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COMMUNICATION

Convergent cascade catalyzed by monooxygenase – alcohol dehydrogenase fusion applied in organic media

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Abstract: With the aim of applying redox-neutral cascade reactions in organic media, fusions of a type II flavin-containing monooxygenase (FMO-E) and horse liver alcohol dehydrogenase (HLADH) were designed. The enzyme orientation and expression vector were found to influence the overall fusion enzyme activity. The resulting bifunctional enzyme retained the catalytic properties of both individual enzymes. The lyophilized cell free extract containing the bifunctional enzyme was applied for the convergent cascade reaction consisting of cyclobutanone and 1,4-butanediol in different micro-aqueous media with only 5% (v/v) aqueous buffer without any addition of external cofactor. Methyl tert-butyl ether and cyclopentyl methyl ether were found to be the best organic media for the synthesis of γ-butyrolactone resulting in ~27% analytical yield.

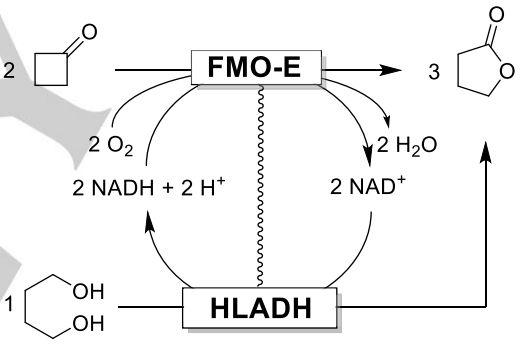
Introduction

Nature uses elegant synthetic strategies by coupling enzymes in metabolic pathways, in which the product of one enzyme is the substrate of the next enzymatic reaction. The catalytically related enzymes often form complexes to increase the efficiency of these enzymatic cascade reactions, such as the pyruvate dehydrogenase complex.[1] The design of artificial multi-enzymatic reactions has been of great interest in biocatalysis during the last decades.[2-4] Cascade reactions have become attractive, especially for redox biocatalysis, since the internal cofactor regeneration can be achieved, creating self-sufficient redox reactions.[5-7] An NADH-dependent redox-neutral convergent cascade reaction composed of a recently discovered type II flavin-containing monooxygenase (FMO-E) and horse liver alcohol dehydrogenase (HLADH) has been established in our previous work.[8] Two model cascade reactions were analyzed for the synthesis of γ-butyrolactone and chiral bicyclic lactones, respectively. In the targeted cascade reaction, FMO-E catalyzes the Baeyer-Villiger oxidation of the cyclic ketone into a lactone at the expense of NADH, while HLADH regenerates NADH while producing the same lactone from the precursor diol substrate (Scheme 1).

In addition to cascade reactions, the use of non-conventional media in biocatalysis has also been attracting great interest since the use of water as reaction medium may have several limitations, such as (i) low solubility of hydrophobic substrates/products, (ii) undesired side reactions, (iii) tedious downstream processing, (iv) enzyme inhibition issues by substrates/products dissolved in water, and (v) microbial contamination.[9] Although a two-liquid-phase system, typically using 50:50 ($v_{organic}$:$v_{aqueous}$) organic-to-aqueous phase, is an approach that takes away some of these limitations, it is necessary to use higher volumetric ratios to achieve higher partitioning in the organic phase under equilibrium conditions.

Another alternative is the use of (predominantly) non-aqueous media. In order to use non-aqueous media i.e. solvent-free systems or organic solvents for redox catalysis, cofactor regeneration is still a challenge to be dealt with. During the 1980s and 1990s, extensive studies on the use of oxidoreductases in water-deficient conditions were reported by the research groups of Klibanov[9-11] and Adlercreutz[12-16]. It has been shown that substrate-coupled cofactor regeneration is possible in low-water media,[11,17-18] whereas the enzyme-coupled cofactor regeneration is still not trivial as the nicotinamide cofactor (oxidized and reduced forms) need to diffuse from one active site to the second one during the course of the reaction.

One potential solution to make the enzyme-coupled cofactor regeneration possible in low-water media is to fuse the two enzymes, so that the "cofactor travel distance" can be kept as short as possible, avoiding the degradation of nicotinamide cofactor by reaction media e.g. organic solvents.[19, 20]



Scheme 1. Fusion of type II flavin containing monooxygenase (FMO-E) and horse liver alcohol dehydrogenase (HLADH) applied in a convergent cascade reaction for the synthesis of γ-butyrolactone as a model lactone product.

The aim of this study is to investigate whether fusing a monooxygenase and an alcohol dehydrogenase generates a bifunctional enzyme that can be used to catalyze a convergent cascade reaction in micro-aqueous media, using predominantly organic solvents.

Results and Discussion

Design and construction of the fusion enzymes

Firstly the FMO-E-encoding gene from *Rhodococcus jostii* RHA1 and the HLADH isoenzyme E-encoding gene from *Equus caballus* were fused in both orientations in vector pET-28a(+). In this way, either of the enzymes being influenced by its fusion partner in one orientation could be identified. The two enzymes were fused using a short glycine-rich peptide linker (SGSAAG), which has been found to be flexible in structure and typically does not influence the functioning of the fused enzymes.[21-24] The two resulting fusion enzymes were overexpressed in *E. coli* BL21 (DE3) and the activities of their cell free extracts (CFEs) were measured and compared with the individual non-fused enzymes (Table 1). The FMO-E oxidation activity was analyzed using 10 mM cyclobutanone as substrate while the HLADH oxidation activity was analyzed with 10 mM 1,4-butanediol.

The enzyme fused with HLADH at the C-terminus side retained much more activity than the reverse one, which showed almost no activity. This phenomenon is consistent with many other studies, in which short-chain dehydrogenases/reductases (SDRs) would lose activity and/or stability when fused as C-terminal fusion protein.[21, 25] This might be caused by the perturbation of the oligomers formed by these ADHs.[26] However, the relative activities of the two enzymes in the best performing fusion enzyme (FMO-E–HLADH) were also much lower (~10%) than their individual ones. The fusion construct with FMO-E fused as the N-terminal fusion partner was then cloned into the pBAD vector and expressed in *E. coli* Top10, since FMO-E was originally expressed in this way.[27] The CFE of *E. coli* Top10 cells expression fusion construct showed approximately three times higher activities for both enzymes (Table 1), perhaps because of higher enzyme expression (Figure S1).

Table 1. Activity assay of individual and fusion enzymes.

Enzyme	Vector	Host	FMO-E		HLADH	
			Specific activity (U/g)[a]	Relative activity	Specific activity (U/g)[a]	Relative activity
FMO-E	pBAD	*E. coli* Top10	296	100%	–	–
HLADH	pET-28a(+)	*E. coli* BL21 (DE3)	–	–	150	100%
FMO-E—HLADH	pET-28a(+)	*E. coli* BL21 (DE3)	40	14%	16	11%
HLADH—FMO-E	pET-28a(+)	*E. coli* BL21 (DE3)	3	1%	3	2%
FMO-E—HLADH	pBAD	*E. coli* Top10	116	39%	45	30%

[a] The specific activity was calculated based on the protein concentration of cell free extract (CFE).

Enzyme purification and steady-state kinetic analysis

The fusion construct FMOE-HLADH cloned in the pBAD vector was then expressed in *E. coli* Top10 and purified *via* nickel affinity chromatography. The purification yield was approximately 40 mg of fusion enzyme per liter of culture broth after optimization of enzyme expression. The purified enzyme displayed a light-yellow color which was indicative of binding of the flavin cofactor in FMO-E. From SDS-PAGE analysis of fusion enzyme purification, it was clear that proteolytic cleavage of a significant part of the purified fusion enzyme was observed (Figure 1), which could occur due to the sonication conditions and purification process. There were two proteins bands detected in the purified fusion enzyme, the upper band (~100 kDa) was the fusion enzyme (FMO-E–HLADH) while the lower one (~64 kDa) was the FMO-E part of the fusion enzyme. Since FMO-E had the His-tag as N-terminal tag, it could be also purified even after the proteolytic cleavage of the fusion enzyme whereas the HLADH part was lost during the purification process. To circumvent the proteolytic cleavage of fusion enzyme due to and during the cell disruption, the French Press method (3 × 13000 psi on ice) was applied instead of sonication.

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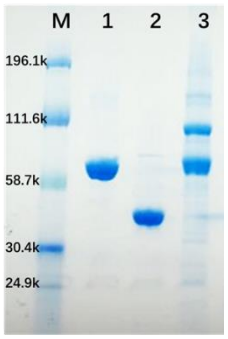

Figure 1. SDS-PAGE analysis of purified individual and fusion enzymes. M: Marker; lane 1: FMO-E; lane 2: HLADH; lane 3: fusion enzyme FMO-E–HLADH.

To verify the influence of fusion on the two enzymes, the kinetic parameters of the fusion enzyme and the individual enzymes were determined (Figure S4). Both enzymes showed approximately 60–70% activities (k_{cat} values) compared to the non-fused enzymes (Table 2), which may be caused by structural effects of bringing the two enzymes together. For FMO-E in the fusion enzyme, the affinity towards the substrate was affected by the fusion since the K_M value for cyclobutanone increased by two times. Conversely, the HLADH in the fusion enzyme showed a somewhat lower K_M value, indicating that the affinity was hardly affected by the fusion. It was gratifying to note that the fusion enzyme displayed a higher K_i value towards 1,4-butanediol, which meant that the inhibition effect of the substrate on HLADH was alleviated. Overall, the fused enzyme largely retained the catalytic properties of the individual non-fused enzymes and was further investigated for its potential application in non-conventional media.

Table 2. Kinetic constants of purified fusion and individual enzymes.

Enzyme	K_M (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	K_i (mM)
FMO-E	2.4 ± 0.7	1.9 ± 0.2	2	–
Fusion_FMO-E[a]	5.9 ± 1.6	1.3 ± 0.2	1.4	–
HLADH	2.4 ± 0.4	4.8 ± 0.2	3.2	2082 ± 755
Fusion_HLADH[b]	2.1 ± 0.8	2.9 ± 0.5	1.9	6026 ± 275

[a] Activity assay applied for the fusion protein for the evaluation of the activity of FMO-E; [b] Activity assay applied for the fusion protein for the evaluation of the activity of HLADH. Fusion_FMO-E: FMO-E–HLADH assayed for FMO-E activity, Fusion_HLADH: FMO-E–HLADH assayed for HLADH activity.

Lyophilization of cell free extract of fusion enzyme

Since the aim of this study is to investigate the application of the fusion enzyme in non-conventional media, lyophilization of the fusion enzyme can facilitate the use of enzymes in non-aqueous media as well as shipping and storage in general.[28] From our previous study,[8] FMO-E was identified as having low thermo- and storage stability. Denaturation and deactivation of enzymes can take place upon freeze drying. However, additives can reduce the aggregation/inactivation during the lyophilization or rehydration process and hence can compensate for the loss of essential water during lyophilization.[29] Therefore, we put effort in optimizing the lyophilization conditions of the fusion enzyme, especially for FMO-E part in the fused protein by using additives i.e. lyoprotectants. Sugars such as sucrose are widely used as lyoprotectants for the lyophilization process.[29-31] Besides sugars, there are also other reported lyoprotectants, such as salts, reducing compounds and amino acids.[30] Based on the investigation of literature, we focused on sucrose and magnesium sulfate ($MgSO_4$), since they have been shown to stabilize in the lyophilization of many BVMOs.[30]

To determine the protective effect of different concentrations of the selected additives, the CFE of fusion enzyme was lyophilized with 10, 20, and 50 mg/mL sucrose, 10, 50, and 200 mM magnesium sulfate as well as a combination of 20 mg/mL sucrose and 25 mM $MgSO_4$. Most of the additives resulted in a positive effect on the protection of HLADH part, while only low concentrations of $MgSO_4$ as additive could preserve activities of FMO-E during lyophilization. The combination of sucrose and $MgSO_4$ did not show any additive effect, but only displayed a compromise of these two components. 50 mM $MgSO_4$ was found to be the best lyoprotectant for both FMO-E and HLADH. This slight improvement was significant enough to perform the lyophilization of the fusion enzyme with 50 mM $MgSO_4$.

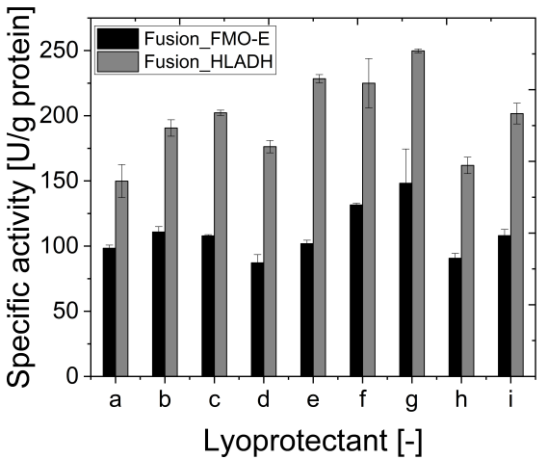

Figure 2. Influence of lyoprotectants on the lyophilization of fusion enzyme. (a) Before lyophilization, (b) No lyoprotectant, (c) 10 mg/mL sucrose, (d) 20 mg/mL sucrose, (e) 50 mg/mL sucrose, (f) 10 mM $MgSO_4$, (g) 50 mM $MgSO_4$, (h) 200 mM $MgSO_4$, and (i) 20 mg/mL sucrose + 25 mM $MgSO_4$. CFE of fusion enzymes were prepared in 10 mM pH 7.5 Tris-HCl buffer. Results are average values from duplicated experiments. Fusion_FMO-E: FMO-E–HLADH assayed for FMO-E activity, Fusion_HLADH: FMO-E–HLADH assayed for HLADH activity.

Employing fusion enzyme in micro-aqueous system

The lyophilized CFE of fusion enzyme (in the presence of $MgSO_4$) was then applied for catalyzing the model convergent cascade reaction in micro-aqueous system with 5% (v/v) buffer. Seven organic solvents, acetonitrile (ACN), isopropanol (IPA), tetra-hydrofuran (THF), ethyl acetate (EtOAc), methyl *tert*-butyl ether (MTBE), cyclopentyl methyl ether (CPME) and *n*-heptane, were selected based on their different polarities (log*P*, logarithmic value of octanol-water partition coefficient) (Table S2) and also as they have been applied in the reactions catalyzed by dehydrogenases and monooxygenases.[32-36] Among these screened organic

solvents, the two ether solvents, MTBE and CPME, gave the highest product concentration (Figure 3), whereby the log*P* value of MTBE is 1.0 and log*P* value of CPME is 1.41. These results indicate that solvent functionality and structure is also an important parameter for enzyme deactivation. On the other hand, there was no product detected in the reaction systems containing ACN, IPA and THF, in which these organic solvents with low log*P* values tended to strip the essential enzyme-bound water from the enzymes and resulted in less molecular flexibility for catalysis. The fused enzyme-catalyzed control reaction in aqueous buffer generated ~3.5-fold less product (1.9 mM) than that synthesized in MTBE (7.2 mM) and CPME (7 mM) micro-aqueous systems (Figure 3). It is worth mentioning here that CPME currently refers to be an environmentally benign solvent. It has been applied in a micro-aqueous system with 10% (v/v) buffer for the reduction of β-carboline harmane and 1-methyl-3,4-dihydroisoquinoline to the corresponding amines catalyzed by an imine reductase (IRED).[37] MTBE has also been applied for the reduction of a series of ketones catalyzed by an ADH and promoted by smart co-substrate 1,4-butanediol in a micro-aqueous system with only 2.5% (v/v) buffer.[34] In that study, MTBE was selected among a series of organic solvents with log*P* ranging from 1.0 to 5.6 owing to its high conversion, low boiling point and good biocompatibility. It is commonly accepted that, solvents with log*P* > 4 cause negligible inactivation of enzymes, while those solvents with log*P* < 2 are highly inactivating, and the effect of log*P* values between 2 and 4 is hard to predict.[38-40] However, in this study, log*P* cannot be the direct/only criterion to choose an organic solvent for enzymatic reactions.

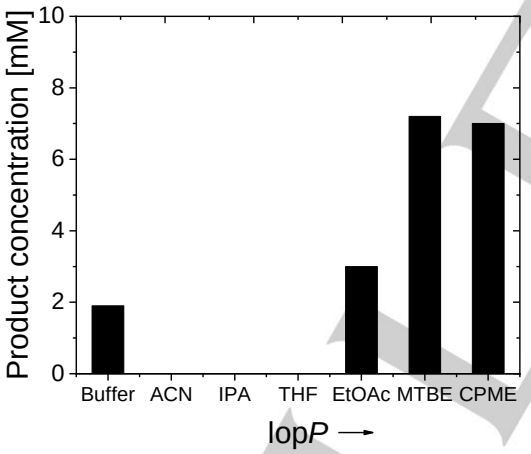

Figure 3. Screening of organic solvents for the convergent cascade reaction. Reaction conditions: *c*(cyclobutanone) = 20 mM, *c*(1,4-butanediol) = 10 mM, 95% (v/v) organic solvent, 5% (v/v) external water (40 mM Tris-HCl, pH 7.5), 50 µL CFE, 20°C, 900 rpm, and 48 h. log*P* (ACN) = -0.33, log*P* (IPA) = 0.05, log*P* (THF) = 0.53, log*P* (EtOAc) = 0.7, log*P* (MTBE) = 1.0, log*P* (CPME) = 1.41, and log*P* (*n*-heptane) = 4.47. 50 mM $MgSO_4$ was used as lyoprotectant for the preparation of the enzymes.

The time-courses of the lyophilized CFE of the fusion enzyme catalyzed model convergent cascade reaction in the micro-aqueous system of MTBE and CPME are shown in Figure 4. The two reaction systems showed almost the same progress curves and both resulted in ~8 mM product (~27% analytical yield) after 48 h. Whereas when unfused enzymes –prepared under the same lyophilization conditions as in the case of fused enzyme– were applied in the micro-aqueous media, lactone concentration was 3.5 mM in MTBE and 2 mM in CPME (Figure 4). This can be attributed to the reduced travel distance of cofactor while using the fused enzyme. On the other hand, the effect of different organic media i.e. MTBE vs CPME became significant while using the unfused enzymes. It is worth mentioning here that these reactions were performed without external nicotinamide cofactor and hence it was only driven by the nicotinamide cofactors present in the CFE. The model reaction was also performed under the same conditions with an additional 0.5 mM NAD^+ in the two systems.

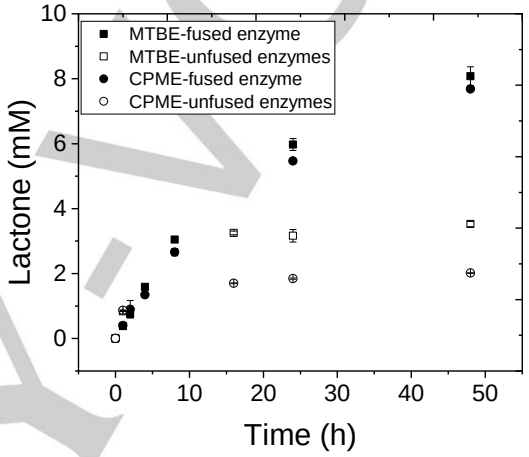

Figure 4. Time-courses of the lyophilized fusion enzyme FMO-E–HLADH and lyophilized unfused individual enzymes catalyzing convergent cascade reaction in the micro-aqueous system of MTBE and CPME. Reaction conditions: *c*(cyclobutanone) = 20 mM, *c*(1,4-butanediol) = 10 mM, 95% (v/v) organic solvent, 5% (v/v) external water (40 mM Tris-HCl, pH 7.5), 1.5 mg/mL lyophilized fusion enzyme or 1.0 mg/mL lyophilized FMO-E and 0.5 mg/mL lyophilized HLADH, 20°C, 900 rpm, and 48 h. Results are average values from triplicated experiments. 50 mM $MgSO_4$ was used as lyoprotectant for the preparation of the enzymes.

The addition of external NAD^+ resulted in a ~20% increase in product formation in the case of fused enzyme (data not shown), which means that the cofactor is a minor limitation for the two reaction systems. Possibly one of the two enzymes was slowing or stopping, as it will cause the other enzyme to stop, since both enzymes rely on the cofactor form (reduced or oxidized) that the other enzyme produces.

Conclusion

In this study, we have demonstrated the first application of a bifunctional fusion enzyme catalyzed convergent cascade in micro-aqueous media. A type II flavin-containing monooxygenase and an alcohol dehydrogenase were combined with a glycine rich linker to be a bifunctional fusion enzyme. Sucrose and magnesium sulfate showed a positive effect on the lyophilization of the CFE containing the overexpressed fusion enzyme. The lyophilized CFE of fusion enzyme was applied for the convergent cascade reaction consisting of cyclobutanone and 1,4-butanediol in micro-aqueous media with only 5% (v/v) aqueous buffer without any addition of external cofactor. MTBE and CPME were found to

be the best organic solvents in the micro-aqueous media for the fused protein FMO-E–HLADH. Overall, the here presented cascade reaction catalyzed by fused oxidoreductase enzymes in predominantly organic media shows the high potential for these fragile enzymes to be employed in non-conventional conditions.

Experimental Section

Chemicals, reagents, enzymes and strains: Chemicals, media components, and reagents were purchased from Sigma–Aldrich (St. Louis, USA), Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland) or Acros Organics (Geel, Belgium) and used without further purification. Nickel-NTA affinity resin was ordered from Expedeon (Cambridgeshire, UK) and BCA protein quantification kit (Pierce™) was obtained from Thermo Scientific (Rockford, USA). The recombinant pET-28b plasmid containing HLADH gene was from Dr. Diederik Johannes Opperman (University of Free State, South Africa). Oligonucleotides were obtained from Sigma-Aldrich (St. Louis, USA). T4 ligase and restriction enzyme BsaI were ordered from New England Biolabs (Ipswich, USA). The PfuUltra Hotstart PCR master mix was purchased from Agilent Technologies (Santa Clara, USA). *E. coli* NEB® 10-beta chemically competent cells were purchased from New England Biolabs (Ipswich, USA) and used as host for cloning of the recombinant plasmids. Chemically competent *E. coli* BL21 (DE3), *E. coli* Top10 cells were purchased from Invitrogen (Carlsbad, USA) and used as host for protein expression. Details on experimental protocols and analytics are found in Supporting Information.

Employing fusion enzyme in a convergent cascade in micro-aqueous media: Lyophilized CFE of fusion enzyme (from 2 mL CFE) was re-dissolved in 500 µL ddH_2O. Cyclobutanone (5.95 µL) at final concentration of 20 mM and 1,4-butanediol (3.6 µL) at final concentration of 10 mM were prepared in aforementioned organic solvents (4 mL) to form substrate stocks. The reactions were started by adding 50 µL enzyme solution in 950 µL substrate stocks. Therefore, the starting concentrations were 20 mM of cyclobutanone, 10 mM 1,4-butanediol, 95% (v/v) organic solvent and 5% (v/v) of aqueous buffer. The total reaction volume was 1.0 mL and the reaction mixtures were kept ay 20°C and 900 rpm. Aliquots samples (50 µL) from the organic phases were taken at definite time intervals and mixed with 250 µL of ethyl acetate, followed by vigorously mixing and drying over anhydrous $MgSO_4$. The samples were then analyzed by GC. If phase separation occurred, the ethyl acetate phase was taken for GC analysis.

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Keywords: Fusion Enzyme • Baeyer-Villiger Monooxygenase • Alcohol Dehydrogenase • Convergent Cascade Reaction • Biocatalysis in Organic Media

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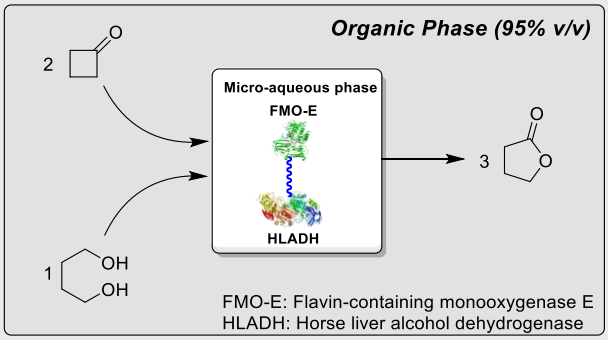

Lei Huang, Friso S. Aalbers, Wei Tang, Robert Röllig, Marco W. Fraaije, Selin Kara**

Page No. – Page No.

Convergent cascade catalyzed by monooxygenase – alcohol dehydrogenase fusion applied in organic media