**Asymmetric reduction of (R)-carvone by cellular envelopes with an immobilized two enzyme system**

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**Highlights**

* Analysis of undesired (2R,5R)-dihydrocarvone isomerization during whole cell-biotransformations
* One-step expression and immobilization of two enzymes in cellular envelopes of *Escherichia coli*
* 66 % reduced (2R,5R)-dihydrocarvone isomerization rates using cellular envelopes compared to whole cells
* The cellular envelope technology can be applied to reduce the effect of undesired side reactions mediated by the host cell metabolism.

**1. Introduction**

(2R,5R)-dihydrocarvone is a chiral building block which can be obtained by asymmetric reduction of the C-C double bond of (R)-carvone using ene-reductases. A purified ene-reductase from *Nostoc sp.* PCC 7120 (NostocER1) is capable of producing (2R,5R)-dihydrocarvone with a diastereomeric excess (de) ≥ 99 %. However, the application of whole cell biocatalysts expressing the cyanobacterial ene-reductase NostocER1 and a NADP+-accepting formate dehydrogenase (FDH) mutant for internal cofactor regeneration led to a decreased de of 81.7% after 5 h which further dropped to the thermodynamic equilibrium of ~60% after 24 h [1]. The implementation of an *in situ* substrate feeding and product removal (SFPR) system based on polymeric adsorbent resins or ionic liquids could increase the achievable de in biotransformations with the whole cell biocatalysts up to 96.5 % [2], albeit the theoretical limit defined by the pure ene-reductase could not be reached. For different organisms than *Escherichia coli* it is suggested in literature that cytoplasmic components might catalyze the isomerization reaction of (2R,5R)-dihydrocarvone [4,5]. Therefore, in this contribution an inventive procedure for the one-step expression and immobilization of enzymes in cellular envelopes of *Escherichia coli* was applied to the two enzyme system comprising NostocER1 and FDH. The concept of cellular envelopes allows the construction of a biocatalyst lacking undesired catalytic interferences caused by host cell cytoplasmic components while keeping the economically beneficial low production and work-up costs of whole cell biocatalysts.

**2. Methods**

For the production of cellular envelopes, the two enzymes NostocER1 and FDH were fused on a genetic level to membrane anchorage domains, which allow a post-translational insertion into the cytoplasmic membrane. In a second step the expression of protein E from phage ΦX174 led to the formation of lysis pores traversing both membranes of *Escherichia coli*. After cross-flow filtration to clean-up the released cytoplasmic components, cellular envelopes with a defined lysis pore and immobilized synthesis enzymes were obtained. Therewith, a biocatalyst with immobilized synthesis enzymes on the one hand but without potentially disturbing soluble host cell components on the other hand could be created.

**3. Results and discussion**

First it was proven that soluble cytoplasmic components of *Escherichia coli* can catalyze the undesired isomerization of (2R,5R)-dihydrocarvone to (2S,5R)-dihydrocarvone making it reasonable to apply the cellular envelope technology hereafter. Thus, seven different membrane anchorage domains were evaluated for the immobilization of NostocER1 in biotransformations with isolated membrane fractions. The highest activities and maintained stereo specificity were displayed for N- or C-terminal fusions of the hydrophobic transmembrane segment of the Vam3 protein from *S. cerevisiae* (Vam3p’) and NostocER1. In these biotransformations FDH was membrane anchored via the C-terminal hydrophobic part of the Ubiquitin-conjugating enzyme 6 (UBC6) from *S. cerevisiae.* Subsequently, both configurations were employed in liter scale fed-batch processes for the production of cellular envelopes with membrane anchored NostocER1 and FDH. For both fusion types of Vam3p’ to NostocER1 (N- and C-terminal) cellular envelopes could be prepared with high lysis yields of 99.93 and 97.25% after 27.5 and 29.7 h process time with maximal biomass concentrations of 12.83 and 10.72 g L-1. During five subsequent washing steps of the cellular envelopes the protein concentration in solution as a measure of released cytoplasmic components in general was reduced to less than 4% (~80 µg mL‑1) of the starting value. In biotransformations the cellular envelopes displayed both NostocER1 and FDH activity and indeed (2R,5R)-dihydrocarvone isomerization rates were reduced up to 66% in comparison to whole cells.

**4. Conclusions**

The application of the one-step expression and immobilization procedure to the two enzyme system allowed the production of cellular envelopes with immobilized NostocER1 and FDH activity and high lysis yields. These cellular envelopes showed in biotransformations decreased (2R,5R)-dihydrocarvone isomerization rates compared to whole cell biocatalysts confirming the assumption that at least a part of the isomerization reaction is catalyzed by cytoplasmic compounds and that the cellular envelope technology can be used to reduce undesired side-reactions catalyzed by soluble host cell enzymes. To further investigate the remaining isomerization activity additional experiments regarding the completeness of the workup process should be considered.

**References**

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