Christin Peters and Rebecca Buller*

Linear enzyme cascade for the production of (–)-*iso*-isopulegol

https://doi.org/10.1515/znc-2018-0146
Received September 26, 2018; revised December 4, 2018; accepted December 9, 2018

Abstract: Biocatalysis has developed enormously in the last decade and now offers solutions for the sustainable production of chiral and highly functionalised asset molecules. Products generated by enzymatic transformations are already being used in the food, feed, chemical, pharmaceutical and cosmetic industry, and the accessible compound panoply is expected to expand even further. In particular, the combination of stereo-selective enzymes in linear cascade reactions is an elegant strategy toward enantiomeric pure compounds, as it reduces the number of isolation and purification steps and avoids accumulation of potentially unstable intermediates. Here, we present the set-up of an enzyme cascade to selectively convert citral to (–)-*iso*-isopulegol by combining an ene reductase and a squalene hopene cyclase. In the initial reaction step, the ene reductase YqjM from *Bacillus subtilis* selectively transforms citral to (*S*)-citronellal, which is subsequently cyclised exclusively to (–)-*iso*-isopulegol by a mutant of the squalene hopene cyclase from *Alicyclobacillus acidocaldarius* (*Aac*SHC). With this approach, we can convert citral to an enantiopure precursor for isomenthol derivatives.

Keywords: biocatalysis; ene reductases; enzyme cascade; (–)-*iso*-isopulegol; squalene hopene cyclases.

1 Introduction

1.1 Biocatalytic cascades

In their broadest sense, cascade reactions are defined as the combination of several chemical reactions in one pot [1]. The main advantages of cascade reactions lie in the elimination of intermediate isolation and purification steps, thus allowing for a reduction of costs, production time and waste compared to classical step-by-step synthesis. Using a cascade strategy may also lead to an overall increase in yield, as unstable intermediates are directly consumed by the next step and are not accumulated in the reaction mixture [2].

In biocatalytic cascades, at least one reaction step is catalysed by a biocatalyst in the form of either isolated enzymes, immobilised enzymes, crude cell extracts or whole microbial cells [3, 4]. Nature frequently uses biocatalytic cascades as an efficient system to allow for life and growth, thus being the best teacher for the set-up of artificial analogues. The design of man-made in vivo and in vitro biocatalysis cascade reactions is additionally facilitated by the fact that most enzymes work in aqueous buffer systems with similar pH and temperature profiles, thus simplifying the establishment of in situ approaches. Due to the decreasing costs for gene synthesis and the development of improved enzyme engineering as well as high-throughput technologies [5], a plethora of artificial biocatalytic cascade reactions have been developed in the last two decades [2–4, 6, 7].

Biocatalytic cascades can be classified by reaction concepts into linear, orthogonal, parallel and cyclic cascades (Figure 1) [8]. Linear cascade reactions are the most prevalent. Here, one substrate is converted via one or more intermediates to the product. An example for a linear biocatalytic cascade reaction is the production of (*R*)- or (*S*)-1-phenyl ethyl amine from ethyl benzene via oxyfunctionalisation by a peroxygenase followed by amination catalysed by a transaminase [9]. In case of parallel cascade reactions, two transformations are coupled via their respective co-factor recycling to generate two products from two different starting materials in a redox neutral fashion. In contrast to parallel cascades, orthogonal cascade reactions employ additional enzymes for co-factor or co-substrate regeneration or side-product elimination. Cyclic cascade reactions are in most cases chemo-enzymatic reactions including a chemical racemisation [8]. Classification into one of the four concepts is not always absolutely clear, in which case the classification depends on the most important part of the cascade reaction.

***Corresponding author: Rebecca Buller,** Competence Center for Biocatalysis, Institute of Chemistry and Biotechnology, Zürich University of Applied Sciences, Einsiedlerstrasse 31, 8820 Wädenswil, Switzerland, E-mail: Rebecca.Buller@zhaw.ch.
https://orcid.org/0000-0002-5997-1616
Christin Peters: Competence Center for Biocatalysis, Institute of Chemistry and Biotechnology, Zürich University of Applied Sciences, Einsiedlerstrasse 31, 8820 Wädenswil, Switzerland

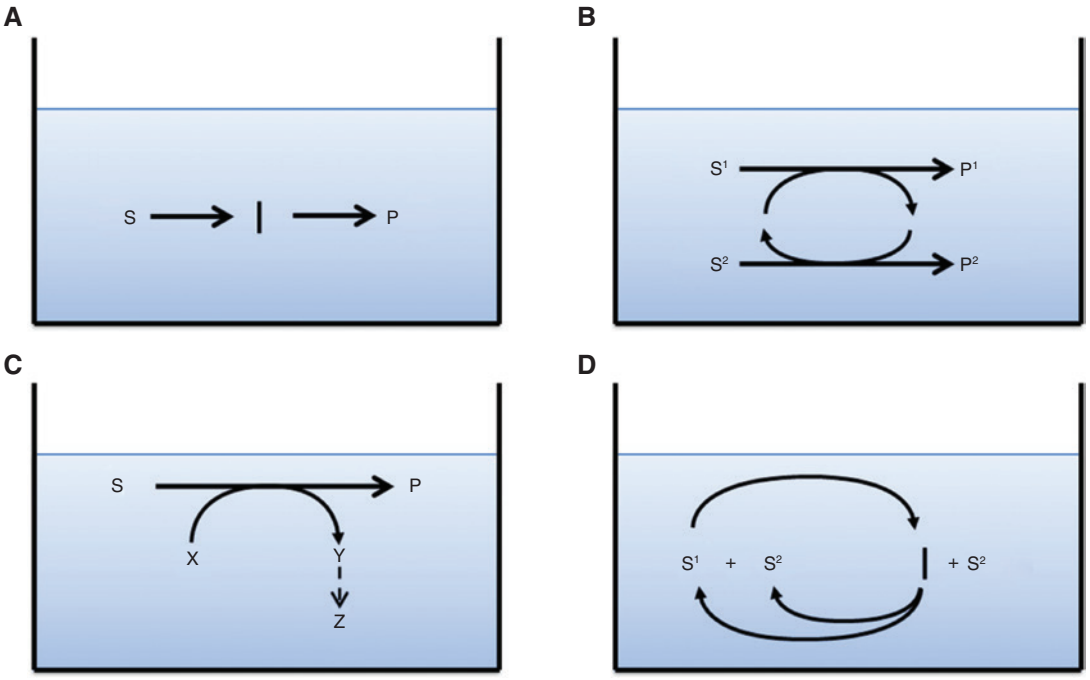

Figure 1: Different types of biocatalytic cascade reactions: (A) linear cascade, (B) parallel cascade, (C) orthogonal cascade, and (D) cyclic cascade.

1.2 Ene reductase

Members of the ene reductase (ER) enzyme class are effective biocatalysts for stereo-selective *trans*-hydrogenation of activated alkenes [10–12] complementing the respective chemical *cis*-hydrogenations via chiral rhodium or ruthenium phosphines (Knowles and Noyori, Nobel Prize in Chemistry 2001) [13, 14]. ERs are NAD(P)H-dependent oxidoreductases and can create up to two stereogenic centres in a single step by catalysing the stereo- and enantio-selective reduction of α,β-unsaturated ketones, aldehydes, nitro alkenes and carboxylic acids [10]. ERs can be divided into several families: old yellow enzymes (OYEs), the Fe-S cluster-depending enoate reductases, medium-chain dehydrogenases and short-chain dehydrogenases. Thanks to their broad substrate scope and favourable physico-chemical properties, members of the OYE family are currently most often used for synthetic purposes [12]. The first enzyme of this family was discovered by Warburg and Christian in bottom-fermented yeast and named "yellow enzyme" [15]. Subsequently, several other members of the yellow enzyme family were found, and the first ER was redefined as "OYE-I" [16]. Since then, various ERs that belong to the OYE family have been described from yeasts, bacteria, plants, fungi, and eukaryotes [17, 18].

The catalytic mechanism that all enzymes of the OYE family follow is well understood [19]. The enzymes use a bi-bi ping-pong mechanism that can be divided into the reductive half-reaction, in which flavin mononucleotide is reduced through hydride transfer from NAD(P)H, and the oxidative half-reaction, in which the hydride is transferred from the reduced flavin to the Cβ-atom of the activated alkene. To complete the reaction, the missing proton for the Cα is provided by a tyrosine residue [19, 20]. This specific mechanism typically leads to an *anti*-addition hydrogenation [21]. For the OYE enzyme family, it has been found that their natural stereo-specificity is nearly always the same [22]. However, the stereo-preference can be changed by point mutations making available stereo-complementary pairs of ERs [23].

All known enzymes of the OYE family have a typical TIM-barrel as basic structure [24]. The enzymes of the OYE family are further subdivided into two main subclasses: the classical OYEs and the thermophilic-like OYEs. The two classes can be distinguished by sequence homology and structural features [10]. One of the first classified thermophilic-like enzymes is YqjM from *Bacillus subtilis* [25, 26]. This well-characterised enzyme shows a broad substrate scope from aliphatic aldehydes to unsaturated esters [27].

1.3 Squalene hopene cyclases

Terpenes comprise the most structurally diverse family of natural products, currently numbering >80,000 members

in a greater family that also includes steroids and carotenoids [28]. Thus, terpene cyclases, the enzymes that catalyse the formation of these polycyclic compounds in a regio- and stereo selective fashion, are an attractive source for generating compound diversity. Terpene cyclases are classified according to the chemical strategy for initial carbocation formation: class I terpene cyclases use a trinuclear metal cluster to break a labile allylic diphosphate ester bond yielding an allylic cation and inorganic pyrophosphate, whereas a class II terpene cyclase relies on a general acid in the active centre to protonate the terminal ispoprene unit [29]. Due to their reaction mechanism, enzymes belonging to class I are restricted to substrates containing diphosphates, while the class II enzymes may have a broader substrate scope.

Squalene hopene cyclases (SHCs) are class II terpene cyclases and initiate cyclisation of their natural substrate squalene, a C_{30} triterpene, via the activity of an unusually acidic aspartic acid residue acting as a natural Brønsted acid. Controlled by the hydrophobic pocket of the enzymes, the polycyclisation reaction proceeds in a predetermined manner and a final deprotonation or water addition leads to the formation of the products hopene or hopanol [30]. Interestingly, SHCs show substantial substrate promiscuity and are also capable of catalysing the cyclisation of small (C_{10} to C_{20}) terpenoids and terpenoid-like molecules [31–33].

The best-characterised SHC, *Aac*SHC, was isolated from *Alicyclobacillus acidocaldarius* and is the only SHC for which a crystal structure could be elucidated [34, 35]. The dimeric thermostable enzyme is capable to catalyse the cyclisation of small compounds such as geranylacetone (C_{13}) but also accepts C_{35} non-natural polyprenoids [29].

Siedenburg et al. showed for citronellal that the inherent catalytic properties of SHCs are more generally utilizable. Using wild-type enzymes or variants with only one amino acid exchange, SHCs were able to convert citronellal to the different isopulegols via a Prins reaction [32, 36, 37]. More specifically, an *Aac*SHC mutant (*Aac*SHC_I261A) was identified that converts 2 mM (*S*)-citronellal to the single diasteromer (–)-*iso*-isopulegol with 8% conversion after 40 h at 30 °C [38, 39].

1.4 Biocatalytic cascade reaction toward (–)-*iso*-isopulegol

To accomplish our goal to transform (*E/Z*)-citral (**1**) selectively to (–)-*iso*-isopulegol (see Figure 2), an important intermediate in the synthesis of isomenthol derivatives [40], we chose an enzymatic cascade approach by coupling the well-known ER YqjM from *B. subtilis* [26] with the SHC variant *Aac*SHC_I261A [38]. The ER YqjM converts **1** with >95% ee and good activity into the desired *S*-product **2** [25, 27, 41], while *Aac*SHC I126A has been reported to exhibit precise conformational control of the cyclisation of (*S*)-citronellal forming exclusively (–)-*iso*-isopulegol (selectivity >95%) [38].

2 Results and discussion

2.1 In vivo enzyme cascade

First, synthetic or isolated genes encoding the selected enzymes for the biocatalytic cascade were cloned into vector pET28b+ before enzymes were expressed individually in *Escherichia coli* BL21 (DE3) to determine best cultivation and reaction conditions. The reactivity optimum of YqjM and *Aac*SHC_I261A in different buffer

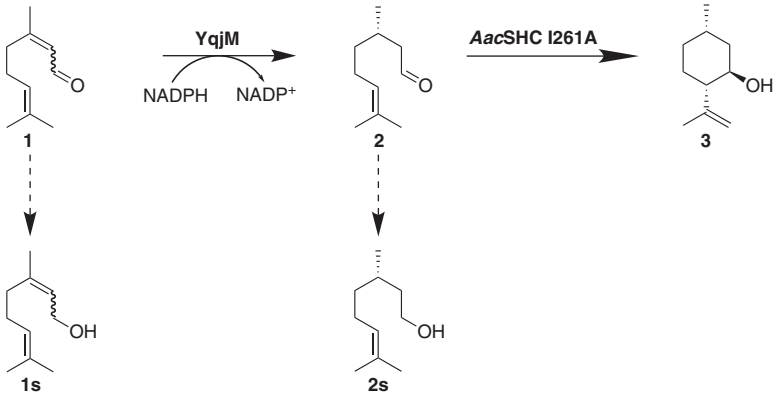

Figure 2: Enzyme cascade capable of converting citral (**1**) via citronellal (**2**) to (–)-*iso*-isopulegol (**3**).

systems was established by carrying out whole-cell biocatalysis reactions in the presence of either 50 mM sodium phosphate buffer (pH 7.5), 50 mM sodium citrate buffer (pH 6.0) or 50 mM sodium citrate buffer (pH 6.0) containing 0.2% Triton X-100. Analysis of the reaction mixtures after 24 h showed that YqjM exhibited the best conversion from **1** to **2** using sodium citrate buffer containing 0.2% Triton X-100 at pH 6.0 (see Table 1). This result is coherent with published pH profiles. It has been reported, for example, that YqjM exhibits the highest activity for ketoisophorone at pH 6.0 [27]. Notably, even when using low concentrations of cell mass (20 mg/mL), we detected production of the alcohol side product **2s**. In parallel to the synthetic biocatalysis reactions, we therefore determined the background reaction of endogenous *E. coli* enzymes by using non-transformed *E. coli* BL21 (DE3) cells as negative control. In this case, we observed very low (*S*)-citronellal **2** production by the *E. coli* ER N-ethylmaleimide reductase [42] but significant formation of side product alcohol **1s**, presumably due to the activity of endogenous alcohol dehydrogenases (ADHs) [43, 44]. Further control reactions included incubation of (*S*)-citronellal **2** and (–)-*iso*-isopulegol **3** with YqjM-containing cells, leading to the formation of side product **2s** or no reaction, respectively.

The whole-cell biotransformations of (*S*)-citronellal **2** employing 200 mg/mL cells expressing *Aac*SHC_I261A exhibited activity only in combination with citrate buffer independent of the presence of Triton X-100. The detected conversions were low (<1%), as after 6 h, the substrate **2** was already quantitatively converted to the unwanted side product **2s**. Further control experiments in which (*E*/*Z*)-citral **1** was incubated with *Aac*SHC_I261A-expressing cells revealed the predominant formation of side product **1s** and low amount of alcohol **2s**.

As expected, initial attempts of in vivo reactions, in which 20 mg/mL YqjM and 200 mg/mL *Aac*SHC_I261A-expressing cells were co-incubated with substrate **1**, confirmed the presence of active endogenous *E. coli* ADHs, as only side product **2s** was detected.

2.2 In vitro enzyme cascade

In an attempt to crudely purify the enzyme solutions and inactivate the problematic endogenous ADHs, we applied heat shock to both cell suspensions for 30 min at 60 °C. As expected, the thermostable *Aac*SHC_I261A was still active after prolonged incubation at 60 °C [45]; however, the ER YqjM did not retain its catalytic activity. Moreover, we also found that some of the endogenous ADHs were still active after the heat treatment. As an alternative approach to reduce the side reactivity of the native ADHs, the pH of the biocatalysis reaction was lowered to pH 4.0. While the *Aac*SHC variant retained its activity, the biocatalytic conversion rates by YqjM dropped dramatically and no product **3** was detected. Having unsuccessfully explored several optimisation strategies in the context of a whole-cell biocatalysis cascade, we decided to set up a biocatalytic cascade based on semi-purified enzyme.

As *Aac*SHC_I261A is a membrane-bound protein, separation of the SHC from contaminating soluble proteins is straightforward. Using the semi-purified *Aac*SHC fraction in a resolubilised form with 2% Triton X-100 (see Figure 3), the intermediate substrate **2** could be converted to product **3** with a final conversion of 7.3% after 24 h. In a next step, we employed semi-purified *Aac*SHC_I261A in combination with 10 mg/mL whole cells containing YqjM, thus reducing the total whole-cell load in the biocatalysis reactions and consequently also the quantity of contaminating ADHs. Using this strategy, the resulting cascade generated 3.4% of product **3** after 24 h; however, we also again detected significant side product **2s** formation (66%) (see Table 2).

To further reduce sources of ADH, we extended the semi-purification approach also to YqjM. As the available YqjM construct did not have any affinity tags, semi-purification was carried out using anion exchange chromatography followed by buffer exchange via size exclusion chromatography. The cascade reactions with both enzymes in a semi-purified form led to product **3** formation of 6.2% after 24 h and 10.8% after 62 h, while

Table 1: Quantification of the conversion of 5 mM citral (**1**) by 20 mg/mL YqjM-expressing cells to the intermediate product **2** and of the formation of the side product **2s** after 24 h.

	Conversion to 2 (%)	Conversion to 2s (%)
1 with 50 mM sodium phosphate buffer (pH 7.5)	19.9 ± 3.6	23.7 ± 1.7
1 with 50 mM citrate buffer (pH 6.0)	45.4 ± 2.9	54.6 ± 2.9
1 with 50 mM citrate buffer (pH 6.0), 0.2% Triton X-100	53.2 ± 0.9	26.2 ± 5.3

All experiments were carried out in triplicates and analysed by GC.

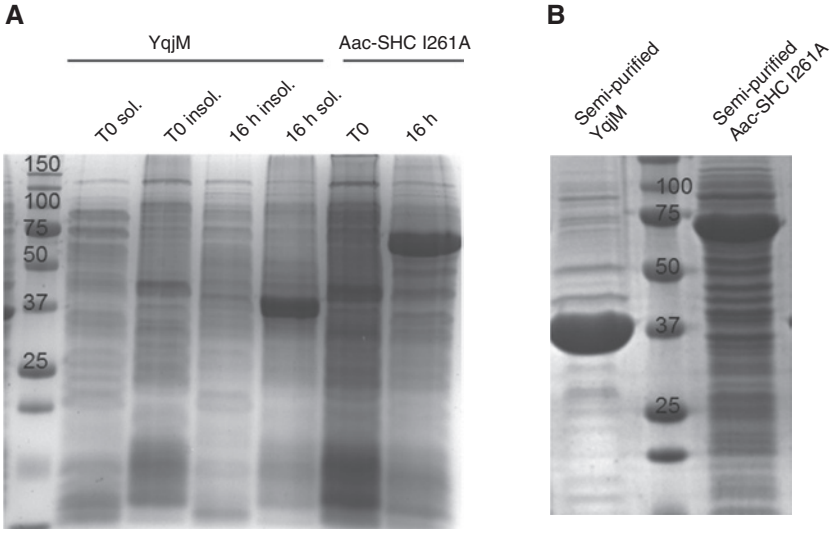

Figure 3: Reducing SDS-PAGE showing the expression levels of enzymes YqjM and *Aac*SHC with 12% acrylamide using a Precision Plus Protein™ dual-colour standard marker: (A) cultivation samples normalised to OD7; (B) samples after the semi-purification of the enzymes.

Table 2: Quantification of the conversion of citronellal **2** or citral **1** to the desired product **3** as well as of the formation of the intermediate product **2** and the side product **2s** by semi-purified *Aac*SHC_I261A.

	Conversion to 3 (%)	Conversion to 2 (%)	Conversion to 2s (%)
2 with *Aac*SHC_I261A 8 h	2.3 ± 0.1		5.8 ± 0.2
2 with *Aac*SHC_I261A 24 h	7.3 ± 0.8		7.8 ± 0.4
1 with *Aac*SHC_I261A 10 mg/mL cells YqjM 8 h	0.6 ± 0.1	44.7 ±0.2	11.8 ± 1.5
1 with *Aac*SHC_I261A 10 mg/mL cells YqjM 24 h	3.4 ± 0.2	24.9 ± 8.2	66 ± 7.3
1 with *Aac*SHC_I261A semi-purified YqjM 8 h	2.1 ± 0.2	54.4 ± 6.0	1.4 ± 0.4
1 with *Aac*SHC_I261A semi-purified YqjM 24 h	6.2 ± 0.5	67.8 ± 4.7	4.4 ± 0.4
1 with *Aac*SHC_I261A semi-purified YqjM 62 h	10.8 ± 0.1	60.1 ± 1.0	4.8 ± 0.1

All experiments were carried out in triplicates and analysed by GC.

generating <5% of side product **2s**. As reported, the use of *Aac*SHC_I261A exclusively led to the formation of (–)-*iso*-isopulegol [38, 39]. This overall yield corresponds closely to results obtained with the semi-purified *Aac*SHC in combination with intermediate substrate citronellal **2**, leading us to believe that the next optimisation steps would have to be carried out on the level of the *Aac*SHC variant. Another possible optimisation will be to increase the temperature to 60 °C after the conversion of **1** to **2** is completed. Going beyond these strategies, background ADH activity could be reduced by the use of a protease-mediated purification strategy, provided both target enzymes are protease resistant [46]. In cascades, fusion proteins [47, 48] have also been considered to facilitate direct transport of intermediates to the following enzyme. However, in the context of the here-described cascade, such a strategy would not only require the fusion of a membrane protein to a soluble protein but would likely pose additional difficulties as the two enzymes are characterised by differing kinetic parameters.

3 Conclusion

At the outset of designing an enzymatic cascade reaction, an in vivo set-up is typically considered as the initial construction strategy. Using the in vivo approach, the internal co-factor regeneration system of the cells can be harnessed and no laborious enzyme purification is necessary. However, the set-up and optimisation of our cascade consisting of an ER and an SHC demonstrates that it is not always straightforward to use interconnected enzymatic transformations in vivo. If any of the substrates or (intermediate) products are accepted by endogenous *E. coli* enzymes, as in the case of aldehydes, the in vivo set-up can become problematic. This challenge is further

compounded, if, as in our case, the second reaction step is rate determining, leading to an accumulation of such a sensitive intermediate. Switching to an in vitro cascade helped us circumvent this side-reactivity problem. Using semi-purified enzymes, we could construct a one-pot biocatalytic cascade able to convert citral stereo-selectively to (–)-*iso*-isopulegol with 10.8% conversion after 62 h. To enhance conversion yields further, optimisation of the *Aac*SHC variant will be targeted next.

4 Materials and methods

4.1 Chemicals and materials

All chemicals were purchased from Merck KGaA (Darmstadt, Germany), Chemie Brunschwig AG (Basel, Switzerland), VWR (Hannover, Germany) or Carl Roth (Karlsruhe, Germany), and were used without further purification unless otherwise specified. NADPH was obtained from OYC Europe (Rotterdam, Netherlands) and also used without further purification. Phusion polymerase was purchased from Thermo Fisher Scientific (Waltham, MA, USA). *Dpn*I was provided by New England Biolabs (Ipswich, MA, USA), and primers were ordered from Microsynth AG (Balgach, Switzerland). The Mono Q columns and the desalting columns were ordered from GE Healthcare (Uppsala, Sweden).

4.2 Bacterial strains and plasmids

Escherichia coli BL21 (DE3) [fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS] was purchased from New England Biolabs (Ipswich, MA, USA). *Escherichia coli* XL1-Blue [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*^{q}Z*ΔM15* Tn*10* (Tet^{r})] was obtained from Agilent Technologies (Santa Clara, CA, USA). The plasmid pet28_AacSHC was ordered from Twist Bioscience (San Francisco, CA, USA) and contained the codon-optimised gene of *Aac*SHC (AAA75452.1) without any tags, cloned into the pet28b+ vector system.

4.3 Cloning

The ER gene *yqjm* was amplified from genomic DNA of *B. subtilis* sp. (strain CCOS014 from Culture Collection of Switzerland, Wädenswil, Switzerland) with the following primers: forward 5′-GAGTGCGGCCGCAAGCTTTTACCAGCCTCTTTCG-3′ and reverse 5′-CTTTAAGAAGGAGATATACCATGGCCAGAAAATTATTTAC-3′. The empty pet28b(+) vector was amplified via forward primers 5′-CGAAAGAGGCTGGTAAAAGCTTGCGGCCGCACTC-3′ and 5′-GTAAATAATTTTCTGGCCATGGTATATCTCCTTCTTAAAG-3′. PCR products were purified using PCR Clean Up Kit from Machery Nagel (Düren, Germany), combined and transformed into *E. coli* XL1 Blue via the Fast-Cloning method [49]. The correctness of the resulting plasmid pet28_YqjM was verified by sequencing with MWG Eurofins (Ebersberg, Germany), and correct construct was subsequently transformed into *E. coli* BL21 (DE3). The *Aac*SHC mutant I261A was created by amplification from pet28_*Aac*SHC with the forward primer 5′-GTAGTTGGGGTGGTGCGCAGCCTCCG-3′ and T7-reverse primer. The PCR product was used as MegaPrimer in a MEGAWHOP PCR [50] using the original pet28_*Aac*SHC as template to introduce the desired mutation. After a 2-h *Dpn*I digest at 37 °C, the PCR product was transformed into *E. coli* XL1 Blue cells. The resulting plasmid, pet28_*Aac*SHC_I261A, was sequenced and a correct construct was transformed into *E. coli* BL21 (DE3).

4.4 Enzyme expression

Bacterial cultures were incubated in baffled Erlenmeyer flasks in a New Brunswick™ Innova® 42 orbital shaker at 200 rpm at 37 °C. Bacteria on agar plates were incubated in a HERATherm Thermo Scientific incubator in air. All materials and biotransformation media were sterilised by autoclaving at 121 °C for 20 min. Aqueous stock solutions were sterilised by filtration through 0.20-mm syringe filters. Agar plates were prepared with LB medium supplemented with 1.5% (w/v) agar and antibiotics. Expression of YqjM in *E. coli* BL21 (DE3) was performed by inoculation of LB medium (400 mL) supplemented with kanamycin (100 μg/mL) with an overnight culture (4 mL, 1:100). The YqjM culture was incubated at 37 °C at 180 rpm until an OD600 of 0.6–0.8 was reached. The next expression was induced by supplying isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.1 mM, and incubation was continued at 25 °C for 16 h. *Aac*SHC_I261A in *E. coli* BL21 (DE3) was cultivated identically except for the use of terrific broth as cultivation medium. The cells were harvested by centrifugation for 10 min at 4000 × *g* and stored at –20 °C.

4.5 Partial purification of the enzymes

The partial purification of *Aac*SHC_I261A was performed according to the protocol of Bastian et al. [38]. Thawed cells of

a 400 mL cultivation were resuspended in 15 mL cell disruption buffer [0.2 M sodium citrate, DNAse 0.1 mg/mL, pH 6.0 (dH_2O)]. Cell disruption was performed by a single passage through a French pressure cell at 2000 psi. Cell debris was collected by centrifugation at 38,500×*g* for 40 min at 10 °C, and the supernatant was discarded. The cell debris was resuspended in 5 mL wash buffer (0.05 M sodium citrate, pH 6.0) and again collected by centrifugation at 38,500×*g* for 40 min at 10 °C. The enzyme was solubilised overnight (16 h) at 4 °C with 5 mL solubilisation buffer (0.05 M sodium citrate, 2% Triton X-100, pH 6.0). Afterwards, the mixture was centrifuged (38,500×*g* for 40 min at 10 °C) and the supernatant was used for the biotransformations (partially purified enzyme). For the partial purification of YqjM, the protocol of Fitzpatrick et al. [26] was adapted: thawed cells of a 400 mL cultivation were resuspended in 15 mL buffer A (50 mM Tris-HCl, pH 7.5). Cell disruption was performed by a single passage through a French pressure cell at 2000 psi, afterwards the cell debris was removed by centrifugation at 8000×*g* for 20 min at 4 °C, and the supernatant was subjected to ion exchange chromatography on a Mono Q column (2.5×10 cm) freshly equilibrated in buffer A. All steps were done on an Äkta Pure device with a 4 mL/min flow rate. After washing with six column volumes of buffer A, bound protein was eluted with a linear gradient of 50 mL each of buffer A and buffer B (same as buffer A but containing 500 mM KCl). The yellow fractions containing YqjM were pooled and concentrated, and the buffer was changed to 0.05 M sodium citrate buffer (pH 6.0) by using three coupled 5 mL HiTrap desalting columns. The protein contents of the crude cell extract as well as that of the partial purified fractions were determined by using the BCA kit. Standard curves were recorded with bovine serum albumin at 0.02–2.0 mg/mL. Samples were measured in triplicate at suitable dilutions with a Tecan Spark device.

4.6 Biotransformations

For biocatalysis, whole-cell suspensions or semi-purified enzyme was used. The appropriate volume of the enzyme solution and 5 mM substrate (stock 1 M in DMF) supplemented with 6 mM NADPH were combined in a sodium citrate buffer (0.05 M, pH 6.0) with 0.2% Triton. A total of 1 or 0.5 mL reaction volume was incubated in closed gas chromatography (GC) vials at 30 °C and 850 rpm. All biocatalysis reactions were done in triplicates, and samples of 500 µL were taken after 8, 24 or 62 h. To determine the background reaction, control experiments with buffer only or *E. coli* cells harbouring no overexpressed protein were performed.

4.7 GC analysis

Samples (500 µL) were extracted two times with 400 and 200 µL ethyl acetate containing 1 g/L octanol as internal standard and dried over sodium sulphate. The extract was subjected to GC-flame ionisation detection analysis. The analysis was done using an Agilent GC 7890B with a DB 5 (30 m×0.25 mm×0.25 µm) column. The separation run was performed at constant pressure of 25 psi with a starting temperature of 75 °C. Next, a temperature ramp of 2 °C/min was carried out. At 110 °C, the heating rate was increased to 90 °C/min up to 300 °C, which was held for 2 min. The method was adapted from Ref. [38]. The conversion calculations were done using the standard curves of the substrate, intermediate product and product.

References

1. Kroutil W, Rueping M. Introduction to *ACS Catalysis* virtual special issue on cascade catalysis. ACS Catal 2014;4:2086–7.
2. Muschiol J, Peters C, Oberleitner N, Mihovilovic MD, Bornscheuer UT, Rudroff F. Cascade catalysis – strategies and challenges en route to preparative synthetic biology. Chem Commun 2015;51:5798–811.
3. Schrittwieser JH, Velikogne S, Hall M, Kroutil W. Artificial biocatalytic linear cascades for preparation of organic molecules. Chem Rev 2018;118:270–348.
4. France SP, Hepworth LJ, Turner NJ, Flitsch SL. Constructing biocatalytic cascades: in vitro and in vivo approaches to de novo multi-enzyme pathways. ACS Catal 2017;7:710–24.
5. Badenhorst CP, Bornscheuer UT. Getting momentum: from biocatalysis to advanced synthetic biology. Trends Biochem Sci 2018;43:180–98.
6. Sperl JM, Sieber V. Multienzyme cascade reactions – status and recent advances. ACS Catal 2018;8:2385–96.
7. Rudroff F, Mihovilovic MD, Gröger H, Snajdrova R, Iding H, Bornscheuer UT. Opportunities and challenges for combining chemo- and biocatalysis. Nat Catal 2018;1:12–22.
8. Ricca E, Brucher B, Schrittwieser JH. Multi-enzymatic cascade reactions: overview and perspectives. Adv Synth Catal 2011;353:2239–62.
9. Zhang W, Fernández-Fueyo E, Ni Y, van Schie M, Gacs J, Renirie R, et al. Selective aerobic oxidation reactions using a combination of photocatalytic water oxidation and enzymatic oxyfunctionalizations. Nat Catal 2018;1:55–62.
10. Toogood HS, Gardiner JM, Scrutton NS. Biocatalytic reductions and chemical versatility of the old yellow enzyme family of flavoprotein oxidoreductases. ChemCatChem 2010;2:892–914.
11. Toogood HS, Scrutton NS. Discovery, characterization, engineering, and applications of ene-reductases for industrial biocatalysis. ACS Catal 2018;8:3532–49.
12. Winkler CK, Faber K, Hall M. Biocatalytic reduction of activated CC-bonds and beyond: emerging trends. Curr Opin Chem Biol 2018;43:97–105.
13. Knowles WS. Asymmetric hydrogenations (Nobel lecture). Angew Chem Int Ed 2002;41:1998–2007.

14. Noyori R. Asymmetric catalysis: science and opportunities (Nobel lecture). Angew Chem Int Ed 2002;41:2008–22.
15. Warburg O, Christian W. Ein zweites sauerstoffübertragendes ferment und sein absorptionsspektrum. Naturwissenschaften 1932;20:688–8.
16. Haas E. Isolierung eines neuen gelben Ferments. Biochem Z 1938;298:369–90.
17. Williams RE, Bruce NC. 'New uses for an Old Enzyme' – the Old Yellow Enzyme family of flavoenzymes. Microbiology 2002;148:1607–14.
18. Scholtissek A, Tischler D, Westphal A, van Berkel W, Paul C. Old yellow enzyme-catalysed asymmetric hydrogenation: linking family roots with improved catalysis. Catalysts 2017;7:130.
19. Breithaupt C, Strassner J, Breitinger U, Huber R, Macheroux P, Schaller A, et al. X-ray structure of 12-oxophytodienoate reductase 1 provides structural insight into substrate binding and specificity within the family of OYE. Structure 2001;9:419–29.
20. Kohli RM, Massey V. The oxidative half-reaction of old yellow enzyme: the role of tyrosine 196. J Biol Chem 1998;273:32763–70.
21. Lonsdale R, Reetz MT. Reduction of α,β-unsaturated ketones by old yellow enzymes: mechanistic insights from quantum mechanics/molecular mechanics calculations. J Am Chem Soc 2015;137:14733–42.
22. Amato ED, Stewart JD. Applications of protein engineering to members of the old yellow enzyme family. Biotechnol Adv 2015;33:624–31.
23. Padhi SK, Bougioukou DJ, Stewart JD. Site-saturation mutagenesis of tryptophan 116 of *Saccharomyces pastorianus* old yellow enzyme uncovers stereocomplementary variants. J Am Chem Soc 2009;131:3271–80.
24. Karplus PA, Fox KM, Massey V. Flavoprotein structure and mechanism. 8. Structure-function relations for old yellow enzyme. FASEB J 1995;9:1518–26.
25. Kitzing K, Fitzpatrick TB, Wilken C, Sawa J, Bourenkov GP, Macheroux P, et al. The 1.3 Å crystal structure of the flavoprotein YqjM reveals a novel class of old yellow enzymes. J Biol Chem 2005;280:27904–13.
26. Fitzpatrick TB, Amrhein N, Macheroux P. Characterization of YqjM, an old yellow enzyme homolog from *Bacillus subtilis* involved in the oxidative stress response. J Biol Chem 2003;278:19891–7.
27. Pesic M, Fernández-Fueyo E, Hollmann F. Characterization of the old yellow enzyme homolog from *Bacillus subtilis* (YqjM). ChemistrySelect 2017;2:3866–71.
28. Christianson DW. Structural and chemical biology of terpenoid cyclases. Chem Rev 2017;117:11570–648.
29. Siedenburg G, Jendrossek D. Squalene-hopene cyclases. Appl Environ Microbiol 2011;77:3905–15.
30. Syrén P-O, Henche S, Eichler A, Nestl BM, Hauer B. Squalene-hopene cyclases – evolution, dynamics and catalytic scope. Curr Opin Struct Biol 2016;41:73–82.
31. Hoshino T, Kumai Y, Kudo I, Nakano S-i, Ohashi S. Enzymatic cyclization reactions of geraniol, farnesol and geranylgeraniol, and those of truncated squalene analogs having C20 and C25 by recombinant squalene cyclase. Org Biomol Chem 2004;2:2650–7.
32. Siedenburg G, Jendrossek D, Breuer M, Juhl B, Pleiss J, Seitz M, et al. Activation-independent cyclization of monoterpenoids. Appl Microbiol Biotechnol 2012;78:1055–62.
33. Seitz M, Syrén P-O, Steiner L, Klebensberger J, Nestl BM, Hauer B. Synthesis of heterocyclic terpenoids by promiscuous squalene-hopene cyclases. ChemBioChem 2013;14:436–9.
34. Reinert DJ, Balliano G, Schulz GE. Conversion of squalene to the pentacarbocyclic hopene. Chem Biol 2004;11:121–6.
35. Wendt KU, Poralla K, Schulz GE. Structure and function of a squalene cyclase. Science 1997;277:1811–5.
36. Siedenburg G, Breuer M, Jendrossek D. Prokaryotic squalene-hopene cyclases can be converted to citronellal cyclases by single amino acid exchange. Appl Microbiol Biotechnol 2013;97:1571–80.
37. Seitz M, Klebensberger J, Siebenhaller S, Breuer M, Siedenburg G, Jendrossek D, et al. Substrate specificity of a novel squalene-hopene cyclase from *Zymomonas mobilis*. J Mol Catal B Enzym 2012;84:72–7.
38. Bastian SA, Hammer SC, Kreß N, Nestl BM, Hauer B. Selectivity in the cyclization of citronellal introduced by squalene hopene cyclase variants. ChemCatChem 2017;9:4364–8.
39. Hammer SC, Marjanovic A, Dominicus JM, Nestl BM, Hauer B. Squalene hopene cyclases are protonases for stereoselective Brønsted acid catalysis. Nat Chem Biol 2014;11:121.
40. Lenardão EJ, Botteselle GV, de Azambuja F, Perin G, Jacob RG. Citronellal as key compound in organic synthesis. Tetrahedron 2007;63:6671–712.
41. Hall M, Stueckler C, Ehammer H, Pointner E, Oberdorfer G, Gruber K, et al. Asymmetric bioreduction of C=C bonds using enoate reductases OPR1, OPR3 and YqjM: enzyme-based stereocontrol. Adv Synth Catal 2008;350:411–8.
42. Williams RE, Rathbone DA, Scrutton NS, Bruce NC. Biotransformation of explosives by the old yellow enzyme family of flavoproteins. Appl Environ Microbiol 2004;70:3566–74.
43. Holland-Staley CA, Lee K, Clark DP, Cunningham PR. Aerobic activity of *Escherichia coli* alcohol dehydrogenase is determined by a single amino acid. J Bacteriol 2000;182:6049–54.
44. Pérez JM, Arenas FA, Pradenas GA, Sandoval JM, Vásquez CC. *Escherichia coli* YqhD exhibits aldehyde reductase activity and protects from the harmful effect of lipid peroxidation-derived aldehydes. J Biol Chem 2008;283:7346–53.
45. Sato T, Hoshino T. Kinetic studies on the function of all the conserved tryptophans involved inside and outside the QW motifs of squalene-hopene cyclase: stabilizing effect of the protein structure against thermal denaturation. Biosci Biotechnol Biochem 1999;63:1171–80.
46. Last D, Müller J, Dawood AW, Moldenhauer EJ, Pavlidis IV, Bornscheuer UT. Highly efficient and easy protease-mediated protein purification. Appl Microbiol Biotechnol 2016;100:1945–53.
47. Aalbers FS, Fraaije MW. Enzyme fusions in biocatalysis: coupling reactions by pairing enzymes. Chembiochem. doi: 10.1002/cbic.201800394 [Epub ahead of print 4 Sep 2018].
48. Elleuche S. Bringing functions together with fusion enzymes – from nature's inventions to biotechnological applications. Appl Microbiol Biotechnol 2015;99:1545–56.
49. Li C, Wen A, Shen B, Lu J, Huang Y, Chang Y. FastCloning: a highly simplified, purification-free, sequence- and ligation-independent PCR cloning method. BMC Biotechnol 2011;11:1–10.
50. Miyazaki K. Chapter seventeen – MEGAWHOP cloning: a method of creating random mutagenesis libraries via MegaPrimer PCR of whole plasmids. Methods Enzymol 2011;498:399–406.