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Asymmetric Bioreduction of Alkenes Using Ene–Reductases YersER and KYE1 and Effects of Organic Solvents

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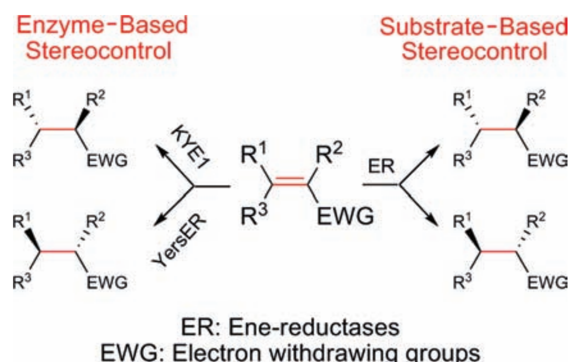
ABSTRACT



Asymmetric *trans*-bioreduction of activated alkenes by KYE1 from *Kluyveromyces lactis* and Yers-ER from *Yersinia bercovieri*, two ene–reductases from the Old Yellow Enzyme family, showed a broad substrate spectrum with a moderate to excellent degree of stereoselectivity. Both substrate- and enzyme-based stereocontrols were observed to furnish opposite stereoisomeric products. The effects of organic solvents on enzyme activity and stereoselectivity were outlined in this study, where two-phase systems hexane and toluene are shown to sustain bioreduction efficiency even at high organic solvent content.

The application of biocatalyst has become an increasingly attractive route in the production of enantiomerically pure compounds in industry due to exquisite regio- and stereoselectivity.[1] One of the emerging platforms is the asymmetric reduction of activated C═C bonds due to the potential to create up to two new stereogenic centers in the process.[2] Ene–reductases from the Old Yellow Enzyme (OYE) family have been reported to catalyze the reduction of a variety of different substrates such as α,β-unsaturated compounds (activated by electron-withdrawing groups such as aldehydes, ketones, imides, nitro groups, nitriles, carboxylic acids, esters) and nitro derivatives such as nitro esters and nitro aromatics.[3] The reduction reaction catalyzed by the OYE family of flavoproteins proceeds in a strict *trans* fashion, with hydride attack derived from the

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Table 1. Asymmetric Bioreduction Study Using Ene−Reductases KYE1 and YersER

enzymes		KYE1		YersER	
		conv %	ee %	conv %	ee %
α,β-alkyl substituted cyclic enones					
1. $R^1 = CH_3$, $R^2 = H$	**1-2**	65	(*S*) >99	45	(*S*) >99
2. $R^1 = H$, $R^2 = CH_3$		6	(*S*) >99	3	(*S*) >99
3. $R^1 = CH_3$, $R^2 = H$	**3-4**	89	(*S*) >99	95	(*S*) >99
4. $R^1 = H$, $R^2 = CH_3$		27	(*S*) >99	5	(*S*) >99
α,β-substituted enolethers					
5. $R^1 = CH_3$	**5-6**, **7**	46	(*S*) 90	51	(*S*) 47
6. $R^1 = CH_2Ph$		0.4	(*S*) >99	26	(*S*) 99
7. $R^1 = CH_3$		nc[a]	-	nc	-
8. $R^1 = CH_3$	**8-10**, **11**	54	(*R*) 71	68	(*R*) 32
9. $R^1 = CH_2Ph$		29	(*S*) 90	100	(*S*) 92
10. $R^1 = CH_2CH_2CH_3$		1	(*S*) >99	68	(*S*) 94
11. $R^1 = CH_3$		0.5	-	nc	-
monoesters					
12. $R^1 = H$	**12-13**	39	-	42	-
13. $R^1 = CH_3$		nc	-	nc	-
dicarboxylic esters					
14	**14**, **15**	74	(*R*) 97	87	(*R*) >99
15		89	(*S*) 94	79	(*R*) 77
16	**16**	3	(*R*) >99	29	(*R*) >99
nitrostyrene					
17. $R^1 = H$, $R^2 = H$	**17-19**	100	-	100	-
18. $R^1 = CH_3$, $R^2 = H$		98	(*R*) 95	98	(*R*) 99
19. $R^1 = H$, $R^2 = CH_3$		99	racemic	99	racemic
maleimide type					
20		80	(*R*) 89	100	(*R*) 95
citral					
21		68	(*R*) 86	96	(*S*) >99

[a] nc: no conversion.

nicotinamide cofactor.[4] Utilizing the coupled-enzyme approach, an efficient cofactor recycling system with enzymes such as glucose dehydrogenase (GDH) is commonly applied to decrease the high costs of redox cofactor for larger scale synthesis.[5,11a] Furthermore, a light-driven cofactor regeneration system[6] and a nicotinamide-independent reduction system[7] were recently reported to yield high substrate conversion and product enantiopurity, again highlighting the potential of OYEs in industrial biotechnological applications.

In a previous work, we began to characterize three ene−reductases: KYE1 from *Kluyveromyces lactis*, YersER from *Yersinia bercovieri*, and XenA from *Pseudomonas putida*. We have shown that these three ene−reductases feature broad but different substrate specificity on various α,*β*-unsaturated carbonyl compounds. XenA was further demonstrated to possess moderate to excellent stereoselectivity and catalyze the reduction of various nitro compounds.[8] In this study, we further explore the catalytic efficiency and stereoselectivity of YersER and KYE1 with a new set of substrates including α,*β*-alkyl-substituted cyclic enones, enol

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ethers, carboxylic esters, nitrostyrenes, and maleimide-type compounds and test both enzymes in aqueous–organic media.

The substrate study for both KYE1 and YersER were performed over 12 h with the GDH cofactor recycling system (Table 1, detailed experimental procedures available in Supporting Information). Both enzymes showed excellent stereoselectivity toward the reduction of α,β-alkyl-substituted cyclic enones (**1**–**4**) and predominantly furnished the corresponding (*S*)-products. Substrate-based stereocontrol was observed for the reduction of α,β-substituted enol ethers.[9] The reduction of five-membered cyclic enol ethers (**5** and **6**) furnished the corresponding (*S*)-products, but upon increasing the ring size to cyclohexenone–enol ether (**8**), the stereopreference switched to (*R*) for both enzymes; furthermore, by changing the side-chain substitution from a methyl group (**8**) to a sterically more demanding *n*-propyl (**10**) or phenyl group (**9**), the substrate was flipped in the enzyme-binding pockets to furnish the corresponding (*S*)-products. The results from substrates **1**–**13** showed that KYE1 and YersER strongly prefer α-substituted substrates, and significantly lower activity was observed for β-substituted substrates (**2**, **4**, **7**, **11**, and **13**). The conversion of dicarboxylic esters, nitrostyrenes, maleimide, and citral (**14**–**21**) revealed a moderate to high degree of enzymatic activity together with excellent product enantiopurity, except for **19**, where a racemic product was observed for both enzymes.[2c] Enzyme-based stereocontrol was again seen in the reduction of **15** and **21**, where KYE1 and YersER formed the corresponding (*R*)- and (*S*)-products, respectively.[10] These results show that prediction of reaction stereo-outcome proves difficult. While KYE1 and YersER share 36% amino acid identity and structures among OYE enzymes are highly conserved, a pair of major residues involved in substrate binding varies from one enzyme to the other (KYE: H191/N194; YersER: H173/H176). Similar variations occur within the OYE family and may be involved in substrate recognition/orientation.[2b]

To enhance the technological utility of ene–reductase for gram-scale synthesis, especially for less water-soluble substrates, we investigated the effects of organic solvents on catalytic efficiency and stereoselectivity. To date, there are few data published addressing solvent effects on OYEs,[11] and this work represents the first detailed study of organic solvent effects on ene–reductase. Water/organic binary co-solvent systems often enhance the catalytic properties of enzymes and potentially offer significant advantages for increasing biocatalyst performance in synthetic chemistry.[12] Twelve different organic solvents (both water miscible and immiscible), arranged according to denaturation capacity scale,[13] were tested with KYE1 and YersER for the bioreduction of 2-cyclohexen-1-one in 20% organic solvent/buffer system (Table 2). Screening results showed that both KYE1 and YersER retained highest enzyme activity in ethylene glycol, dimethyl sulfoxide (DMSO), and in the hexane– and toluene–buffer systems. Given the low level of amino acid identity and similarity between KYE1 and YersER (36% and 53%, respectively), the result also suggests that, in this case, the effect of organic solvents on enzyme stability is independent of both the denaturing capacity scale and the protein amino acid sequences. The experimentally determined threshold concentration (C_{50}), at which half inactivation of the enzyme is observed, for both KYE1 and YersER was 43.3% for ethylene glycol/buffer and 27.5% for dimethyl sulfoxide/buffer (see the Supporting Information). For the two-phase hexane and toluene systems, the catalytic reaction does not seem to be impaired even at 70% organic solvent level, corresponding to a phase ratio of organic to aqueous solvent of 2.33 (0.7:0.3) (detailed results in the Supporting Information). Since both hexane and toluene are immiscible with water, the enzymes are expected to be in the aqueous phase with minimum contact of organic solvents and thus retained full enzyme activity. However, there is only one phase for ethylene glycol and DMSO systems; therefore, the enzymes were much more exposed to the organic solvent, which resulted in decreased activity. A similar trend was observed for the bioreduction of citral with KYE1 and YersER, where the conversion and product enantiopurity were monitored in the presence of 0 – 50% organic solvent (Figure 1). Surprisingly, the product enantiopurity decreased tracking the diminishing enzyme activity (lower conversion) for ethylene glycol (Figure 1a) and DMSO (Figure 1b). The hexane– and toluene–buffer systems again showed constant

Table 2. Catalytic Activity for Bioreduction of 2-Cyclohexen-1-one in 20% Organic Solvent System

solvent	DC[a]	log P[b]	conv (%)	
			YersER	KYE1
ethylene glycol	**18.7**	**−1.43**	**100**	**98.9**
methanol	30.5	−0.74	61.8	23.7
1,2-propanediol	38.8	−0.74	3.1	3.2
ethanol	54.4	−1.35	6.9	6.6
dimethyl sulfoxide	**60.3**	**−0.32**	**100**	**98.5**
dimethylformamide	63.3	−1.35	5.1	4.1
acetonitrile	64.3	−0.32	0.6	0.2
acetone	78.2	3.5	9.8	5.3
1,4-dioxane	92.1	−0.27	10.7	9.1
tetrahydrofuran	100	0.46	1.0	0.7
toluene	**137.9**	**2.46**	**98.1**	**97.8**
hexane	**144.4**	**3.5**	**94.8**	**95.3**

[a] DC: denaturing capacity. [b] log P: partition coefficient.

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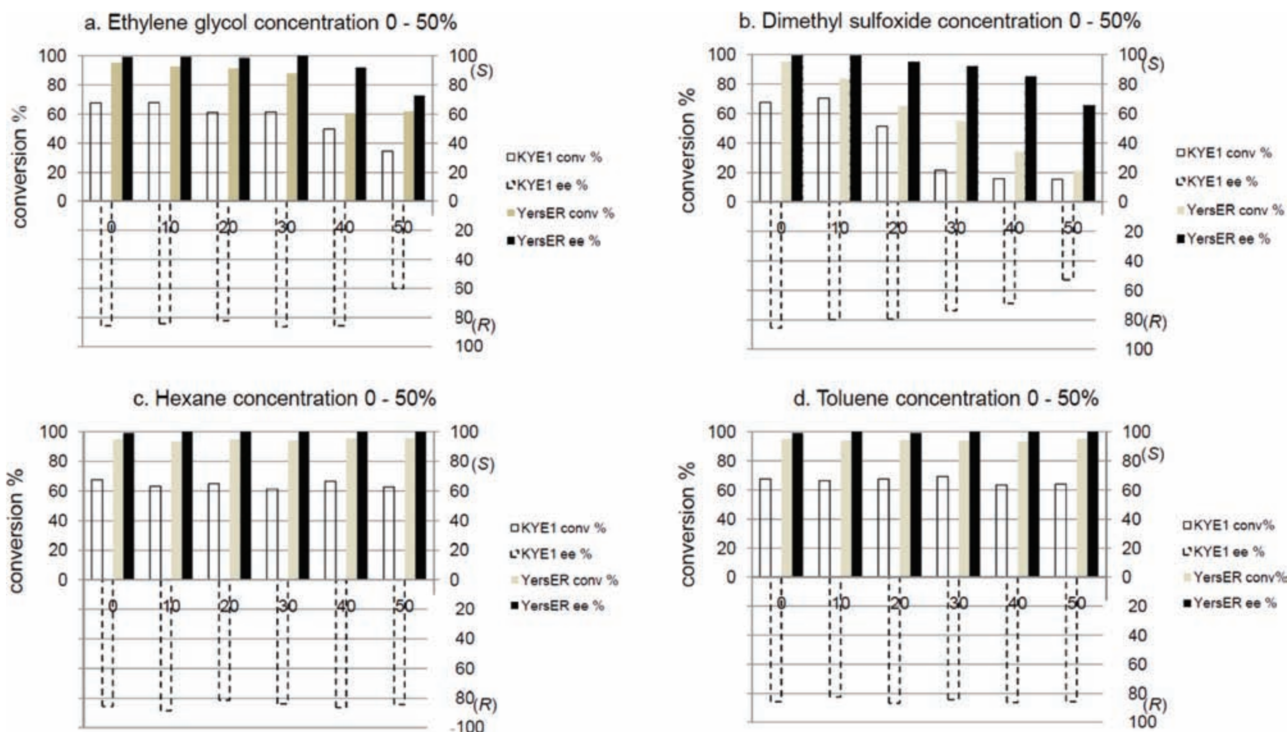

Figure 1. Dependence of organic solvent concentration on the catalytic activity and stereoselectivity of KYE1 and YersER in the asymmetric bioreduction of citral (**21**).

enzymatic activity and product enantiopurity in the investigated phase ratios (Figure 1c,d).

In conclusion, KYE1 and YersER displayed both substrate-based (enol ethers, diesters) and enzyme-based (citral, lactones) stereocontrol. A biphasic organic solvent/buffer system revealed no effect of the phase ratio (in the range of 0−2.3) on catalytic efficiency or stereoselectivity. In monophasic DMSO− and ethylene glycol−buffer systems, the activity declined markedly beyond 20% and 35% solvent, respectively, and stereoselectivity of citral bioconversion declined as well with rising solvent content. To further enhance the applicability of ene−reductases, future research efforts should focus on predictability and effective enhancement of product enantiopurity.

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Supporting Information Available. General synthesis and analytical methods, with additional results. This material is available free of charge via the Internet at http://pubs.acs.org.