Investigation of the co-metabolic transformation of 4-chlorostyrene into 4-chlorophenylacetic acid in *Pseudomonas fluorescens* ST

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ABSTRACT

The side-chain oxygenation of styrene is able to yield substituted phenylacetic acids from corresponding styrenes by co-metabolic transformation. This co-metabolization was investigated in *Pseudomonas fluorescens* ST using 4-chlorostyrene as co-substrate. It was shown that non-substituted styrene is necessary to ensure the co-metabolic process. Furthermore, aspects affecting the co-transformation were studied, e.g. cell density, amount of inducer, pH, effects of co-substrate/co-product. It was demonstrated that 4-chlorophenylacetic acid and 4-chlorostyrene are able to inhibit the reaction. But, these inhibitions are influenced by salt and trace elements. Finally, a protocol was established which considers all findings. Therewith, about $6.7\,g\,L^{-1}$ co-product were obtained after 451 h. Compared to previous studies, the co-product concentration was improved by the factor 1.4 while the reaction time was decreased by the factor 18.5. The study offers also aspects for prospective improvements in order to establish an efficient way to gain substituted acids without genetic manipulation.

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1. Introduction

Substituted and non-substituted phenylacetic acids are natural ingredients in many plants and fruits. This compounds represent important chemicals for various industries and were used as fragrances and flavors in the cosmetic or food industry [1] as well as in the pharmaceutical sector as virostatic agents [2], receptor agonists and antagonists [3,4], precursors [5], analgesics [6,7] or others.

This widespread applicability of these acids initiated several studies about their production and numerous strategies to gain such compounds have been described, e.g. the carbonylation of benzyl chlorides [8] or the hydrolysis of phenylacetonitrile [9]. Beside chemical strategies also biotechnological processes have become popular such as the hydrolysis of arylacetonitriles by a recombinant arylacetonitrilase [10] or the transformation of 2-phenylpropionitrile by nitrile hydratase and a stereoselective amidase [11]. A novel study has recently reported the co-metabolic production of various substituted and halogenated phenylacetic acids from styrenes [12].

The latter strategy uses organisms which harbor enzymes of the styrene-degrading pathway of side-chain oxygenation [12,13].

During this pathway, styrene is transformed into styrene oxide and phenylacetaldehyde by a styrene monooxygenase and styrene oxide isomerase. During a last step, a phenylacetaldehyde dehydrogenase catalyzes the oxidation of the aldehyde into phenylacetic acid. This acid is commonly degraded by further enzymatic steps [14,15,25]. Remarkably, it was shown that different substituted and especially halogenated styrenes were successfully transformed into the corresponding phenylacetic acid, but the subsequent metabolization of the acid was not observed or was significantly declined compared to non-substituted phenylacetic acid [12]. Especially *Pseudomonas fluorescens* ST has been described to be a promising candidate for such co-metabolic studies and for further optimization. At least $3150\,\mu mol$ 4-chlorphenylacetic acid were produced from $3630\,\mu mol$ 4-chlorostyrene within 348 days during preliminary experiments using strain ST [12]. This corresponds to a product concentration of 27.5 mM.

During the present study, the co-metabolism of halogenated styrenes in *Pseudomonas fluorescens* ST was investigated in more detail with 4-chlorostyrene as exemplified substrate. Different parameters were determined to enhance the product yields and transformation rates. Furthermore, some of the complex inter-actions of different parameters were revealed and are helpful to understand this co-metabolic process in more detail. These results offer opportunities for a further optimization of this process during future studies.

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2. Material and methods

2.1. Cultivation media and specifications

For cultivation and transformation, modified 1x phosphate buffer [16] or LB medium [17] was used. The phosphate buffer was modified with a salt and/or trace element stock solution. Therefore, the receipts of [16] for 1000-fold trace element solution 6 (TES6) and 50-fold salt solution (SL) were used. Usually, SL contains already TES6 in the original receipt. Nevertheless, the SL stock solution used for all experiments below was not supplemented with TES6 and the trace elements were added to each culture directly by the TES6 stock solution. This was preferred to ensure an easier variation of the medium ingredients. The total amount of TES6 and SL, which was added to the phosphate buffer, is given below for each experiment. Phosphate buffer (1x) containing 1x SL and 1x TES6 is designated as mineral medium during this study (based on [16]).

2.2. Bacterial strains and initial culture conditions

Pseudomonas fluorescens ST (DSMZ 6290) [18–20] was used during this study. The strain was initially cultivated on solid mineral medium [16] containing 20 g L^{-1} glucose or on LB plates (pH 7, 5 g L^{-1} NaCl) [17] and was incubated at 30 °C. Colonies obtained were used to inoculate liquid medium.

2.3. Inducing effect of different styrenes on the styrene metabolism

300–400 mL of mineral medium (pH 7) with 5 mM glucose and 0.1% (w/v) yeast extract were inoculated with biomass of strain ST and the culture was incubated at 100–120 rpm and 30 °C. All 1–3 days 2.5–5 mM glucose were added and the biomass was cultivated up to an optical density (OD_{600}) of 2.6. Afterwards, the cells were harvested by centrifugation ($5000 \times g$, 30 min, 4 °C) and suspended in fresh mineral medium without glucose and yeast extract. This suspension (OD_{600} of 2.6) was distributed in 25-mL portions to 500-mL baffled flasks. Afterwards, one of the following substrates was supplied to the cultures by a vaporizer (as described by [21]): styrene, 4-chlorostyrene, 4-fluorostyrene, α-methylstyrene, 3-chlorostyrene. In total 46.2–52.4 μmol (=1.85–2.10 mM) substrate were added over 72 h in portions of 15.4–17.5 μmol (=0.62–1.17 mM). Before addition of a fresh portion of substrate, the flasks were aerated for 1–2 h under sterile atmosphere. After 72 h, 5–10 mL of the biomass were sampled, centrifuged at 5000–6800 × g for up to 10 min, and re-suspended in 1 mL of 1x phosphate buffer (pH 7). These samples were used for SOI activity measurements as described previously [22] and product formation was quantified by HPLC directly from the supernatants as described earlier, too [21,23]. The determination of the protein concentrations was performed with the method of Bradford [24].

2.4. Production of styrene-induced cells of Pseudomonas fluorescens ST

Production of styrene-induced biomass for all subsequent experiments was performed in 3-L baffled flasks or in the fermenter. Therefore, 20 mL or 500 mL mineral medium (pH 7) including 5 mM of glucose and 0.1% (w/v) yeast extract were inoculated by solid-grown biomass or by a frozen stock culture. The cultures were incubated in 100-mL or 500-mL flasks for 3–11 days at 30 °C and 120 rpm. These cultures served as preculture for the cultivations mentioned below.

A part of the biomass for subsequent experiments was produced in 3-L baffled flasks. Therefore, 20–40 mL of the preculture were used to inoculate 600–1000 mL mineral medium (pH 7) containing one initial portion of 0.1% (w/v) yeast extract. The biomass was cultivated at 120 rpm and 30 °C for 7–9 days. During the initial 4-5 days, in total 12–30 mmol (=20–30 mM) glucose was added in portions of 3–5 mmol (=5 mM) all 1–2 days resulting in an OD_{600} of about 3. Afterwards, the pH was adjusted to 7.8–8.0 with 12.5% NH_3. Finally, in total 393–655 μmol styrene (=655 μM) were added in portions of 87.4–306 μmol (=87–510 μM) over the last 22–48 h. Before the addition of each portion of styrene, the pH was re-adjusted to pH 8 with 12.5% NH_3 and the flasks were aerated with 1 bar of oxygen for 60–90 s. After styrene induction, the cells (OD_{600} of 1.9–2.7) were harvested by centrifugation ($5000 \times g$, 30 min, 4 °C) and concentrated to higher cell densities by re-suspension into new mineral medium or new 1x phosphate buffer supplemented with different amounts of TES6 or SL ([16]; modified as described above). These suspensions were subsequently used for further experiments.

For fermenter cultivation, a 5-L fermenter (ED/ES5, B. Braun Biotech AG, Melsungen, Germany) containing 5 L of mineral medium (pH of 7) with 0.1% (w/v) yeast extract was used. 100–140 mL of a preculture were used for inoculation and an initial OD_{600} of 0.1–0.15 was adjusted. The cultures were incubated for 12–15 days at 460–600 rpm and 30 °C. During a first step of the cultivation, glucose served as substrate to obtain high cell densities. Therefore, in total 352–487 mmol glucose (=78–97 mM) were added in portions of 250–375 μmol (=50–75 μM) all 7–25 min. In some cases, an initial portion of 26 mmol (=5.25 mM) was provided during the first 20 h. During this cultivation the aeration was applied to 2–12 standard liters per minute (slpm) to ensure aerobic culture conditions. The pH was automatically adjusted to pH 7 with 8.5% H_3PO_4 and 12.5% NH_3. Finally, OD_{600} values of 12–14 were reached after 8–11 days which correspond to a cell dry weight of 4–5 mg mL^{-1}. Afterwards, these cells were adapted with styrene to activate the styrene metabolism. Therefore, the cell suspension at pH 7 was used directly for enzyme adaptation or was previously adjusted to pH 8 with 12.5% NH_3. Afterwards, the culture was automatically or manually re-adjusted to pH 7 or 8 during styrene adaptation. Airflow was commonly adjusted to 0 slpm during styrene metabolization. Thus, styrene degradation was indicated by a significant decrease in the oxygen level of the culture. After styrene was metabolized and the oxygen level increased or stagnated again, the culture was aerated with 6–15 slpm of oxygen until the oxygen level reached a level >50% followed by the addition of the next portion of styrene. This non-continuous aeration was also useful to avoid that styrene was exhausted over time. Styrene was initially added in single or in multiple portions of 1750–6990 μmol (=368–1400 μM) during the first 20–30 h. Afterwards, smaller portions of 437–655 μmol (=92–131 μM) were added all 1.1–3.5 h. In total 6.49–22.7 mmol (=1.44–4.54 mM) styrene were added within 20–85 h. To investigate the influence of fresh trace elements on the styrene induction, at least 0.33–0.5x TES6 (referred to culture volume) were added in some cases during adaptation. Finally, 3.5–5 L of an induced culture with an OD_{600} of 10–12 (=cell dry weight of 3–4 mg mL^{-1}) were obtained. The cells were subsequently harvested and suspended into new medium as described above.

2.5. Influence of the pH on the co-metabolization of 4-chlorostyrene

Biomass from the bioreactor was re-suspended in 125 mL of fresh mineral medium (pH 7) and an OD_{600} of 27 was adjusted. The cells were cultivated for 188 h in a 3-L baffled flask at 30 °C and 200 rpm. During this initial cultivation, styrene was added in portions of 437, 219 or 109 μmol each day (=3.50, 1.76–1.78, 0.89–0.90 mM; in total: 1640 μmol/13.3 mM). Furthermore, 4-chlorostyrene was supplied in portions of 786 or 393 μmol (=6.29 or 3.16–3.24 mM) each 1–2 days. In total 3150 μmol (=25.5 mM) of this co-substrate were

added over time. The substrate and the co-substrate were supplied via a vaporizer as described earlier [21]. The pH was re-adjusted to pH 7 with 12.5% NH_3 (if necessary) and the culture was aerated for 30–45 s by 1 bar of O_2 prior addition of the substrate and co-substrate portions. Afterwards, this culture was harvested as described above and the cells were re-suspended in 25 mL of mineral medium with an initial pH of 6,7,8 or 9, respectively. The suspensions were cultivated in 1-L baffled flasks for 434 h at 120–130 rpm and 30 °C. During this second incubation, styrene was added directly to the medium in portions of 87.4 µmol (=3.50–4.10 mM) each day. 4-chlorostyrene was supplied all 24–72 h by portions of 157 µmol (=6.29–7.38 mM). In total 1570 µmol (=69.2 mM) styrene and 1730 µmol (=76 mM) 4-chlorostyrene were added. During incubation the cells were aerated again as described above. The pH was daily re-adjusted with 7 M or 0.2 M NaOH and 50% H_3PO_4. During the complete incubation, samples of 125–500 µL were regularly taken from the cultures. The samples were diluted by methanol and used for HPLC analysis as described below.

2.6. Influence of the daily amount of styrene and the cell density on the co-metabolization of 4-chlorostyrene

Biomass for these experiments was obtained by initial reactor cultivation or by cultivation in 3-L baffled flasks as described above. The biomass was harvested and the cells were subsequently re-suspended in mineral medium (pH 8). 25-mL volumes of the suspensions were incubated in 1-L flasks at 120 rpm and 30 °C. All cultures were aerated each day by 1 bar of O_2 for 30–45 s and the pH was re-adjusted daily to 8 by 0.2–7 M NaOH, 50 or 85% H_3PO_4, and particularly by 12.5% NH_3.

During one experiment cell suspensions with an OD_{600} of 5, 10, 15, and 20 were incubated for 482–504 h with an equal amount of styrene each day. Therefore, portions of 87.4 µmol (=3.50–6.21 mM) styrene were supplied [totally 1660–1840 µmol (=75.7–97.9 mM)]. 4-chlorostyrene was added in portions of 157 µmol (=6.29–10.2 mM) and later particularly also in portions of 78.6 µmol (about 4.9–5.4 mM) all 1–2 days. In total 118–163 mM co-substrate were added during time.

In another experiment the daily amount of styrene was adjusted to the OD_{600} of the cultures in a constant ratio of 8.74:1 (µmol$_{styrene}$ day^{-1}: OD_{600}; estimated as described in the results and discussion section). Hence, the culture with an OD_{600} of 5 was supplied with daily portions of 43.7 µmol styrene (=1.75–2.59 mM). In total 830 µmol (=40.2 mM) styrene were added during 456 h. Further cultures with an OD_{600} of 10, 15 and 20 were incubated with daily amounts of 87.4 µmol (=3.50–4.49 mM), 131.1 µmol (=5.24–6.56 mM) or 175 µmol (=7.16–11.6 mM) styrene. Total amounts of 1570 µmol (=71.2 mM), 2100 µmol (=93.7 mM) and 2970 µmol (=154 mM) were reached after 451 h, 382 h and 411 h, respectively. In all cases portions of 157 µmol (6.29–10.6 mM) co-substrate were supplied all 1–2 days (in total 99.7–145 mM).

During all cultivations mentioned above, samples of 125–500 µL were regularly taken from the batches, diluted by methanol, and used for HPLC analysis as described below.

2.7. Is the co-metabolism influenced by 4-chlorostyrene and 4-chlorophenylacetic acid?

The cells for these investigations were gained by initial reactor cultivation or by cultivation in 3-L baffled flasks as described above. Cells were harvested and re-suspended in mineral medium (pH 8). 25-mL portions of the cell suspension were incubated in 1-L flasks at 120 rpm and 30 °C. All cultures were aerated daily as described above and the pH was re-adjusted to 8 by 0.2–7 M NaOH and 50 or 85% H_3PO_4.

During the first part of these investigations, cells with an OD_{600} of 14–15 were incubated under different initial conditions for 431 h. One culture was cultivated without additional amounts of 4-chlorostyrene at the beginning while another culture was supplemented with 88 mM (=2202 µmol) 4-chlorostyrene. A third culture was incubated with an initial concentration of 30 mM (=5.08 g/L, 744 µmol) 4-chlorophenylacetic acid. All cultures were subsequently fed by portions of 87.4 µmol styrene each day (=3.50–5.62 mM, totally 1570 µmol/76.6–79.2 mM). Portions of 157 µmol (=6.29–10.1 mM) 4-chlorostyrene per day served as co-substrate. In total 2830 µmol (=139–143 mM) or 4880 µmol (=220 mM) co-substrate were added over time in the case of the cultures without or with an initial portion of 4-chlorostyrene, respectively.

During a second experiment the influence of different initial amounts of 4-chlorostyrene on a cell suspension with an OD_{600} of 10 was investigated for 334–380 h. The reference culture was incubated without an additional initial amount of 4-chlorostyrene while further cultures were incubated with 53 mM (=1340 µmol) or 79 mM (=1970 µmol) co-substrate. Afterwards, the cultures were incubated on a similar way as described above and portions of 87.4 µmol (=3.50–5.39 mM) styrene were added each day (totally 1220–1400 µmol/60.4–67.1 mM). Besides the initial amount of 4-chlorostyrene in some cases, portions of 157 µmol (=6.29–9.12 mM) co-substrate were added to each culture all 24–120 h (totally 2200–2750 µmol/103.3–123 mM). Sampling and sample preparation for HPLC was performed as described above. Determination of the co-product by HPLC is mentioned below.

2.8. Is the co-metabolism influenced by salt or trace elements?

Biomass obtained from fermenter cultivation as described above was re-suspended in mineral medium (pH 8) yielding an OD_{600} of 10. This suspension was distributed in 25-mL portions to 1-L flasks. Some cultures were supplied with additional amounts of TES6 and SL (up to 2x). The cells were cultivated at 120 rpm and 30 °C. Aeration was performed as described above. The pH was re-adjusted to 8 by 0.5 or 7 M NaOH and 85% H_3PO_4. The following medium compositions of SL and TES6 were tested: 1x and 1x (=normal mineral medium); 1x and 2x; 2x and 1x; 2x and 2x. The cultures were incubated for 500 h. Furthermore, 1x fresh SL and 1x fresh TES6 were additionally added after 525 h to the culture initially containing 1x SL and 1x TES6 to investigate the influence of fresh salt and trace elements on the transformation. This culture was cultivated for additional 193 h (in total 693 h). Styrene was fed in all cases in portions of 87.4 µmol (=3.50–8.31 mM) each day and 4-chlorostyrene was added in portions of 157 µmol (=6.29–15.0 mM) all 1–3 days. In total 1840 µmol (96.7–103 mM) styrene and 2670 µmol (135–143 mM) 4-chlorostyrene were added over a period of 500 h while the culture with an extended cultivation time of 693 h reached total amounts of 2540 µmol (=152 mM) styrene and 3620 µmol (=212 mM) 4-chlorostyrene.

Furthermore, biomass obtained from a cultivation in 3-L flasks was used to investigate the influence of lacking SL on the transformation of 4-chlorostyrene. The cells were re-suspended in 1x phosphate buffer (pH 8) to an OD_{600} of 14–15 and 25-mL portions of the suspension were distributed to 1-L flasks. The reference culture was supplied with 1x SL and 1x TES6 (=mineral medium) while the remaining culture was only supported by 1x TES6. Aeration and cultivation was performed as described above and pH was re-adjusted by 0.2 or 7 M NaOH and 50% H_3PO_4. The cultures were incubated for 431 h. All cultures were fed by portions of 87.4 µmol (=3.50–5.45 mM, totally 1570 µmol/77.0–78.1 mM) styrene and 157 µmol (=6.29–9.81 mM, totally 2830 µmol/139–141 mM) 4-chlorostyrene each day. Sampling and sample preparation for HPLC was performed as described above while the analysis via HPLC is mentioned below.

2.9. Further investigation of the co-metabolism by reactor-based cultivations

Biomass obtained by reactor cultivation was harvested and re-suspended in 1.5 L of 1x phosphate buffer (pH 8) initially containing either 1x SL and 1x TES6 (=mineral medium) or only 1x TES6. An OD_{600} of 10 was adjusted and the suspensions were cultivated in the fermenter for 406–692 h. The suspensions were aerated by pressured air each day after consuming the styrene portions added. Furthermore, the pH was manually re-adjusted by 1 M NaOH and 8.5% H_3PO_4. Each suspension was cultivated at 30 °C and 600 rpm. Aeration, oxygen supply, stirrer speed, consumption of base and acid as well as pH was monitored automatically by the control unit of the reactor. Furthermore, the OD_{600} was determined during sampling.

Run 1 and 2 were performed in presence of mineral medium. Styrene was added by an initial amount of 3500 µmol and subsequently in portions of 3060 µmol (=2.33–2.88 mM) all 24 h. 4-Chlorostyrene was provided by an initial portion of 6290 µmol followed by 5,510-µmol portions all 24–72 h (=4.19–5.05 mM). In total 52.4 mmol (=41.3 mM) and 83.0 mmol (=62.9 mM) styrene as well as 83.4 mmol (=64.8 mM) and 99.9 mmol (=73.0 mM) 4-chlorostyrene were fed during Run 1 (cultivation time of 406 h) and Run 2 (646 h) over time. Compared to Run 1 additional amounts of fresh SL and TES6 were added during Run 2 to investigate the influence of these additives on the co-metabolic transformation. The following concentrations of fresh supplements were added (related to the culture volume): 1x TES6 after 237 h (in total 2x TES6 in the medium), 1x SL after 404 h (totally 2x SL), and 1x SL with 1x TES6 after 547 h (totally 3x SL and TES6).

Run 3 was performed with cells suspended in 1x phosphate buffer with only 1x TES6 and without SL. Styrene and 4-chlorostyrene were added in the same way and in the same µmol-portions as described for Run 1 and 2 corresponding to 2.33–3.23 mM substrate and 4.19–5.70 mM co-substrate. In total 86.1 mmol (=73.6 mM) styrene and 116 mmol (=96.4 mM) 4-chlorostyrene were added within 692 h. During cultivation, fresh SL and TES6 were added in the following final concentrations (related to culture volume): 0.75x SL and 0.75x TES6 after 258 h (in total 0.75x SL, 1.75x TES6) as well as 0.33x SL and 0.33x TES6 after 621 h (in total 1.08x SL, 2.08x TES6).

Samples were regularly taken from the cultures and prepared for HPLC as mentioned above. The analysis via HPLC is described below.

2.10. HPLC analysis

The determination of the product formation by SOI as well as the analysis of the samples taken during all transformation studies were performed by reversed-phase high performance liquid chromatography (HPLC). Therefore, a DIONEX Ulti-Mate3000 UHPLC system with a diode array detector (Thermo Scientific) was used. HPLC was performed with 50% (v/v) methanol containing 0.1% (w/v) phosphoric acid as mobile phase at a flow rate of 0.7 mL min^{-1} and a Europhere C18-column (125 mm × 4 mm, 5 µm particle size; Knauer) was used as solid phase [12,21,23]. The peaks obtained were compared to standards with respect to retention volume and UV-spectrum. A wavelength of 205 nm was used for quantification. For all concentrations determined by HPLC, an estimated mistake of 5% of the average value determined from the measurements was additionally considered and added to the standard error. Therewith, unspecific smaller differences during the transformations and the initial adaptation with styrene should be considered.

3. Results and discussion

3.1. Initial overview over the experimental design

During this study, several experiments were performed to investigate the co-metabolism of 4-chlorostyrene in *Pseudomonas fluorescens* ST. A detailed overview about the experimental design is given in Fig. 1. After some preliminary experiments investigating the induction of the styrene-degrading pathway by various styrenes, the influence of the pH on the co-transformation of 4-chlorostyrene was determined as first parameter. Afterwards, the optimal cell density and the optimal daily amount of inducer were investigated in dependence of each other. Based on these results, initial culture-depending parameters were identified and used for further studies. Subsequent experiments investigated inhibiting effects mediated by the co-substrate or the co-product as well as possibilities to affect the transformation and the inhibitions by salt- and trace elements. Finally, the knowledge of all of these experiments was applied to establish a protocol for an enhanced transformation in the fermenter. During this up-scaling, further details about the oxygen consumption and the preference of substrate or co-substrate were revealed. Details of all experimental results are described below.

3.2. Induction of styrene-degrading genes by different styrenes in Pseudomonas fluorescens ST

During this initial step, the inducing effect of substituted styrenes compared to non-substituted styrene was investigated to reveal the conditions under which the styrene-degrading genes (*sty*-gene cluster) are expressed. The activity of the styrene oxide isomerase (SOI, StyC) – one key enzyme of this pathway – was exemplarily used to evaluate the *sty*-gene expression in strain ST in presence of the styrenes.

The incubation of cells in presence of non-substituted styrene resulted in an SOI activity of 6.13 U mg^{-1} (=100%) while 4-chlorostyrene, 4-fluorostyrene, α-methylstyrene, and 3-chlorostyrene led only to relative activities of 10.5, 6.93, 10.4, and 0.41% compared to styrene-induced cells, respectively. This strongly indicates that the expression of the styrene-degrading gene cluster is significantly linked to the main substrate styrene. Therefore, the cultures, which were investigated for the co-metabolization of the halogenated co-substrate, were initially pre-induced only with styrene as described in the material and methods section to ensure that all cells are adapted with an active *sty* operon (cultivation example shown in Fig. S1). The initial portion of styrene (commonly 0.52–1.05 mM) was normally depleted after 19–27 h. Afterwards, further portions of styrene were metabolized significantly faster. Interestingly, the addition of 0.33–0.5x TES6 at the beginning of the styrene adaptation step is suitable to accelerate the induction and the first portion of styrene was already consumed after 10–14 h (detailed data not shown).

The cells adapted were subsequently used for the co-metabolic transformation with the chosen co-substrate 4-chlorostyrene. The formation of the co-product 4-chlorophenylacetic acid was only observed over time if styrene was added daily to the culture during the complete transformation (Fig. S2; experimental details are given in the figure description). These results strongly proved again that the transformation of substituted styrenes is a co-metabolic process and depends on the availability of the main substrate styrene.

3.3. pH influence on the co-metabolic process

The influence of a pH of 6, 7, 8, or 9 on the reachable co-product concentrations was investigated as described in the material and
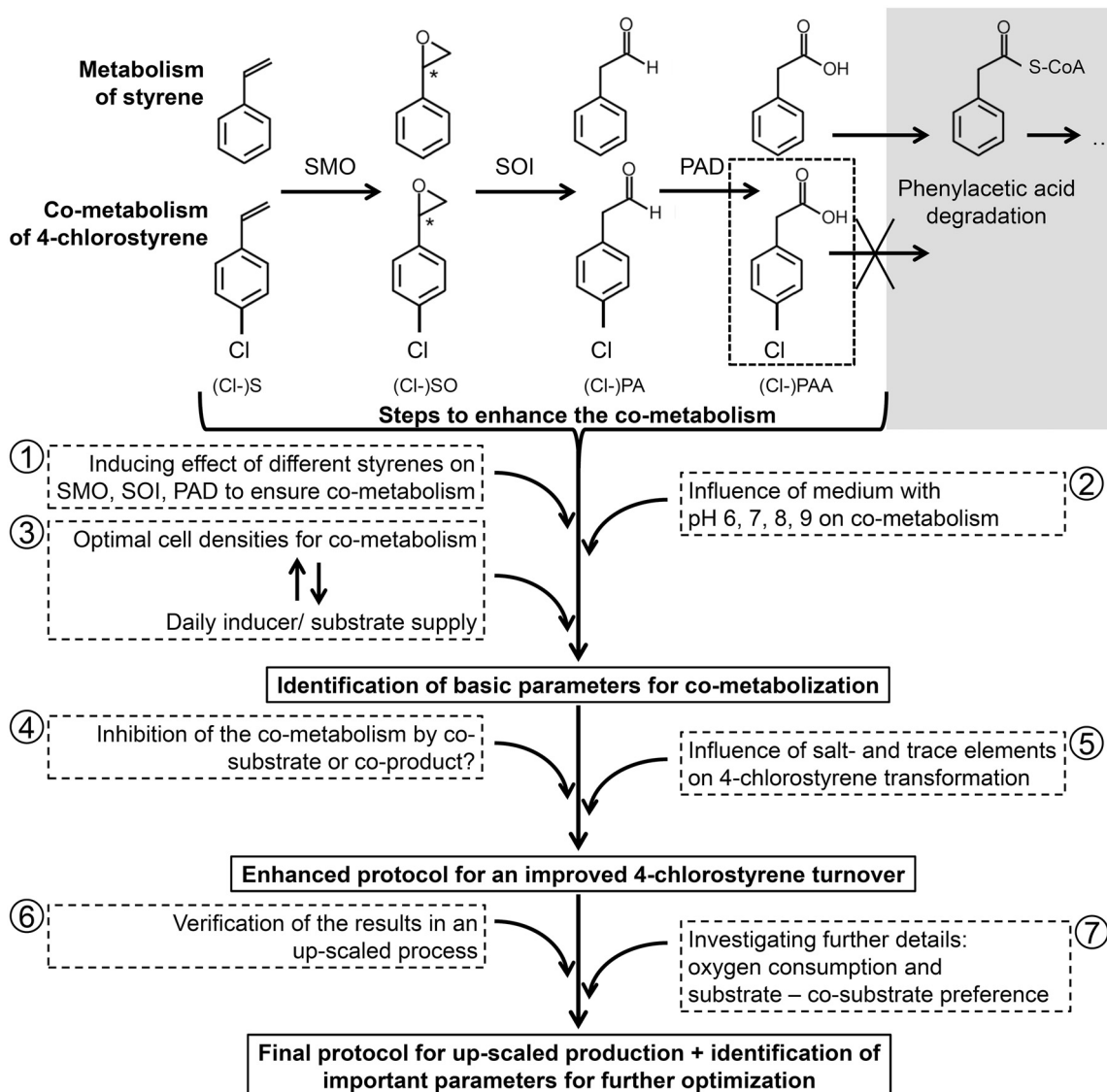
Fig. 1. Overview about the experimental design of this study.
The co-metabolic transformation of 4-chlorostyrene was investigated during this study applying cells of *Pseudomonas fluorescens* ST. While styrene is metabolized completely by the cells, the co-substrate 4-chlorostyrene funnels through the pathway of side-chain oxygenation, but undergoes no subsequent metabolization by phenylacetic acid degradation. The experimental steps of this study in order to investigate several parameters influencing the co-metabolism are shown by the numbers 1–7 yielding several milestones and finally a protocol for an up-scaled process to gain 4-chlorophenylacetic acid. The following abbreviations are included in the figure: SMO – styrene monooxygenase; SOI – styrene oxide isomerase; PAD – phenylacetaldehyde dehydrogenase; (Cl-)S – (4-chloro-)styrene; (Cl-)SO – (4-chloro-)styrene oxide; (Cl-)PA – (4-chloro-)phenylacetaldehyde; (Cl-)PAA – (4-chloro-)phenylacetic acid.

methods section. The results are shown in Fig. 2 (additional information given in the Supplemental part at Fig. S3). Total co-product concentrations of 3.76 g L^{-1} (=22.0 mM) were achieved at pH of 8 and slightly lower yields of 3.40 g L^{-1} (=19.9 mM) were obtained at pH 7. Significantly lower values were determined for pH 6 and pH 9.

As also shown in Fig. 2, the pH decreased during the biotransformation of styrene and 4-chlorostyrene in the batches and the transformation is inhibited at a pH of lower than 6. To avoid that the pH falls to fast below 6, a pH of 8 is preferred towards 7. Furthermore, a pH of 8 allowed also slightly higher amounts of co-product compared to pH 7. The short lag-phase in Fig. 2 after adjusting the pH to 8 can be avoided by adapting the cells with styrene at pH 8 instead of 7 during biomass production.

For all subsequent transformation studies, a pH of 8 was used for all experiments described below and the cells were initially induced at pH 8, too.

3.4. Influence of the cell density and the amount of styrene on the co-metabolic process

During an initial experiment the daily amount of 87.4 μmol of styrene yielded the fastest formation of co-product using cultures with a cell density (OD_{600}) of 10 (see Fig. S4; experimental details are given in the figure description). Thus, this feeding rate with the corresponding OD-referred ratio of 8.74:1 (μmol$_{styrene}$ day^{-1}: OD_{600}) was used as basis for further optimization.

During a second experiment, 87.4 μmol$_{styrene}$ day^{-1} were added to cultures with OD_{600} values of 5, 10, 15, and 20 to evaluate the influence of the optical density on the transformation. The results are shown in Figs. Fig. 33 A and S5A. The most promising transformation rates of 5.97 g L^{-1} (=35.0 mM) and 5.89 g L^{-1} (=34.5 mM) after about 307–312 h were determined for an OD_{600} of 10 and 15. Lower rates of 5.71 g L^{-1} (=33.5 mM) and 5.69 g L^{-1} (=33.4 mM) within 357 h and 386 h were obtained using cell densities (OD_{600}) of 5 and 20.
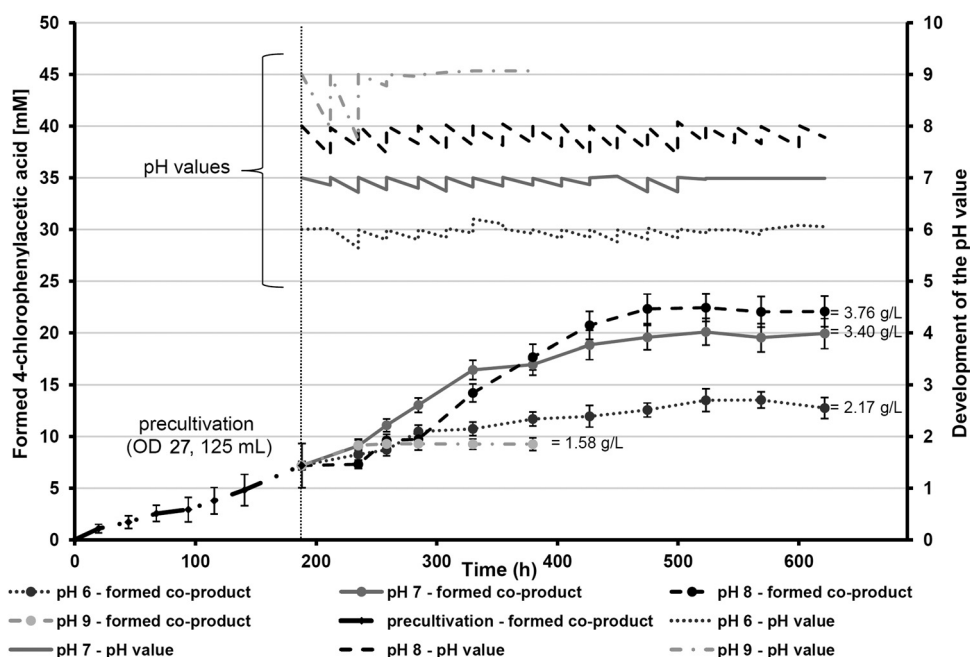
Fig. 2. Influence of the pH on the co-metabolic turnover of 4-chlorostyrene.
Biomass with an OD_{600} of 27 was pre-cultivated for 188 h in 125 mL mineral medium (pH 7) together with styrene and 4-chlorostyrene as described in the material and methods section. Afterwards, the cells were harvested and distributed to 25 mL of mineral medium with a pH of either 6–8 or 9. The cells were incubated with styrene and 4-chlorostyrene for 434 h as described in the methods section. The development of the pH and the formation of 4-chlorophenylacetic acid are illustrated. Co-product formation was determined via HPLC and means of 3–4 measurements are given under consideration of error bars. Further details about the addition of styrene and 4-chlorostyrene are provided in Fig. S3.

During a third experiment an 8.74:1 ($\mu mol_{styrene}$ day^{-1}: OD_{600}) ratio was considered for cell suspensions with an OD_{600} of 5, 10, 15 and 20 to ensure the comparable molar amount of inducer per cell. The results are shown in Fig. 3B (additional information in Fig. S5B). For the culture with an OD_{600} of 20, the formation of 5.15 g L^{-1} (=30.2 mM) co-product was observed under these conditions within 267 h. The adjustment of the amount of styrene to the OD_{600} of 20 (175 $\mu mol_{styrene}$ day^{-1}) yielded an 1.4-fold faster transformation compared to the previous study mentioned above (with 87.4 $\mu mol_{styrene}$ day^{-1}; Fig. 3A). Adjusting the amount of styrene to the OD_{600} was also favorable for a cell density (OD_{600}) of 5. This culture yielded 5.79 g L^{-1} (=33.9 mM) co-product within 380 h after providing 43.7 $\mu mol_{styrene}$ day^{-1}. Interestingly, this

represents as very similar transformation rate compared to the above-mentioned cultivation with 87.4 $\mu mol_{styrene}$ day^{-1} (Fig. 3A), but only the half of the styrene was needed. Remarkably, a ratio of 8.74:1 ($\mu mol_{styrene}$ day^{-1}: OD_{600}) was not preferable for a culture with an OD_{600} of 15 and a significantly reduced transformation rate with only 1.97 g L^{-1} (=11.5 mM) 4-chlorophenylacetic acid within 262 h was determined.

In summary and with respect to the highest absolute co-product concentrations, the most favorable transformation rates were observed during the experiments mentioned above for cell suspensions with an OD_{600} of 10 and 15 using about 87.4 $\mu mol_{styrene}$ day^{-1}. Thus, these combinations were used for subsequent experiments.
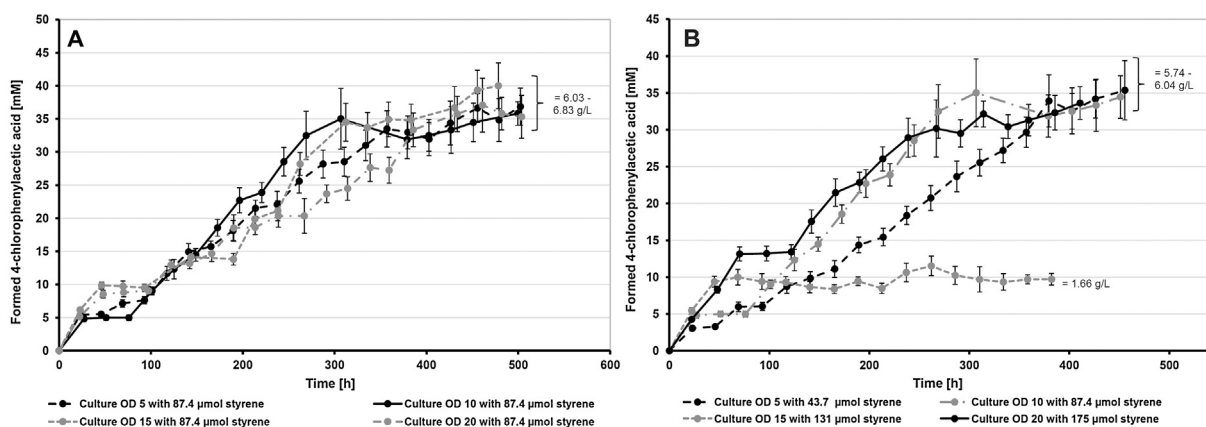

Fig. 3. Influence of the cell density under consideration of the daily amount of styrene on the formation of 4-chlorophenylacetic acid.
Styrene-induced cells were suspended in mineral medium (pH 8) adjusting an OD_{600} of 5, 10, 15, or 20. 25 mL of each suspension were incubated for 382–504 h as described in the material and methods section. A: Styrene was added in daily portions of 87.4 μmol. B: The cultures were fed daily with the following amount of styrene: OD_{600} of 5 with 43.7 μmol; OD_{600} of 10 with 87.4 μmol; OD_{600} of 15 with 131 μmol; OD_{600} of 20 with 175 μmol. 4-Chlorostyrene was commonly added in all cases (A, B) in portions of 157 μmol. Co-product formation was determined via HPLC and the averages of 3–4 measurements under consideration of the error bars are illustrated. Further details about the addition of styrene and 4-chlorostyrene are provided in Fig. S5.

3.5. Influence of 4-chlorostyrene and 4-chlorophenylacetic acid on the co-metabolization

During the investigations mentioned above a reduced or even stopped co-transformation was observed after the co-product concentration reached about 4–6 g L^{-1}. This indicates an inhibition by the co-product or even by accumulated non-transformed co-substrate. This aspect was investigated with a culture (OD$_{600}$ of 15, 87.4 μmol$_{styrene}$ day^{-1}) containing either the co-substrate 4-chlorostyrene with a starting concentration of 88 mM (2200 μmol) or the co-product 4-chlorophenylacetic acid with an initial concentration of 30 mM (5.1 g L^{-1}) (Fig. 4A, S6A). A culture without an additional concentration of co-substrate or co-product served as reference.

4-Chlorophenylacetic acid was identified as an important inhibitor of the co-metabolic process and only about 2.2–2.6 g L^{-1} (=12.9–15.4 mM) new co-product were formed after initial addition of 5.1 g L^{-1} of the acid. In contrast, it was clearly demonstrated that a larger initial amount of 4-chlorostyrene together with a frequent addition of 157 μmol co-substrate supported the transformation under the conditions mentioned and 6.79 g L^{-1} (=39.8 mM) co-product were formed after 262 h. This corresponds to 15% more co-product as achieved about 50 h later in the reference culture.

Nevertheless, 4-chlorostyrene can be a disturbing factor if cell suspensions with lower densities were used. This was revealed with biomass of an OD$_{600}$ of 10 and initial co-substrate concentrations of 53 mM (1340 μmol) and 79 mM (1970 μmol) compared to a reference culture (Figs. 4B, S6B). During 262 h, about 11.5% more co-product were achieved for the culture starting with an initial portion of 53 mM co-substrate compared to the reference culture. Remarkably, only 22.6% of the co-product obtained with initially 53 mM co-substrate were formed after 262 h if an initial amount of 79 mM 4-chlorostyrene was provided. As shown by the previous experiment with higher cell densities (OD$_{600}$ of 15), the increasing initial amounts of 4-chlorostyrene principally improved the transformation rate at an OD$_{600}$ of 10, too. Nevertheless, an initial amount of about 80 mM co-substrate inhibits the transformation in the latter case while cultures with an OD$_{600}$ of 15 tolerates more than 120 mM of the co-substrate (see Figs. 4A and S6A). This indicates that the co-substrate can serve as inhibitor at high concentrations, but the inhibiting effect

seems strongly depend to the optical cell density applied. Based on these data, the inhibiting effect of accumulated 4-chlorostyrene is also most probably responsible for the reduced transformation after 262 h in Fig. 4B. In all cases the transformation was strongly declined or inhibited when the residual 4-chlorostyrene accumulates to about 70–80 mM (see also Fig. S6B). Remarkably, a lower optical density resulted not principally in a lower tolerance of a culture towards critical co-substrate concentrations. Interestingly, another culture with an OD$_{600}$ of 5 also tolerates about 80 mM non-converted 4-chlorostyrene as shown in Fig. 3A (see also Fig. S5A). This similar tolerance of a cell suspension with an OD$_{600}$ of 5 compared to cultures with an OD$_{600}$ of 10 is maybe caused by a higher oxygen supply or – as shown below – by higher amounts of SL and TES6 for each cell at lower cell densities. In contrast, higher cell densities of 15 seem to tolerate higher co-substrate concentrations because the critical level is maybe distributed to a large number of cells. Nevertheless, further experiments are needed to prove these assumptions.

In contrast, the inhibiting effect of 4-chlorophenylacetic acid depends not significantly on the cell density as consecutively shown. For a culture with an OD$_{600}$ of 10 in Fig. 3A, an inhibition was observed after 307 h resulting in finally 5.98 g L^{-1} (=35.1 mM) co-product. The transformation was inhibited although 4-chlorostyrene accumulated only to about 53.9 mM and the critical concentration of about 70–80 mM was not reached. Similar results were determined for the cultures with an OD$_{600}$ of 20 in Fig. 3A and B. Both cultures were inhibited after 385 and 267 h in presence of only 64.0 and 41.9 mM of accumulated 4-chlorostyrene, respectively. But, similar final co-product concentrations of 5.69 and 5.15 g L^{-1} (33.4 mM and 30.2 mM) were determined in both cases. Furthermore, it was shown above that cultures with an OD$_{600}$ of 15 tolerate higher 4-chlorostyrene concentrations of more than 120 mM (Figs. 4A, S6A). Nevertheless, the reference culture in Fig. 4A was inhibited after 312 h at a residual 4-chlorostyrene concentration of about 67.6 mM and a co-product concentration of 5.89 g L^{-1} (=34.5 mM). It is obvious that the 4-chlorostyrene concentrations differ significantly between the cultures and are comparably low in most cases. Thus, a 4-chlorostyrene-mediated inhibition is probably not the reason for the reduced formation rates in these cases. But, very similar co-product concentrations of about 5–6 g L^{-1} (29–35 mM) were observed in these cultures which strongly indicates co-product inhibition.
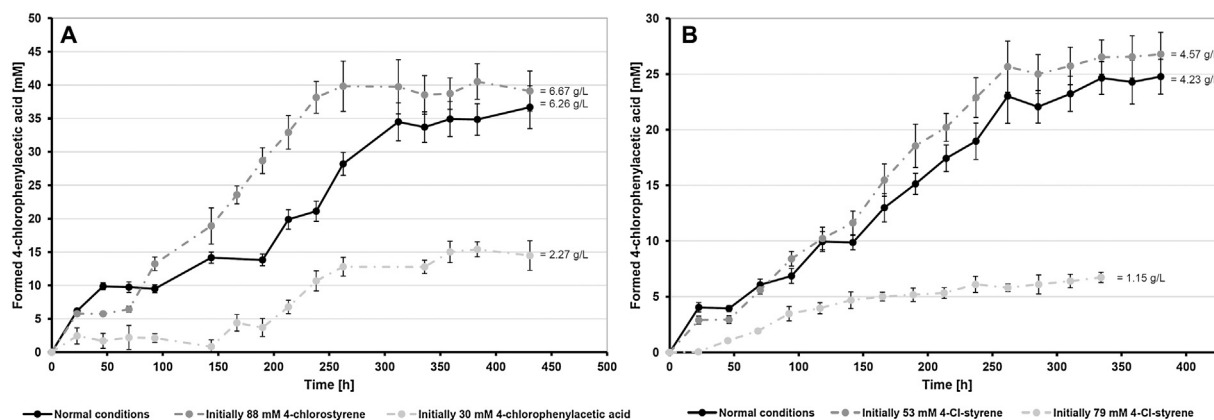

Fig. 4. Inhibiting effects of 4-chlorostyrene and 4-chlorophenylacetic acid on the co-metabolism.
Styrene-adapted cells were suspended in fresh mineral medium (pH 8) adjusting an OD$_{600}$ of 14–15 (A) and 10 (B). The cultivation parameters are mentioned in the material and methods section. A: Cultures of 25 mL were incubated for 431 h starting with an initial amount of 2200 μmol (88 mM) 4-chlorostyrene or 744 μmol (30 mM) 4-chlorophenylacetic acid. A culture without additional amounts of co-substrate or co-product served as reference. Styrene and further portions of 4-chlorostyrene were supplied as described in the methods section and as illustrated in Fig. S6A. B: 25 mL-cultures were incubated for 334–380 h with initial 4-chlorostyrene portions of 1340 μmol (53 mM) and 1970 μmol (79 mM). A culture without an initial amount of additional co-substrate served as reference. Styrene and 4-chlorostyrene were added as mentioned in the methods section and as illustrated by Fig. S6B. Co-product quantification for A and B was performed as described in material and methods. The data points represent the mean of 3–4 independent measurements and standard deviations are given. Note: Only the formation of new 4-chlorophenylacetic acid is illustrated in A while the initial portion of 30 mM co-product is not shown.

It was shown that the co-metabolic process depends strongly on the co-substrate and the corresponding product. However, the inhibiting effect described above is reversible and can be overcome by re-suspending the cells in fresh medium (data not shown).

3.6. Influence of salt and trace elements on the co-metabolic process

The influence of different amounts of salt solution (SL) and trace element solution 6 (TES6) was investigated with a cell suspension of an OD_{600} of 10 as described in the material and methods section. About 4 g L^{-1} (=23.4 mM) co-product were formed after 262 h applying cells in medium containing 1x SL and 1x TES6 or 1x SL and 2x TES6 (Figs. 5 A; S7A). Afterwards, only a slight increase to finally 4.37 g L^{-1} and 4.55 g L^{-1} (25.6 mM and 26.7 mM) co-product was determined for both cultures after in total 500 h. Remarkably, the application of 2x SL and 1x TES6 as well as 2x SL and 2x TES6 decreased the transformation rate by 22% and 14% during the first 262 h. Nevertheless, higher co-product amounts were achieved for the culture with 2x SL and 2x TES6 reaching finally 5.73 g L^{-1} (=33.6 mM) co-product after 500 h. As also shown in Fig. 5A, higher co-product yields were reached by the addition of fresh 1x SL and 1x TES6 after 525 h to the culture which starts with 1x SL and 1x TES6. While the co-product concentration was almost constant for >150 h with about 4.19-4.56 g L^{-1} (=24.6–26.7 mM), the addition of fresh SL and TES6 improved the co-product yield by 15% to 5.26 g L^{-1} (=30.8 mM) within 48 h.

These results strongly indicate – under the conditions mentioned – that an increasing initial amount of SL principally reduces the co-metabolic transformation rate while initially 2x TES6 has no significant effect on the conversion of 4-chlorostyrene. Remarkably, higher SL concentrations, especially together with an equally elevated TES6 concentration in a relative ratio of 1:1, allowed finally the formation of larger amounts of co-product over time. These results were also proven by a second experiment which was performed with a cell suspension of an OD_{600} of 15. The cultures were incubated in medium containing *a)* 1x SL and 1x TES6 or *b)* only 1x TES6 (Figs. 5B, S7B). It was shown that a co-product concentration of about 5.7–5.9 g L^{-1} (=33.4–34.6 mM) was obtained in both cases, but the transformation was successfully finished about 50 h earlier when no salt components were present. Afterwards, a slow co-product formation was still measureable in the culture, which was incubated in presence of 1x SL and 1x TES6,

while no new co-product was formed in the culture with only 1x TES6. About 12% more co-product were determined after 431 h for the culture containing SL.

Remarkably, SL seems to be important to reduce inhibiting effects of large amounts of 4-chlorostyrene. This was also proven by another experiment. Two cultures (OD_{600} of 15) containing 1x TES6 and 0x or 1x SL were incubated with an initial amount of 78.6–88.1 mM 4-chlorostyrene (1970–2200 µmol) under comparable conditions as described for the OD_{600}-15-cultures in the methods section. Only about 2.32 g L^{-1} (=13.6 mM) co-product were formed after 221 h in the culture initially containing only 1x TES6 while 5.62 g L^{-1} (=32.9 mM) co-product were obtained after 213 h in the culture containing 1x SL and 1x TES6 (detailed data not shown).

In summary, the results indicate that SL supported the co-transformation if larger concentrations of co-substrates accumulates or are present. But, too large amounts of SL principally reduce the transformation rate. Thus, no or only a low salt-content seems to be preferable during a first stage of the transformation, in which the co-substrate concentrations are low. But, the addition of SL together with TES6 is beneficial in the later process when co-substrate accumulates. Further investigations with respect to SL and TES6 were subsequently performed in an up-scaled experiment using the fermenter.

3.7. Co-metabolic transformation of 4-chlorostyrene in the fermenter

The results from the preliminary experiments shown above were investigated in the fermenter to reveal more details about these findings. During these experiments, 1.5 L of a cell suspension with an OD_{600} about 10 were cultivated as described in the material and methods section. Compared to the preliminary experiments, lower than 60% of the daily amounts of co-substrate and styrene were fed during the cultivation in the fermenter. This was necessary to ensure a complete aerobe transformation in the bioreactor and avoid a complete consumption of the solved oxygen. However, the ratio of styrene and 4-chlorostyrene was the same as in the experiments above.

During the first experiment mineral medium was used (see Fig. 6, Run 1; supplemental information in Fig. S8). During the initial 68 h, about 1.42 g L^{-1} (=8.32 mM) 4-chlorophenylacetic acid were formed. Finally, 3.22 g L^{-1} (=18.9 mM) co-product were
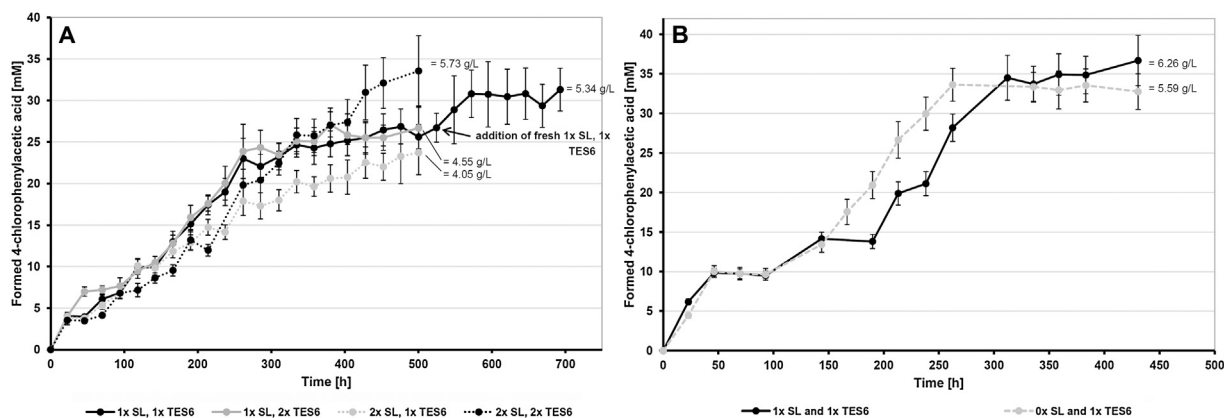

Fig. 5. Influence of salt and trace elements on the transformation of 4-chlorostyrene.
Styrene-adapted biomass was harvested and suspended in mineral medium (pH 8, A, B) or 1x phosphate buffer with 1x TES6 (pH 8, B) to obtain an OD_{600} of 10 (A) or 14–15 (B). A: 25-mL cultures were partially supplemented with additional concentrations of SL and TES6. The following SL and TES6 combinations were tested: 1x and 1x (=mineral medium); 1x and 2x; 2x and 1x; 2x and 2x. The cultures were incubated for 500 h as described in the material and methods section. The culture with 1x SL and 1x TES6 was supplemented after 525 h with fresh SL and TES6 (1x of both referred to the culture volume) and was cultivated for further 168 h. B: 25-mL cultures in 1x TES6-containing phosphate buffer (1x) or in mineral medium were incubated for 431 h as described in the material and methods section. The formation of co-product (A, B) was determined as described in material and methods. The means of 3–4 independent measurements are illustrated and standard deviations are given. Further details about the addition of styrene and 4-chlorostyrene are also provided in Fig. S7.
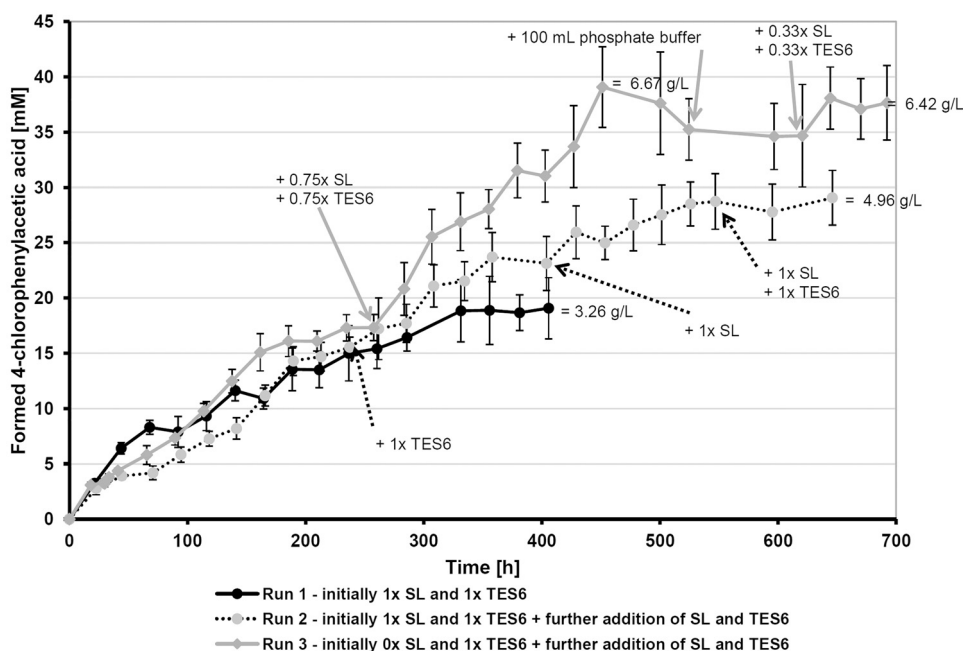
Fig. 6. Fermenter-based studies on the co-metabolization of 4-chlorostyrene by strain ST.
Biomass cultivated and adapted in the fermenter was harvested and suspended in 1.5 L of fresh medium to obtain a cell density (OD_{600}) of 10. Therefore, either mineral medium (pH 8) in the case of Run 1 and 2 or 1x phosphate buffer with 1x TES6 (pH 8) during Run 3 was used. The biomass was cultivated and fed with styrene and 4-chlorostyrene for 406–692 h as described in the material and methods section. During Run 2 and 3 additional amounts of SL and TES6 were added as illustrated. 4-Chlorophenylacetic acid was quantified as described in material and methods. The averages of 3–4 measurements are illustrated and standard deviations are given. Further information about all three fermenter cultivations are provided in Figs. S8–S10. Note: The reduced co-product concentration in Run 3 after 500 h is caused by a dilution of the medium by a factor of 1.1 to ensure the minimum water level in the reactor.

determined after 331 h. Afterwards, no further co-product formation was observed while non-substituted styrene was completely metabolized over the complete time. This styrene metabolization was clearly indicated by the oxygen consumption (see Fig. S8) and proved by HPLC. The co-transformation of 4-chlorostyrene during styrene metabolization was additionally investigated during this run and is illustrates in Fig. 7. Remarkably, it was shown that 4-chlorostyrene is particularly transformed into the corresponding acid after styrene was completely metabolized. Styrene metabolization is clearly linked to oxygen consumption while 4-chlorostyrene led not to a significant change in the oxygen level. The maximum turnover of the substituted co-substrate was observed about 3 h after the complete transformation of styrene and was down-regulated after a further period of 4 h. This co-transformation remains more and more incomplete over time indicating that the co-transformation is increasingly inhibited by 4-chlorphenylacetic acid or accumulated 4-chlorostyrene.

The initial cultivation in the fermenter was repeated (Run 2), but different amounts of SL and TES6 were additionally supplemented during the transformation (see Fig. 6, Run 2; details are shown in Fig. S9). During the first 237 h, the co-metabolic transformation of 4-chlorostyrene was similar to Run 1 and resulted in 2.55–2.65 g L^{-1} (=14.9–15.5 mM) co-product. Afterwards, fresh TES6 (1x referred to the culture volume) was added. The addition of fresh TES6 resulted in 25% higher co-product concentrations after 358 h. Afterwards, no transformation was observed again and fresh SL (1x) was added after 404 h. This further portion improved the co-product yield by 21% and resulted in finally 4.90 g L^{-1} (=28.7 mM) co-product after 547 h. Further portions of SL and TES6 caused no supporting effect anymore.

The results of Run 2 showed again that SL and TES6 are able to improve the transformation in a certain range. Remarkably, it was shown in preliminary studies (Fig. 5A) that there was no significant effect between initially 2x TES6 and 1x SL compared to initially 1x

TES6 and 1x SL. Nevertheless, addition of 1x fresh TES6 during Run 2 (Fig. 6) to a culture initially containing 1x SL and 1x TES6 improved the co-product concentration by about 0.7 g L^{-1} (=4.1 mM) compared to Run 1 (Fig. 6; only initially 1x SL and 1x TES6). These results strongly indicate that especially fresh TES6 is able to improve the transformation in certain range and that the additional amount of trace elements should not be supplied directly at the beginning of the transformation.

Based on the preliminary experiments it was supposed that a culture medium without SL could be beneficial during the first days of the cultivation, because SL is not necessary as long as critical co-substrate concentrations are not reached. Therefore, a third run in the fermenter was performed starting with 0x SL and 1x TES6 (see Fig. 6, Run 3; details are shown in Fig. S10). After 186 h, about 2.75 g L^{-1} (=16.1 mM) co-product were determined and the transformation stopped as also observed during Run 1 and 2. Remarkably, the oxygen consumption after styrene addition was significantly reduced after 186 h indicating also a limitation in the styrene degradation (see Fig. S10). This was not observed in this extent in Run 1 and 2 (see Figs. S8 and S9) and strongly indicated that SL is necessary during this stage of transformation. After about 260 h, a fresh portion of SL and TES6 was added (0.75x of both referred to the culture volume) which resulted in a significant increase in the co-product concentration to finally 6.67 g L^{-1} (=39.1 mM) after 451 h. Afterwards, the co-transformation stopped again. The addition of further amounts of fresh SL and TES6 (0.33x of both) improved the co-product yield only slightly by about 9%.

Finally, Run 3 resulted in 107% more co-product compared to Run 1 and 36% more halogenated acid as in Run 2. Starting without SL is suitable, but SL is important for a subsequent transformation after a certain time in all cases (see Run 3 in Fig. 6). Remarkably, the last run showed that only 0.75x fresh SL together with an equal amount of fresh TES6, which were added at the beginning of the inhibition, are sufficient enough to ensure an efficient synthesis of
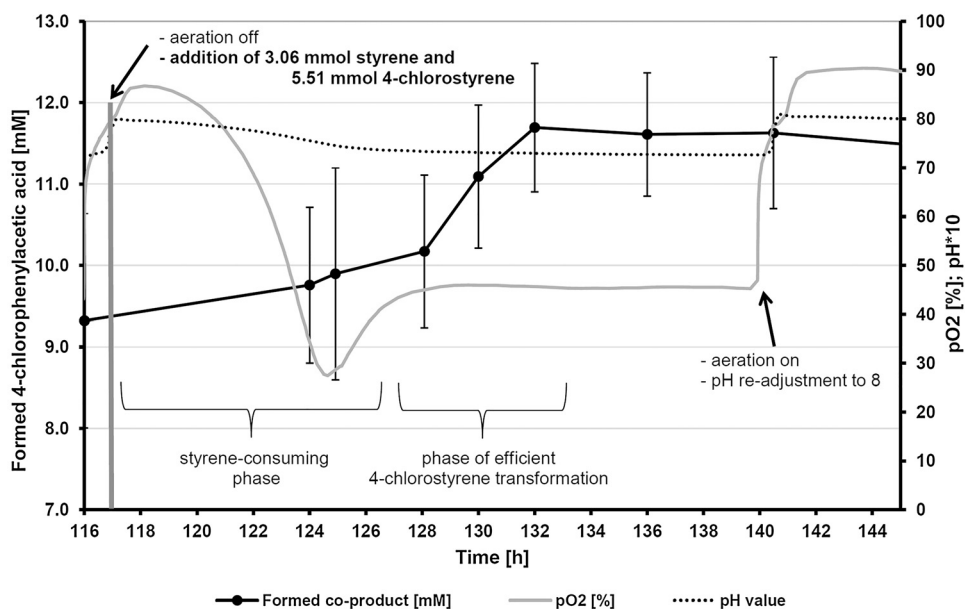
Fig. 7. Detailed insights into the co-metabolization of 4-chlorostyrene in presence of styrene.
The details of this cultivation are given in the description of Run 1 in the material and methods section, in Figs. 6 and S8. But, only a short time period of this cultivation is illustrated. The co-metabolization of one portion of 5510 μmol 4-chlorostyrene in presence of 3060 μmol styrene is shown. The change of the pH and the oxygen depletion as indicators for styrene consumption are given. Co-product formation was determined via HPLC as described in the methods section and is illustrated as mean of 3–4 measurements. Error bars are additionally given.

4-chlorophenylacetic acid (see Run 3 in Fig. 6). This appears on the first view unusual, because it was demonstrated and discussed that a 2-fold amount of SL supports the transformation at a later point of time. But, this can be probably explained by a faster transformation in presence of only 0–0.75% SL in contrast to 1–2% SL (see Figs. 5A and B, 6). Hence, somewhat lower amounts of residual 4-chlorostyrene accumulate over time (see Run 3 in Figs. 6, S10). Because of the lower concentration of accumulated co-substrate, the inhibiting effect by 4-chlorostyrene is delayed and there is no need for further amounts of SL to overcome a 4-chlorostyrene-caused inhibition over a distinct runtime. Because no further SL is added, the transformation rate remains relatively fast. Finally, the inhibition observed is mediated more probably by the co-product as by the co-substrate and further amounts of fresh SL and TES6 have no significant effect anymore (see Run 3 in Fig. 6).

Based on the results obtained some suggestions can also be made for the type of co-metabolic inhibition. Because styrene is commonly degraded by a similar rate over the complete time (estimated by oxygen consumption, see Figs. S8–S10) despite of an inhibited 4-chlorostyrene transformation, a specific inhibition of the co-substrate uptake into the cell or of at least one enzyme of the side-chain oxygenation after the metabolization of styrene can explain the down-regulated co-metabolic process. For the latter putative reason an inhibition of the SMO and therewith of the first step of this synthesis is most probably because no accumulation of halogenated epoxides or aldehydes was detected in the medium over time (detailed data not shown). Furthermore, this would indicate that styrene is able to bypass this enzymatic inhibition during its own transformation until it is depleted. Based on the results obtained it has been shown that styrene degradation is not significantly influenced by the concentrations of 4-chlorostyrene, 4-chlorophenylacetic acid, and TES6 in the ranges investigated, so far. Only 2x SL slightly reduces the transformation rate of styrene (as also shown for 4-chlorostyrene turnover; Fig. 5A) by about 20% (calculated based on Fig. S9). Nevertheless, other inhibiting effects, e.g. on the biosynthesis, cannot ruled out and further experiments are needed for clarification.

4. Conclusion

The co-metabolic transformation of 4-chlorostyrene was investigated in strain ST and important parameters were identified which affect the co-metabolism. It has been shown that these parameters interact in a very complex way and are able to improve or inhibit the reaction. Based on all results, a protocol was established which allows an enhanced transformation of 4-chlorostyrene. With this protocol about 6.7 g L^{-1} (about 39 mM) co-product were determined after 451 h in the fermenter. Compared to previous studies, the co-product concentration was improved herein by a factor of 1.4 while the reaction time was decreased significantly by a factor of 18.5 (compared to [12]). Furthermore, this study identified parameters which could enable a further optimization, e.g. the potential of SL and trace elements. Studying the single components of SL and TES6 with respect to their effect on supporting or inhibiting the reaction should allow a further improvement of the co-metabolism by the right elements and avoid a reduced transformation rate by disturbing salt ingredients. Finally, an efficient process to gain substituted phenylacetic acids from corresponding styrenes could be feasible without the need of genetic modifications.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.btre.2018.e00248.

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