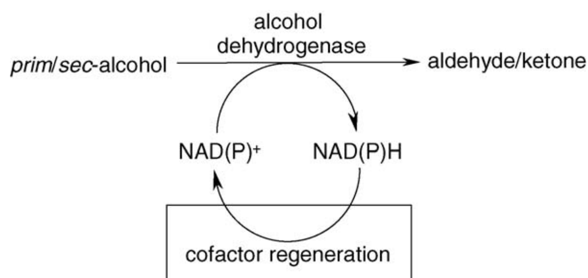
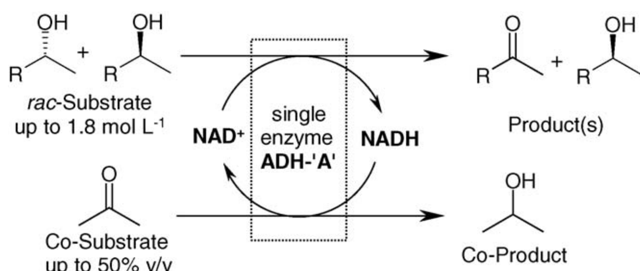


Scheme 1. General reaction scheme for Oppenauer oxidation and (biocatalytic) hydrogen transfer.



Scheme 2. Cofactor-recycling for alcohol oxidation employing $NAD(P)^+$ dependent dehydrogenases.



Scheme 3. Coupled substrate approach for co-factor recycling in the kinetic resolution via biocatalytic hydrogen transfer.

at 20% v/v with whole-cell preparations and up to 50% v/v with partially purified enzyme (Fig. 1) [29,30]. Elevated acetone-concentration not only shifts the equilibrium towards high conversion, but also increases the solubility of lipophilic substrates, thus ensuring enhanced reaction rates.

Due to the intrinsic asymmetry of enzymes, the oxidation of chiral *rac*-*sec*-alcohols inherently leads to kinetic resolution,

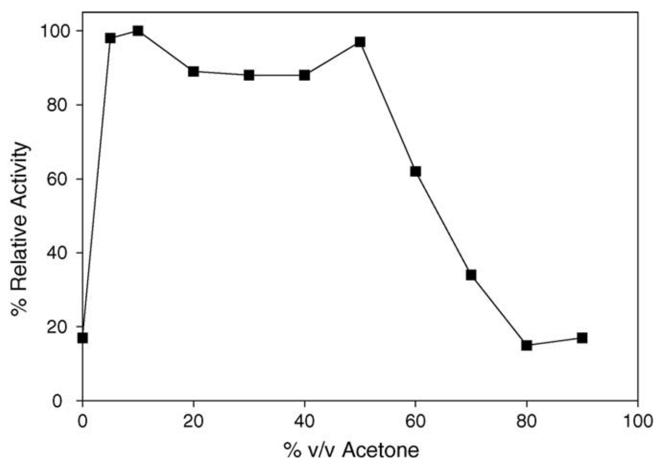
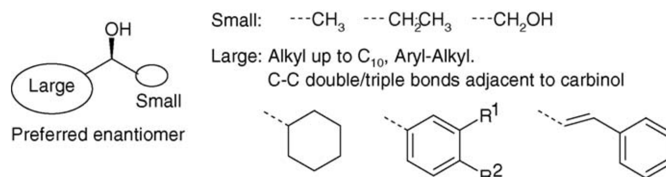


Fig. 1. Relative activity of partial purified ADH-′A′ at varied co-substrate (acetone) concentration (substrate: *rac*-6-methyl-5-hepten-2-ol).



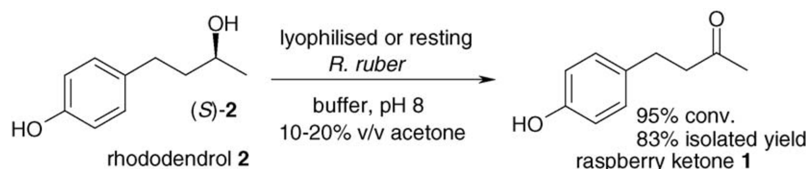
Scheme 4. Examples for substrate for 'ADH-A' from *Rhodococcus ruber* DSM 44541.

whereby one enantiomer out of the racemate is transformed at a faster rate than its mirror image counterpart. As a result, the non-reacting enantiomer can be obtained in 50% theoretical yield in high e.e. The efficiency of such a kinetic resolution depends on the enantioselectivity (expressed as *E*-value) of the enzyme [31]. Quite frequently, the second enantiomer is not transformed at all within a reasonable time. However, if the ketone is the favored product, complete oxidation of the racemate is desired, which is impeded by the fact that oxidation of the second enantiomer generally proceeds at a reduced reaction rate requiring extended reaction times to reach completion. In this case, the benefit of the high chemo-, regio- and enantioselectivity of enzymes may turn into a drawback when non-(enantio)selective oxidation is required.

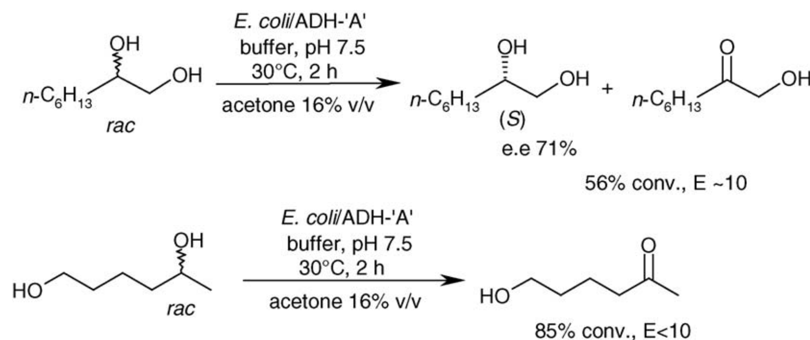
Alcohol dehydrogenase 'ADH-A' from *R. ruber* DSM 44541 [29] does not only show good stability toward elevated concentration of the co-substrate acetone, but also allows remarkably high substrate concentrations of up to 1.8 mol/l (for 2-octanol) [27]. Besides its exclusive regioselectivity for *sec*- over *prim*-alcohols, 'ADH-A' preferentially oxidizes (*S*)-alcohols (CIP-priority small < large), thereby leaving the (*R*)-enantiomer behind. Typical substrates which are oxidized with high activity and high enantioselectivity bear a small group (e.g. methyl, ethyl, hydroxymethyl) and a large group (up to C_{10}) attached to either side of the carbinol moiety (Scheme 4, Table 1) [32–34]. Strict limitations regarding steric requirements exist: while cyclohexanol is not oxidized, cyclopentanol is a good substrate.

As an example for the application of ADH-'A' catalyzed alcohol oxidation in the preparation of a flavor and fragrance-compound, the bio-oxidation of rhododendrol **2** obtained from natural sources, such as the bark of various birch spp., represents the key step in the synthesis of *natural* raspberry ketone **1** [35] (Scheme 5).

Following regulations issued by the European Community, flavor substances labeled 'natural' may only be obtained either by physical means (such as distillation and extraction of natural sources) or by enzymatic/microbial transformations of precursors isolated from Nature [36]. The main natural source of raspberry ketone **1** is raspberry (*Rubus idaeus*, up to 3.7 mg/kg of berry fresh weight) although the compound is also present in other fruits, such as peach, grapes, apples, various berries, vegetables (e.g. rhubarb), and in the bark of trees (e.g. yew, maple, and pine) [37,38]. However, the amount of raspberry ketone obtainable from these sources is too low or too cost intensive to be of industrial significance. Due to the wide area of application (e.g. in aroma formulations of kiwi, cherry, strawberry) as well as the high price for the natural flavor compound

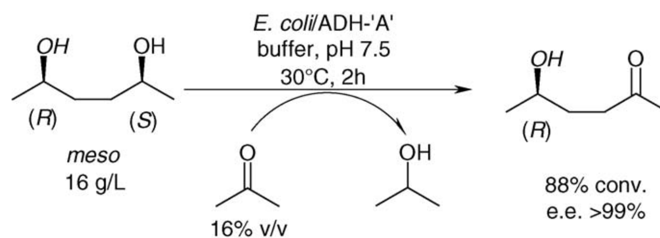


Scheme 5. Biocatalytic oxidation of rhododendrol **2** to yield raspberry ketone **1**.



Scheme 6. Regio-selective bio-oxidation of *sec*- over *prim*-alcohol moieties.

(EUR 600–2000 kg^{-1}) in comparison to the synthetic analogue (EUR 10 kg^{-1}) [39], the food industry shows a strong interest in an economic processes for its 'natural' production [40]. Besides its application in flavor and fragrance formulations, raspberry ketone **1** is also used in an entirely new section of the food and cosmetics industry, called 'cosmaceutical industry', a combination of *cosm*etics and nutr*aceuticals*. Therein, it is a constituent of skin-lightening cosmetics, and weight loss advancing dietary supplements (Vitarosso®) due to its capability to burn subcutaneous fat [41–43]. Since the consumers' preference for

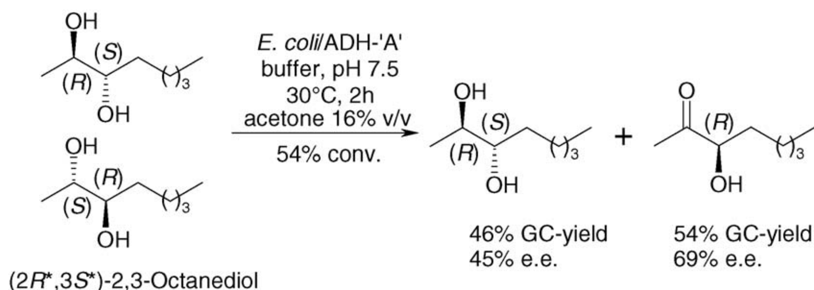


Scheme 7. Stereo-differentiation between a (*S*)- and (*R*)-configured *sec*-alcohol centers in a *meso*-diol.

Table 1
Selected substrates for biocatalytic oxidation employing lyophilized cells of *R. ruber* DSM 44541 (see Scheme 4) via hydrogen transfer [32,33]

Large	Small	Time [h]	Conversion [%]	e.e. [%]	*E*-value
n-C_8H_{17}	CH_3	24	55	97	34
n-C_7H_{15}	CH_3	24	56	99.0	43
n-C_6H_{13}	CH_3	24	50	>99	>100
n-C_5H_{11}	CH_3	24	52	>99	>100
n-C_4H_9	CH_3	24	49	>99	>100
n-C_3H_7	CH_3	24	56	97.5	33
$HC{\equiv}CCH_2$–	CH_3	38	<1.0	1.5	n.d.
$CH_3C{\equiv}C$–	CH_3	38	37	64	>100
$CH_2{=}CHCH_2$–	CH_3	38	48[a]	58	7.6
(*E*)–$CH_3CH{=}CH$–	CH_3	38	53	>99	>100
(*Z*)–$CH_3CH{=}CH$–	CH_3	38	5	2.3	n.d.
C_2H_5	CH_3	24	96	34	1.3
$HC{\equiv}C$–	CH_3	38	n.c.	n.d.	n.d.
$(CH_3)_2C{=}CH(CH_2)_2$–	CH_3	24	50	>99	>100
n-C_5H_{11}	–C_2H_5	22	49.5	33	2.7
n-C_5H_{11}	–$CH{=}CH_2$	20	2.7	n.d.	n.d.
n-C_5H_{11}	–$C{\equiv}CH$	20	n.c.	n.d.	n.d.
(*E*)-$CH_3(CH_2)_2CH{=}CH$–	–C_2H_5	20	42	67	49.2
(*Z*)-$CH_3(CH_2)_2CH{=}CH$–	–C_2H_5	20	n.c.	n.d.	n.d.
$CH_3(CH_2)_2C{\equiv}C$–	–C_2H_5	22	41	84	>100
n-C_4H_9	n-C_3H_7	24	37	0	~1

n.d. not determined due to low conversion.
[a] Isomerization of 4-penten-2-one to 3-penten-2-one.



Scheme 8. Biocatalytic oxidative hydrogen transfer of (2R^*,3S^*)-2,3-octanediol employing ADH-'A'.

'natural' labeled food/cosmetics additives is constantly increasing, an economic biocatalytic process for raspberry ketone from natural sources is needed. One possible natural precursor of raspberry ketone is 4-(*p*-hydroxyphenyl)-2-butanol **2** (rhododendrol, betuligenol), which can be found as the corresponding 2-glycosides (glucoside, mannoside) in the bark of silver birches (*Betula* spp.) [44–46]. Deglycosilation is achieved using β-glycosidase from various micro-organisms or plants [38] to obtain **2**, which is subjected to bio-oxidation to obtain natural raspberry ketone. To simulate a possible oxidative production of 'natural' raspberry ketone, starting with one gram enantiomerical pure (*S*)-alcohol (*S*)-**2** (30 g/l), which is present in nature (see above), the biocatalytic oxidation using acetone as hydrogen acceptor (20% v/v) was performed to yield ketone **1** after 17 h at 95% conversion in 83% isolated yield [35].

Very recently the alcohol dehydrogenase ADH-'A' was sequenced and cloned and can now efficiently be over-expressed in *E. coli* [47]. The regio- and enantioselectivity of the cloned enzyme was studied using a lyophilized cell preparation of *E. coli* containing the over-expressed ADH-'A'. Terminal *vic*-glycols were regioselectively oxidized to the corresponding 1-hydroxy-2-ones with low enantioselectivity (Scheme 6); similar results were obtained with 1,5-hexanediol, which gave 6-hydroxy-2-hexanone within 2 h at 85% conversion.

Oxidation of a diol possessing two symmetrical *sec*-alcohols in ($\omega-1$)-position, such as *meso*-2,5-hexandediol (Scheme 7) proceeded in a stereoselective fashion, exclusively the (*S*)-configured center was oxidized yielding valuable (*R*)-5-hydroxy-2-hexanone at 88% conversion within 2 h (e.e. > 99%).

The picture became more complex with diols possessing two secondary alcohol moieties in non-equal positions: in general the less hindered hydroxy moiety in the ($\omega-1$)-position was preferentially oxidized with (*S*)-preference. For instance the oxidation of (2R^*,3S^*)-2,3-octanediol yielded the corresponding (*R*)-3-hydroxy-2-octanone (Scheme 8).

In summary, we have presented an efficient and highly stable biocatalyst for hydrogen transfer oxidations employing acetone as hydrogen acceptor at concentrations up to 50% v/v. The 'green' oxidation method allows exclusive oxidation of *sec*-alcohols beside *prim*-alcohol moieties with a strong preference for (*S*)-configured centers. The catalyst is stable over several months at 4 °C and enables the oxidation of *sec*-alcohols at ambient conditions in aqueous solution with a strong regiopreference for ($\omega-1$)-carbinols.

1. Experimental

In a typical oxidation procedure employing *R. ruber*, lyophilized cells (0.6 g) were rehydrated in phosphate buffer (6.0 ml, 50 mM, pH 8.0) for 30 min at 30 °C. Co-substrate acetone (1.0 ml + 2.0 ml after 6 h) and substrate *rac*-2-octanol (1.0 g, 7.7 mmol) were added and the mixture was shaken at 30 °C for 16 h. The mixture was extracted with EtOAc, the solvent was removed and the products were analyzed by GC using a chiral stationary phase.

In a typical analytical oxidation procedure employing *E. coli*/ADH-'A' lyophilized cells of *E. coli* Tuner^{TM} (DE3)/pET22b + -ADH-'A' (2 mg) were rehydrated in Tris-buffer (500 μl, pH 7.5, 50 mM) for 1 h at 30 °C in Eppendorf tubes (1.5 ml) by shaking at 130 rpm. Substrate (10 μl) and acetone (100 μl, 1.7 mmol) was added and the mixture was agitated at 30 °C (130 rpm) for 2 h. The mixture was extracted with EtOAc, the solvent was removed and the products were analyzed by GC using a chiral stationary phase.

The cultivation of the *E. coli* over-expressing ADH-'A' and its optimization will be published in due course. The diols and reference compounds were prepared and analyzed as previously reported [48].

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