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Heterologous production of different styrene oxide isomerases for the highly efficient synthesis of phenylacetaldehyde

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ABSTRACT

The styrene oxide isomerase (SOI, StyC) represents a key enzyme of the styrene-degrading pathway and has been discussed as promising biocatalyst during recent studies. The enzyme enables the synthesis of pure phenylacetaldehyde from styrene oxide. In this study the native as well as the corresponding codon-optimized genes of three different SOIs from *Rhodococcus opacus* 1CP (StyC-1CP), *Sphingopyxis fribergensis* Kp5.2 (StyC-Kp5.2), and *Pseudomonas fluorescens* ST (StyC-ST) were investigated for the expression in *Escherichia coli* BL21(DE3)pLysS. Specific enzyme activities of 61.9 ± 7.5 U mg^{-1}, 23.2 ± 2.8 U mg^{-1}, and 10.9 ± 1.2 U mg^{-1} were achieved after 6–9 h for the codon-optimized gene of strain 1CP and the native genes of Kp5.2 and ST, respectively. Afterwards, these enzymes were enriched and applied for biotransformation studies. A complete conversion of 150 mM styrene oxide to phenylacetaldehyde was observed for the enzyme StyC-Kp5.2 indicating a significantly improved stability towards product inactivation. Remarkably, more than 300 mM product (> 36 g L^{-1}, yield of about 80%) were finally synthesized from 400 mM substrate with 150 U of this enzyme within 60-120min. This represents the highest product concentration which has been reached with this type of enzymes, so far.

1. Introduction

Phenylacetaldehyde represents an important compound for the flavor and perfume industry and is used for the synthesis of pharmaceuticals, insecticides, and disinfectants (Hölderich and Barsnick, 2001). The industrial production of this aromatic aldehyde is based on the isomerization of styrene oxide in presence of alkali-treated silica alumina, hydrotalcite-derived solids, or different zeolites (Goetz et al., 1993; Kochkar et al., 2002; Neri and Buonomo, 1985; Zaccheria et al., 2011), and on the oxidation of 2-phenylethanol with hexavalent chromium compounds or rhodium complexes (Fujitsu et al., 1981; Goetz et al., 1993; Lou et al., 1997). An additional biotechnological strategy for the production of phenylacetaldehyde from 2-phenylethanol was described by Çelik et al. (2004) and Molinari et al. (1999) applying cells of *Gluconobacter* or *Acetobacter* strains. Furthermore, the biotechnological application of cell-free styrene oxide isomerases has been described as a highly promising way to gain such aromatic aldehydes from epoxides (Miyamoto et al., 2007; Oelschlägel et al., 2012, 2014a).

The styrene oxide isomerase (StyC, SOI) is involved in the microbiological styrene degradation *via* side-chain oxidation (O'Leary et al., 2002) and catalyzes the transformation of styrene oxide into phenylacetaldehyde. The metabolic route of side-chain oxidation has been reported for some representatives of the genus *Pseudomonas* (Marconi et al., 1996; O'Connor et al., 1995; Panke et al., 1998; Velasco et al., 1998), *Rhodococcus* (Oelschlägel et al., 2012, 2014b; Toda and Itoh, 2012), or *Sphingopyxis* (Oelschlägel et al., 2014b), so far. Because the styrene monooxygenase, the initial enzyme of this pathway, catalyzes the highly specific transformation of styrene into (*S*)-styrene oxide, it is reasonable that SOIs also prefer the transformation of the (*S*)-styrene oxide to phenylacetaldehyde (Itoh et al., 1997; Oelschl & gel et al., 2012). In the case of SOIs from *Pseudomonas fluorescens* ST, *Sphingopyxis fribergensis* Kp5.2, and *Rhodococcus opacus* 1CP, relative activities of about 40% were determined for the (*R*)-enantiomer compared to the activity towards (*S*)-styrene oxide (Oelschlägel et al., 2014b). Nevertheless, both enantiomers were completely transformed by the enzyme over time.

In former studies different StyCs were investigated with respect to an efficient production of various phenylacetaldehydes from the corresponding styrene oxides (Hartmans et al., 1989; Itoh et al., 1997; Miyamoto et al., 2007; O'Leary et al., 2002; Oelschl & gel et al., 2012). During these studies, two limitations for a biotechnological application were revealed. One limitation based on a distinct sensitivity of these membrane-bound enzymes towards high product concentra-

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tions (Oelschlägel et al., 2012, 2014b). It has been shown by previous studies that SOIs are irreversibly inactivated by the accumulated aldehyde probably by covalent modification of crucial amino acids (Oelschlägel et al., 2014a, 2014b). Hence, the highest product concentrations, which have been reported so far, were restricted to about 80 mM of phenylacetaldehyde (Oelschlägel et al., 2012). These product yields were obtained within 60 min using 150 U of an SOI from the Gram-positive *Rhodococcus opacus* 1CP.

Another limitation for an application of these enzymes based on their production process. Promising specific SOI activities of about 10 U mg^{-1} and 21 U mg^{-1} have been reported for *Pseudomonas fluorescens* ST and *Rhodococcus opacus* 1CP, but the expression of the genes in the wild-type hosts was very time-consuming and needed about 7–28 days (Oelschlägel et al., 2014a, 2014b). Furthermore, some SOIs have been described to be difficult to obtain. For the SOI from *Sphingopyxis fribergensis* Kp5.2, for example, only activities of up to 3.1 U mg^{-1} have been reported after the native production of this enzyme in the wild-type strain (Oelschlägel et al., 2014b).

Nevertheless, chances to overcome these limitations have been given by former studies, too. A recent study, for example, has indicated that StyC of *Sphingopyxis fribergensis* Kp5.2 seems to be less sensitive to product inactivation and maybe offers an opportunity to reach higher phenylacetaldehyde concentrations (Oelschlägel et al., 2014b). Furthermore, the efficient recombinant production of an SOI of *Rhodococcus opacus* 1CP has recently been reported (Oelschlägel et al., 2015a). Specific SOI activities of up to 44.5 U mg^{-1} were reached within one day applying an *Escherichia coli* strain as expression host. The results of this study have shown that the recombinant expression seems to be an useful way for a significantly faster production of other SOIs, too.

Based on this knowledge, the initial aim of the present study was the recombinant expression of SOI genes from *Rhodococcus opacus* 1CP (StyC-1CP), *Sphingopyxis fribergensis* Kp5.2 (StyC-Kp5.2), and *Pseudomonas fluorescens* ST (StyC-ST) in an *E. coli* strain (based on Oelschlägel et al., 2015a). Therefore, native *styC* genes and codon-optimized genes (designated as CU-*styC*s) were investigated with respect to an improved SOI production. Afterwards, the enzymes obtained were enriched and subsequently characterized with respect to the reachable product concentrations under consideration of the product inactivation to evaluate the most promising candidates for a biotechnological application.

2. Material and methods

2.1. Chemicals and enzymes

Enzymes, buffers, vectors, and kits were purchased from Thermo Fisher Scientific (Waltham, USA), Invitrogen (Carlsbad, USA), Novagene (Darmstadt, Germany), and Jena Bioscience (Jena, Germany). Further standard chemicals, substrates, styrene oxide, and phenylacetaldehyde were purchased from Sigma-Aldrich (Steinheim, Germany), Carl Roth (Karlsruhe, Germany), AppliChem (Darmstadt, Deutschland), FLUKA (Buchs, Switzerland), and TCI Deutschland GmbH (Eschborn, Germany).

2.2. Construction of styC- or CU-styC-containing E. coli hosts for protein production

The gene sequences of all native *styC* genes investigated in this study have been published before (Beltrametti et al., 1997; Oelschl & gel et al., 2014b). In this study the *styC* genes of *Pseudomonas fluorescens* ST (Beltrametti et al., 1997; DSM 6290, DSMZ; *styC*-ST sequence: Acc. No. **Z92524**), *Rhodococcus opacus* 1CP (Oelschlägel et al., 2012; VKM Ac-2638, Russian strain collection; *styC*-1CP: Acc. No. **KF540254**), and *Sphingopyxis fribergensis* Kp5.2 (Oelschlägel et al., 2015c; DSM 28731, DSMZ; *styC*-Kp5.2: Acc. No. **KF540258**) were considered.

The SOI-encoding genes were amplified directly from disrupted cells by SOI-specific PCRs. Therefore, colonies of the strains mentioned above grown on NB or LB broth (Sambrock et al., 2001) were picked and suspended separately in water. The cells were disrupted by incubation at 98–99 °C for 15 min. Afterwards, 1.0 or 2.0 µL crude extract were added to 20 µL- or 40 µL-PCR mixtures consisting of 1 × PCR buffer (includes 2.0 mM $MgCl_2$), 0.2 mM of each deoxynucleoside triphosphate, 0.8 µM of each primer, and 2.0 or 4.0 U of DreamTaq polymerase. The following primers were used for specific amplification: FW-styC-ST 5′-TTCGCCATGGGAATGCTTCATGCCTTCGAACG-3′ and REV-styC-ST 5′-AATGCGGCCGCTCATTCCGCAGTAGCGTGC-3′ for *styC*-ST; FW-styC-Kp5.2 5′-TTCGCCATGGGAATGAGGAGAGATGTTATGGCAAC-3′ and REV-styC-Kp5.2 5′-AATGCGGCCGCGTGATTGACTTGAAATCCGTCG − 3′ for *styC*-Kp5.2; FW-styC-1CP 5′-ATTCGCCATGGGAATGAAAACGCTCGAACGGAAG-3′ and REV-styC-1CP 5′-AATGCGGCCGCTCAGGCATTTGCCGATTCG-3′ for *styC*-1CP. The PCR was performed by an initial step at 95 °C for 4.5 min followed by 31 cycles of amplification (95 °C for 30 s, 62 °C for 30 s, and 72 °C for 60 s). During the last step, the PCR mixtures were incubated at 72 °C for 5 min. The PCR products were directly purified with the UltraClean® PCR Clean-Up-Kit (MOBIO, Carlsbad, USA).

About 50 ng of the purified PCR product and 50 ng of the expression vector pET16bP [pET16b (Novagene) with altered multicloning site, U. Wehmeier, personal communication] were added to the restriction mixture containing buffer O and 12 U of the enzyme NcoI and 3 U of NotI (both Thermo Scientific). Digestion of PCR products was able because the forward primers shown above include an NcoI restriction site and the reverse primers a NotI restriction site. The mixtures were incubated for 4–12 h at 37 °C and the DNA was subsequently purified by gel electrophoresis (1% agarose, 90 V). The DNA fragments were visualized with SYBR Gold (Invitrogen) and isolated from the gel by the DNA Isolation Spin Kit (AppliChem).

6 ng of each PCR product obtained and 16 ng digested pET16bP (corresponds to a molar ratio of 4:1) were ligated with 1 U of T4 ligase in 1× T4 ligase buffer (both Thermo Scientific) for 30 min at 22 °C. After ligation, the vectors constructed were transferred immediately into *Escherichia coli* NEB5α *via* heat-shock transformation for propagation. After propagation, the vectors were purified from the cells − grown in LB medium with 100 µg mL^{-1} ampicillin at 37 °C over night − by the Gene JET Plasmid Miniprep Kit (Thermo Fisher Scientific). The DNA sequences of the inserts in the plasmids were finally checked by subsequent sequencing with the pET-check primers 5′-CATCACAGGCAGCGGCCATATCGAAG-3′ and 5′-CAGCTTCCTTTCGGGCTTTGTTAG-3′ by MWG Eurofins (Ebersberg, Germany). Afterwards, the results were aligned with the Staden Package [England (Bonfield et al., 1995)] and the consensus sequence of each insert was compared to the NCBI databases by the Basic Local Alignment Search Tool [BLAST (Altschul et al., 1990, 1997)]. The vectors with the correct inserts were subsequently transformed into *E. coli* BL21(DE3)pLysS as described above. The expression hosts obtained were designated as *E. coli* BL21(DE3)pLysS + pET16bP + *styC*-ST, *E. coli* BL21(DE3)pLysS + pET16bP + *styC*-Kp5.2, and *E. coli* BL21(DE3)pLysS + pET16bP + *styC*-1CP.

Besides the native genes the codon-optimized SOI genes (= CU-*styC*-ST, CU-*styC*-1CP, CU-*styC*-Kp5.2) were investigated during this study, too. Therefore, the codon usage of the native *styC* genes cloned above (sequences shown in Supplemental Fig. S1) was rewritten into that of *Acinetobacter baylii* ADP1 by the OPTIMIZER tool (Puigbò et al., 2007, 2008) as reported by Oelschl & gel et al. (2015a). This *Acinetobacter* codon usage has been described to be useful for the expression in a broad range of hosts (Oelschlägel et al., 2015a). The construction of the *E. coli* BL21(DE3)pLysS host, which contains the optimized *styC* of strain 1CP (= CU-*styC*-1CP) in a pET16bP expression vector, has already been described in a previous study (Oelschlägel et al., 2015a) while the expression hosts with CU-*styC*-Kp5.2 and CU-*styC*-ST were constructed as described below.