http://dx.doi.org/10.1595/205651316X692572 *Johnson Matthey Technol. Rev.*, 2016, **60**, (4), 243–249

JOHNSON MATTHEY
TECHNOLOGY REVIEW

www.technology.matthey.com

Reduction of Activated Carbon-Carbon Double Bonds using Highly Active and Enantioselective Double Bond Reductases

ENEs from Johnson Matthey's enzyme collection provide a suitable alternative to metal-catalysed hydrogenation

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The use of enzymes for the asymmetric reduction of activated C=C double bonds is a viable and straightforward alternative to chiral hydrogenation. The number of isolated and characterised double bond reductases (ENEs) has grown significantly over the past fifteen years and the use of this enzyme class in organic synthesis has increased accordingly. In this article we examine the ENE-catalysed reduction of a number of activated alkenes using enzymes from Johnson Matthey's collection. These reductions proved to be scalable: they can be run at high substrate concentration, delivering the reduced product in high yield and high chemical purity.

1. Introduction

The use of enzymes in organic synthesis offers an alternative to the use of metal catalysts and allows a high degree of chemo-, regio- and enantioselectivity. Among the redox enzymes commonly employed in organic synthesis there is the group known as ene-reductases (ENEs) that is used for the reduction of activated C=C double bonds. Traditionally, whole cell microorganisms were used for this purpose but a recent increase in the number of isolated and characterised ENEs means that recombinantly expressed enzyme preparations are now generally favoured over whole cells, as a number of recent publications show (1–5).

One well characterised type of ENEs is old yellow enzyme (OYE). Although OYE was first isolated from *Saccharomyces pastorianus* back in 1932 (6), it was not until 1993 that the reduction of activated alkenes by this enzyme class was reported (7, 8). Since then, OYE family members have been found widely distributed in fungi, bacteria and plants (9).

The enzymatic reduction of alkene derivatives using isolated ENEs has been broadly reported in the literature (1–5, 10–17). Examples of reductions carried out at large, preparative scale are however scarce. To the best of our knowledge, the first reported example of an ENE-catalysed enantiospecific reduction at large scale (70 g) was published in 2012 (18). An initial enzyme screening programme followed by further optimisation of the reaction conditions allowed the authors to achieve acceptable conversion (53% after 20 h, 73% after 44 h) on the reduction of an ester-activated olefin at volume efficiency of 200 mM. Previously to that, low

productivities have been overcome by implementing an *in situ* substrate feeding product removal (SFPR) strategy (19). This strategy proved very successful, allowing the substrate loading to be increased from 15 to 30 g l^{-1} and achieving productivities of 59.4 g l^{-1} day^{-1} in the reduction of an α,β-unsaturated aldehyde, a chiral intermediate in the synthesis of antidiabetic drug ethyl (*S*)-2-ethoxy-3-(p-methoxyphenyl)propanoate (EEHP). The reaction was demonstrated at preparative scale (1 g substrate) in the presence of Amberlite™ XAD 1180 ion-exchange resin (1 g, $X_{r/s}$ = 1).

Alternative strategies to increase the productivity of ENEs in order to make them more attractive for transformations at commercial scale have resorted to protein engineering, including generation of chimeric enzymes. These efforts have led to increased enzyme thermostability, solvent stability or both, resulting in up to ten-fold improvements in conversion rates compared to the naturally occurring enzymes (20). These engineered enzymes have been used at substrate concentrations up to 300 g l^{-1}.

The present article reports the use of wild-type ENEs from Johnson Matthey's enzyme collections, which are particularly promising and economically viable for industrial applications owing to their tolerance to high substrate concentrations, up to 1.5 M (equivalent to 257 g l^{-1}).

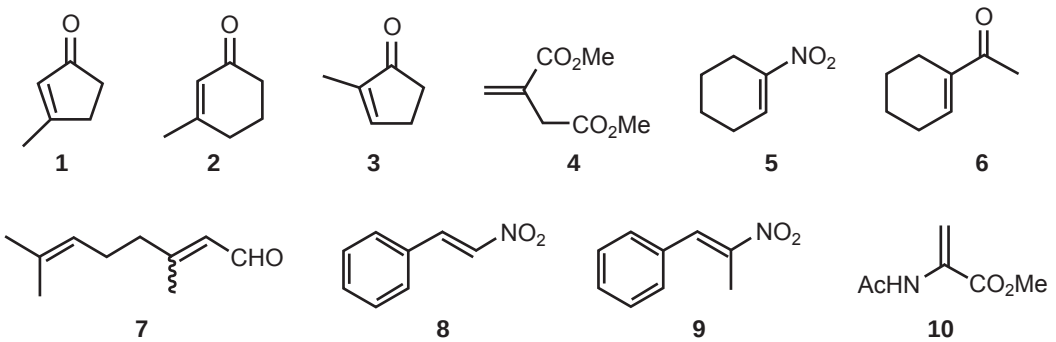
2. Results and Discussion

2.1 Substrate Scope Characterisation

ENE-101™, ENE-102™ and ENE-103™ OYEs have been tested for the reduction of a number of electron-deficient double bonds, conjugated to acyl, carboxy, acyloxy, nitro and acylamino groups (**Figure 1**). This initial test helped us to define the substrate scope of these enzymes. Results from these reactions are described in **Table I**.

Reduction of substrates **1** and **2** proved slow and only partial conversion was obtained with ENE-101™ and ENE-102™ (**Table I**). The formation of side products was not detected for these reactions and enantioselectivity was excellent for both substrates. Substrate **3** was rapidly reduced by ENE-101™, ENE-102™ and ENE-103™. Racemisation of the corresponding reduction product, 2-methylcyclopentanone, under the reaction conditions resulted in disappointing enantioselectivity values. The fact that OYEs are better at reducing α-substituted than β-substituted enones has been extensively reported in the literature (21–23). Good to excellent conversions were observed for substrates **4**, **5**, **6**, **7**, **8** and **9** and, again, we did not detect any side product formation.

Substrates **3**, **4**, **5**, **6** and **7** were subsequently tested at higher concentrations: 50, 100 and 300 mM (**Table II**). The amount of enzyme and other reagents were scaled up accordingly, so the number of equivalents remained constant. The reactions were run in 250 mM phosphate buffer pH 7.0. It should be noted that gluconic acid is generated as the reaction takes place, therefore the buffer strength may not be sufficient for the more concentrated reactions and the pH of the media may turn too acidic for the enzymes to remain active. Although the evolution of the pH of the reaction media with conversion was not specifically measured for these experiments, we observed that, at 100 mM substrate concentration, the media pH had decreased from 7 to *ca*. 5 when 40% conversion was reached and to *ca*. pH 4 at 75% conversion.



1 2 3 4 5 6 7 8 9 10

Fig. 1. Activated alkenes tested in this study

Table I Conversion Percentage[a] and Enantiomeric Excess Percentage[b] at 20 mM Substrate Concentration, pH 7, 35°C, 18 h

Substrate	ENE-101™	ENE-102™	ENE-103™
1	10.2 (>99.9 *S*)	37.3 (>99.9 *S*)	0
2	18.7 (>99.9 *S*)	41.6 (>99.9 *S*)	0
3	100 (34.6) [c, d]	100 (49.2) [c, e]	100 (75.4) [c, d]
4	91.1 (>99.9 *R*)	100 (>99.9 *R*)[c]	85.2 (>99.9 *R*)
5	97.7	100	100
6	80.2	97.6	100
7	72.7[f]	88.8[f]	35.9[f]
8	100	100	97.4
9	91.6[f]	80.5[f]	80.9[f]
10	33.0[f]	97.7[f]	40.1[f]

[a] Integration of the product peak in the GC (uncorrected AUC), values below 100% indicate that unreacted starting material was detected; no side products were detected for these reactions

[b] Stereo. refers to the assigned stereochemistry of the product

[c] Erosion of the product ee in the reaction media has been observed; this erosion is not enzymatically-catalysed

[d] Full conversion was achieved in 6 h

[e] Full conversion was achieved in 3 h

[f] ee has not been determined

Table II Conversion Percentage[a] and Enantiomeric Excess Percentage[b] at 50, 100 and 300 mM Substrate Concentration, pH 7, 35°C, 18 h

Substrate	Enzyme	50 mM	100 mM	300 mM
3	ENE-101™	70.1 (14.9)[c]	67.2 (26.8)[c]	36.4 (16.0)[c]
3	ENE-102™	68.8 (38.1)[c]	44.1 (31.8)[c]	43.9 (31.8)[c]
3	ENE-103™	74.5 (55.2)[c]	87.3 (48.7)[c]	51.5 (67.8)[c]
4	ENE-101™	–	46.3 (>99.9 *R*)	17.6 (>99.9 *R*)
4	ENE-102™	–	–	37.3 (>99.9 *R*)
4	ENE-103™	–	69.7 (>99.9 *R*)	47.1 (>99.9 *R*)
5	ENE-101™	52.8	68.5	39.4
5	ENE-102™	61.9	49.8	39.5
5	ENE-103™	96.8	83.1	47.0
6	ENE-101™	7.3	49.9	23.0
6	ENE-102™	3.4	5.9	11.3
6	ENE-103™	94.9	95.0	56.2
7	ENE-101™	92.4[d]	81.2[d]	52.3[d]
7	ENE-102™	85.9[d]	75.0[d]	46.8[d]
7	ENE-103™	14.2[d]	21.6[d]	21.8[d]

[a] Integration of the product peak in the GC (uncorrected AUC), values below 100% indicate that unreacted starting material was detected; no side products were detected for these reactions

[b] Stereo. refers to the assigned stereochemistry of the product

[c] Erosion of the product ee in the reaction media has been observed; this erosion is not enzymatically-catalysed

[d] ee has not been determined

These results encouraged us to repeat these reactions at higher than 300 mM substrate concentration introducing strict external pH control of the reaction media.

2.2 Scale-up Experiments

The reduction of electron-deficient alkenes by ENE-101™, ENE-102™ and ENE-103™, so far demonstrated only at screening scale in **Tables I** and **II**, was successfully repeated at preparative scale for substrates **4** (5.9 g) and **6** (4.7 g) at relatively high substrate concentration (from 0.73 to 1.5 M).

The reduction of **4** (5.9 g) at 0.73 M was especially fast with ENE-102™ and full conversion was achieved in 7 h (**Figure 2**). Additionally, ENE-101™ and ENE-103™ could convert more than 90% of substrate, although over a longer time period (**Figure 2**). The enantioselectivity of the reaction, 99.9% towards the (*S*) enantiomer, remained unchanged during the reaction time.

The reduction of **6** was demonstrated at 0.75 M at similar scale (4.7 g). In this case ENE-101™ was the most active enzyme towards the substrate, while ENE-102™ achieved less than 50% conversion (**Figure 3**). In order to challenge further this enzymatic process, the reduction of **6** was attempted with ENE-101™ at 1.5 M concentration, reaching more than 90% conversion in 96 h (**Figure 3**).

3. Experimental

3.1 General

All reagents were purchased from Sigma-Aldrich or Alfa Aesar and were of the highest available purity.

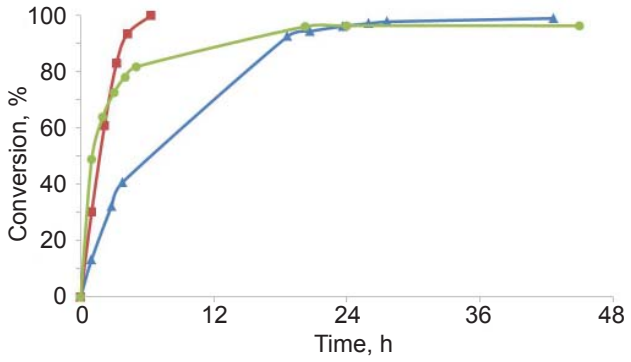


Fig. 2. Conversion profiles based on the integration of the product peak in the GC (uncorrected AUC) for the reduction of **4** (0.73 M substrate concentration) by: ENE-101™ (blue triangles), ENE-102™ (red squares) and ENE-103™ (green circles). No side products were detected for these reactions

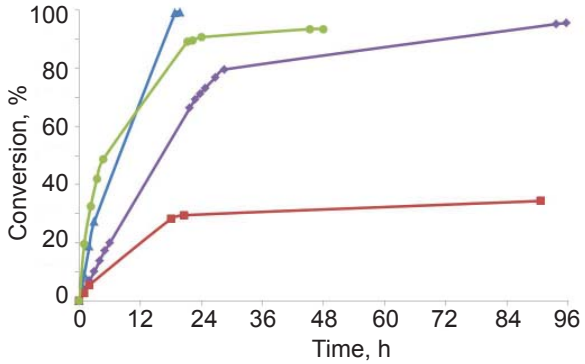


Fig. 3. Conversion profiles based on the integration of the product peak in the GC (uncorrected AUC) for the reduction of **6** (0.75 M substrate concentration) by: ENE-101™ (blue triangles), ENE-102™ (red squares) and ENE-103™ (green circles) and for the reduction of **6** (1.5 M substrate concentration) by ENE-101™ (purple diamonds). No side products were detected for these reactions

3.2 Enzyme Preparations

Genes coding for Johnson Matthey ENEs (ENE-101™, ENE-102™ and ENE-103™) were ordered codon-optimised from GeneArt® (Thermo Fisher Scientific Inc) and cloned into T5 vector pJEx401 (DNA2.0). Enzymes were expressed recombinantly in *Escherichia coli* BL21 in both shake flasks and fed batch fermentations, whereby induction was carried out with isopropyl β-D-thiogalactopyranoside (IPTG) at 30°C. Harvested biomass was resuspended in 100 mM potassium phosphate buffer (pH 7) and cells were broken up either by sonication or homogenisation. The so-obtained cell lysate was clarified by centrifugation and filtrated prior to lyophilisation. Protein expression was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and chromatographic activity assays.

3.3 Small Scale Reactions for Substrate Scope Characterisation

50 µl of substrates **1**–**10** solution in toluene (400 mM) followed by 50 µl of a solution of ENE-101™, ENE-102™ or ENE-103™ enzymes in water (100 mg ml^{-1}; 5 mg enzyme per test) were added to reaction vials containing 900 µl of aqueous media at pH 7 (250 mM potassium phosphate buffer pH 7, 1.1 mM nicotinamide adenine dinucleotide (NAD^+), 30 mM D-glucose, 10 U ml^{-1} glucose dehydrogenase (GDH)) to give a final concentration of substrate of 20 mM. The vials were shaken at 35°C for 18 h. After adding 1 ml of ethyl acetate the reaction vials were vortexed

and centrifuged. Samples of the organic phase were analysed by gas chromatography (GC) to measure conversion and enantiomeric excess (ee). When higher concentrations of substrate were added to the reaction (50, 100 and 300 mM) the substrate was added neat without co-solvents and 1.1 equivalents of co-substrate D-glucose were added.

3.4 Scale-up Reactions

In a magnetically stirred 50 ml round bottom flask, equipped with a pH controlled dosing pump, were introduced deionised water (36.3 ml), dipotassium hydrogen phosphate (K_2HPO_4) (597 mg) and potassium dihydrogen phosphate (KH_2PO_4) (27 mg), D-glucose monohydrate (1.1 equiv.), sodium chloride (1.68 g) (24), ENE (ENE-101™, ENE-102™ or ENE-103™, 1.0 g), GDH (103 mg; 4.88 U mg^{-1}; 500 U), NAD^+ (166 mg) and substrate (**4** 5.9 g; 37.3 mmol; **6** 4.67 g, 37.6 mmol or 9.34 g, 75.2 mmol). By default we add NaCl to the reaction media, according to Chaplin and Bucke:

> "In general, proteins are stabilised by increasing their concentration and the ionic strength of their environment. Neutral salts compete with proteins for water and bind to charged groups or dipoles. This may result in the interactions between an enzyme's hydrophobic areas being strengthened causing the enzyme molecules to compress and making them more resistant to thermal unfolding reactions" (24).

The reaction was stirred at 40°C until full conversion was observed by GC analysis. In order to maintain a constant pH at 7.0 the reaction was dosed with a 45% sodium hydroxide solution.

3.5 Gas Chromatography Methods

GC analysis of conversion and ee was performed on a Varian CP-3800 using γ-DEX™ 225 capillary column (30 m × 0.25 mm × 0.25 µm) and using helium as carrier gas. The conversion percentage was measured by integration of the product peak in the GC (uncorrected area under curve (AUC)), values below 100% indicate that unreacted starting material was detected. No side products were detected in any of the reported reactions. GC program parameters: injector 250°C, flame ionisation detector (FID) 250°C, constant flow 5 ml min^{-1} unless specified otherwise.

1: 60°C for 5 min then 5°C min^{-1} up to 110°C, then 20°C min^{-1} up to 180°C, hold 0.5 min (total time 19 min). t_R 7.8 min (*R*-3-methylcyclopentanone), 7.9 min (*S*-3-methylcyclopentanone) and 14.0 min (substrate **1**)

2: 80°C for 5 min then 10°C min^{-1} up to 130°C, then 15°C min^{-1} up to 180°C, hold 1.67 min (total time 15 min). t_R 6.8 min (*S*-3-methylcyclohexanone), 7.0 min (*R*-3-methylcyclohexanone) and 10.0 min (substrate **2**)

3: 60°C for 5 min then 5°C min^{-1} up to 90°C, then 20°C min^{-1} up to 180°C, hold 0.5 min (total time 16 min). t_R 7.0 min, 7.2 min (*R*- and *S*-2-methylcyclopentanone) and 9.6 min (substrate **3**)

4: 90°C for 5 min then 0.5°C min^{-1} up to 94°C, then 20°C min^{-1} up to 180°C, hold 0.7 min (total time 17 min). t_R 6.9 min (dimethyl *R*-2-methylsuccinate), 7.2 min (dimethyl *S*-2-methylsuccinate), 10.1 min (substrate **4**)

5: 120°C for 2 min then 10°C min^{-1} up to 190°C, hold 1 min (total time 10 min). t_R 4.7 min (nitrocyclohexane) and 5.8 min (substrate **5**)

6: 110°C for 1 min then 15°C min^{-1} up to 160°C, hold 1.67 min; flow 2.5 ml min^{-1}. t_R 3.1 min (1-cyclohexylethanone) and 3.7 min (substrate **6**)

7: 100°C then 5°C min^{-1} up to 135°C, then 15°C min^{-1} up to 170°C, hold 1.67 min. t_R 5.8 min (citronellal), 6.1 min and 6.5 min (substrate *Z/E*-**7**)

8: 80°C then 30°C min^{-1} up to 145°C, then 0.5°C min^{-1} up to 150°C, then 15°C min^{-1} up to 180°C (total time 14.16 min). t_R 5.7 min (2-nitropropyl benzene) and 8.11 min (substrate **8**)

9: as in **8**. t_R 5.6 min (2-nitroethylbenzene) and 7.53 min (substrate **9**)

10: 120°C for 2 min then 1°C min^{-1} up to 140°C, then 20°C min^{-1} up to 180°C hold 0.7 min (total time 24.7 min). t_R 8.14 min (methyl 2-acetamidopropanoate) and 4.8 min (substrate **10**).

4. Conclusions

The substrate scope of Johnson Matthey's ENE-101™, ENE-102™ and ENE-103™ for the reduction of electron-deficient double bonds has been defined. In addition, the scalability of these reactions has been demonstrated: dimethyl itaconate **4** (5.9 g, 0.73 M) was reduced with excellent enantioselectivity by ENE-102™ to achieve full conversion in 7 h, while the reduction of 1-cyclohexenylethanone **6** (9.34 g, 1.5 M) with ENE-101™ reached more than 90% conversion in 96 h.

These examples demonstrate the synthetic potential of ENE-101™, ENE-102™ and ENE-103™ for the synthesis of chiral and achiral intermediates.

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Beatriz Domínguez gained her PhD in Synthetic Organic Chemistry from the University of Vigo, Spain, and then moved to the UK where she worked with Professor Tom Brown at the University of Southampton, UK, and with Professor Guy Lloyd-Jones at the University of Bristol, UK. In 2002 she joined Synetix, soon to become Johnson Matthey Catalysts and Chiral Technologies and has worked at the company's facilities in Cambridge since. For the last fifteen years Beatriz has gained broad experience in the application of metal catalysis, particularly hydrogenation and cross-coupling, working closely with pharmaceutical companies to deliver optimal catalysts for chemical processes. Her interests have more recently expanded to the area of biocatalysis.

Ursula Schell completed her PhD in Molecular Microbiology in 1998 from the University of Stuttgart, Germany, and then focused on engineering gene clusters for polyketide biosynthesis in streptomycetes. Between 2004 and 2007 she was involved as a postdoctoral fellow in the Engineering and Physical Sciences Research Council (EPSRC) funded Bioconversion – Chemistry – Engineering Interface (BiCE) programme at University College London (UCL), UK, where she recruited transaminases for chiral aminodiol synthesis. She later gained experience at GlaxoSmithKline, UK, where her team improved a microbial expression platform and contributed to the early process development of microbially derived drug candidates. Ursula joined Johnson Matthey's catalysis team in 2012 and supervises both enzyme engineering and bioproduction projects.

Serena Bisagni completed her MSc in Industrial Biotechnology from the University of Pavia, Italy, in 2010 and then moved to Lund University, Sweden, for her postgraduate studies. In 2014 she obtained her PhD in Biotechnology in which she focused on the identification of new Baeyer-Villiger monooxygenases for fine chemicals synthesis within the Marie Curie Innovative Training Networks (ITN) 'Biotrains'. In 2015 Serena joined Johnson Matthey Catalysts and Chiral Technologies where she is a Research Chemist. Her main interests are enzyme screening for synthesis of active pharmaceutical ingredients and fine chemicals and identification of new biocatalysts to add to Johnson Matthey's enzyme portfolio.

Thomas Kalthoff graduated in Chemical Engineering from Niederrhein University, Germany. He co-founded Jülich Fine Chemicals GmbH, Germany, while working on process development in biocatalysis. After the acquisition by Codexis Inc in 2005, Thomas was involved in technology integration and scale-up projects for the production of chiral intermediates before he joined Johnson Matthey Catalysis and Chiral Technologies in 2010 as Senior Process Engineer.