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Immobilization of an integral membrane protein for biotechnological phenylacetaldehyde production

Michel Oelschlägel[a,*], Anika Riedel[a], Aurelia Zniszczoł[b], Katarzyna Szymańska[b], Andrzej B. Jarzębski[b,c], Michael Schlömann[a], Dirk Tischler[a]

[a] Interdisciplinary Ecological Center, Environmental Microbiology Group, TU Bergakademie Freiberg, Leipziger Str. 29, 09599 Freiberg, Germany
[b] Department of Chemical Engineering, Silesian University of Technology, M. Strzody 7, 44-100 Gliwice, Poland
[c] Institute of Chemical Engineering, Polish Academy of Sciences, Bałtycka 5, 44-100 Gliwice, Poland

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ABSTRACT

Styrene oxide isomerase (SOI) has previously been shown to be an integral membrane protein performing a highly selective, hydrolytic ring opening reaction of epoxides to yield pure aldehydes. Earlier studies had also shown a high sensitivity of SOIs toward their product phenylacetaldehyde which caused an irreversible inhibition and finally complete loss of activity at higher aldehyde concentrations. Here we report on the covalent immobilization of a styrene oxide isomerase (SOI) on SBA-15 silica carriers. The production of the SOI from a *Rhodococcus* strain was optimized, the enzyme was enriched and immobilized, and finally the biocatalyst was applied in aqueous as well as in two-phase systems. Linkage of the protein to epoxide or amino groups on the SBA-based carriers led to relatively poor stabilization of the enzyme in an aqueous system. But, improved stability was observed toward organic phases like the non-toxic phthalate-related 1,2-cyclohexane dicarboxylic acid diisononyl ester (Hexamol DINCH) which here to our knowledge was used for the first time in a biotechnological application. With this two-phase system and the immobilized SOI, 1.6–2.0× higher product yields were reached and the lifetime of the biocatalyst was tremendously increased.

1. Introduction

Immobilization of enzymes is a common procedure to improve their stability, functional efficiency, and reproducibility with respect to an application in biocatalysis (Datta et al., 2013; Sheldon, 2007).

Polymeric organic materials are commonly used for enzyme immobilization (Bryjak, 2003; Buchholz et al., 2005). Especially for conventional silica gels, a high potential was demonstrated during previous studies (Bryjak et al., 2012; Jarzębski et al., 2007; Pandya et al., 2005; Petri et al., 2004; Rekuć et al., 2010; Vansant et al., 1995). These carriers are environmentally acceptable, structurally more stable and more resistant to microbial attacks. The pore sizes of these silica-based materials can be diversified during their preparation and the surface of such carriers can be covered with various anchor groups for enzyme linkage (Jarzębski et al., 2007; Szymańska et al., 2007, 2009; Zhao et al., 1998).

Covalent immobilization is predicated on amino-acid residues like aspartate, cysteine, histidine, and arginine as well on reactive groups on the carrier surface, e.g. epoxy or amino groups etc. (Pandya et al., 2005; Petri et al., 2004; Rekuć et al., 2010; Singh, 2009). Glutaraldehyde can also serve as a cross-linker itself or as a spacer arm between protein and the carrier matrix. As potential carrier, several mesoporous silicas such as SBA-15 have been tested during previous studies with various pore sizes (Jarzębski et al., 2007; Szymańska et al., 2007, 2009). Rekuć et al. (2010) could covalently immobilize a laccase from *Cerrena unicolor* on mesoporous silicas modified with amino or epoxy groups. Remarkably, this treatment allowed a significantly improved stability, e.g. against higher temperature.

An enzyme with the potential for an application in fine-chemical production is styrene oxide isomerase (SOI) (Itoh et al., 1997; Miyamoto et al., 2007). This protein is involved in the aerobic styrene degradation of several soil bacteria, e.g. of *Rhodococcus opacus* 1CP, *Pseudomonas fluorescens* ST, or *Xanthobacter* sp. 124X (Beltrametti et al., 1997; Hartmans et al., 1989; Oelschlägel et al., 2012), and it catalyzes the isomerization of styrene oxide to phenylacetaldehyde. The enzyme mentioned – commonly induced during growth on styrene, styrene oxide, or phenylacetaldehyde in the wild-type strains (O'Connor et al., 1995; Oelschlägel et al., 2012) – has been identified as an integral membrane-bound enzyme (Oelschlägel et al., 2012). Membrane localization stabilizes SOI against environmental conditions and a number of chemicals. This integration in the cell membrane also allows a simple enrichment

* Corresponding author. Tel.: +49 3731 394015; fax: +49 3731 393012.
E-mail address: michel.oelschlaegel@ioez.tu-freiberg.de (M. Oelschlägel).

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of the insoluble membrane-embedded SOI by centrifugation to high specific activities of >370 U mg^{-1}. Additionally, the independence from cofactors suggested an application in the production of phenylacetaldehydes which were used as flavors or fragrances and represent important building blocks for pharmaceutical industry (Hölderich and Barsnick, 2001). Up to 760 μmol (=76 mM) pure phenylacetaldehyde have been previously obtained in presence of 130 U enriched SOI from strain 1CP applied in an aqueous reaction system (Oelschlägel et al., 2012). In spite of the high stability of the SOI, we found that the enzyme is subject to product inhibition at higher product concentrations because of the high reactivity of the formed aldehyde (Oelschlägel et al., 2012). Therefore, either the reaction system could be changed into a two-phase system so that the product is in situ extracted by an organic phase or the catalyst itself could be stabilized.

Here we report on the first covalent immobilization of a membrane-bound SOI under various conditions on SBA-15-based carriers to improve its stability for the production of phenylacetaldehyde. Additionally, applicability of non-immobilized and covalently linked enzyme in a two-phase system was shown. However, production of the enzyme with *R. opacus* 1CP has certain drawbacks as slow growth of the cells on styrene and handling of a toxic and volatile substrate to improve the provision of sufficient amounts of SOI. That issue and the identification of alternative inducers were also a part of the present study.

2. Material and methods

2.1. Chemicals and enzymes

Standard chemicals, detergents, styrene oxide, and phenylacetaldehyde were purchased from Sigma Aldrich (Steinheim, Germany), Appli Chem GmbH (Darmstadt, Germany), Merck KGaK (Darmstadt, Germany), Riedel-de Haen (Seelze, Germany), Fisher Scientific (Loughborough, UK), VWR International GmbH (Darmstadt, Germany), BioRad Laboratories GmbH (München, Germany), or Carl Roth (Karlsruhe, Germany) in highest available purity.

2.2. Bacterial strains and culture conditions

R. opacus 1CP (Gorlatov et al., 1989) was kept on mineral medium plates (Dorn et al., 1974, modified) in presence of 5 mM benzoate for preservation at 30 °C (Oelschlägel et al., 2012). Otherwise it was cultivated at room temperature on plates containing 20 g l^{-1} glucose or on solid mineral medium without carbon source in a 5-l desiccator in which an evaporating aliquot of 50 μl styrene was provided.

Fed-batch cultivation of strain 1CP was performed in a 5-l fermenter (ED/ES5, B. Braun Biotech AG, Melsungen, Germany) containing 4–5 l mineral medium. Biomass growth was achieved by using phenylacetic acid or glucose as the initial substrate.

Cells grown on phenylacetic acid in the fermenter were used for growth and induction studies or for SOI enrichment. In total 248 mmol of phenylacetic acid were added in 15–20 mmol portions (T = 30 °C, 400–600 rpm, pH = 7.0–7.3, aeration 2–4 standard liters per minute) obtaining an OD_{546} of 10–15 within 15 days. After cell densities mentioned had been reached and the substrate phenylacetic acid had been consumed, the culture was adapted with styrene and subsequently used for an SOI enrichment. Styrene was added in portions of 0.8–0.9 mmol every 3–20 h over at least 8 days (total 13 mmol). Air flow was temporarily stopped after each addition of substrate till the aliquot was metabolized by the cells as indicated by a stagnation of the oxygen consumption measured online. Afterwards, air flow was re-adjusted till another styrene aliquot was added. At last, cells were harvested by centrifugation (5000 × g, 30 min, 4 °C), washed with 25 mM phosphate buffer (pH 7.0), and suspended in 45 ml of new phosphate buffer. Biomass was stored at −80 °C.

To investigate SOI induction, 100 ml of phenylacetic acid-grown biomass were transferred into 500-ml baffled flasks and incubated with 0.1 mmol putative inducer. The inducer was added either directly into the medium or supplied via an evaporation system. Cells were incubated at 30 °C and 120 rpm over 5 days. Cultures were daily ventilated for 2 h and inducer was added afterwards (total 0.5 mmol). The following compounds were tested as inducer: styrene (reference), styrene oxide, phenylacetaldehyde, phenylacetic acid, 1-phenyl-, and 2-phenylethanol, cinnamic acid, benzoate, ethylbenzene, 3-phenylpropene, 1-phenyl-1,2-ethanediol, glucose, tetradecane, 4-vinylpyridine, and indene. After incubation in presence of a presumed inducer, 50 ml of the cultures were harvested by centrifugation (5000 × g, 30 min, 4 °C). Pellets obtained were washed with 50 mM phosphate buffer (pH 7.0), suspended in 1 ml fresh 50 mM phosphate buffer (pH 7.0), and stored at −20 °C. For determination of SOI activity, 400 μl of the harvested cells were mixed with 200 μl 50 mM phosphate buffer (pH 7.0), 600 mg zirconia beads, and 8 U DNAse I and then subjected to cell disruption. For that the batches were vigorously shaken for 1 h at 30 s^{-1} and 4 °C in a swing mill (MM-200, Retsch, Germany) and afterwards crude extracts were investigated for SOI activity.

Cells obtained from the bioreactor were also used for growth experiments, which were performed in 500-ml baffled flasks containing 100 ml mineral medium and the putative substrates in a final amount of 0.5 mmol. An initial OD_{546} of 0.5 was adjusted and cultures were incubated at 30 °C and 120 rpm for two days. With this adapted preculture, new 500-ml baffled flasks containing 100 ml mineral medium and 0.1 mmol substrate were inoculated with an initial OD_{546} of 0.4. Cultures were incubated as described above and the OD_{546} was determined every hour. The following putative substrates were tested: glucose, cinnamic acid, 2-phenylethanol, benzoate, tetradecane, and phenylacetic acid.

To consider toxic effects of inducers, test tubes were filled with 10 ml of a cell suspension of strain 1CP in 25 mM phosphate buffer (pH 7.2) with an OD_{600} of 0.3. 5 μmol substrate/inducer were added and tubes were cultivated for 20 h as previously described. Afterwards, 50 μl 1:5 diluted sample of each tube were spread out on mineral-medium plates containing 20 g l^{-1} glucose and the plates were incubated at 30 °C. After three days, colony-forming units (CFUs) were counted. An untreated sample served as reference.

The most promising substrate und the most efficient inducer were applied for an improved cultivation in the fermenter. In total 357 mmol glucose were added in 18-mmol portions (T = 30 °C, 400–600 rpm, pH = 7.0–7.3, aeration 2–4 standard liters per minute) within 14 days. Afterwards, cells were adapted with small aliquots of 0.8–0.9 mmol of styrene every 10–20 h for 14 days (total 15.5 mmol). Styrene addition was stopped after 28 days to determine how long SOI activity remains induced. Cell harvesting and disruption to investigate SOI induction were performed as described above.

2.3. Enzyme assays and protein quantification

Activity of SOIs was determined with gas (GC) or high-performance liquid chromatography (HPLC) by quantification of the formed reaction product phenylacetaldehyde from the substrate styrene oxide. GC and HPLC analysis and sampling were performed as described earlier (Oelschlägel et al., 2012). For HPLC, 50% (v/v) methanol containing 0.2% (w/v) phosphoric acid were used as mobile phase in an isocratic mode at a flow rate of 0.7 ml min^{-1}. The net retention volume of phenylacetaldehyde was 3.1 ml under these conditions.