Method Article

A simple and efficient method for lyophilization of recombinant *E. coli* JM109 (DE3) whole-cells harboring active Rieske non-heme iron dioxygenases

Theresa Farr, Julian L. Wissner, Bernhard Hauer*

Department of Technical Biochemistry, Institute of Biochemistry and Technical Biochemistry, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany

ABSTRACT

Rieske non-heme iron dioxygenases are a class of intriguing enzymes covering a broad reaction and substrate spectrum and have been studied extensively in the last decades. In nature, these biocatalysts are essential for the production of *cis*-dihydroxylated metabolites, as a first step during the degradation of aromatic compounds in microorganisms. The enzymes are able to produce relevant amounts of compounds in short reaction times, but the effort for constant cultivation of recombinant cells and production of cell mass for biotransformations is high. To overcome the steady production process, our task was to find a way to make the biocatalysts durable and storable. In this way, laboratories lacking equipment for microbiology, *e.g.* chemistry laboratories, can be supplied with the enzymes to open up new possibilities in the production of molecules. We present a quick and efficient method that uses lyophilization to freeze-dry recombinant whole-cells that harbor the enzyme of interest. By washing the cells with a cryoprotectant before lyophilization, we could conserve the enzyme activity to the level of freshly harvested cells. Moreover, this simple to apply method enables subsequent steps like storage of the cell powder for transportation and on demand use in biotransformations. The method was established with the cumene dioxygenase (CDO) of *Pseudomonas fluorescens* IP01 and its variant CDO M232A expressed in *E. coli* JM109 (DE3) cells, employing *R*-limonene and naphthalene, respectively, as substrates in biotransformations. The method could be successfully applied in the analytical and semi-preparative reaction scale.

- Preservation of biocatalysts in recombinant whole-cells.
- Ready-to-use enzymatic reaction.
- Semi-preparative biotransformation with lyophilized whole-cells.

* Corresponding author.
  E-mail address: bernhard.hauer@itb.uni-stuttgart.de (B. Hauer).

Specifications Table

Subject Area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	Preservation of enzymes in freeze-dried whole-cells for biotransformations
Method name:	A simple and efficient method for lyophilization of recombinant *E. coli* JM109 (DE3) whole-cells harboring active Rieske non-heme iron dioxygenases
Name and reference of original method:	**Preparation of competent cells and transformation:**
	Wissner, J. L., Escobedo-Hinojosa, W., Heinemann, P. M., Hunold, A. & Hauer, B. Methods for the detection and analysis of dioxygenase catalyzed dihydroxylation in mutant derived libraries. Methods in Enzymology vol. 644 (Elsevier Inc., 2020).
	Treatment with cryoprotectants:
	Whittall, J., Sutton, P. W., Kroutil, W. Practical Methods for Biocatalysis and Biotransformations 3. (John Wiley & Sons, 2016).
	Wessman, P., Håkansson, S., Leifer, K. & Rubino, S. Formulations for freeze-drying of bacteria and their influence on cell survival. J. Vis. Exp. (2013).
	Vila, M. A. et al. Production of *cis*-1,2-dihydrocatechols of high synthetic value by whole-cell fermentation using *Escherichia coli* JM109 (pDTG601): A detailed study. J. Mol. Catal. B Enzym. 96, 14–20 (2013).
	Biotransformation and analytics:
	Gally, C., Nestl, B. M. & Hauer, B. Engineering Rieske Non-Heme Iron Oxygenases for the Asymmetric Dihydroxylation of Alkenes. Angew. Chemie Int. Ed. 54, 12,952–12,956 (2015).
	Wissner, J. L., Escobedo-Hinojosa, W., Heinemann, P. M., Hunold, A. & Hauer, B. Methods for the detection and analysis of dioxygenase catalyzed dihydroxylation in mutant derived libraries. Methods in Enzymology vol. 644 (Elsevier Inc., 2020).
Resource availability:	*N/A*

Method details

Background

Biocatalysis has become an exciting alternative to conventional chemical catalysis, and provides an eco-efficient use of alternative raw materials. It offers advantages in many ways: Not only do the reactions take place under milder conditions, but through enzyme engineering and directed evolution activity and selectivity of the biocatalysts can be improved and non-natural reactions can be tapped [1–4]. Rieske non-heme iron dioxygenases (ROs) catalyze stereo- and regiospecific mono- or dihydroxylation reactions and naturally play a vital role in pathways for bacterial degradation of aromatics and recycling of organic matter in the environment [5–7]. As the first step of such pathways, ROs generate *cis*-dihydroxylated metabolites by incorporation of molecular oxygen to form *cis*-dihydrodiols [5,8,9]. In the last decades, this enzyme class has been extensively studied, and it could be shown, that besides oxygenation, these enzymes are also able to catalyze distinct reactions, for instance sulfoxidations, desaturations, dealkylations and the transfer of nitrene [5,10–15]. ROs consist of a multi-component system containing an oxygenase, reductase and ferredoxin coordinating the electron transfer [5,7,16]. Despite the high activity and selectivity of ROs, the lifespan of the enzymes is short. For the toluene dioxygenases from *Pseudomonas putida* F1 (TDO) an activity loss of 90% was shown after 48 h in crude cell extracts. Additionally, these results could be confirmed by studies with the naphthalene dioxygenase (NDO) from *Pseudomonas* sp. 9816-4, where enzyme

activity decreased sharply only after a few hours of reaction [17,18]. This short lifespan causes a demand for constant production of cell mass. Since the process of cell transformation, cultivation and harvest is of expenditure, we aimed to find a simple solution to overcome this obstacle. Our objective was to find a way for the production of durable biocatalysts, that can be delivered and stored easily to be available for a ready-to-use application in chemical or pharmaceutical laboratories. This enables the production of small amounts of molecules of interest without needing knowledge and equipment for microbiological works. Lyophilization or freeze-drying is a well-known technique in the food and pharmaceutical industry that enables stabilization, storage and transport of unstable materials by dehydration [19,20]. Vila and colleagues could show, that recombinant *E. coli* JM109 cells expressing the TDO cluster, exhibited a remarkable enzyme activity after freeze-drying and were active for at least one month [21]. In this work, we report a method that covers the full procedure from protein expression to biotransformation with lyophilized *E. coli* JM109 (DE3) whole-cells containing the cumene dioxygenase (CDO) cluster [10]. The method is easy to apply and effective in making biocatalysts storable and available for on demand usage in biotransformations. We tested different cryoprotectants to conserve enzyme activity (see method validation) and describe biotransformation protocols for analytical and semi-preparative reaction scale.

Method

Transformation of competent E. coli *JM109 (DE3) cells*

1. Competent *E. coli* JM109 (DE3) cells were prepared according to Wissner et al., 2020 [22].
2. Thaw one aliquot of competent cells (50 μL) by placing the tube on ice for 15 min.
3. Add 1 μL of pIP107D (100–200 ng μL^{-1}) to competent cells and mix by gentle shaking.
4. Incubate reaction tube on ice for 30 min.
5. Heat shock the cell-plasmid mixture at 42 °C for 1 min (in Eppendorf thermomixer or water bath).
6. Place reaction tube immediately back on ice and cool transformation mixture for 2 min.
7. Add 500 μL LB liquid medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 5 g L^{-1} NaCl) to the transformation mixture and incubate at 37 °C, 650 rpm for 60 min (Eppendorf thermomixer).
8. Spread 100 μL of the cell suspension on LB agar plates (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 5 g L^{-1} NaCl, 15 g L^{-1} agar) containing 100 μg mL^{-1} ampicillin and incubate at 37 °C for 16 h.

Cultivation of cells and protein expression

1. A starter culture was inoculated from a single colony in 5 mL LB liquid medium supplemented with ampicillin (100 μg mL^{-1}) and was incubated at 37 °C and 180 rpm for 6 h. The whole starter culture was transferred to 30 mL LB containing 100 μg mL^{-1} ampicillin and cultivated at 37 °C and 180 rpm for 16 h on an orbital shaker (Multitron, Infors, Switzerland).
2. The main cultures were inoculated with 6% (*v/v*) preculture. The preculture (30 mL) was added to 500 mL TB medium (5 g L^{-1} glycerol, 12 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 0.17 M KH$_2$PO$_4$, 0.72 M K$_2$HPO$_4$), supplemented with 100 μg mL^{-1} ampicillin and incubated at 37 °C and 180 rpm until OD$_{600}$=0.6–0.8 was reached.
3. Enzyme expression was induced with 1 mM IPTG and the cultures were incubated for 20 h at 25 °C and 180 rpm.
4. Cells were harvested by centrifugation (6000 x g, 20 min, 4 °C, Beckman Coulter, USA).
5. This cultivation protocol yields about 8 g L^{-1} cells (cell wet weight).

Preparation for lyophilization

1. 0.5 g of freshly harvested cells were transferred to a 50 mL reaction tube.
2. Cell pellets were resuspended in 10 mL 100 mM potassium phosphate buffer pH 7.4 supplemented with 10% (*v/v*) glycerol by shaking at 65 rpm on a rotator mixer (Intelli-Mixer RM-2 M, ELMI, USA) at 4 °C for 30 min.

3. The cells were separated by centrifugation (3500 rpm/959 x g, 20 min, 4 °C, Centrifuge 5810 R, rotor F-34–6–38, Eppendorf, Germany) and the supernatant was discarded subsequently.
4. Cell pellets were frozen at −80 °C for at least 4 h.
5. The lyophilization step was conducted for 24 h at −86 °C and 0.0044 mbar in a freeze-dryer (Martin Christ Gefriertrocknungsanlagen, Germany).
6. Lyophilized cell pellets were stored at −20 °C until further use.
7. Tubes with cell pellets were weighed before and after lyophilization to determine the factor of water loss between cell wet weight and cell dry weight.

Biotransformation

Whole-cell biotransformations were performed with living cells as well as lyophilized cells. *In vivo* biotransformations with living cells were performed right after cell harvest to measure the fresh cells' enzymatic activity without any loss. Biotransformations with lyophilized cells were carried out with rehydrated cells, using the amount of the cells (cell dry weight, *cdw*) equivalent to the cell wet weight (*cww*). In our experiments 0.5 g living cells were weighed into a 50 mL reaction tube, washed with 10 mL cryoprotectant solution, centrifuged and lyophilized. To obtain the wished cell concentration according to the *cww* (here exemplary shown for 0.05 g_{cww} mL^{-1}) the freeze-dried cell pellets were rehydrated in 10 mL potassium buffer to a final cell concentration. The cell suspensions were used for biotransformations in 1 mL volume performed in triplicates.

Biotransformation in analytical volume (1 mL biotransformation)

1. 0.05 g_{cww} mL^{-1} recombinant JM109 (DE3) cells, harbouring pIP107D, were resuspended in 100 mM potassium phosphate buffer pH 7.4 by vortexing and pipetting.
2. To a volume of 970 µL cell suspension 20 µL glucose (1 M in ddH$_2$O, final concentration 20 mM, for *in situ* cofactor regeneration) and 10 µL *R*-limonene (1 M in dimethyl sulfoxide/DMSO, final concentration 10 mM) were added (*R*-limonene is volatile, thus the reaction vessels have to be closed properly).
3. Biotransformations were conducted in 20 mL glass vials sealed with a gastight screw cap for 4 h at 30 °C and 180 rpm in a shaking incubator (vials must be incubated horizontally to assure a sufficient oxygen saturation).
4. After the reaction, the 1 mL biotransformation was transferred to a 2 mL reaction tube and 1 mL of *tert*-butyl methyl ether (MTBE, containing 1 mM biphenyl as internal standard) was added. For extraction of the analytes, the samples were vortexed and then centrifuged in a microcentrifuge (5 min, 12,000 rpm at room temperature, centrifuge 5424 R, Eppendorf, Germany).
5. 800 µL of the solvent phase was transferred to 2 mL crimp top vials for analysis by GC-FID or HPLC-DAD.

Biotransformation in semi-preparative volume (50 mL biotransformation)

For upscaled biotransformations, the enzyme variant CDO M232A was expressed in JM109 (DE3) as described above. Semi-preparative biotransformations were conducted in 1 L reaction vessels in a reaction volume of 50 mL, to maintain the required gas volume.

1. To obtain a concentration of 0.05 g_{cww} mL^{-1} cells, the equivalent amount of dry cell weight (here 0.803 g_{cdw} of lyophilized cell powder, see Determination of cell concentration) was added to 48.5 mL 100 mM potassium phosphate reaction buffer pH 7.4 (without resuspension of the cell powder).
2. Add 1 mL glucose (1 M, final concentration 20 mM) and 500 µL naphthalene substrate stock (0.5 M in ethanol, final concentration 5 mM).
3. Incubate at room temperature on a magnetic stirrer (700 rpm).
4. Extract 1 mL of biotransformation 1:1 (*v/v*) with MTBE and run on LC-MS to monitor the reaction progress.

5. For extraction, transfer 25 mL of biotransformation to 50 mL reaction tube (50 mL reaction: 2 × 50 mL tubes).
6. Mix 25 mL of biotransformation 1:1 (*v/v*) with MTBE (without internal standard), shake vigorously and vortex.
7. Centrifuge 5 min, 7000 rpm at room temperature (Centrifuge 5810 R, rotor F-34–6–38, Eppendorf, Germany).
8. Collect organic solvent phase in a round-bottom flask.
9. Extraction of the product is repeated three times.
10. Evaporate MTBE on the rotary evaporator.

Determination of cell concentration

To ensure a constant and comparable cell concentration, the required cell quantity for each reaction condition was weighed in a 50 mL reaction tube (cell wet weight), washed with buffer and cryoprotectant, and subsequently lyophilized. Proper centrifugation before discarding the buffer-cryoprotectant mixture prior to freeze-drying was crucial to avoid loss of cells and to guarantee a consistent cell concentration. The dried samples were rehydrated in the same 50 mL tubes to the adequate concentration and used for biotransformations. To determine the weight difference between cell wet weight and cell dry weight due to water loss, the tubes were weighed before and after freeze-drying. Analysis of $n = 66$ tubes led to an average factor of 3,11 ± 0,6 meaning 1 g_{cww} equals 0,322 g_{cdw}.

Determination of viable cell count

To determine the number of living cells after lyophilization, the Miles and Misra method was applied [23]. Samples were taken from 0.1 g_{cww} mL^{-1} cell suspensions (living cells and lyophilized cells equivalent to *cww*, treated with 10% (*v/v*) glycerol in 100 mM potassium phosphate buffer pH 7.4). For the living cells, sample dilutions from 10^{-5} to 10^{-8}, and for lyophilized cells, dilutions from 10^{-2} to 10^{-5} were plated. Serial dilutions were made in triplicates and 20 μL were dropped onto LB-ampicillin plates and incubated overnight at 37 °C. The following day, the number of colonies was determined in the most countable dilution. The number of colony-forming units (CFUs) for the living cells was $10.8 × 10^8$ while the number of CFUs was clearly reduced after freeze-drying to $23.3 × 10^4$. To compare the amount of total protein between living and lyophilized cells, samples of the cultures were taken before cell harvest (living cells) and from rehydrated cells after lyophilization (0.1 g_{cww} mL^{-1} lyophilized cells before biotransformation). The optical density of both samples was set to OD$_{600}$=10 and compared by SDS-PAGE gel analysis. 12 μL of the cell suspensions was denatured by heating to 99 °C in SDS buffer for 10 min and then applied to a 12% SDS-PAGE gel. The proteins were subsequently visualized with a Coomassie blue staining solution.

Analytics

Peak analysis and product standards
The product standards for (1*R*,5*S*)-carveol (1 M in DMSO) and (1*R*,2*S*)-*cis*-1,2-dihydro-1,2-naphthalenediol (0.5 M in ethanol) were diluted in 100 mM potassium phosphate buffer pH 7.4 to final concentrations of 1, 2.5, 5 and 10 mM and were treated equally to biotransformations. Each data point was performed in technical triplicates and analyzed by GC-FID and HPLC-DAD, respectively. The graphs were integrated manually, and the corresponding areas were normalized with the internal standard. The concentration was determined using the standard curves (see Figs. S2 and S3).

Analysis of R-limonene and (1R,5S)-carveol
Products were extracted with MTBE containing 1 mM biphenyl (100 mM in ethanol) as internal standard and analyzed by gas chromatography (GC). GC analyses were performed on a GC-2010 gas chromatograph (Shimadzu, Japan) equipped with FID detector using a HP-1 ms UI column (30 m x 0.25 mm x 0.25 μm, Agilent, USA) with hydrogen as carrier gas (linear velocity 30 cm/s). 1 μL sample

was injected at a temperature of 250 °C with a split ration of 1:15 and analyzed with the following program: 3 min at 120 °C, 13 °C min^{-1} to 190 °C, 250 °C min^{-1} to 290 °C, hold 250 °C for 2 min. The temperature of the flame ionization detector (FID) detector was set to 330 °C.

Analysis of naphthalene and (1R,2S)-cis-1,2-dihydro-1,2-naphthalendiol

Products were extracted with MTBE containing 1 mM biphenyl as internal standard (100 mM in ethanol) and analyzed by high-performance liquid-chromatography (HPLC, Agilent, USA). Naphthalene and (1*R*,2*S*)-*cis*-1,2-dihydro-1,2-naphthalendiol were detected following the protocol for HPLC-DAD (262 nm) analysis described by Wissner et al., 2020.

Conclusion

In this work we describe a practical and easy-to-use method to produce and provide lyophilized recombinant cells that can be utilized on demand for biotransformations. Through this approach, biocatalysts can be made available to consumers who do not have the microbiological expertise and equipment available to cultivate recombinant cells. In this way broader access to green chemistry can be created. This availability can open up new fields of application and production methods of molecules with Rieske non-heme iron dioxygenases.

Method validation

Establishment of a cell treatment prior to lyophilization

This study aimed to develop a method to simplify the use of biocatalysts and make them more accessible ((Fig. 1). The method development was carried out with the Rieske non-heme iron dioxygenase CDO and the substrate *R*-limonene. The monoterpene *R*-limonene is an interesting precursor substance, because the microbial oxidation of this molecule gives access to bioactive natural compounds. One significant advantage of this precursor molecule is the availability and the inexpensive production, as the substance is obtained as a waste product from orange peel [24,25]. Enzymatic reactions of CDO in combination with *R*-limonene as a substrate have been extensively studied and it is known that the CDO wildtype (WT) is forming (1*R*,5*S*)-carveol as only product (Fig 2A, hereinafter referred to as carveol) [13]. Due to the availability of established protocols and expertise, this enzyme-substrate combination was chosen to develop the here described method. To achieve a quick and easy way to make recombinant whole-cells durable, we chose lyophilization as method of choice. The advantage of this method is, that the freeze-drying process takes place in only one step and the resulting cell powder can be shipped, stored and be efficiently rehydrated and instantly used. Another gain of using freeze-dried whole-cells is the effect of *in situ* co-factor regeneration during biotransformation. As the first step before lyophilization, cells have to be sufficiently frozen to guarantee that the solid ice is directly vaporized during the freeze-drying process. Freshly harvested cells were frozen at three different temperatures for 4 h and afterwards lyophilized, then biotransformations were performed. Enzyme activity was reduced by more than 60% in all conditions, and no major difference between the freezing temperatures could be seen (Fig. 2B). All following experiments were performed with a freezing step at −80 °C for 4 h before lyophilization, to guarantee that the samples were frozen thoroughly. As the process of freeze-drying is impairing the integrity of the cells and proteins, different osmotic stabilizers, called cryoprotectants, can be added to the cells prior to the lyophilization process. Various cryoprotectants are known in literature and for TDO, the effects of glycerol, glucose and skim milk have been investigated [19,21,26]. To establish the method, we tested the two sugars sucrose and trehalose, and glycerol, which are well known for effective cryopreservation of cells. 1 g of the harvested cell pellet was transferred to a 50 mL tube, and resuspended in 10 mL 100 mM potassium phosphate buffer pH 7.4, enriched with a final concentration of 5% (*w/v*) sucrose, 5% (*w/v*) trehalose or 10% (*v/v*) glycerol. Cell suspensions were centrifuged, the supernatant was discarded and the pellets were subsequently frozen for 4 h at −80 °C and then lyophilized. Biotransformation revealed that the activity of the treated and lyophilized enzymes was restored and even increased at least by a factor 2 compared to fresh cells directly after cell harvest (Fig. 2C). Several tests showed that the addition of 10% (*v/v*) glycerol to reaction buffer led to the
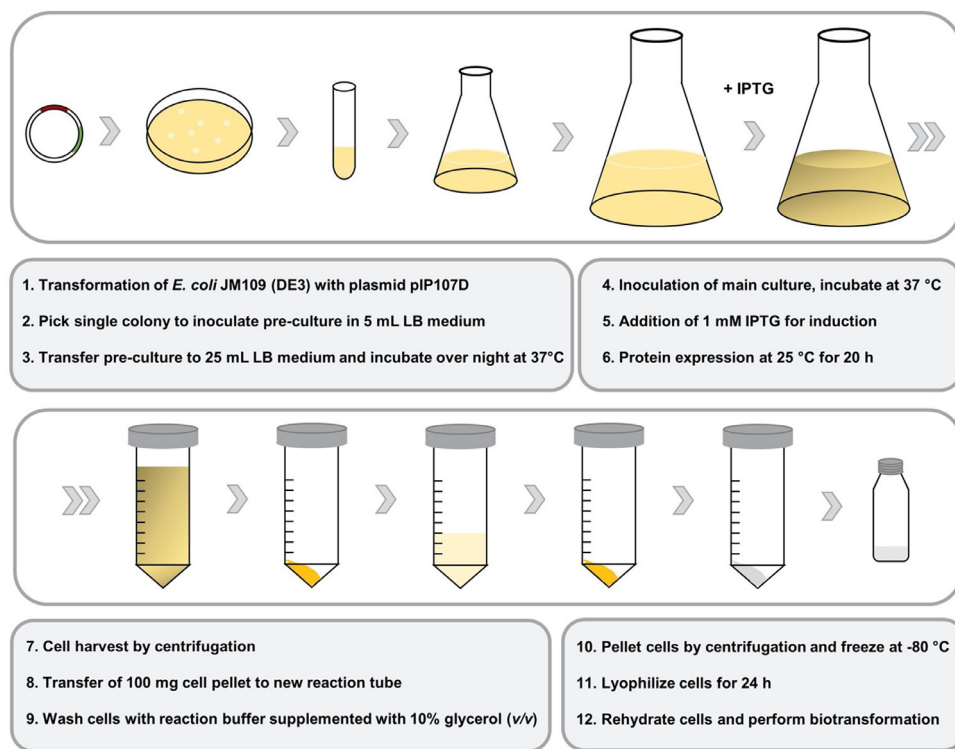
Fig 1. Graphical display of the described method, starting from transformation of the cells, cell cultivation and harvest, treatment with cryoprotectant and lyophilization step.

highest product formation of carveol. Therefore, this condition was selected for further experiments. The addition of 20% (*v/v*) glycerol led to a rise in activity but also caused a change of consistency of the freeze-dried cell pellets (Figs. 1 and 2D). Instead of a gray and dusty texture, the pellets were sticky, challenging to resuspend, and showed a brownish color. Determination of the viable cell count revealed a reduction of CFU of a factor 10^4 in lyophilized cell suspensions, whereas the total protein of the cell did not differ from living cells. Therefore, enzyme levels should also not be impaired (Fig. 2E+F). Note that the best results were obtained with freshly transformed cells. The dioxygenase catalyzed dihydroxylation is highly dependent on the co-substrate oxygen and therefore for the air to liquid ratio of the reaction vessel. In our experience the highest product formation can be achieved with a liquid to air ration of 1:19 (1 mL reaction volume in 20 mL reaction vessel).

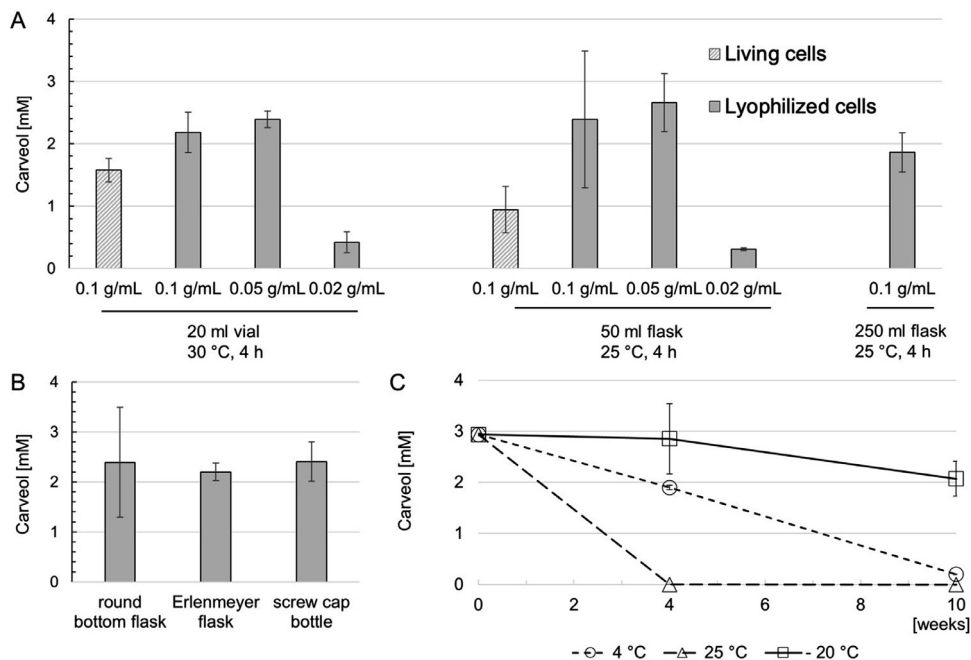*Study of biotransformation conditions*

After investigating the effect of different cryoprotectants on enzyme activity, the next goal was to determine the optimal conditions for biotransformations in upscaled volume to ensure optimal application conditions. To address this subject, different reaction volumes, vessels, temperatures and cell concentrations were tested in biotransformations. The formation of carveol by adding 10 mM *R*-limonene to 0.1, 0.05 and 0.02 g_{cww} ml L^{-1} was compared under different reaction conditions. The cell concentration of 0.05 g_{cww} L^{-1} led to the highest product formation (Fig. 3A). The formation of carveol was also not impaired by the change of reaction vessel and reaction temperature. While control reactions (1 mL reaction in 20 mL vial at 30 °C) were performed in gas tight vials to avoid *R*-limonene and carveol evaporation, the upscaled reactions were performed in round-bottom flasks sealed with stoppers with ground glass joint and laboratory film (Parafilm, USA). To maintain the 1:19 liquid to
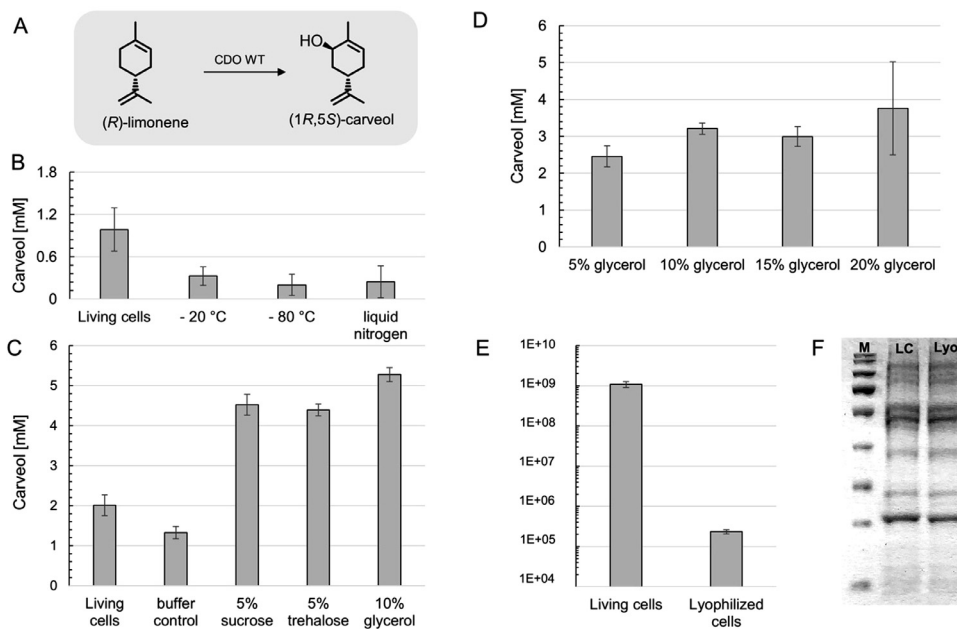
Fig. 2. Study of different cell treatments before lyophilization. The CDO cluster was expressed in JM109 (DE3) cells and after cell harvest, the cells were frozen and subsequently lyophilized. R-limonene was used as a substrate and (1R,5S)-carveol is formed as the only product by CDO WT (A). Different freezing temperatures (−20 °C, −80 °C and −196 °C / liquid nitrogen) before lyophilization did not lead to a difference in enzyme activity (B). After cell harvest, the cell pellets were washed with 100 mM potassium phosphate buffer pH 7.4, that was supplemented with three different cryoprotectants to test the protection efficiency on the expressed enzymes, frozen at −80 °C and lyophilized. Cells were rehydrated and enzyme activity was measured. Supplementation with 10% (v/v) glycerol could restore the highest enzyme activity, but no clear difference between glycerol and the sugars sucrose and trehalose was observed (C). A follow-up experiment showed, that different glycerol concentrations (5%, 10%, 15%, 20%, (v/v) each) did not lead to strong differences in enzyme activity (D). Biotransformations with 10 mM R-limonene were performed in technical triplicates with a concentration of 0.1 g_{cww} mL^{-1} for 4 h at 30 °C. They were subsequently 1:1 (v/v) extracted with MTBE and analyzed on a GC-FID system. Determination of the live cell count by the Miles and Misra method [23] revealed a reduction of CFUs. Cell dilutions were plated in triplicates, average shown in (E). Comparison of the total protein amount extracted from the living cell (LC) and lyophilized cells (Lyo) did not show differences (F). The use of recombinant cells from different batches led to a divergence of the total product formation (2 B, C, D). Within one experiment, only cells from one batch were used to assure comparability.

air ratio, the reaction volume was scaled up to 2.5 mL in a 50 ml flask and 12.5 mL in a 250 mL flask. The round-bottom flasks were incubated at room temperature on magnetic stirrers and after 4 h 1 mL of every replicate was extracted and analyzed. The results showed, that biotransformations were successfully performed in upscaled conditions at room temperature (in Fig. 3A, referred to as 25 °C), showing that the reactions can be performed in laboratories that are not equipped with temperature-controlled shaking incubators. Reactions performed in 50 mL screw cap bottles and Erlenmeyer flasks sealed with parafilm did not differ from round-bottom flasks, leading to the statement that there is no restriction in the choice of reaction vessel, despite the volatility of the examined molecules (Fig. 3B). Samples of lyophilized cell powder were stored under different temperature conditions for several weeks and enzyme activity was tested after four and ten weeks (Fig. 3C). By storage at room temperature (25 °C), no enzyme activity could be detected anymore after four weeks. The storage of the samples in a fridge (4 °C) led to a drop of around 35% activity, and after ten weeks to an almost complete loss. The enzymatic activity was very well preserved when stored in a freezer at −20 °C, and after ten weeks still 70% of the activity compared to the starting point could be detected. These results suggest that the lyophilized recombinant cells can be stored up to 2.5 months in freezers without serious restrictions in terms of activity.

Fig. 3. Investigation of biotransformation reaction conditions. The activity of the CDO cluster expressed in JM109 (DE3) cells was compared before and after lyophilization by biotransformations with *R*-limonene. The treatment with 10% (*v/v*) glycerol before freeze-drying led to a rise in the activity. The yield of the product carveol was compared between different cell concentrations. The upscaling of the reactions to 2.5 mL in 50 mL round-bottom flasks and 12.5 mL in 250 mL round-bottom flasks yielded product concentrations in the same range, showing that the incubation temperature and reaction vessel and volume did not negatively influence the reaction (A). Besides, no disadvantage could be detected by the use of different reaction vessels (B). Storage of lyophilized cell powder at −20 °C showed best to avoid losing enzyme activity for at least 2.5 months (C). Biotransformations were performed as follows: (A) 0.1 g_{cww} mL^{-1} of living cells and 0.1, 0.05, 0.02 g_{cww} mL^{-1} of lyophilized cells (treated with 10% (*v/v*) glycerol before freeze-drying) were resuspended in 100 mM potassium phosphate buffer pH 7.4. 1 mL reactions in 20 mL vials were performed as described in the method section, larger reaction volumes were upscaled linear to the 1 mL reaction. 10 mM *R*-limonene as substrate were used, incubation was conducted for 4 h at 30 °C at 180 rpm in shaking incubator and for 4 h at room temperature (25 °C) on a magnetic stirrer (700 rpm), respectively. 1 mL per triplicate of 2.5 mL and 12.5 mL reaction was extracted and analyzed. (B) Biotransformations with 0.1 g_{cww} mL^{-1} lyophilized cells (treated with 10% (*v/v*) glycerol) were performed in 2.5 mL reaction in 50 mL reaction vessel with 10 mM *R*-limonene and incubated for 4 h at room temperature on a magnetic stirrer. 1 mL per triplicate of 2.5 mL reaction was extracted and analyzed. (C) Lyophilized cells (treated with 10% (*v/v*) glycerol) were stored in the dark at room temperature (25 °C), in a lab fridge (4 °C) and freezer (−20 °C). A cell concentration 0.05 g_{cww} mL^{-1} was used in 1 mL reaction in 20 mL reaction vessel, with 10 mM *R*-limonene for 4 h at 30 °C and 180 rpm in shaking incubator.

Semi-preparative biotransformation

To validate the developed method for its application on a larger scale, biotransformations were carried out in a semi-preparative volume. In this context, a different enzyme-substrate combination was used than in the previous experiments. The bicyclic aromatic compound naphthalene is known as the natural substrate of the NDO of *Pseudomonas spec.* strain 9816–4, and is also successfully converted by the TDO of *Pseudomonas putida* F1 in the only product (1*R*,2*S*)-*cis*-1,2-dihydro-1,2-naphthalenediol (DHND) [11,16,21]. It could also be shown, that the CDO is able to catalyze the reaction from naphthalene to (1*R*,2*S*)-*cis*-1,2-dihydro-1,2-naphthalenediol (Fig. 4A) [27]. The advantage of naphthalene as a substrate, compared to *R*-limonene, is the low volatility of the substrate and lack of volatility of the product DHND. The compounds can be isolated easily, and are thus feasible for an exemplary upscaling reaction to a semi-preparative scale. The CDO variant M232A has been
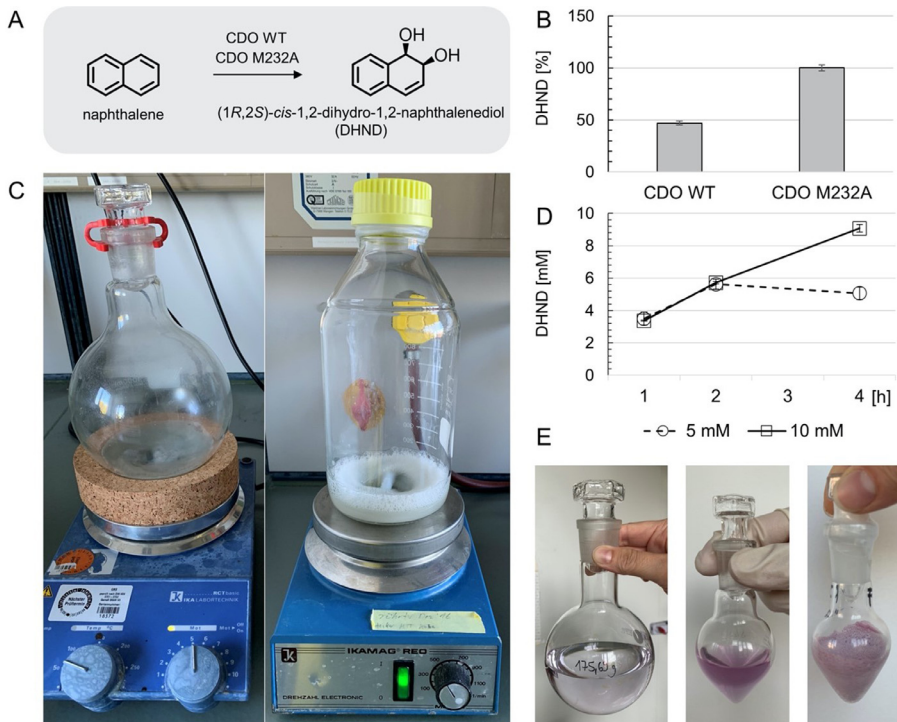
Fig 4. Biotransformations were upscaled to semi-preparative reaction volume with the substrate naphthalene. In pre-experiments CDO WT and CDO variant M232A, expressed in JM109 (DE3) cells, showed to convert naphthalene to (1R,2S)-cis-1,2-dihydro-1,2-naphthalenediol (DHND) and no additional product could be detected by HPLC-DAD analysis (see supplementary material, Fig. S1). CDO M232A was used for upscaled reaction volumes of 50 mL in round-bottom flasks and screw-cap bottles (C). Biotransformations: (B)+(D) Biotransformations in analytical volume, 0.05 g_{cww} mL^{-1} lyophilized cells (treated with 10% (v/v) glycerol) in 1 mL reaction, incubation for 4 h at 30 °C, with 10 mM naphthalene (B) and 5 mM and 10 mM Naphthalene (D). The reactions were extracted once 1:1 (v/v) with MTBE. (C) Biotransformations in semi-preparative volume, 0.05 g_{cww} mL^{-1} lyophilized cells (treated with 10% (v/v) glycerol, from the same cell batch as used in (D)), were incubated for 4 h at room temperature on a magnetic stirrer with 5 mM naphthalene in a total volume of 50 mL potassium phosphate buffer pH 7.4. The reactions were extracted three times 1:1 (v/v) with MTBE. The product DHND was separated by evaporating the solvent MTBE (steps are depicted in the pictures). The weight of the extracted product was determined after complete drying (E).

described previously and is known for its increased activity to enhance conversions of different substrates [13]. A comparison between lyophilized cells harboring CDO WT and CDO M232A showed doubled product yields after reactions with CDO M232A within 4 h (Fig. 4B). Two different substrate concentrations were tested to validate the activity of CDO M232A before the 50 mL upscale reaction was performed. Biotransformations with 5 mM naphthalene were shown best for a full conversion after 2 h (Fig. 4D). Biotransformations were performed on a magnetic stirrer for 4 h to make sure all substrate was converted. To test different reaction vessels, the reactions were performed in round-bottom flasks (2 × 50 mL reaction volume) and screw-cap bottles (1 × 50 mL, Fig. 4C). Product quantity of 120 mg/100 mL (round-bottom flask) and 80 mg/100 mL (screw-cap bottle) could be isolated (Fig. 4E). The product identity was confirmed by NMR analysis (see supplemental material). The purple color of the extracted product is caused by a side reaction that is catalyzed by dioxygenases: the dihydroxylation of indole, which spontaneously dehydrates and forms the blue dye indigo [28]. It is known that the reaction is mediated by a tryptophanase (TnaA) in E. coli and can be avoided by introducing a deletion of the tnaA gene. Using a tnaA deficient strain like BW25113 ΔtnaA,

that is available in the Keio collection, could be a solution to address the cross contamination of the product [22,29,30,31].

Material and equipment

1. HT Multitron Infors Shaker (Infors HT, Switzerland).
2. Beckman Coulter centrifuge Avanti J-26S XP, Rotor JA-10 (Beckman Coulter, USA).
3. Microcentrifuge 5424 R (Eppendorf, Germany).
4. Centrifuge 5810 R (Eppendorf, Germany), fixed-angle rotor F-34–6–38 (Eppendorf, Germany).
5. Freeze-dryer (Martin Christ Gefriertrocknungsanlagen, Germany).
6. GC-2010 gas chromatograph (Shimadzu, Japan).
7. Agilent 1260 Infinity II LC system (Agilent, USA).
8. Ultrospec 3100 pro photometer (Amersham Biosciences, United Kingdom).
9. Thermomixer comfort 1.5 ml (Eppendorf, Germany).
10. Vortex-Genie 2 mixer (Scientific industries, USA).
11. Rotator mixer (Intelli-Mixer RM-2 M, ELMI, USA).
12. Rotary evaporator Laborota 4000 efficient (Heidolph. Germany).
13. 2 mL reaction tubes (Eppendorf, Germany).
14. 50 mL reaction tubes (Sarstedt, Germany).
15. 20 mL head space vials (Wicom, Germany).
16. Screw cap bottles (Schott, Germany).
17. Glass ware (round-bottom flask and Erlenmeyer flask, Schott, Germany).

Chemicals *and culture media*

- *R*-(+)-Limonene (Sigma Aldrich, USA)
- (-)-Carveol, mixture of isomers (Sigma Aldrich, USA)
- Naphthalene (Fluka, USA)
- (1*R*,2*S*)-*cis*-1,2-dihydro-1,2-naphthalenediol (Santa Cruz Biotechnology, USA)
- Biphenyl (Sigma Aldrich, USA)
- Ampicillin sodium salt (Carl Roth, Germany)
- Agar-agar (Carl Roth, Germany)
- Yeast extract (Carl Roth, Germany)
- Tryptone (Sigma Aldrich, USA)
- NaCl (Honeywell Fluka, USA)
- Glycerol (Fisher Scientific, USA)
- D-(+)-Glucose monohydrate (Acros organics, Belgium)
- D-(+)-Trehalose Dihydrat (Carl Roth, Germany)
- Sucrose (Sigma-Aldrich, USA)
- Potassium dihydrogen phosphate (KH_2PO_4, Carl Roth, Germany)
- Dipotassium phosphate (K_2HPO_4, Carl Roth, Germany)
- Dimethylsulfoxide (DMSO, Carl Roth, Germany)
- Ethanol (Merck, Germany)
- *tert*-butyl methyl ether (MTBE, Honeywell Riedel-de-Haën, Germany)

All chemicals were purchased at the highest purity available.

Strains and constructs

The CDO cluster was expressed in *E. coli* strain JM109 (DE3). The *cumA1, cumA2, cuma3* und *cuma4* of *Pseudomonas fluorescens* IP01 forming the CDO cluster were expressed under the *lac*-promoter in the vector pIP107D. The enzyme variant M232A expressed in pIP107D_M232A (pITB1012) harbors the mutation M232A in *cumA1*, the oxygenase α-subunit [10,13,30].

Acknowledgements

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10. 1016/j.mex.2021.101323.

References

[1] B. Hauer, Embracing nature's catalysts: a viewpoint on the future of biocatalysis, ACS Catal. 10 (15) (2020) 8418–8427, doi:10.1021/acscatal.0c01708.

[2] F.H. Arnold, Directed evolution: bringing new chemistry to life, Angew. Chemie - Int. Ed. 57 (16) (2018) 4143–4148, doi:10. 1002/anie.201708408.

[3] F.H. Arnold, Innovation by evolution: bringing new chemistry to life (Nobel Lecture), Angew. Chemie - Int. Ed. 58 (41) (2019) 14420–14426, doi:10.1002/anie.201907729.

[4] M.D. Truppo, Biocatalysis in the pharmaceutical industry: the need for speed, ACS Med. Chem. Lett. 8 (5) (2017) 476–480, doi:10.1021/acsmedchemlett.7b00114.

[5] D.J. Ferraro, L. Gakhar, S. Ramaswamy, Rieske business: structure-function of Rieske non-heme oxygenases, Biochem. Biophys. Res. Commun. 338 (1) (2005) 175–190 Biochem Biophys Res CommunDec. 09, doi:10.1016/j.bbrc.2005.08.222.

[6] C. Perry, E.L.C. De Los Santos, L.M. Alkhalaf, G.L. Challis, Rieske non-heme iron-dependent oxygenases catalyse diverse reactions in natural product biosynthesis, Nat. Prod. Rep. 35 (7) (2018) 622–632, doi:10.1039/c8np00004b.

[7] L.P. Wackett, Mechanism and applications of Rieske non-heme iron dioxygenases, in: Enzyme and Microbial Technology, 31, Elsevier, 2002, pp. 577–587, doi:10.1016/S0141-0229(02)00129-1. Oct. 03.

[8] S.M. Barry, G.L. Challis, Mechanism and catalytic diversity of rieske non-heme iron-dependent oxygenases, ACS Catal. (2013), doi:10.1021/cs400087p.

[9] T.D. Bugg, S. Ramaswamy, Non-heme iron-dependent dioxygenases: unravelling catalytic mechanisms for complex enzymatic oxidations, Curr. Opin. Chem. Biol. 12 (2) (2008) 134–140 Curr Opin Chem Biol, doi:10.1016/j.cbpa.2007.12.007.

[10] H. Aoki, T. Kimura, H. Habe, H. Yamane, T. Kodama, T. Omori, Cloning, nucleotide sequence, and characterization of the genes encoding enzymes involved in the degradation of Cumene to 2-Hydroxy-6-Oxo-7-Methylocta-2,4-Dienoic Acid in Pseudomonas fluorescens IP01, J. Ferment. Bioeng. (1996), doi:10.1016/0922-338X(96)82207-0.

[11] R.E. Parales, K. Lee, S.M. Resnick, H. Jiang, D.J. Lessner, D.T. Gibson, Substrate specificity of naphthalene dioxygenase: effect of specific amino acids at the active site of the enzyme, J. Bacteriol. (2000), doi:10.1128/JB.182.6.1641-1649.2000.

[12] G.J. Zylstra, D.T. Gibson, Toluene degradation by Pseudomonas putida F1, J. Biol. Chem. (1989).

[13] C. Gally, B.M. Nestl, B. Hauer, Engineering Rieske Non-Heme Iron Oxygenases for the Asymmetric Dihydroxylation of Alkenes, Angew. Chemie Int. Ed. 54 (44) (2015) 12952–12956 Oct., doi:10.1002/anie.201506527.

[14] J.M. Halder, B.M. Nestl, B. Hauer, Semirational Engineering of the Naphthalene Dioxygenase from *Pseudomonas* sp. NCIB 9816-4 towards Selective Asymmetric Dihydroxylation, ChemCatChem 10 (1) (2018) 178–182 Jan., doi:10.1002/cctc. 201701262.

[15] M.A. Vila, V. Steck, S. Rodriguez Giordano, I. Carrera, R. Fasan, C−H amination via nitrene transfer catalyzed by mononuclear non-heme iron-dependent enzymes, ChemBioChem (2020), doi:10.1002/cbic.201900783.

[16] G.J. Zylstra, W.R. McCombie, D.T. Gibson, B.A. Finette, Toluene degradation by Pseudomonas putida F1: genetic organization of the tod operon, Appl. Environ. Microbiol. (1988), doi:10.1128/aem.54.6.1498-1503.1988.

[17] W.K. Yeh, D.T. Gibson, T.N. Liu, Toluene dioxygenase: a multicomponent enzyme system, Biochem. Biophys. Res. Commun. (1977), doi:10.1016/0006-291X(77)91268-2.

[18] G.F. Catterall, P.A. Williams, Some properties of the naphthalene oxygenase from Pseudomonas sp. NCIB 9816, J. Gen. Microbiol. (1971), doi:10.1099/00221287-67-1-117.

[19] S. Piszkiewicz, G.J. Pielak, Protecting Enzymes from Stress-Induced Inactivation, Biochemistry (2019), doi:10.1021/acs. biochem.9b00675.

[20] R. Pisano, A. Arsiccio, L.C. Capozzi, B.L. Trout, Achieving continuous manufacturing in lyophilization: technologies and approaches, Eur. J. Pharma. Biopharma. (2019), doi:10.1016/j.ejpb.2019.06.027.

[21] M.A. Vila, et al., Production of cis-1,2-dihydrocatechols of high synthetic value by whole-cell fermentation using Escherichia coli JM109 (pDTG601): a detailed study, J. Mol. Catal. B Enzym. 96 (2013) 14–20, doi:10.1016/j.molcatb.2013.06.003.

[22] J.L. Wissner, W. Escobedo-Hinojosa, P.M. Heinemann, A. Hunold, B. Hauer, Methods for the Detection and Analysis of Dioxygenase Catalyzed Dihydroxylation in Mutant Derived Libraries, 644, 1st ed., Elsevier Inc., 2020.

[23] A.A. Miles, S.S. Misra, J.O. Irwin, The estimation of the bactericidal power of the blood, J. Hyg. (Lond). (1938), doi:10.1017/S002217240001158X.
[24] J. Schrader, Biokatalyse: mikrobielle oxidation von monoterpenen, BioSpektrum (2010).
[25] N. Oberleitner, et al., From waste to value-direct utilization of limonene from orange peel in a biocatalytic cascade reaction towards chiral carvolactone, Green Chem. 19 (2) (2017) 367–371, doi:10.1039/c6gc01138a.
[26] S.B. Leslie, E. Israeli, B. Lighthart, J.H. Crowe, L.M. Crowe, Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying, Appl. Environ. Microbiol. (1995), doi:10.1128/aem.61.10.3592-3597.1995.
[27] J. Halder, "Naphthalen Dioxygenase aus Pseudomonas sp. NCIB 9816-4: systematische Analyse der aktiven Tasche," p. 163, 2017.
[28] A.N. Fabara, M.W. Fraaije, An overview of microbial indigo-forming enzymes, Appl. Microbiol. Biotechnol. (2020), doi:10.1007/s00253-019-10292-5.
[29] S. Brixius-Anderko, F. Hannemann, M. Ringle, Y. Khatri, R. Bernhardt, An indole-deficient Escherichia coli strain improves screening of cytochromes P450 for biotechnological applications, Biotechnol. Appl. Biochem. (2017), doi:10.1002/bab.1488.
[30] G. Li, K.D. Young, Indole production by the tryptophanase TnaA in escherichia coli is determined by the amount of exogenous tryptophan, Microbiol. (United Kingdom) (2013), doi:10.1099/mic.0.064139-0.
[31] T. Baba, et al., Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection, Mol. Syst. Biol. (2006), doi:10.1038/msb4100050.