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Production of a recombinant membrane protein in an *Escherichia coli* strain for the whole cell biosynthesis of phenylacetic acids

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ABSTRACT

The styrene oxide isomerase (SOI) represents a membrane-bound enzyme of the microbial styrene degradation pathway and has been discussed as promising biocatalyst. It catalyzes the isomerization of styrene oxide to phenylacetaldehyde. In this study a *styC* gene, which encodes the SOI of *Rhodococcus opacus* 1CP, was optimized for optimal expression in *Escherichia coli* BL21(DE3) pLysS. The expression of this synthetic *styC* was investigated and subsequently optimized. Highly active biomass was obtained yielding an SOI activity of 44.5 ± 8.7 U mg^{-1} after 10 h. This represents the highest SOI activity reported for crude cell extracts of SOI-containing bacterial strains. Remarkably, this biomass can be applied as whole cell biocatalyst for the production of phenylacetic acids from styrene oxides. In the case of non-substituted styrene oxide, nearly 730 mg l^{-1} phenylacetic acid (~85% yield) was formed over a period of 20 days.

1. Introduction

Phenylacetaldehyde and phenylacetic acid represent fine chemicals with a high industrial demand. Both compounds are used for the synthesis of pharmaceuticals, insecticides, disinfectants, and products for the perfume industry [7,8,17,18,26,38].

Processes for industrial phenylacetaldehyde production are established on two basic reactions: the oxidation of 2-phenylethanol and the isomerization of styrene oxide [15]. For the oxidation of 2-phenylethanol, hexavalent chromium compounds [24], silver(II) picolinate [6], cationic rhodium complexes [12], and *N*,*N*-dibromo-*p*-toluenesulfonamide [39] have been reported to be useful catalysts. The isomerization of styrene oxide can be catalyzed by acetic zeolite [15], alkali-treated silica alumina [46], hydrotalcite-derived basic solids [22], or titanium-containing synthetic zeolite [29] beside others. Biotechnological processes of phenylacetaldehyde synthesis have been described by several previous studies, e.g., applying a cell-free styrene oxide isomerase (SOI) in presence of styrene oxide [27,32,34] or whole cells of *Gluconobacter* or *Acetobacter* strains for the transformation of 2-phenylethanol [5,28].

Phenylacetic acids are produced industrially by the hydrolysis of phenylacetonitriles in presence of mineral acids at temperatures of 80 °C to 250 °C [20]. Furthermore, the carbonylation of benzyl chlorides catalyzed by tetracarbonyl nickel at 80 °C and 10–60 bar [2,4] leads to the formation of phenylacetic acids. Ruthenium(III) EDTA complexes [43] or rhodium-based catalysts [14] are also used to transform such benzyl halogenides. Some biochemical strategies have been reported applying a nitrile hydratase (EC 4.2.1.84) and an amidase (EC 3.5.1.4) of *Rhodococcus equi* TG328 [13] or an arylacetonitrilase (EC 3.5.5.1) from *Pseudomonas fluorescens* EBC191 [42]. Koma et al. [23] have reported a further way to obtain phenylacetic acid and 4-hydroxyphenylacetic acid from glucose by a metabolically engineered *Escherichia coli* strain with an expanded shikimate pathway.

Phenylacetaldehyde and phenylacetic acid have been found to be intermediates of the microbiological styrene degradation via side-chain oxidation [31]. This degradation route has been reported for several bacteria, e.g., representatives of the genus *Pseudomonas* [25,30,36,45], *Rhodococcus* [32,35,44], or *Sphingopyxis* [35]. In order to establish a biotechnological process for the synthesis of phenylacetaldehyde, previous investigations were performed with styrene oxide isomerases (SOI, encoded by *styC*). These enzymes are involved in the microbiological styrene degradation and convert styrene oxide into phenylacetaldehyde [16,19,27,31,32]. Despite of the high SOI activities in some wild-type strains [35] the production of SOIs by these strains is very time-consuming [34]. This aspect and the inhibition of these enzymes by their product at higher concentrations [32,35] limits their application and need further optimization.

The aim of the present study was the heterologous expression of a synthetic *styC* gene in *E. coli* BL21(DE3) pLysS in order to

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improve the production of this biocatalyst for the enzymatic synthesis of phenylacetaldehydes as well as to apply SOI-active biomass for the whole-cell biotransformation of styrene oxides into phenylacetic acids.

2. Material and methods

2.1. Chemicals and enzymes

All standard chemicals, substrates, non-substituted and substituted styrene oxides, and phenylacetic acids were purchased from Sigma–Aldrich (Steinheim, Germany), Carl Roth (Karlsruhe, Germany), AppliChem (Darmstadt, Deutschland), FLUKA (Buchs, Switzerland), and TCI Deutschland GmbH (Eschborn, Germany). Enzymes, related buffers, vectors, and kits were purchased from Thermo Fisher Scientific (Waltham, USA), Invitrogen (Carlsbad, USA), Novagene (Darmstadt, Germany), and Jena Bioscience (Jena, Germany).

2.2. Plasmids and bacterial strains

The genetic information of the native *styC* gene encoding a styrene oxide isomerase in *Rhodococcus opacus* 1CP has recently been reported by a former study [35]. During present study, a synthetic gene was constructed by gene synthesis encoding StyC from strain 1CP. To that end, the native gene *styC* was optimized for expression by rewriting the codon usage of *R. opacus* 1CP into that of *Acinetobacter baylii* ADP1 by the OPTIMIZER online tool [37]. This codon usage was used because to our experience it is also applicable for the protein expression in *E. coli* strains and further hosts (data not shown). Additionally, NotI, KpnI, EcoRI, and NcoI restriction sites were appended at both ends of the gene to facilitate its cloning into various expression vectors. The gene sequence constructed (Supplemental Fig. S1) was subsequently synthesized by MWG Eurofins Operon (Ebersberg, Germany) and provided in the cloning vector pEX-A.

The synthetic gene *styC* was ligated into the expression vector pET16bP (pET16b [Novagene] with altered multicloning site, U. Wehmeier, personal communication) and the construct subsequently transformed into *E. coli* DH5α and finally into *E. coli* BL21 (DE3) pLysS as described below in detail.

The pEX-A-*styC* construct was transferred into *E. coli* DH5α via heat–shock transformation for propagation. Afterwards, plasmid DNA was purified by the Gene JET Plasmid Miniprep Kit (Thermo Scientific). 0.5 μg pEX-A-*styC* and 0.3 μg pET16bP were digested in 1 × buffer O containing 12 units (U) of the enzyme NcoI and 3 U of NotI (Thermo Scientific). The DNA was incubated for 4 h at 37 °C. Subsequently, the products were purified by gel electrophoresis (1% agarose, 90 V), stained with SYBR Gold (Invitrogen), and isolated from the gel by the DNA Isolation Spin Kit (AppliChem).

Afterwards, 6 ng of the *styC*-containing insert and 16 ng digested pET16bP (molar ratio 4:1) were ligated in 1 × T4 ligase buffer containing 1 U of T4 ligase (Thermo Scientific). After a 30 min incubation at 22 °C, ligation product was immediately used for the transformation in strain DH5α as described above. After propagation of the construct pET16bP-*styC*, DNA was purified by the GeneJET Plasmid Miniprep Kit. The transformation of purified DNA into *E. coli* BL21(DE3) pLysS was also performed as described above.

Clones harboring the synthetic gene *styC* have been designated as *E. coli* BL21(DE3) pLysS pET16bP-*styC*. Biomass was initially investigated for SOI activity by a pre-incubation of the cells in 100 ml baffled flasks containing 10 ml of LB medium (pH 7, 5 g l^{-1} NaCl [40]) at 37 °C. Cells were grown up to an optical density at 600 nm (OD_{600}) of about 1. Afterwards, cultures were induced by addition of 0.5 mM IPTG, cultivated for 20–22 h at 22 °C, and the cells subsequently disrupted and screened for SOI activity as described below. Quite high IPTG concentrations of 0.5 mM were used during all experiments to ensure complete *styC* induction in all cells.

2.3. Optimization of the SOI expression in E. coli BL21(DE3) pLysS

LB broth was used to cultivate *E. coli* BL21(DE3) pLysS pET16bP-*styC*. If not stated otherwise, 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} chloramphenicol were additionally added to the culture medium.

Expression of *styC* in *E. coli* BL21(DE3) pLysS pET16bP-*styC* was investigated with respect to pH, temperature, NaCl concentration, the presence of antibiotics, the influence of glucose, and the effect of a pre-incubation at different temperatures before gene expression. For that, the precultures were incubated at 30 °C for 17–19 h at 120 rpm in 1 l baffled flasks containing LB medium (pH 7, 5 g l^{-1} NaCl). These cultures were harvested by centrifugation (15 min, 3000 × *g*), once washed with 10–15 ml of 25 mM phosphate buffer (pH 7), and the pellets resuspended and diluted with fresh LB medium to adjust an OD_{600} of 0.7. Expression in LB medium (5 g l^{-1} NaCl) with pH values of 6, 7, and 8 was initially investigated at 25 °C to evaluate the optimal (best) pH for subsequent experiments. Afterwards, the influence of the expression temperature on SOI activity at 14 °C, 30 °C, and 37 °C was investigated applying LB medium (pH 7) with 5 g l^{-1} NaCl. Furthermore, the effect of the NaCl concentration on *styC* expression was determined using LB medium (pH 7) with either 5 g l^{-1} or 10 g l^{-1} NaCl at 30 °C. The effect of antibiotics on the SOI gene expression at 30 °C was investigated by a cultivation of biomass in LB medium (pH 7, 10 g l^{-1} NaCl) with and without ampicillin and chloramphenicol. The influence of in total 20 mM glucose (added in 10 mM-portions during enzyme expression) on the SOI activity was determined in LB medium (pH 7, 5 g l^{-1} NaCl) at 30 °C. Furthermore, the effect of a one-step cultivation (initial biomass growth and subsequent expression at 30 °C) or a two-step cultivation (initial biomass growth at 37 °C, expression at 30 °C) was investigated applying LB medium (pH 7) with 5 g l^{-1} NaCl. In all cases the cultivation was performed in 250 ml or 500 ml baffled flasks containing 25 ml or 50 ml of liquid medium. SOI gene expression was induced by addition of IPTG in a final concentration of 0.5 mM.

Up-scaling of the biomass production under more defined conditions was performed by fed-batch cultivation of *E. coli* BL21 (DE3) pLysS pET16bP-*styC* in a 5 l biofermenter (ED/ES5; B. Braun Biotech AG, Melsungen, Germany). For that purpose, 4 l of LB broth (pH 7, 5 g l^{-1} NaCl) were used as culture medium. Precultures were incubated as described above and used to inoculate the fermenter. An initial OD_{600} of 0.05 was adjusted. Afterwards, the culture was incubated at 37 °C, 300–400 rpm, and with an aeration of 6 standard liters per min (slpm) for about 4 h to obtain biomass with an OD_{600} of about 1.4. The expression of *styC* was subsequently induced by addition of IPTG in a final concentration of 0.5 mM. During the expression of *styC*, biomass was incubated at 30 °C, 400–500 rpm, and with 6 slpm for several days. Additionally, in total 15 mM glucose were added in 5 mM portions. To follow the growth of the culture and the expression of *styC* after addition of IPTG, the culture was frequently sampled to determine OD_{600}, specific SOI activity, and cell dry weight.

2.4. Applicability of E. coli BL12(DE3) pLysS pET16bP-styC as whole-cell biocatalyst

To investigate the applicability of *E. coli* BL21(DE3) pLysS pET16bP-*styC* for the transformation of styrene oxide, SOI-active biomass was obtained from cultures incubated in flasks or in the fermenter under conditions as described above. 6–24 h after addition of IPTG, 50–

400 ml culture samples were harvested by centrifugation (5000 × *g*, 15 min, 10 °C), the cells were washed once with 25 mM phosphate buffer (pH 7.0), and centrifuged again. The pellet was subsequently resuspended in 50–400 ml fresh 25 mM phosphate buffer (pH 7.0) and distributed to baffled flasks. These suspensions were immediately used for biotransformation experiments.

For the biotransformation of styrene oxide either 15 ml or 200 ml SOI-active biomass were incubated in 250 ml or 1 l baffled flasks with in total 63 or 600 μmol styrene oxide which were added in portions of 7.9 μmol or 200 μmol (initially correspond to 0.5–1 mM in the medium) over 19–20 days. The cultures were ventilated all 2–3 days for 1–4 h. Samples were regularly taken from the cultures and the product formation quantified by reversed-phase high-performance liquid chromatography (reversed-phase HPLC) as described below.

To determine the substrate spectrum, 10 μmol of one of the following compounds were added to 250 ml flasks containing 25 ml cell suspension: styrene oxide, 4-fluorostyrene oxide, 4-chlorostyrene oxide, or 4-bromostyrene oxide. Substrate formation was monitored by reversed-phase HPLC.

2.5. Enzyme assays and protein quantification

The SOI activity was measured in units (U) and one unit is defined as the amount of SOI which converts 1 μmol styrene oxide to phenylacetaldehyde per min at 21–22 °C. For that purpose, 1.5 ml samples were taken from the expression cultures and subsequently centrifuged (13,000 × *g*, 5 min). Each cell pellet obtained was suspended in 500 μl 25 mM phosphate buffer (pH 7) and 500 mg zirconia beads as well as 8 U DNAse were added. The batches were vigorously shaken for 30 min at 30 Hz in a swing mill (MM-200, Retsch, Germany). The extracts were subsequently analyzed for SOI activity by incubation with styrene oxide and sampling as well as

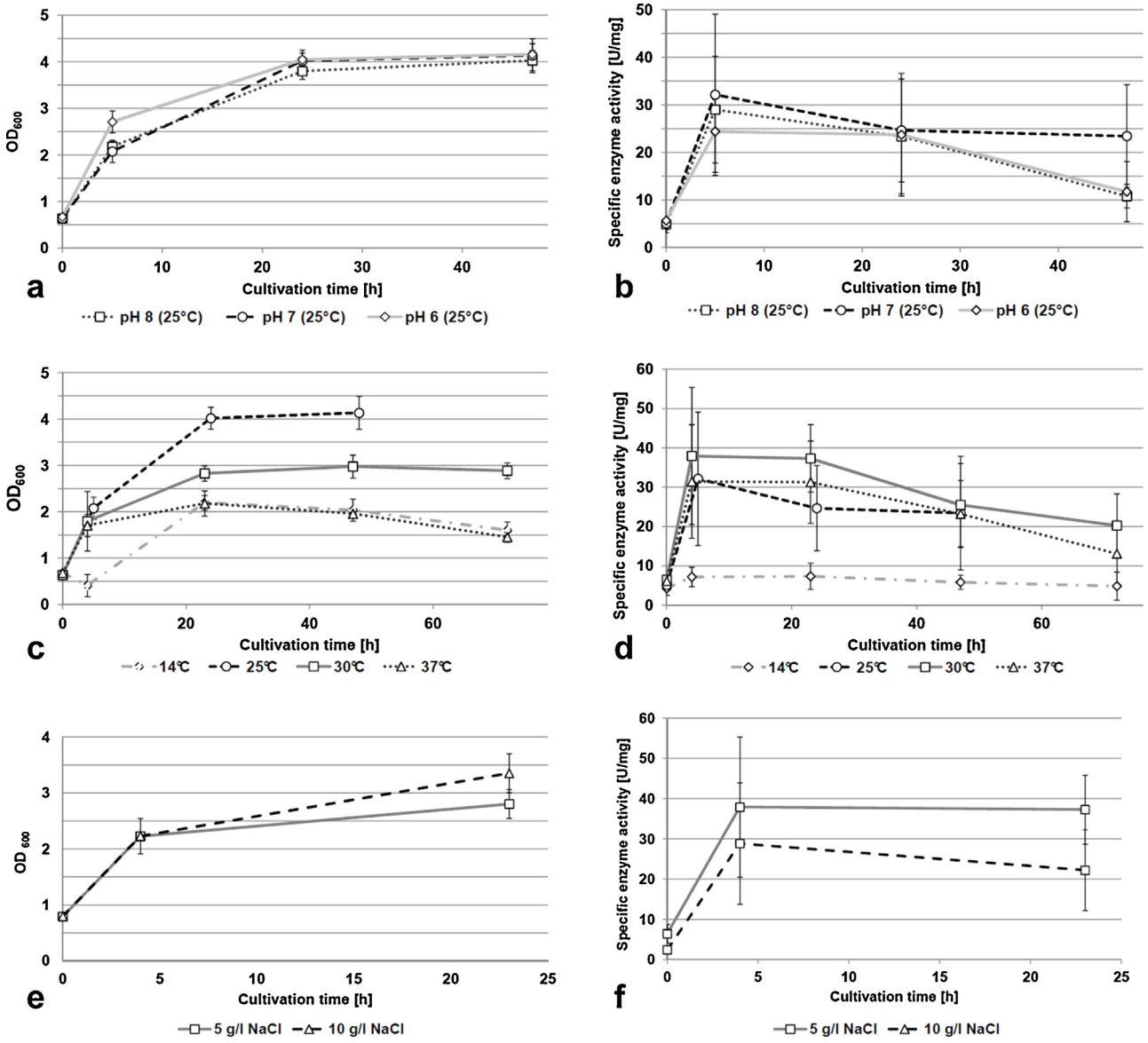

Fig. 1. Influence of different parameters on the *styC* expression in *E. coli* BL21(DE3) pLysS pET16bP-*styC*. The data presented illustrates: the influence of different pH values (6, 7, and 8) in the culture medium on (a) bacterial growth and (b) expression of active SOI; the influence of different cultivation temperatures (14, 25, 30, or 37 °C) on (c) growth and (d) expression; the influence of NaCl (5 or 10 g l^{-1}) on (e) growth and (f) expression. The cultivation was performed in baffled flasks at 120 rpm for 24–72 h. Further incubation conditions for each experiment are described in the Material and Methods section. Protein expression was induced by addition of 0.5 mM IPTG. Data points represent the means of four or six independent measurements and the standard deviations are given.

quenching with H_3PO_4 as previously described [32]. Afterwards, the acidified samples were directly used for reversed-phase HPLC [35].

To detect products formed by whole-cell biotransformation, 500 μl of the culture medium was sampled and diluted with an equal volume of methanol. The mixture was shaken for 1 min, centrifuged (5 min, 13,000 × *g*), and supernatant analyzed by reversed-phase HPLC. HPLC analysis was performed as previously described [33] applying a mobile phase containing 50% methanol (v/v) and 0.1% (w/v) phosphoric acid at a flow rate of 0.7 ml min^{-1}.

For the determination of the protein concentration, the method established by Bradford [3] was performed.

3. Results and discussion

3.1. Determination of conditions suitable for the improved SOI expression in E. coli BL21(DE3) pLysS

For the expression of *styC* in strain BL21(DE3) pLysS pET16bP-*styC*, no significant influence of pH 6–8 was observed in the present study (Fig. 1a and b). In all cases the highest SOI activities were measured about 4–5 h after addition of the inducer IPTG (pH 6: 24.4 ± 8.6 U mg^{-1}; pH 7: 32.1 ± 16.9 U mg^{-1}; pH 8: 28.9 ± 11.2 U mg^{-1}). However, the decrease of the enzyme activity was found to be lower under neutral conditions (pH 7) than under the other conditions. Thus, a pH of 7 was considered for all subsequent experiments.

In a second step, the cultivation temperature was investigated for its influence on SOI gene expression after addition of the inducer IPTG. With respect to the temperature the cell densities reached showed larger differences (Fig. 1c). The highest OD_{600} values of about 4 and 3 were reached at 25 °C and 30 °C. The highest SOI activities were measured 4–5 h after addition of IPTG (Fig. 1d). A specific SOI activity of 37.9 ± 17.4 U mg^{-1} was reached at 30 °C and only marginally lower specific activities of 31–32 U mg^{-1} were observed after incubation at 37 °C or 25 °C. The expression of *styC* at 14 °C resulted only in a low SOI activity of 7.1 ± 2.5 U mg^{-1}. This indicates that SOI activity of *E. coli* BL21(DE3) pLysS pET16bP-*styC* can obviously not be enhanced by slowering transcription and translation rate by lowering the cultivation temperature as observed for other proteins [1,10,11,41].

Additionally, the NaCl concentration of the medium was varied in order to investigate the influence of osmolarity on protein production (Fig. 1e and f). Expression of *styC* in the presence of medium containing 5 g l^{-1} NaCl led to the formation of less biomass with higher SOI activity (OD_{600} of 2.8 reached after 23 h; maximum SOI activity of 37.9 ± 17.4 U mg^{-1} after 4 h) compared to LB medium containing 10 g l^{-1} (OD_{600} of 3.3 reached after 23 h, maximum SOI activity of 28.8 ± 15.0 U mg^{-1} after 4 h). Thus, the lower concentration was preferred for further investigations.

Furthermore, it was observed that the antibiotics ampicillin and chloramphenicol have no significant effect on the expression and the highest activities were also reached about 4 h after addition of IPTG (with antibiotics: 28.8 ± 15.1 U mg^{-1}; without antibiotics: 32.7 ± 15.7 U mg^{-1}). Initial cultivation of *E. coli* BL21(DE3) pLysS at 37 °C and subsequent induction of the biomass with IPTG at 30 °C did not affect the SOI activity (maximum SOI activity of 24.7 ± 10.1 U mg^{-1}) negatively compared to a one-step cultivation at a constant temperature of 30 °C (maximum SOI activity of 26.3 ± 8.3 U mg^{-1}). In both cases the highest SOI activities were reached about 4 h after addition of IPTG. But the two-step cultivation allowed a faster growth during the first step of cultivation whilst IPTG was not added (data not shown). Furthermore, the addition of glucose in portions of 10 mM (in total 20 mM within 5 h) did not influence the SOI gene expression and the activity (data not shown). Based on these preliminary experiments, an up-scaled cultivation in a fermenter was performed.

3.2. Up-scaled production of biomass with high SOI activity

The cultivation of strain BL21(DE3) pLysS pET16bP-*styC* was performed in the fermenter under optimal conditions for SOI expression (30 °C, LB medium with pH 7 and 5 g l^{-1} NaCl) (Fig. 2). The fermenter cultivation enabled an improved reproducibility of the cultivation as well as the production of larger amounts of SOI-active biomass.

The highest SOI activity of 44.5 ± 8.7 U mg^{-1} was determined about 6 h after addition of ITPG (Fig. 2) which is also similar to the preliminary experiments mentioned above (Fig. 1). This recombinant specific activity corresponds to our knowledge to the highest SOI activity ever reported for crude cell extracts of SOI-containing wild-type strains [16,19,30,34,35]. In contrast to the cultivation in battled flasks, culture volume was significantly extended from 25 ml to 4 l. Thus, in total 2380 mg ± 262 mg protein and 106,100 ± 9200 U of SOI were obtained after in total 10 h applying strain BL21(DE3) pLysS pET16bP-*styC* in the fermenter. A previous study has reported the production of a wild-type SOI in *R. opacus* 1CP [34]. The cultivation of strain 1CP was performed in the same fermenter containing also 4 l of medium. In the former study, a total SOI activity of 18,000 U and in total about 1000 mg protein were obtained which corresponds to a specific SOI activity of only 18 U mg^{-1} [34]. Furthermore, the cultivation time of this *Rhodococcus* strain was significantly longer and the production of active biomass needed 28 days. In summary, the recombinant production of the SOI improved the specific enzyme activity by the factor 2.5 and reduced the cultivation time by a factor of 67 compared to the application of the wild-type strain 1CP. The recombinant SOI obtained can be applied for the cell-free production of phenylacetaldehyde from styrene oxide as described previously [27,32,34].

3.3. Applicability of E. coli BL21(DE3) pLysS pET16bP-styC as whole-cell biocatalyst for the production of phenylacetic acid

E. coli BL21(DE3) pLysS pET16bP, which only contained the pET16bP-vector without *styC*, served as negative control for the whole-cell biotransformation of styrene oxide. No product formation was observed in this case (data not shown). The integration of an SOI in *E. coli* BL21(DE3) pLysS pET16bP-*styC* enables whole cells to transform styrene oxide via

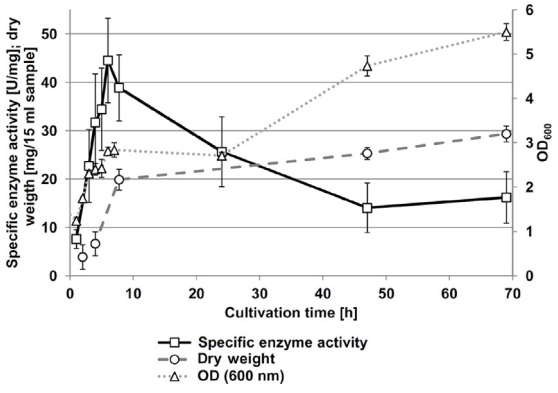

Fig. 2. Expression of SOI activity in *E. coli* BL21(DE3) pLysS pET16bP-*styC* during cultivation in the bioreactor. 4 l of biomass were cultivated at 37 °C for 4 h during which an OD_{600} of 1.4 was reached. Afterwards, cultivation temperature was decreased to 30 °C and 0.5 mM ITPG were added to express the synthetic *styC*. The figure illustrates the development of the SOI activity in the *E. coli* cells and of two growth parameters (OD_{600}, dry weight) after addition of IPTG. Portions of 5 mM glucose were additionally added one, four, and 5 h after addition of ITPG. The data illustrated represent the averages of two or three independent measurements and the standard deviations are given.

phenylacetaldehyde to phenylacetic acid (Figs. 3 and 4). The latter reaction is commonly catalyzed by phenylacetaldehyde dehydrogenases (PAD). A gene encoding such an aldehyde dehydrogenase (NCBI Acc. no. ACT43241) has also been found on the genome of *E. coli* BL21 (Acc. no. CP001509) in previous studies Jeong et al., 2009; [47]. Further metabolization of phenylacetic acid was not observed which is in accordance with the fact that corresponding genes, which are involved in the further degradation of phenylacetic acid [9], are not present on the genome of strain BL21.

Preliminary results showed that a high phenylacetaldehyde concentration of about 1.35 mM (~162 mg l^{-1}), which was formed after addition of three 1 mM portions of styrene oxide within 30 h, caused an irreversibly reduced metabolic activity in *E. coli* BL21(DE3) pLysS pET16bP-*styC* and prevented the formation of higher amounts of phenylacetic acid (Fig. 3). Such a toxic effect of phenylacetaldehyde in similar concentration of ~1.3 mM (156 mg l^{-1}) has also been reported by Kang et al. [21].

To prevent the inactivation of the biomass, a less frequent addition of styrene oxide was performed in final concentrations of 0.5–1.3 mM each 2–4 days (Fig. 4). The application of 15 ml of SOI-active biomass of *E. coli* BL21(DE3) pLysS pET16bP-*styC* (14.3 ± 4.3 U mg^{-1} SOI activity, OD_{600} ~ 2.9, 1.36 $mg_{cell\ dry\ weight}$ ml^{-1}, cells resuspended in phosphate puffer [25 mM, pH 7]) resulted in 3.32 ± 0.42 mM phenylacetic acid (452 ± 57 mg l^{-1}) after 15 days which corresponds to a yield of 79.6% (Fig. 4). After this period, 4.3% of 2-phenylethanol (0.18 ± 0.01 mM, 2.0 ± 1.2 mg l^{-1}) and 13.7% phenylacetaldehyde (0.57 ± 0.13 mM, 68.5 ± 15.6 mg l^{-1}) were present. After 20 days, the following yields were obtained: 85.4% for phenylacetic acid (5.36 ± 0.85 mM, 729 ± 116 mg l^{-1}) and 2.5% for 2-phenylethanol (0.16 ± 0.04 mM, 19.5 ± 4.9 mg l^{-1}). These results indicate that phenylacetic acid is formed as the main product. Additionally, the results described above also confirmed that the biomass still possesses SOI activity after 20 days despite of the absence of IPTG during the biotransformation.

3.4. Applicability of E. coli BL21(DE3) pLysS pET16bP-styC for the production of substituted phenylacetic acids by whole-cell biotransformation

E. coli BL21(DE3) pLysS pET16bP-*styC* was also applied for the transformation of mono-halogenated styrene oxides. For that, 25 ml biomass (OD_{600} of 1.8, 0.85 $mg_{cell\ dry\ weight}$ ml^{-1}) with a specific SOI activity of 19.6 ± 6 U mg^{-1} were incubated with

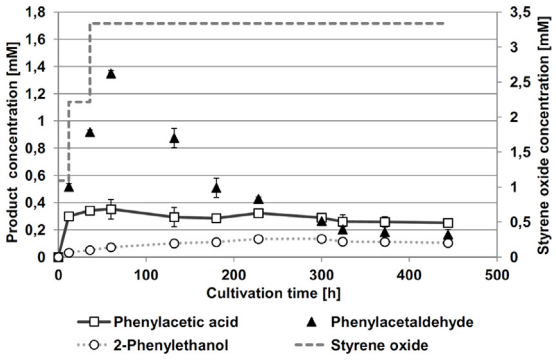

Fig. 3. Inhibition of the whole-cell biocatalyst by rapid production of phenylacetaldehyde after a too frequent addition of styrene oxide. 200 ml biomass of *E. coli* BL21(DE3) pLysS pET16bP-*styC* (OD_{600} ~ 2.3, 1.08 $mg_{cell\ dry\ weight}$ ml^{-1}, specific SOI activity of 19.7 ± 5.7 U mg^{-1}) were cultured for 19 days (444 h). Within the first 36 h, in total 0.6 mmol styrene oxide were added in portions of 0.2 mmol. Afterwards, the biomass was cultured without further addition of styrene oxide. Formation of phenylacetic acid, 2-phenylethanol, and phenylacetaldehyde is illustrated. Data points represent the means of four independent measurements and the standard deviations are given.

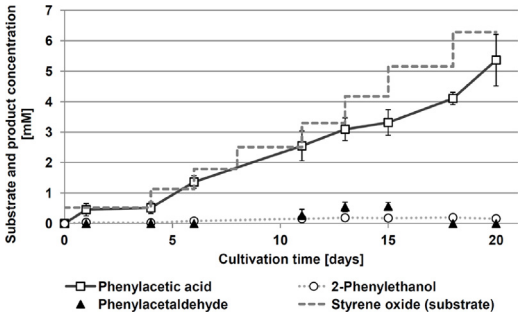

Fig. 4. Whole-cell biotransformation of styrene oxide applying *E. coli* BL21(DE3) pLysS pET16bP-*styC*. 15 ml of *E. coli* BL21(DE3) pLysS pET16bP-*styC* (OD_{600} ~ 2.9, 1.36 $mg_{cell\ dry\ weight}$ ml^{-1}, specific SOI activity of 14.3 ± 4.3 U mg^{-1}) in 25 mM phosphate buffer (pH 7) were cultivated over 20 days. Within the cultivation time in total about 63 μmol styrene oxide were added in portions of about 7.9 μmol. Formation of phenylacetic acid, 2-phenylethanol, and phenylacetaldehyde is illustrated. Data points represent the means of two independent measurements and the standard deviations are given.

10 μmol non-substituted or substituted styrene oxide. Beside the phenylacetic acids, also the corresponding phenylacetaldehydes and phenylethanols were determined during the first hours in all cases. Amounts of 6.2 μmol, 3.6 μmol, and 3.1 μmol were obtained for phenylacetic acid, 4-bromophenylacetic acid, and 4-fluorophenylacetic acid after 75 min, respectively. A lower yield of 2.3 μmol was achieved in the case of 4-chlorophenylacetic acid. Further cultivation without addition of fresh substrate led to the complete transformation of the respective substrates and formed aldehydes to the corresponding phenylacetic acids. Besides small amounts of the corresponding phenylethanols, the substrates styrene oxide and 4-bromostyrene oxide were transformed completely to the corresponding acids after 150 min while 4-chlorostyrene oxide was completely metabolized to 4-chlorophenylacetic acid after 360 min. Remarkably, the transformation of 4-fluorostyrene oxide occurred somewhat slower, but was also completed in 24 h.

4. Conclusion

A promising biocatalyst was constructed by using *E. coli* BL21 (DE3) pLysS as host for the heterologous expression of a synthetic SOI gene (*styC*) which encodes the SOI of *R. opacus* 1CP. An expression temperature of 30 °C and LB medium with a pH of 7 and 5 g l^{-1} NaCl were suitable to obtain biomass with a specific SOI activity of up to 44.5 ± 8.7 U mg^{-1} within 6 h after addition of IPTG. The recombinant biomass provides a promising source for the preparation and enrichment of the SOI for cell-free applications [32,34]. *E. coli* BL21(DE3) pLysS pET16bP-*styC* was found to be additionally suitable as whole-cell biocatalyst for the production of (substituted) phenylacetic acids from corresponding styrene oxide (s). In the case of phenylacetic acid, a product amount of about 730 mg l^{-1} (85% yield) was reached within 20 days.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2015.05.002.

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