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A Rapid and High-Throughput Assay for the Estimation of Conversions of Ene-Reductase-Catalysed Reactions

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A fast and sensitive colorimetric assay (FRED, fast and reliable ene-reductases detection) that allows the estimation of levels of conversion of ene-reductase (ER)-catalysed reactions has been developed. The activated olefin is reduced by ER at the expense of NAD(P)H cofactor, whose regeneration is carried out in situ by the glucose/glucose dehydrogenase system. Subsequently, the consumption of the co-substrate glucose is determined colorimetrically by a multienzymatic system. The FRED assay offers a wide range of possible applications, from enzyme fingerprinting and kinetic analysis, to primary screening of enzyme libraries and optimisation of ERs' performances under different reaction conditions.

Ene-reductases (ERs) of the Old Yellow Enzyme (OYE) family catalyse the stereospecific *anti* hydrogenation of activated olefins by the transfer of a formal hydride from a flavin mononucleotide ($FMNH_2$) prosthetic group (regenerated by NAD(P)H cofactor) to the β-carbon, followed by the delivery of a proton from the hydroxy group of a tyrosine residue to the α-carbon of the resulting enolate.[1] In the last decade, these biocatalysts have proven to be a real alternative to the classical metal-based hydrogenation methods for the preparation of enantiopure chiral compounds. Thus, the ER-mediated bioreduction of various prochiral substrates (α,β-unsaturated aldehydes/ketones, nitroalkenes, maleimides) has been intensively investigated during the last years, although mostly on a lab scale.[2]

Many wild-type OYEs isolated from different sources (bacteria, yeasts, plants etc.), showed relatively broad spectra of accepted substrates, usually combined with good to excellent stereoselectivity. As well as by screening naturally diverse ERs, access to optically pure products can be achieved by exploiting different enzyme-based or substrate-based stereocontrol strategies;[3] this has in some cases led to significant improvement, or even to a switch of the stereochemical outcome.[4]

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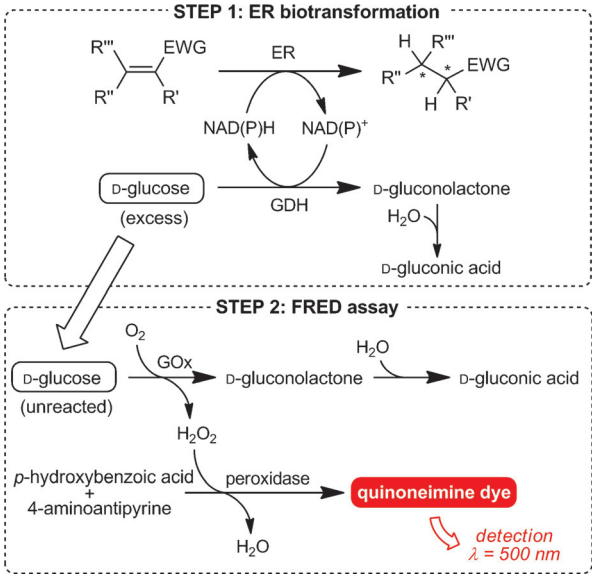
Even though the performances of these biocatalysts in terms of stereoselectivity are often close to optimal from the very first stages of the investigations, there is still some room for improvement as far as other relevant aspects of their synthetic application are concerned. For example, in different cases the tolerance shown by ERs to high substrate/product concentrations,[2b–c] as well as the specific activity toward selected target substrates,[2d,5] are inadequate for large-scale applications.

Enzyme engineering and process development in ER-mediated applications demand rapid, convenient and reliable activity assays.[6] If possible, the use of colorimetric assays also suitable for microscale analyses is preferred because they allow parallel screening of many samples and reduce time and reagent consumption. In contrast, ER performances are currently most often evaluated by monitoring the reactions through GC or HPLC analyses, which in some cases advantageously provides a simultaneous evaluation of conversion and stereoselectivity. However, these methods are time-consuming, and a simpler conversion-based assay might be very useful as a primary screening method during, for example, evaluation of enzyme libraries and optimization of reaction conditions. In the development of a conversion-based screening assay, we avoided the use of substrate- or product-based assays, as well as of synthetic chromogenic substrates that lack general applicability.

Direct spectrophotometric detection of NAD(P)H consumption at 340 nm has previously been used for mechanistic studies and characterization of ERs,[7a] and more recently for the development of a high-throughput screening (HTS) method based on steady-state kinetic analyses.[7b] However, very often the maximum absorbance peak of the unsaturated substrate overlaps with that of NAD(P)H (especially for compounds containing extended conjugated systems, such as one or more aromatic rings), so this detection method becomes inapplicable, except for the rare cases in which the analysis can be carried out at a slightly higher wavelength.[7b] Overall, this kind of assay intrinsically suffers from poor flexibility and requires tedious and time-consuming setup. Alternatively, a fluorescent intensity (FLINT) assay[7c] has been proposed for HTS.

Here we describe a simple and fast colorimetric method (Scheme 1) for the determination of levels of conversion in ER-catalysed reductions in a substrate-independent way (FRED, fast and reliable ene-reductase detection).

The activated olefin is reduced by the ER at the expense of the NAD(P)H cofactor, regeneration of which is carried out in situ with the aid of the glucose/glucose dehydrogenase (GDH) system (Scheme 1, step 1), thus allowing its use in catalytic



Scheme 1. Reactions involved in the FRED assay.

amounts. Additionally, the coupled cofactor regeneration system can be exploited to establish the level of conversion achieved in the bioreduction by determining either the formation of the co-product gluconic acid or the consumption of the co-substrate glucose.

Although a simple pH-based assay can be used for the estimation of gluconic acid formation in the presence of pH indicators, this method is not very sensitive or reliable, due to possible interference with acidic/basic substrates.

Instead, the alternative option—that is, the detection of residual glucose—offers a wide range of already developed chemical and enzymatic methods that show different advantages and drawbacks. Of these methods, we chose the well-established system based on the enzymatic oxidation of glucose catalysed by a glucose oxidase (GOx), which results in the production of H_2O_2. H_2O_2 can be detected colorimetrically by the addition of a peroxidase and a suitable chromogenic mixture of substrates, such as *p*-hydroxybenzoic acid and 4-aminoantipyrine (Scheme 1, step 2).[8] Advantageously, ready-to-use assay solutions are commercially available from several companies as colorimetric kits for quantification of glucose in food, blood, etc. and show high sensitivity and reproducibility, as well as exquisite specificity, with very low risk of interference with contaminant substances (in our case, substrates/products of the ER-catalysed reaction). Moreover, the ER-catalysed reaction can be carried out under any desired set of conditions, because the reaction and the detection steps are uncoupled, and the step of product extraction with organic solvents is no longer necessary.

To validate the FRED assay and to assess its general applicability, we performed a comparison of the conversion values estimated by this method with those obtained by GC analyses in the bioreduction of substrates **1**–**4** (with different electron-withdrawing groups for the activation of the C=C bond) catalysed by the ER OYE1 (Figure 1).

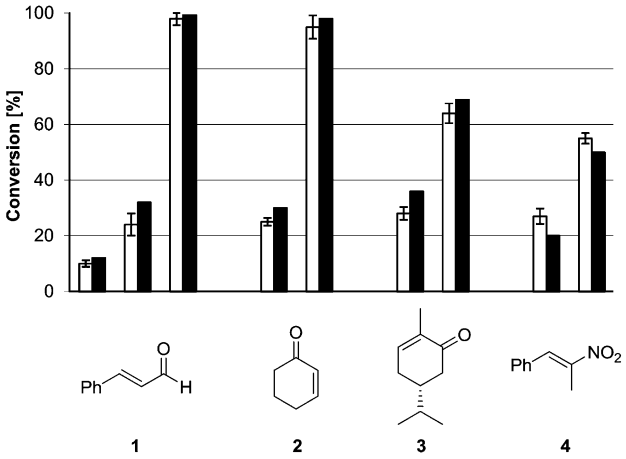


Figure 1. Validation of the FRED assay (□) with GC analysis (■). Sampling times were chosen to give very different conversion values.

Reactions were carried out in the presence of $NADP^+$, GDH and glucose (1.2 equiv). Samples were withdrawn at scheduled times and incubated with the assay solution (1 mL), and then the absorbance at 500 nm was measured to estimate the amount of residual glucose.[9] In parallel, samples were extracted with EtOAc, and conversions were determined by GC analysis.

A very good estimation of the levels of conversion was shown, over the full range of values from low to quantitative. Control experiments carried out in the absence either of OYE1 or of the substrates showed negligible consumption of glucose after 24 h (see the Supporting Information). In this regard, the use of purified enzymes in the reduction reaction is essential, to avoid unproductive glucose consumption due to the presence of contaminating activities, such as alcohol dehydrogenases. However, this requirement is common to all the known assays for ER activity.[7]

Both the reduction reactions and the assay reactions were easily scaled down to 96-well microtiter plate format (200 μL) with comparable results.[9] The suitability of this method for high-throughput measurements was evaluated by statistical treatment of the experimental data and calculation of the *Z*-factor, a dimensionless statistical parameter conceived for the validation of HTS methods.[10] In particular, a *Z*-factor of 0.84 was obtained, this being an indicator of an excellent assay quality (Supporting Information).

This colorimetric assay can also be used to monitor the progress of ER-catalysed reductions over time. In the example shown in Figure 2, conversion rates of the OYE1-catalysed reduction of cinnamaldehyde (**1**) and (*R*)-carvone (**3**) were easily estimated by collecting samples from the corresponding reactions at scheduled times and, subsequently, measuring the concentrations of residual glucose in all samples at once in a 96-well microtiter plate. The possibility of running several samples in a high-throughput format allows for parallel substrate profiling of ERs as well as for estimation of conversion rates in ER-catalysed reductions under different experimental conditions.

Therefore, the assay is suitable for parallel and/or combinatorial evaluation of the performances of coupled ER-GDH sys-

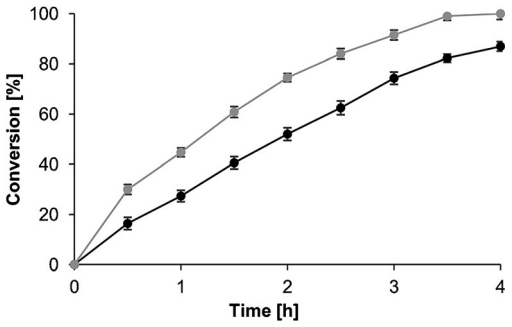


Figure 2. Application of the FRED assay to the estimation of conversion rates of **1** (●) and **3** (●).

tems under different reaction conditions and optimization of the biotransformation parameters. For instance, the efficiency of the OYE1-GDH cascade in the reduction of **1** as a model substrate was tested in the presence of 5–20% (*v/v*) of different organic cosolvents (Figure 3). Both the OYE1-catalysed reaction and the detection by the FRED assay were carried out in 96-well plates, and the outcome was validated by GC analysis (Supporting Information). The screening allowed fast identification of well-tolerated organic solvents—DMSO and methanol, for example—and of totally unsuitable solvents such as DMF, without the need to perform time-consuming GC analyses. Moreover, the assay provided reliable information about the maximum concentrations of the various solvents that can be used to obtain measurable levels of conversion in the ER-catalysed reductions.

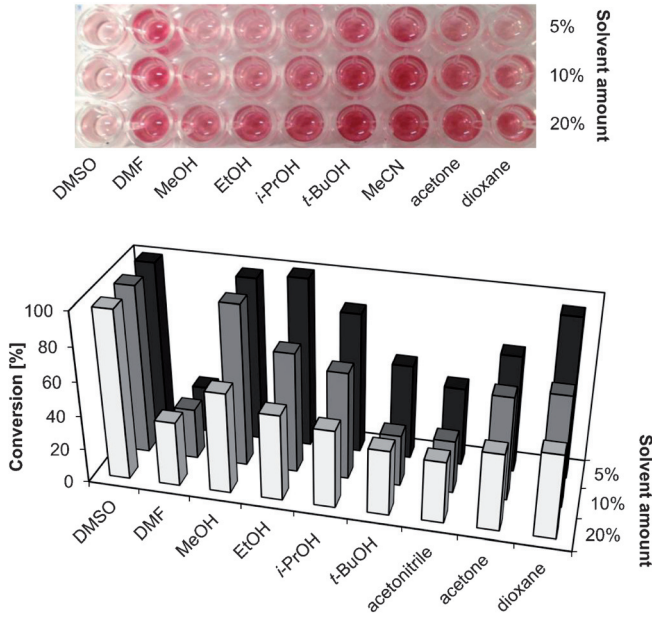


Figure 3. Application of the FRED assay to the screening of different solvent systems for the bioreduction of **1**.

In conclusion, the described FRED assay proved suitable for quick and reliable determination of conversion values of ER-catalysed reductions. It offers a wide range of possible applications, from enzyme fingerprinting and conversion rate measurements to primary screening of enzyme libraries and optimization of ERs performances under different reaction conditions.

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[9] Standard biotransformation and assay procedure: the substrate (5 μmol), dissolved in DMSO (10 μL), was added to a solution of glucose (6 mM, 1.2 equiv with respect to the substrate), $NADP^+$ (0.1 mM), GDH (5 U mL^{-1}) and OYE1 (0.5–100 μg mL^{-1}) in KP_i buffer (1.0 mL, 50 mM, pH 7.0). The mixture was stirred in an orbital shaker (160 rpm, 30 °C), and samples (30 μL for the cuvette-based assay, 6 μL for the plate-based assay) were withdrawn after specified time intervals. Each sample was thoroughly mixed with the assay solution (970 μL for the cuvette-based assay, 194 μL for the plate-based assay). The mixtures were incubated at 40 °C for 20 min in a static incubator, and then absorbance was read at 500 nm.

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