Spheroplasts preparation boosts the catalytic potential of a terpene cyclase

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Abstract

Squalene-hopene cyclases (SHCs) are a highly valuable and attractive class of membrane-bound enzymes as sustainable biotechnological tools to produce aromas and bioactive compounds at industrial scale. However, their application as whole-cell biocatalysts suffer from the outer cell membrane acting as a diffusion barrier for the highly hydrophobic substrate/product, while the use of purified enzymes leads to dramatic loss of stability. Here we present an unexplored strategy for biocatalysis: the application of SHC spheroplasts. By removing the outer cell membrane, we produced stable and substrate-accessible biocatalysts. SHC spheroplasts exhibited up to 100-fold higher activity than their whole-cell counterparts for the biotransformations of squalene, geranyl acetone, farnesol, and farnesyl acetone. Their catalytic ability was also higher than the purified enzyme for all high molecular weight terpenes. In addition, we introduce a new concept for the carrier-free immobilization of spheroplasts via crosslinking, CLS (crosslinked spheroplasts). The CLS maintained the same catalytic activity of the spheroplasts, offering additional advantages such as recycling and reuse. These timely solutions contribute not only to harness the catalytic potential of the SHCs, but also to make biocatalytic processes even greener and more cost-efficient.

Introduction

Biocatalysis as the use of enzymes to speed-up organic reactions has become a sustainable and efficient alternative to replace or complement traditional chemical catalysis.[1] Typically, biocatalysts can be applied as cell-free enzymes or as whole-cell (mostly bacteria, fungi, yeast) biocatalysts. Cell-free enzymes are preferred to avoid secondary reactions that may happen within the cell resulting in a decrease of the desired product yield.[2] However, the purification protocols clearly add time and costs to the process. Partial purification of enzymes can also be carried out by simpler methods such as heat shock (for thermophilic proteins) and ammonium sulfate precipitation, often at the expense of the purity degree of the cell-free enzyme.[3] On the other hand, whole-cell biocatalysts are desirable for multi-step transformations that require several enzymes, or for cofactor-dependent reactions.[4] Likewise, whole

cells often protect enzymes from exterior stresses and grant catalytic activity in a more natural environment.[5,6]

One of the most challenging biocatalysts to handle are monotopic membrane-bound enzymes.[7] The purification of these enzymes is tedious given that the non-soluble enzymes require the addition of detergents and stabilizers to extract the enzymes from the lipid bilayer. Upon solubilization, the additives must still be maintained in the enzyme solution to preserve the structure integrity and activity of the enzyme outside its biological environment. Among the membrane-bound enzymes, squalene-hopene cyclases (SHC) are a class of enzymes with a great potential as biocatalysts to produce high-value flavors, fragrances, and precursors for bioactive molecules.[8,9] The biocatalytic potential of SHCs in *E. coli* whole-cell environment have been recently reported.[10] Nevertheless, it is well-known that the cell membrane is a diffusion barrier for the highly hydrophobic substrates/products, hampering the enzymatic activity.[11] To alleviate the diffusion issues, strategies such as the introduction of transporter enzymes into the cell membrane or the use of additives (e.g. SDS) are applied to increase cell permeability. However, such approaches entail time-consuming molecular cloning, tedious downstream steps, and increased process costs.[10–12] Consequently, efficient strategies to circumvent mass transfer issues are of high interest in whole cell biocatalysis.

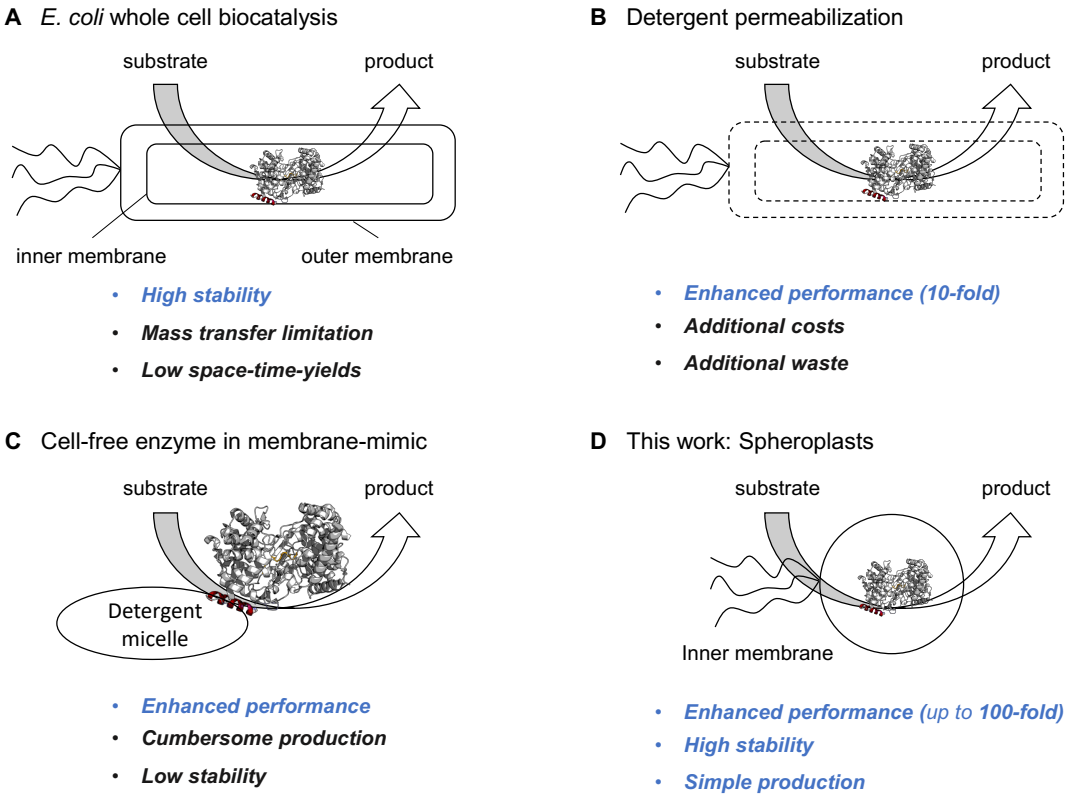


Fig. 1 Strategies applied in SHC catalysis to avoid mass transfer issues. **a** *E. coli* whole cell biocatalysis with SHC benefits from high stability of the enzyme in its host. However, mass transfer limitations of the membrane hamper the productivity.[13] **b** Deliberate permeabilization of the cell membrane by using detergents enhances the mass transfer but increases the process costs and creates additional waste.[10] **c** The application of islolated enzyme in an artificial membrane mimic enhances the mass transfer but the enzymes suffer from low stability.[14] **d** Spheroplasts comprise a promising alternative to previous applications.

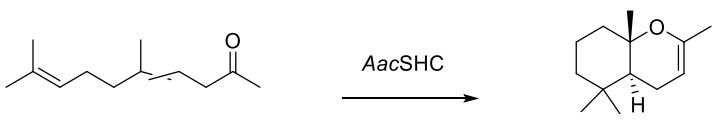
Flow biocatalysis is an emerging technology which improves the reaction productivity while minimizing waste and energy consumption.[15] To integrate the biocatalysts into a continuous flow reactor, both cell-free enzymes and whole cells are generally immobilized on a carrier.[16] Since no universal protocol suits the immobilization of every enzyme, diverse strategies have been developed over the last decades which can generally be classified in three main approaches: binding to a premade support, entrapment into a polymer network, and crosslinking.[17,18]

A compromise between cell-free enzymes and whole cells is offered by spheroplasts preparations: these are gram negative bacterial cells in which the outer membrane has been partially or completely removed. Despite some applications of spheroplasts reported in biomedicine and cell biology research, their potential role as biocatalysts has been overlooked.[19,20]

Here, we present an easy and quick preparation of *E. coli* spheroplasts and their novel application as biocatalysts to tackle issues of substrate/product diffusion, additives requirement, costly preparation of biocatalysts, and integration into a flow reactor. The performance of the three types of biocatalyst preparation (whole cells, cell-free enzymes, and spheroplasts) have been compared under different reaction conditions. As a proof of concept, we have employed a membrane-bound enzyme from *Alicyclobacillus acidocaldarius* (*Aac*SHC) for the cyclisation of various terpenoids. To optimize the application of this industrially relevant enzyme, different immobilization protocols have been tested. As an innovative alternative to the traditional immobilization techniques, we introduce the crosslinking of spheroplasts (CLS) as an optimal, cost-effective, and sustainable strategy.

Results

***Aac*SHC as a whole-cell biocatalyst in batch and flow.** Initially, and based on previous studies on SHC biocatalysis, we defined the cyclization of *E*/*Z*-geranyl acetone **1t** (trans) **/c** (cis) into the bicyclic product **2t/c** as a model reaction to examine the performance of the *Aac*SHC enzyme in the *E. coli* whole-cell environment (Scheme 1).[8,9] Noteworthy, *Aac*SHC WT presents a much higher preference for the cyclization of the *E*-isomer **1t**, thus the maximum conversion that can be expected is 50%.[8] The product formation was analyzed by gas chromatography, revealing only 7% conversion at 10 mM scale after 24 h at 30ºC corresponding to a space-time yield of 0.006 g/L·h^{-1}. However, it is a common drawback of SHC biocatalysis that smaller substrate analogues suffer from up to 10^3 slower cyclization rates.[14]



Geranylacetone **1t/c** Hexahydrochromene **2t**

Scheme 1. Biotransformation of geranyl acetone with the wild-type *Aac*SHC. Only the *E*-isomer **1t** is converted into the product **2t** by the enzyme *Aac*SHC.

As our first strategy, we hypothesized that the enhanced mass transfer that takes place in continuous flow reactors could benefit the SHC catalytic rate.[21] In order to implement the enzymatic reaction into continuous flow, the whole cells were immobilized by entrapment into alginate hydrogel

beads. Firstly, the entrapped cells were tested in batch biotransformations reaching the same product formation as the non-entrapped cells (7%) (Table S1A). Longer reaction times 48-72 h were also tested, with no improvement (Table S1B). Packing of the entrapped cells into a flow reactor and running the cyclization of **1t/c** in continuous mode led however to even lower conversions (Table S2). Alternative strategies such as immobilization on agarose and methacrylate did not improve the overall reaction yield (Fig. S1A-C) which was impacted also by the substrate partial affinity for the resin material (in addition to the cell membrane) (Fig. S2A-B).

***Aac*SHC as a cell-free enzyme biocatalyst in batch and flow.** To tackle the problem of substrate/product detection when using whole cells, we tested cell-free enzymes as biocatalysts. *Aac*SHC was partially purified according to a previously reported method,[22] and applied in batch biotransformations for the cyclization of **1t/c**. In this case, while the conversion was low (7%) the substrate was fully and reproducibly extracted unlike with whole cells (Fig. S3). Then, *Aac*SHC was immobilized on methacrylate microbeads, maintaining up to >99% of its activity when compared to the soluble form (Fig. S4). However, yet again the conversion only reached 7%, despite testing several immobilization chemistries and conditions (Fig. S5). An additional attempt to perform the biotransformation in flow was done with the best immobilized system, *Aac*SHC covalently immobilized on methacrylate microbeads (HFA403) (Table S3). To facilitate the substrate/product elution from the resin, the flow biotransformation was carried out in a biphasic system (2:1:1 buffer, ethyl acetate and cyclohexane) [23] without any significant improvement (4% conversion). Triton X-100 has been reported to mimic the natural environment of membrane associated proteins, as well as increasing the water solubility of organic molecules, and it could also prevent the non-specific binding of organic molecules to the resin.[24–26] When this was tested in flow, it did not lead to any improvement (Table S3). Likewise, increasing the retention time and temperature had only minimal effect (8% conversion) (Table S3).

***Aac*SHC spheroplasts: an innovative and more efficient type of biocatalysts.** As monotopic enzymes such as the SHC are strongly dependent on an intact membrane,[27,28] we envisioned a hybrid biocatalyst combining the natural environment of cellular lipidic layer while minimizing the entrapment of the substrate/product in the cell wall. Previous insights on the subcellular location of SHCs indicated that these enzymes are bound to the cytoplasmic membrane when expressed in bacteria.[22] Taking advantage of this circumstance, the SHC represented an ideal candidate to explore spheroplasts as a novel type of biocatalysts. The *E. coli* outer membrane could be easily dissolved by simple membrane digestion with lysozyme and EDTA (Fig. 1).[29] Under the microscope, the resulting spheroplasts showed the typical, more circular shape, confirming the loss of the outer membrane.[30] The presence of active *Aac*SHC within the spheroplasts was confirmed by SDS-PAGE analysis and activity test (Fig. S6). The biotransformation yield obtained with the spheroplasts was 6-fold higher than using whole-cell biocatalysts, reaching full conversion of the *E*-isomer **1t** to the bicyclic product **2t** at 1-2 mM scale (Table 1). Remarkably, the productivity was 17-fold higher with spheroplast biocatalysts achieving 0.19 g/L of **2t**. Indeed, GC analyses showed that whole-cell biocatalysts retained both substrate and product, while this was completely avoided with the spheroplast preparation (Fig. S2B, S7, and GC chromatograms in ESI).

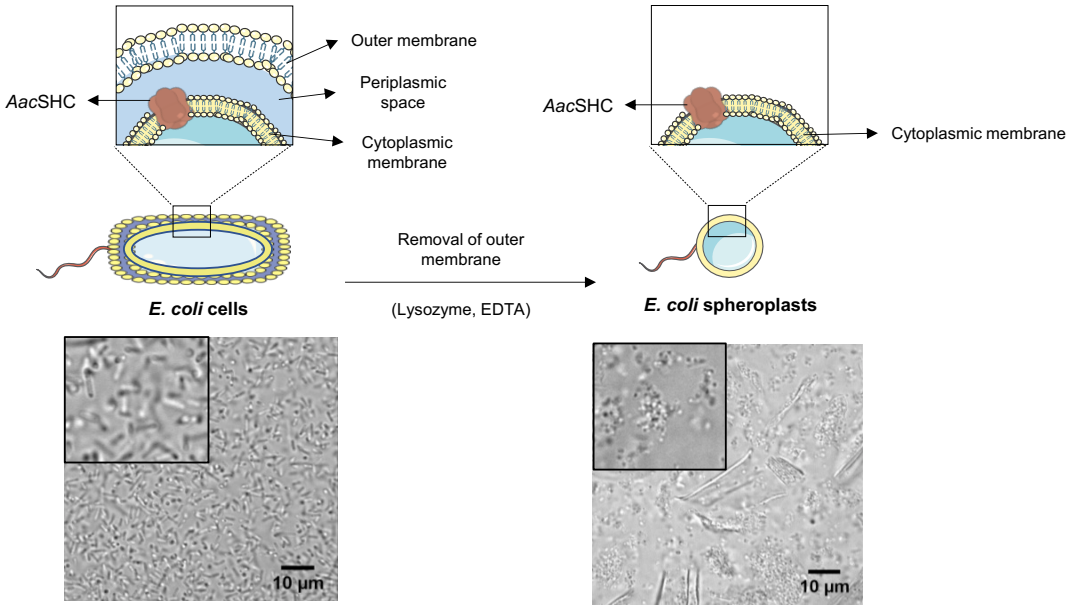


Fig. 1. Preparation of spheroplasts by removing the outer membrane of *E. coli* cells. The microscopy images show the change of shape from whole cells to spheroplasts.

As the substrate/product extraction was no longer an issue when using spheroplast biocatalysts, we investigated whether the addition of surfactants and other molecules that are typically required to extract the substrate/product from whole-cell biotransformations could be avoided.[9] We found that neither the addition of SDS or cyclodextrins (CD) influenced the detection of both substrate **1t/c** and product (Table S4) and therefore are no longer essential. Furthermore, the robustness of the spheroplasts under freeze-drying conditions was assessed. After lyophilization and re-hydration, *Aac*SHC spheroplasts showed the same biocatalytic activity as before lyophilization (Table S5), offering an excellent storing methodology.

Table 1. Comparison of the efficiency of whole-cell biocatalysts and spheroplast biocatalysts. Biotransformations with 10 mg of biocatalyst (whole cells or spheroplasts, or 10 µL of supernatant) in 1 mL of 10 mM **1t/c**, 1% DMSO and 20 mM citric acid buffer at pH 6.0. The reactions were incubated at 30 ºC for 24 h. The amount protein was determined after extraction and solubilization of *Aac*SHC. The specific activity was calculated from biotransformations (24h) and it is defined as µmol of product per minute and mg of enzyme. m.c.: molar coversion.

Fraction	Protein (mg)	m.c. (%)	Productivity in 24h ($x10^{-3}$ mmol product/mg enzyme)	Specific activity (U/mg)
Whole cells	0.8	8	0.2	0.14
Spheroplasts	0.28	49	3.5	2.4
Supernatant	<0.1	n.d.	n.d.	n.d.

Spheroplast biocatalysts for the efficient cyclization of terpenes. With these excellent results, we expanded the application of *Aac*SHC spheroplasts as well as two additional SHC variants (*Aac*SHC-G600F and *Aac*SHC-F365C) which were previously developed for the cyclization of geraniol.[31] We compared the enzymes specific turnover frequencies (TOFs) with those of the whole cells, whole cells supplemented with SDS,[10] and the cell-free enzyme (Fig. 2). First, the natural reaction of squalene **3** towards hopene **4** was evaluated. Due to its high hydrophobicity, this substrate is barely converted with a TOF of 0.18 h^{-1} with whole cell.[8] Treating the cells with SDS slightly improved the TOF to 0.2 h^{-1}. The application of the cell-free enzyme in the presence of the membrane mimic CHAPS improved the reaction 37-fold to 6.6 h^{-1}. However, the spheroplasts showed a remarkable improvement of 98-fold in TOF to >17 h^{-1}. Next, we examined the promiscuous cyclization of *E*,*E*-farnesol **5** towards drimenol **6** using the *Aac*SHC-G600F. Whole cells as well as whole cells treated with SDS displayed TOFs of ~2.5 h^{-1}. The isolated enzyme and the spheroplasts improved the performance more than double to 7-8 h^{-1}. Pure *E*-geranyl acetone **1t** was converted to **2t** with increasing TOF in the order of whole cells, whole cells treated with SDS, isolated enzyme and spheroplasts with a maximum of 12-fold improvement to >17 h^{-1}. The substrate *E*,*E*-farnesyl acetone **7** displayed the same tendency with the spheroplasts showing the maximal improvement of 25-fold in TOF to >17 h^{-1}. Finally, we tested *E*-geraniol **9** as the smallest substrate analog. In this case, the isolated enzyme *Aac*SHC-F365C was the only preparation to convert this substrate with a TOF of 3 h^{-1}.

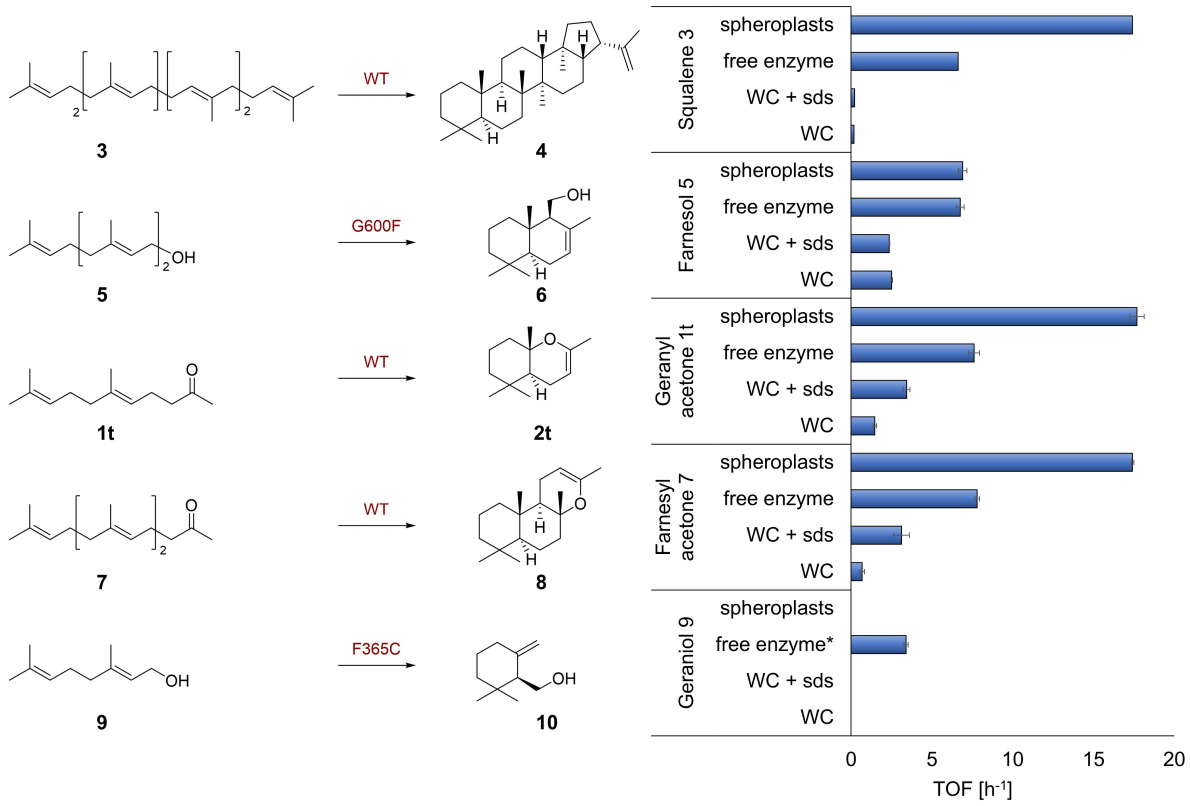


Fig. 2 Squalene-hopene catalyzed cyclization of the substrates, squalene **3**, *E*-farnesol **5**, *E*-geranyl acetone **1t**, *E*,*E*-farnesyl acetone **7** and *E*-geraniol **9** employing the WT enzyme and the variants G600F or F365C as whole cells, whole cells treated with SDS, cell-free enzyme or spheroplasts. The biocatalyst preparations were compared regarding their turnover frequency per hour (TOF). Reaction conditions: 40 g_{CWW}/L whole cells or spheroplasts, 2

mM substrate. The cell-free enzyme was used in 0.2 % CHAPS as a membrane mimic. For details see supporting methods. Error bars indicate the S.D. of technical triplicates (see SI for more details). The cell-free enzyme was prepared by the protocol described by Hammer *et al.*[31]

To push the reaction further, we increased the substrate concentration to 10 mM. However, congruently to the earlier experiments, the spheroplasts did not show better performance at higher substrate concentrations (Fig. S8).

Entrapped spheroplasts into hydrogel beads. As spheroplasts showed such an excellent performance, we investigated if they could be suited for flow applications. To integrate the spheroplasts into the flow reactor, immobilization by entrapment was applied. Three hydrogel materials (alginate, agarose, and polyacrylamide) were tested in batch. Agarose entrapped spheroplasts showed the best catalytic activity for the cyclization of **1t/c** into **2t** (45%)**,** followed by the alginate entrapped spheroplasts, while the polyacrylamide entrapped spheroplasts were not successful (Fig. S9). In addition, the entrapped spheroplasts were tested in the synthesis of **4** and **8**, the conversion levels for these two cyclizations achieved 40% and 30% respectively (Fig. S8). Hence, the spheroplasts entrapped into alginate beads were selected for the integration into the flow reactor to produce **2t**, but surprisingly no product formation was detected despite different attempted conditions (Table S6 and S7). Nevertheless, the unreacted substrate was observed in the output of the flow reactor.

A novel technique for enzyme immobilization: crosslinked spheroplasts (CLS). Inspired by the concept of CLEAS (crosslinked enzyme aggregates) which are carrier-free immobilized enzymes,[32] we devised the preparation of crosslinked spheroplasts (CLS). Glutaraldehyde (GA), polyethyleneimine (PEI), and 1,4-butanediol diglycidyl ether (BDE) were employed as crosslinkers to bind the proteins located on the cytoplasmic membrane of different spheroplasts creating a network of spheroplasts (Fig. 3A). Then, the CLS were applied in biotransformations for the cyclization of **1t/c** in batch with identical performance as of the original spheroplasts (Fig. 3B). No loss of activity or efficiency was observed following crosslinking chemistry. The reusability of the CLS was then trialed in consecutive reactions, observing that CLS crosslinked with either GA or PEI were remarkably stable even after washing with buffer and filtration (Fig. 3C and Fig. S10). BDE, on the other hand, did not yield a reusable biocatalyst.

A clear advantage of CLS is the lack of a solid support. We therefore challenged the requirement of the costly cyclodextrins (CD) which helped before to solubilize the substrate and preventing binding to the resin material in immobilized preparations. Biotransformations with CLS with or without CD resulted in fact in very similar conversion and CD addition could be avoided altogether (Table S8).

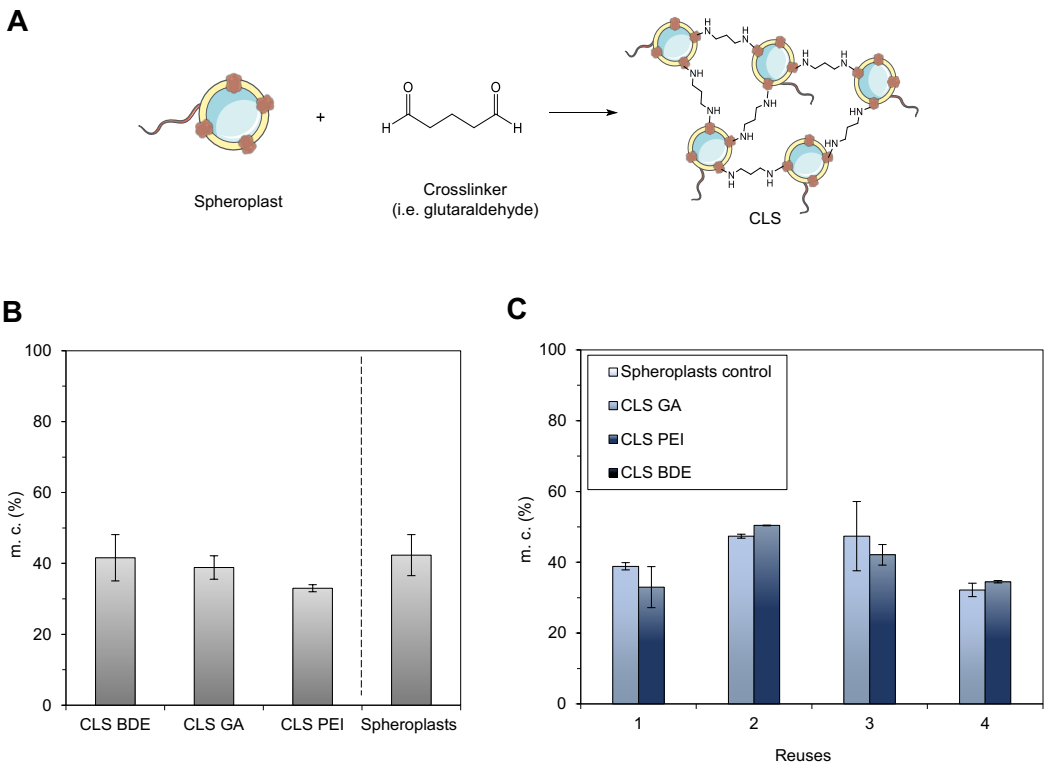


Fig. 3 Crosslinked spheroplasts (CLS) as biocatalysts. A Scheme of the crosslinking of the proteins located on the cytoplasmatic membrane. Glutaraldehyde crosslinking is depicted. **B** Biotransformations in batch with CLS in 1 mL of 2 mM geranyl acetone **1t/c**, 2 mM cyclodextrin, 0.2% SDS and 20 mM citric acid buffer at pH 6.0 after 24h at 30ºC. **c** Reuses of the CLS for consecutive biotransformations. Each reuse corresponds to 24 h at 30ºC. After each reuse the reaction mix was filtered, and the reaction media was replaced by fresh substrate solution.

Deciphering the challenge to perform SHC reactions in the flow system. With the efficient spheroplasts biocatalysts and a robust technique for their cross-linking we again attempted to intensify the process to produce **1t/c** in the flow reactor. In this case, the reaction was performed in absence of additives (i.e. SDS, CD) as we observed before that spheroplasts preparations do not require them. Surprisingly, we detected only traces of both the substrate and the product in the output (Fig. S11). Considering that the tubing is made of a plastic polymer (PTFE), it was plausible that the substrate may in fact adhere to the surface of the tubing due to its elevated hydrophobicity. In fact, as an incise, it is important to mention that all batch reactions must be carried out in glass vials rather than plastic vessel for the same reason. To prove such hypothesis, the flow reactor was fed with a substrate solution in the absence of additives, in presence of CD, and in presence of SDS. Samples were taken at different points along the tubing of the flow system before reaching the reactor (Fig. S12A). We observed that only the addition of SDS prevented to some extent the adhesion of the substrate **1t/c** to the plastic of the tubing (Fig. S12B). However, the substrate detected was consistently decreasing along the tubing regardless of the presence of any additive and, with our flow system set-up this reaction could simply not be implemented.

Discussion

To overcome the drawbacks of the outer cell membrane while maintaining SHC in its membrane environment, we have found a “goldilocks” compromise between the use of cell-free enzymes and the use whole-cell biocatalysts: the spheroplasts. We have adapted a known solution for a novel application as efficient biocatalysts for the cyclization of geranyl acetone **1t/c** into **2t,** reaching 7-fold higher specific activity with 29 times less amount of biocatalyst (Table 1). The straightforward and quick protocol to prepare spheroplasts make this strategy highly attractive to expand its application scope to other membrane-bound enzymes and for its implementation in industrial processes. This is particularly relevant for SHC reactions which are getting great attention in the flavor industry to synthesize enantiopure cyclic terpenoids but have remained a challenging catalyst until now.[33] Moreover, the use of spheroplast biocatalysts is a significantly more cost-efficient and sustainable alternative since no additives are needed in the biotransformation set-up (Table S8). Besides, their proved stability after lyophilization makes them storable and their application is as simple as for batch chemistry.

The potential of the SHC spheroplast biocatalysts was also proved with four additional terpenoid substrates, obtaining valuable information in a comparative evaluation of four biocatalyst preparations (whole cells, whole cells + SDS, cell-free enzymes, and spheroplasts). First, the treatment with SDS did not result in better TOFs compared to the whole cells using the substrates squalene **3** and *E*-farnesol **5**, which discloses a limitation of the membrane permeabilization using detergents (Fig. 2). Indeed, Eichhorn *et al.* demonstrated that among various tested detergents, SDS was the only one that permitted the cyclization of homofarnesol towards (–)-Ambroxide.[10] Second, despite using cell-free enzyme with different substrates and different variants, the TOFs resulted in around 7 h^{-1} which suggests that the diffusion through the membrane mimic CHAPS is still limited in the overall biotransformation. *E*-geraniol **9** represents an exception in this regard, as the cell-free enzyme set-up for this substrate was prepared using a different protocol.[31] A screening of alternative detergents by high-throughput methods such as described by Kotov *et al.*[34] could improve the diffusion. Interestingly, the natural substrate **3** was very poorly converted using SHC whole cells, due to the known challenge of substrate diffusion through the outer membrane.[35] Noteworthy, the spheroplasts improved the reaction in almost every case by up to ~100-fold, which highlights this superior hybrid enzyme preparation. The only exception was the cyclization of **9**, which was exclusively transformed by tailor-made cell-free enzyme.

Regarding the immobilization of SHCs, no previous studies have been reported to date. Herein, we performed an extensive analysis of different immobilization strategies, from entrapment into hydrogels to attachment to a solid support. Whereas the hydrogel entrapment showed good results to keep the enzymatic activity after immobilization, this strategy failed the stability test (Fig. S9). More robust biocatalysts have been developed by attachment to solid supports (methacrylate and agarose microbeads). However, we found a strong unfavorable affinity of the substrate/product to the support. This issue could be alleviated by using more hydrophilic materials such as agarose, but still some ‘sticking’ effect happened. In some of our previous works, we have observed a similar phenomenon when using non-polar substrates that can interact with hydrophobic supports.[36,37] Therefore, we can conclude that SHC-catalyzed reactions are not particularly compatible with carriers used to immobilize

the biocatalyst. As an innovative alternative, we developed the concept of crosslinked spheroplasts (CLS), which overcomes the stability and reusability issues of the catalysts, it avoids any interaction between the substrates/products and the carrier, and of course also eliminates costs and waste management linked to the use of a carrier (Fig. 3).

Overall, the strategy to enhance the catalytic activity of the SHC by implementing it in flow mode did not result in satisfying findings. Surprisingly, longer residence times did not improve the overall conversion in any case, which could suggest strong inhibitory effects on the SHC as devised by Neumann and Simon.[14] However, in our opinion, the major challenge of these specific reactions is the hydrophobicity of the substrates/products which readily diffuse in the *E. coli* cell membrane.[38,39] In this regard, segmented flow techniques as presented by Tang *et al.* may offer one solution.[40] However, that study was based on hydrophilic terpenes and a soluble class I cyclase, while *Aac*SHC is strongly dependent on the membrane structure. More difficult to overcome are the inherent limitations of the plastic tubing material of many flow systems which also sequester the substrate/product, even when the catalyst could be prepared as CLS.

In summary, we bring a novel approach to develop more efficient and sustainable biocatalysts by removing the outer layer of gram-negative bacteria. During this research journey, we have also collected valuable insights into the optimal operation conditions of membrane-bound enzymes, and their limitations, specifically about SHCs. Thus, the notoriously challenging stereoselective head-to-tail cyclization could be finally added to the chemical toolbox and release terpene synthesis from the classical ex chiral pool approach.[41,42] Furthermore, spheroplasts are not limited to membrane-bound enzymes: they can be a potential solution for other relevant biotransformations mediated by cytoplasmic enzymes in whole cells systems which suffer from the drawbacks of the outer cell membrane barrier. Finally, we have introduced a new immobilization strategy for spheroplasts (CLS) with potential application to any other biocatalyst.

Methods

Materials. All the reagents used for syntheses, buffer preparation, culture media preparation and biochemical work were purchased from Carl-Roth (Karlsruhe, DE), VWR (Pennsylvania, US), Sigma-Aldrich (St. Louis,US) and Alfa-Aesar (Ward Hill, US). The substrate (E/Z)-geranyl acetone was obtained from Combi-blocks (San Diego, USA). All the other substrates were chemically synthesized and analyzed by ^{1}H-NMR, ^{13}C-NMR and GC/MS.

Protein expression.[9] The plasmid pET22b(+) harboring the gene of *AacSHC* (UniProt: P33247) or a variant was transformed into *E. coli* BL21(DE3) by heat-shock at 42°C for 45 s followed by ice cooling for 3 min. Individual colonies were picked from generated agar plates and cultivated in 10 mL LB medium with 100 µg/mL Ampicillin overnight at 37 °C, 150 rpm. Then, 1 L flasks containing 300 mL of of T-DAB autoinduction medium with lactose as the inductor and 100 µg/mL kanamycin were inoculated with 3 mL of the overnight culture. The cultures were incubated for 20 h at 37 °C, 150 rpm and harvested afterwards (4000 g, 20 min).

Enzyme purification by thermolysis.[3,9] The cells were resuspended in 1 mL of Lysis buffer (200 mM citric acid, 0.1% EDTA, pH 6.0) and incubated for 1 h at 70 °C. The cell suspension was centrifuged (14000 g, 1 min) and the supernatant was discarded. As the enzyme is membrane-bound 1 mL of 1% CHAPS buffer was added to extract it from the cell pellet by shaking at room temperature for at least 1 day at 600 rpm. After centrifugation (14000 g, 1 min), the supernatant containing the *Aac*SHC was transferred to a new tube followed by SDS-PAGE analysis and determination of enzyme concentration by using the EPOCH2 (nanodrop Tek3 plate). Therefore the "Protein A280" mode was chosen with MW= 71439 Da and molar extinction coefficient ε = 185180 as protein specific data.

Preparation of spheroplasts. Based on previous protocols,[29,43] 100 mg harvested or lyophilized cells were resuspended in 1 mL of 20 mM citric acid at pH 6.0 with 10% sucrose and 150 mM NaCl. After centrifugation (15000 g, 3 min), the cells were resuspended in 20 mM citric acid at pH 6.0 with 10% sucrose, 1 mM EDTA and 1 mg/mL of lysozyme. The suspension was incubated for 30 min at room temperature followed by centrifugation for (15000 g, 5 min). The supernatant containing the outer membrane was discarded. Finally, the resulting spheroplasts were washed (3x) with 2 mL of 20 mM citric acid at pH 6.0.
The change of the cell shape corresponding to the removal of the outer membrane was confirmed by microscopy. *E. coli* cells (rod shape) and the spheroplasts (circular shape) were visualized using transmission light in a Nikon Ti2 Eclipse microscope. Objective 60x (oil) was used.

SDS-PAGE. After protein purification and extraction 20 µL of the enzyme/cell solution were mixed with 20 µl SDS loading buffer and heated to 95°C for 5 min. Afterwards 5-15 µL of the preparation were loaded on the 12% SDS-PAGE.

Biotransformations in batch mode. 1mL of the reaction mix containing the substrate (from a stock solution in DMSO) and citric acid buffer at pH 6.0 was added to a glass vial. The reaction mix could also contain SDS, cyclodextrins, or triton as specified for each experiment. Typically, 10 mg of whole/cells or spheroplasts were added to the reaction, unless otherwise specified. The biotransformations were incubated at 30ºC for 24 h with shaking, unless otherwise specified.

Preparation of CLS (crosslinked spheroplasts). 100 mg of lyophilized spheroplasts were added in 2 mL of crosslinked solution at pH 6.0. The reaction was incubated at room temperature for 5 h (16 h for BDE) under shaking, and after centrifugation (15000 g, 2 min), the supernatant was discarded. The resulting crosslinked spheroplasts (CLS) were washed (5x) with 2 mL of 20 mM citric buffer at pH 6.0.

Biotransformation in continuous flow mode. Flow reactions were performed using a R2S/R4 Vapourtec flow reactor equipped with a V3 pump and an Omnifit glass column (6.6 mm i.d. ×100 mm length) filled with the immobilized enzyme (1 g with 4 mgg 1 of protein loading) as a packed-bed reactor (PBR). A first equilibration step was performed by running 100 mm phosphate buffer pH 7.5 buffer at 0.5 mLmin 1 for 10 min. Then, the solutions of substrates at different concentrations were mixed in a T-tube and pumped through the PBR containing the immobilized biocatalyst. The flow rate was adapted depending on the desired residence time for each reaction. Samples were collected after each column volume and analyzed by GC.

Gas chromatography (GC). Samples were extracted with ethyl acetate:cyclohexane (1:1) in a final volume of 1 mL and the resulting organic phase was submitted to GC analysis. Agilent GC8860 equipment was employed for the analyses, with an Agilent19091J-413 column (30 m x 320 µm x 0.25 µm) and nitrogen as carrier gas (pressure: 12.816 psi), unless otherwise specified. Injections (1 µL) were performed in split mode (5:1). The following temperature profile was used: 1 min at 155 °C, 11 °C/min to 205 °C, 0.6 min at 205 °C; inlet and detector temperature: 250 °C.

The m. c. (%) were calculated directly from GC spectra by integration-quotient of substrates and products. The molar conversions were also calculated by using standard curves of the substrates and products (1-15 mM) when possible.

The results presented in Figure 3 were obtained by GC analysis with an Agilent 7820A equipped with a mass spectrometer MSD5977B and a HP-5MS capillary column (Agilent, 30 m x 250 µm x 0.25 µm) and helium as carrier gas with a constant pressure of 14.168 ψ. Injections (1 µL) were performed in split mode (10:1). Relative conversion rates were calculated directly from GC-MS spectra by integration-quotient of substrates and products.

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Author contributions

F.P. and B.H. conceived and guided the project. A.I.B.M. performed most of experiments and drafted the manuscript. A.S. and E.H. contributed to the experimental work. A. S. contributed to the draft writing. All authors discussed and agreed to the final version of the manuscript.

Competing interests

The authors declare no conflict of interest.

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