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Photoenzymatic Reduction of C=C Double Bonds

Maria Mifsud Grau,[a] John C. van der Toorn,[a] Linda G. Otten,[a] Peter Macheroux,[b] Andreas Taglieber,[c] Felipe E. Zilly,[d] Isabel W. C. E. Arends,[a] and Frank Hollmann[a,*]

[a] Delft University of Technology, Department of Biotechnology, Biocatalysis and Organic Chemistry Group, Julianalaan 136, 2628 BL Delft, The Netherlands
Fax: (+31)-152-781-415; phone: (+31)-152-781-957; e-mail: f.hollmann@tudelft.nl
[b] Graz University of Technology, Institute of Biochemistry, Petersgasse 12, 8010 Graz, Austria
[c] Firmenich SA, Corporate R&D, Biotechnology & Bioengineering, Route des Jeunes 1, 1211 Geneva 8, Switzerland
[d] Max-Planck-Institut für Kohlenforschung, Kaiser-Wilhelm-Platz 1, 45470 Mülheim an der Ruhr, Germany

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Abstract: A simplified procedure for cell-free biocatalytic reductions of conjugated C=C double bonds using old yellow enzymes (OYEs) is reported. Instead of indirectly regenerating YqjM (an OYE homologue from *B. subtilis*) or NemA (*N*-ethylmaleimide reductase from *E. coli*) *via* regeneration of reduced nicotinamide cofactors, we demonstrate that direct regeneration of catalytically active reduced flavins is an efficient and convenient approach. Reducing equivalents are provided from simple sacrificial electron donors such as ethylenediaminetetraacetate (EDTA), formate, or phosphite *via* photocatalytic oxidation. This novel photoenzymatic reaction scheme was characterized. Up to 65% rates of the NADH-driven reaction were obtained while preserving enantioselectivity. The chemoselectivity of the novel approach was exclusive. Even when using crude cell extracts as biocatalyst preparations, only C=C bond reduction was observed while ketone and aldehyde groups remained unaltered. Overall, a simple and practical approach for photobiocatalytic reductions is presented.

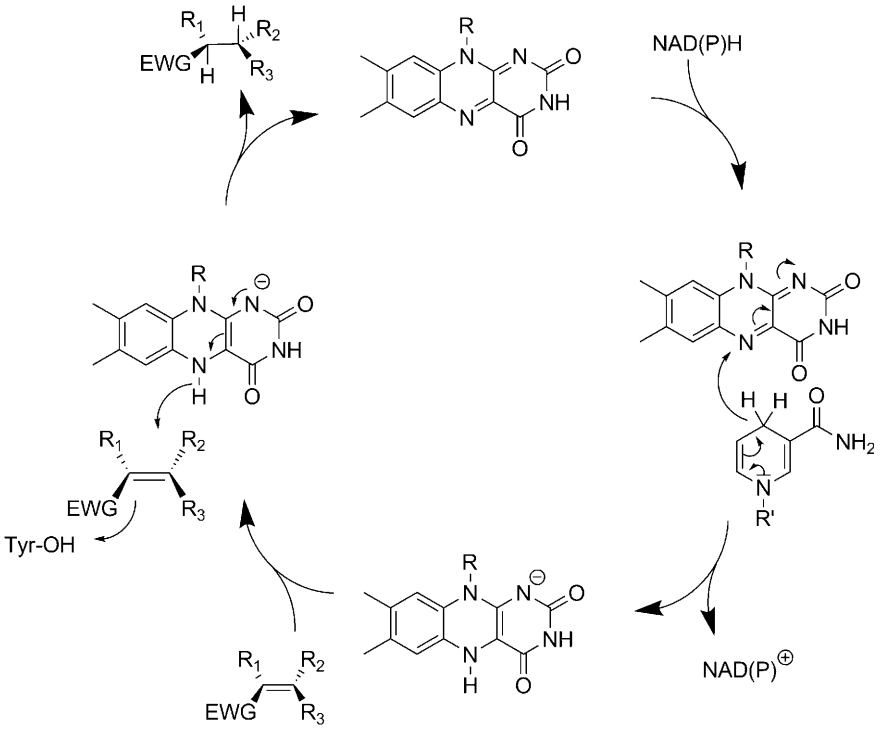
Keywords: alkene reduction; asymmetric catalysis; cofactor regeneration; enzyme catalysis; old yellow enzymes; photoenzymatic reduction

Introduction

Asymmetric reduction of C=C double bonds is one of the most widely used methodologies for the generation of chiral molecules and synthons.[1] In addition to the well-established transition metal-catalyzed (transfer) hydrogenations[2,3] new methodologies such as organocatalytic[4] or biocatalytic[5] approaches are constantly being added to the toolbox. For the latter, so-called enoate reductases [E.C. 1.3.1.X] (or old yellow enzymes, OYEs)[6] have recently experienced a renewed interest as catalysts for the asymmetric reduction of activated C=C-double bonds.[5] For example, enantiospecific reductions of α,β-unsaturated aldehydes and ketones,[7–12] acids and esters,[13,14] as well as nitro compounds[15–17] or nitriles[18] have been reported. Especially their broad substrate scope combined with the often excellent enantioselectivity makes OYEs attractive biocatalytic counterparts to the existing chemical methods.

The active reductant within OYEs is a reduced flavin (reduced flavin adenine mononucleotide, $FMNH_2$)[19,20] transferring a hydride in a Michael-type reaction to the substrate's β-C-atom. The resulting enolate anion is protonated in a *trans*-fashion (by a tyrosine residue) at α-C, leaving the reduced product and the oxidized flavin prosthetic group. A new catalytic cycle is initiated by nicotinamide [NAD(P)H]-dependent reduction of the flavin (Scheme 1).

This dependence on NAD(P)H, however, constitutes a major challenge for OYEs *en route* to efficient biocatalytic reductions. NAD(P)H is expensive therefore necessitating catalytic use and efficient *in situ* regeneration from $NAD(P)^+$. In principle, a broad range of NAD(P)H regeneration systems are available from which the most suitable for the given reaction can be chosen.[21–26] To avoid additional efforts and expenditures of external NAD(P)H regeneration, asymmetric bioreductions using OYEs are preferentially performed *in vivo*, thus utilizing the metabolic



Scheme 1. Catalytic cycle of OYEs.[19] EWG = electron-withdrawing group, for example, −CHO, −COR, −COOH/R, $-NO_2$ etc.

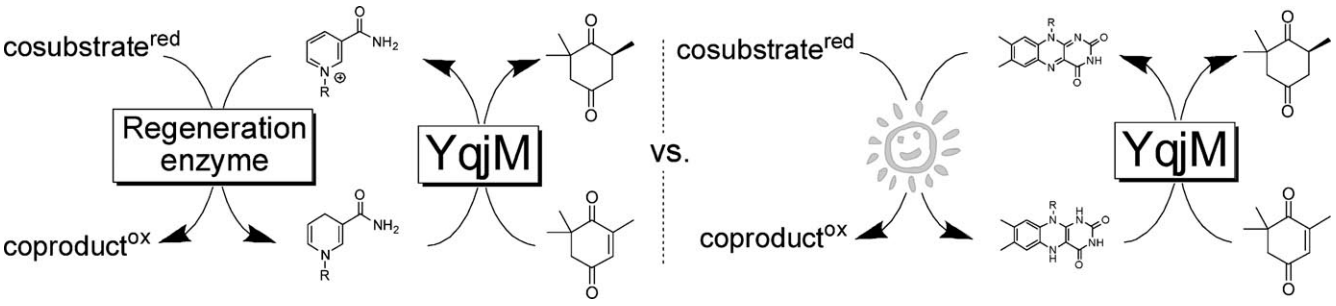
NAD(P)H regeneration capacity of the living cell.[9–11,14,27] However, ubiquitous NAD(P)H-dependent ketoreductases can cause undesired carbonyl reduction of substrates and products leading to lower yields and complex product mixtures. Even using isolated OYEs together with an enzymatic NAD(P)H regeneration system the procedure can suffer from this side reaction due to contaminating ketoreductases.[8]

An alternative approach circumventing this overreduction challenge would be to directly regenerate the flavin prosthetic group of OYEs. So, ketoreductases present in the reaction mixture would not be regenerated due to their exclusive NAD(P)H dependency. For this, 3 methods are available: (1) chemical regeneration of $FMNH_2$ for example, by transition metals such as $[Cp^*Rh(bpy)(H_2O)]^{2+}$,[28–31] (2) direct electrochemical regeneration,[32] and, (3) light-driven reduction exploiting the photoexitability of flavins.[33–36,39,48]

Due to its simplicity, we decided to evaluate the latter strategy to directly regenerate OYEs (Scheme 2).[34] Such an approach circumvents the costly and instable nicotinamide cofactor as well as a corresponding enzymatic regeneration system. As a result a highly simplified and more chemoselective production system is obtained (Scheme 2).

As a model enzyme we chose the OYE homologue from *Bacillus subtilis* (YqjM, E.C. 1.6.99.1).[37,40,41] As a model substrate we chose ketoisophorone.[8,34]

The aim of this study was to evaluate the efficiency of this photoenzymatic reduction and to investigate



Scheme 2. Comparison of: the 'traditional' regeneration approach for OYEs utilizing (enzymatic) regeneration of NAD(P)H (*left*) and the proposed light-driven direct regeneration of OYEs utilizing the prosthetic group as photocatalyst (*right*). For an overview over the proposed catalytic mechanisms, please refer to the Supporting Information.[35]

the influence of the single reaction parameters on its performance.

Results and Discussion

Enzyme Preparation

Envisaging a most simple catalyst preparation, we decided to heterologously express YqjM in *Escherichia coli* (*E. coli*) and use the crude cell extracts (CE) as biocatalyst preparation. The CE contained approximately 27% YqjM (w/w of total soluble protein, Supporting Information). We estimated the concentration of active YqjM to be 0.17 mg mL^{-1} (4.57 μM).

Reaction Set-Up

Initial photoenzymatic experiments were performed at ambient temperature and atmosphere using FMN as externally added photocatalyst. Under these conditions only trace amounts of product were observed. We attribute this to the high reactivity of reduced flavins with O_2 and concomitant formation of hydrogen peroxide.[42] Deaeration of the reaction mixture proved to be essential to achieve conversion.

Also the light source had a significant influence on the rate of the photoenzymatic reaction. Changing the light source from a conventional projector lamp (40 W, set-up 1) to a more intensive light bulb (250 W, set-up 2) increased the productivity, under otherwise identical conditions, by more than 50% (Supporting Information). This set-up was used for all subsequent reactions.

Chemo- and Enantioselectivity

Especially aldehydes are subject to undesired ketoreductase-catalyzed reduction to alcohols.[8] Therefore, we tested citral (3,7-dimethyl-2,6-octadienal) as substrate in the photoenzymatic reduction. Full conversion into citronellal was observed without detectable traces of either citronellol or geraniol or nerol. Thus we conclude that even by using crude *E. coli* cell extracts containing various endogeneous ketoreductases, chemoselective photobiocatalytic reduction of the C=C-double bond is feasible. By selectively reducing the flavin cofactor only enoate reductases such as YqjM are regenerated while NAD(P)H-dependent ketoreductases remain inactive.

The photoenzymatic reduction of ketoisophorone proceeded with comparably poor enantioselectivity (69% *ee*). Therefore, we investigated a range of potential causes. The optical purity of the product decreased by only 1–2% *ee* over the time course of the experiments; therefore, we exclude *in situ* racemization as a major factor influencing enantioselectivity. The rate of the non-enzymatic reduction was examined either in the absence of CE or using thermally inactivated CE (4 h at 90 °C). In both cases, a background activity of less than 0.27 turnovers per minute was determined for FMN in the non-enzymatic reduction, also not significantly contributing to the non-exclusive enantioselectivity. Furthermore, increasing [FMN], increasing the rate of the background reaction, did not negatively influence enantioselectivity (*vide infra*). However, using stoichiometric NAD(P)H as source of reducing equivalents, practically the same enantioselectivity (72% *ee*) was observed. Hence, we concluded that the enantioselectivity of the biocatalyst preparation was not optimal (*vide infra*) and that the 'unnatural reaction conditions' of the simplified reaction set-up did not impair enantioselectivity.

The Photocatalyst

Changing the photocatalyst from FMN to FAD or riboflavin as photocatalyst resulted in slight changes of both rate and enantioselectivity (Figure 1).

There was, however, no clear correlation between rate and enantioselectivity on the one side and structure of the photocatalyst on the other side. We attribute this variability to differences in the electron transfer rates between external reduced flavins and YqjM-bound FMN (the K_D value of FMN in YqjM lays in the lower μM range[37]).

In all cases, anaerobic conditions were crucial to observe conversion. This becomes obvious considering the high reactivity of reduced flavins with molecular oxygen yielding oxidized flavins and hydrogen peroxide.[42] However, when using synthetic, O_2-stable 5-deazaflavin as photocatalyst,[38,39] rapid reduction of

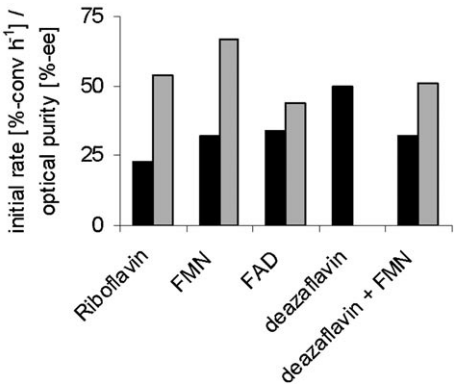


Figure 1. Influence of flavin type on the rate (black) and enantioselectivity (grey) of the photoenzymatic reduction reaction. Riboflavin, FMN, and FAD (100 μM each): anaerobic conditions; deazaflavin and deazaflavin + FMN (100 μM each): aerobic conditions.

ketoisophorone was observed even in the presence of O_2 albeit forming racemic product. Enantioselectivity could partially be reconstituted (50% *ee*, Figure 1) by addition of calaytic amounts of FMN to saturate YqjM's active site. This may be explained by the specificity of YqjM for FMN on the one hand and by more negative reduction potential of deazaflavin (>100 mV more negative than, for example, riboflavin)[46] on the other hand. Reduced deazaflavin seems to be a potent reductant for at least ketoisophorone without being recognized at the chiral active site of YqjM. Thus, in the absence of FMN only non-enantioselective background reduction occurs. If YqjM is saturated with FMN, also electron transfer from deazaflavin to YqjM-bound FMN occurs resulting in enzyme-related reduction competing with deazaflavin-mediated non-enantioselective reduction.

Influence of Reaction pH and Temperature

The influence of pH was examined between pH 5.1 and 9.4 showing a relatively broad activity maximum in the slightly alkaline range. Enantioselectivity slightly decreased with increasing pH, which is most likely explained by increased racemization *via* enolization (Figure 2).

Interestingly, also at pH 5, a significant reduction activity was observed contradicting earlier observations by Frisell et al.[35] who explained poor photocatalytic activity at low pH values with unfavourable N-protonation resulting in 'masked' N-lone pairs required for efficient electron transfer to the photoexcited oxidized FMN (FMN^+). Obviously, in case of EDTA [$pK_a(NH^+)=6.13$ and 10.37] already at pH 5 the concentration of unprotonated nitrogen is sufficiently high to enable efficient reduction of FMN^+. The activity decrease at elevated pH (>8.5) is most likely to be explained by decreasing YqjM activity. It should be mentioned, however, that so far a distinct pH (and *T*) profile of YqjM is lacking.

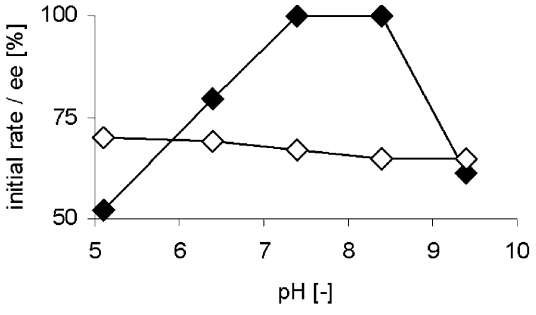


Figure 2. Influence of pH on activity (◆) and enantioselectivity (◇) of the photoenzymatic reduction of ketoisophorone. 100% corresponds to a initial productivity of 6.38 mM h^{-1}

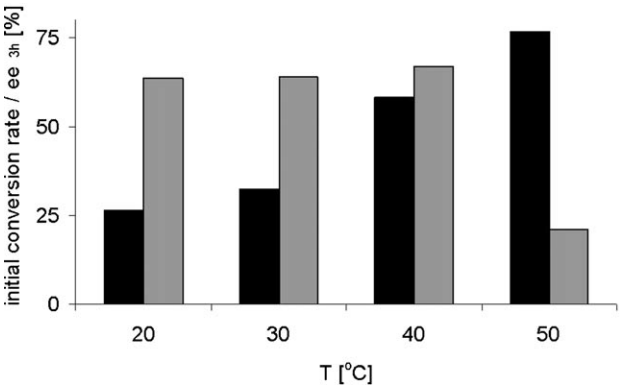


Figure 3. Influence of the reaction temperature on the maximal conversion rate (black) and optical purity (grey).

Initial rates correlated with the reaction temperature between 20 and 50 °C (Figure 3). However at 50 °C, productivity caved-in by more than 90% after only 30 min. Likewise, only racemic product was produced from this time on resulting in an overall *ee* of less than 25% after 4 h. Obviously, the enzyme stability was greatly impaired at 50 °C leading to fast inactivation. Below 50 °C, only a slight decrease of enantioselectivity was observed over time indicating significantly higher enzyme stability.

Rate-Dependence on [EDTA] and [FMN]

In the experiments performed so far, a nominal YqjM activity of approximately 0.5 U mg^{-1} was estimated corresponding to 7.1% of the activity determined in the NAD(P)H-dependent activity assay under comparable conditions. We suspected [$FMNH_2$] to be significantly below saturation concentration for YqjM. Therefore a series of experiments investigating the effect of varying [EDTA] and [FMN] on the overall reduction rate were performed to clarify this issue (Figure 4 and Figure 5).

The concentration of the sacrificial electron donor ([EDTA]) had no influence on the initial rate of the photoenzymatic reduction [$A_{spec}(YqjM)=0.64\pm0.03$ U mg^{-1}, Figure 4]. Apparently, under the given conditions [EDTA] does not influence the formation rate of $FMNH_2$ suggesting flavin photoexcitation to be rate-limiting.

EDTA contains per molecule 4 oxidizable *N*-alkylamino acid moieties. Therefore, principally 0.25 equivalents (based on substrate) should be sufficient to obtain full conversion. Under these conditions, however, the conversion rate leveled off significantly above 40–50% indicating that only 2 of the 4 oxidizable functionalities are available for FMN photoreduction.

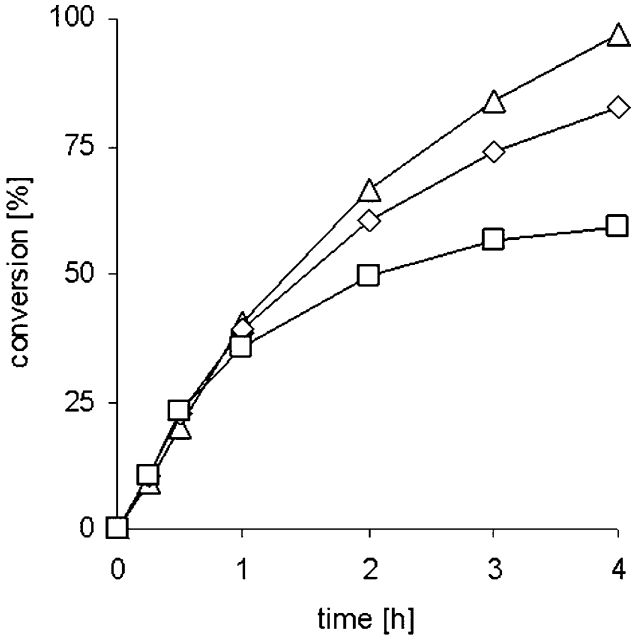


Figure 4. Influence of [EDTA] on the rate of the photoenzymatic reduction. [EDTA] = 2.5 mM (□), 5 mM (◇), 10 mM (△).

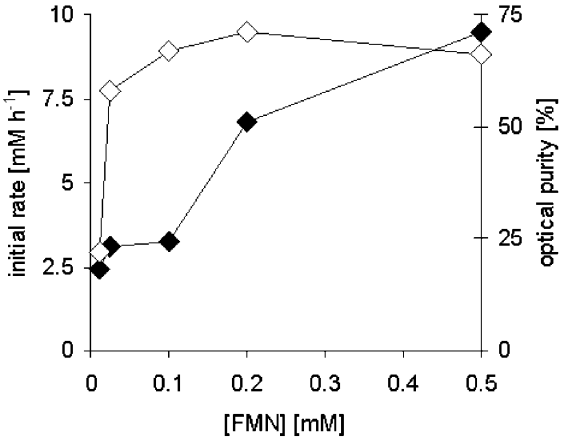


Figure 5. Influence of [FMN] on the rate (◆) and enantioselectivity (◇) of the photoenzymatic reduction reaction.

A plausible explanation for this may be the increased basicity of the secondary amines generated in the photooxidation process. Thus, the protonation equilibrium may be shifted towards the ammonium salts thereby slowing down the rate of the next electron transfer step to photoexcited FMN (Supporting Information).

Quite surprising results were obtained varying [FMN] (Figure 5). In accordance with our initial hypothesis, the overall rate correlated with [FMN] suggesting the formation rate of $FMNH_2$ to be overall rate-limiting. Accordingly, the specific activity determined for YqjM increased to 4.6 U mg^{-1} (65% of the nominal activity) in the presence of 0.5 mM FMN.

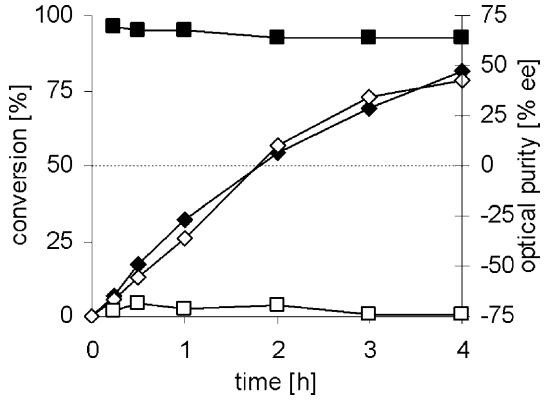


Figure 6. Comparison of rates (diamonds) and enantioselectivities (squares) photoenzymatic reductions of ketoisophorone using crude extracts of *E.coli* containing YqjM (filled symbols) and devoid of YqjM (open symbols).

Most interestingly, however, was that the enantioselectivity of the photoenzymatic reduction increased with increasing FMN concentration. This was unexpected assuming an increased background reduction at increased $FMNH_2$ concentrations. Obviously, *in situ* formed $FMNH_2$ is efficiently used by YqjM for reduction. Even more astonishingly, inversion of enantioselectivity was observed at FMN concentrations below 10 μM. One plausible explanation involves the presence of another, stereocomplementary biocatalyst with higher affinity towards or faster electron transfer rates with $FMNH_2$ than YqjM (*vide infra*).

Enoate Reductase Activity in *E. coli*

As stated above, the peculiar inversion of enantioselectivity at low [FMN] may be explained by the presence of an endogeneous *E. coli* enoate reductase exhibiting inverse enantioselectivity and higher affinity to $FMNH_2$. In fact, a putative OYE homologue, NemA, had been reported in *E. coli* by Miura et al.[44] Therefore, as a control experiment, we performed the light-driven reduction of ketoisophorone using a crude cell extract from *E. coli* cells devoid of the plasmid encoding for YqjM as biocatalyst. Indeed, under these conditions reduction of ketoisophorone occurred with opposite enantioselectivity compared to the use of YqjM as biocatalyst (Figure 6).

Thus, we have strong indications that NemA is the actual reason for the comparably poor enantioselectivity observed in the reduction of ketoisophorone.

Alternative Electron Donors

Finally, we performed a preliminary screening for suitable electron donors for the photoenzymatic re-

Table 1. Alternative sacrificial electron donors tested.

Electron donor	Rate [%][a]	*ee* [%]
EDTA	100	69
$NaHCO_2$	51	1
Na_2HPO_3	26	0
Methionine	14	n.d.
Triethyl phosphite	20	30
Nicotine	20	n.d.
Sarcosine	>1	n.d.

[a] The rate of using EDTA was set to 100%.

duction. EDTA may not be the optimal sacrificial reductant for a variety of reasons. From an atom efficiency point-of-view the necessity of at least 0.6 equivalents for full conversion appears questionable. Furthermore, formaldehyde and amines as side products are also not unproblematic from an environmental and toxicological point of view. We therefore performed a preliminary screening of other potentially more suited electron donors such as formate or phosphite (Table 1). In fact, EDTA could be substituted by HCO_2^- or HPO_3^{2-} to achieve photoenzymatic reduction.

Rates were somewhat decreased compared to EDTA but, more importantly, enantioselectivity was reduced as well (Supporting Information). Currently we are lacking a plausible explanation for this observation. Perhaps the enzyme enantioselectivity is impaired by the presence of these salts. Alternatively, the reduced *in situ* concentration of $FMNH_2$ (due to reduced reduction rate) may have a similar effect on enantioselectivity as observed with low total FMN concentrations.

Conclusions

Light-driven reduction of flavins is a feasible method for direct, cofactor-independent regeneration of OYEs such as YqjM and NemA. Thus, photoenzymatic reductions circumvent the need for the nicotinamide cofactor and a corresponding (enzymatic) nicotinamide regeneration system. Using the simplified, artificial regeneration system rates of up to 65% of the 'native', NADH-driven reaction have been reached while preserving the enantioselectivity. Another benefit of the simplified system is that NAD(P)H-dependent reduction of keto groups is circumvented yielding a more chemoselective alternative to 'classical' regeneration approaches. Unfortunately, enantioselectivity in the present system is impaired by the presence of two, enantiocomplementary enoate reductases (recombinant YqjM and endogeneous NemA). Using purified enzyme preparations, we expect also to obtain a highly enantioselective system.

Table 2. Performance summary of the photobiocatalytic reduction system.

	TF [min^{-1}]	TTN [$mol\,mol^{-1}$]
FMN	4	1000
YqjM	210	10.900

The influence of various reaction parameters on rate and enantioselectivity has been investigated putting a solid basis for further preparative-scale applications of the photoenzymatic reduction reaction. Already at this early stage of development, the catalytic performance of the photo- and biocatalyst are promising, as shown in Table 2. A direct comparison with the reported traditional approaches is not straightforward due to a lack of exact values.

One point of interest concerns the stability of the flavin photocatalyst. Isoalloxazines such as Rf, FMN and FAD have been reported to be rather labile under irradiation conditions.[47] In contrast, the high total turnover number observed for FMN in this study and some of our recent work[48] suggest that the reported photoinstability does not pose a severe limitation for our system. This is also in line with the proposed flavin-degradation products being photoactive isoalloxazines.[47]

Further work will concentrate on detailed analysis of the reaction mechanism including photodegradation and enzyme kinetics using purified preparations of YqjM and NemA. Furthermore, we will substantiate the very promising results using alternative, environmentally benign electron donors such as formate or phosphite.

Thus, we are convinced to obtain a simple, preparatively and economically attractive methodology for enantioselective C=C bond reductions.

Experimental Section

Production of YqjM

Fermentative production of YqjM was performed according to the method reported by Macheroux et al.[37] In brief, *E. coli* BL21-codon plus™-DE3(RIL) (Stratagene) containing pET21a encoding the gene for YqjM was fermented on 10-L scale at 37 °C using LB medium. YqjM production was induced at $OD_{600} = 0.6$ with 1 mM_{final} IPTG and fermentation was continued for 4 h. The harvested cells were resuspended in potassium phosphate buffer (50 mM, pH 7.5) and broken by two passages through a French press and centrifuged. The resulting clear crude cell extract was supplemented with 5 μM_{final} FMN and stored at −20 °C. Under these conditions enzyme activity was not impaired for at least 6 months.

Activity Assay

NADH-dependent YqjM activity was assessed spectrophotometrically at 340 nm ($\varepsilon = 6.22\ mM^{-1}$) using cyclohexenone 100 mM) as substrate. A specific C=C-bond reduction of $1.2\ U\,mL^{-1}$ was determined by subtracting substrate-independent aerobic NADH oxidation from the total activity. Based on these numbers and literature values [M(YjqM) = 37.4 kDa, $A_{spec} = 7.03\ U\,mg^{-1}$][37], the concentration of YqjM in the stock solution was estimated to be 4.57 μM).

Light-Driven Reduction Reactions

Unless stated otherwise photoenzymatic reduction reactions were performed on a 10-mL scale using potassium phosphate (100 mM, pH 7) at ambient temperature supplemented with 100 μM_{final} FMN, 25 mM EDTA, and 10 mM ketoisophorone. Prior to enzyme addition, the reaction mixture was degassed by leading N_2 gas through the reaction mixture for 15 min. Throughout the reaction, a gentle N_2 stream was passed over the reaction mixture. Afterwards, 0.914 μM_{final} YqjM was added and the reaction was started with insetting illumination (250 W bulb, Philips 7748 XHP). At intervals, samples were withdrawn, extracted with one aliquot of ethyl acetate (containing 5 mM dodecane as internal standard), dried over $MgSO_4$ and analyzed by GC. Alterations to this protocol are indicated in the text. Semi-quantitative determination of hydrogen peroxide was performed using Quantofix (Sigma–Aldrich).

Synthesis of Deazaflavin

5-Deazariboflavin (deazaflavin) was synthesized according to literature methods.[45]

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