Thomas Eixelsberger

Graz University of Technology

Scale-up of unit operations in whole-cell biocatalytic production of *S*-1-(2-chlorophenyl)-ethanol: *E. coli* fermentation, bioreduction and downstream processing

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Univ.-Prof. Dipl.-Ing. Dr. techn. Bernd Nidetzky

Dipl.-Ing. Dr. techn. Regina Kratzer

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Acknowledgement

I would like to express my appreciation to my supervisors, Univ.-Prof. Dipl.-Ing. Dr. Bernd Nidetzky and Dipl.-Ing. Dr. Regina Kratzer, for their support throughout this whole work. They always had the time and patience to answer my questions and I could count on their help any time I needed it.

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Kurzfassung

Die Bioreduktion von *o*-Chloracetophenon durch einen *Escherichia coli*-Ganzzell-Biokatalysator ist ein effizienter Weg, um *S*-1-(2-chlorphenyl)-ethanol in hoher Stereoselektivität (>99 %) und Ausbeute herzustellen. Dieses Produkt wird als chirale Schlüsselverbindung in der Synthese von Polo-like kinase 1-Inhibitoren, einer neuen Klasse von chemotherapeutischen Medikamenten, verwendet. Die Produktion des Alkohols im Multi-Gramm-Maßstab erfordert das Scale-up von drei Einheitsoperationen: Produktion des Biokatalysators (Fermentation), Biotransformation und Downstream Processing (Produktisolierung). Die Kultivierung der Zellen in einem 7-L-Bioreaktor führte zu einer 10-fachen Erhöhung der Zelldichte im Vergleich zur Kultivierung im Schüttelkolben, unter Erhalt der intrazellulären Enzymaktivitäten. Beim Scale-up der Biotransformation in einem Rührkesselreaktor konnten eine Vergrößerung des Reaktionsvolumens um das 500-fache, eine Erhöhung der Produktkonzentration um das 3-fache und vollständige Umsetzung des Substrats erreicht werden. Scale-up und Optimierung der Produktisolierung, basierend auf der Extraktion mit Hexan, ergaben eine Produktausbeute von 86 %. Der neu entwickelte Prozess senkte die Produktionskosten um 80 % und ermöglicht die Produktion von *S*-1-(2-chlorphenyl)-ethanol innerhalb vorher definierter wirtschaftlicher Grenzen. Ein einfacher und effizienter Weg zur Synthese von *S*-1-(2-chlorphenyl)-ethanol in einer isolierbaren Ausbeute von 20 g pro Reaktionsansatz wurde gezeigt.

Schlüsselwörter: Ganzzellbioreduktion; Maßstabsvergrößerung in der Biokatalyse; *E. coli*-Fermentation; asymmetrische Reduktion von *o*-Chloracetophenon; *S*-1-(2-chlorophenyl)-ethanol; *Candida tenuis* Xylosereduktase

Abstract

Bioreduction of *o*-chloroacetophenone by an *Escherichia coli* whole-cell biocatalyst is an efficient way to synthesize *S*-1-(2-chlorophenyl)-ethanol in high stereoselectivity (>99.9 %) and yield. The product is used as chiral key intermediate in the synthesis of polo-like kinase 1 inhibitors, a new class of chemotherapeutic drugs. Production of the alcohol in multi-gram scale requires scale-up of three unit operations: biocatalyst production (fermentation), bio-transformation and downstream processing (product isolation). Cell cultivation in a 7-L-bioreactor led to >10-fold increases in cell densities as compared to shaken flask cultivation while maintaining intracellular enzyme activities. A 3-fold higher product concentration, a 500-fold larger reaction volume and full conversion of the substrate were achieved by bio-reduction scale-up from 1 mL to 500 mL in a stirred tank reactor with pH and temperature control. Scale-up and optimization of downstream processing based on hexane extraction yielded 86 % of isolated product. The developed process lowered production costs by 80 % and enables *S*-1-(2-chlorophenyl)-ethanol production within previously defined economic boundaries. A simple and efficient way to synthesize *S*-1-(2-chlorophenyl)-ethanol in an isolated amount of 20 g product per reaction batch was demonstrated.

Keywords: Whole-cell bioreduction; biocatalysis scale-up; *E. coli* fermentation; asymmetric *o*-chloroacetophenone reduction; *S*-1-(2-chlorophenyl)-ethanol; *Candida tenuis* xylose reductase

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Scale-up of unit operations in whole-cell biocatalytic production of *S*-1-(2-chlorophenyl)-ethanol: *E. coli* fermentation, bioreduction and downstream processing

Thomas Eixelsberger[1], Regina Kratzer[1] and Bernd Nidetzky[1,§]

[1] Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Petersgasse 12/1, 8010 Graz, Austria

[§] Corresponding author

Phone: +43 316 873 8400; bernd.nidetzky@tugraz.at

Running title: Scale-up in whole-cell biocatalysis

Main Part

Results and discussion

Whole-cell biocatalysis is an emerging field in the production of optically pure pharmaceutical intermediates (Marques et al., 2010; Pollard et al., 2006). Process flow from biocatalyst production to product isolation can be divided into three unit operations: cell cultivation (fermentation), biotransformation and downstream processing. Approaches for scale-up of cell cultivation include constancy of k_La value, volumetric power input or impeller tip speed (Islam et al., 2008; Junker et al., 2004). The number of reports related to scale-up of the whole cell biotransformation is limited so far (Marques et al., 2010). Cull et al. identified the maintenance of the interfacial area (i.e. mixing efficiency) as a crucial point in scale-up of biotransformations in aqueous/organic two-phase-systems (Cull et al., 2002). Scale-up of product extraction turned out as most important step during product isolation (downstream processing). Mixing time and width-to-height ratio of the vessel are main factors contributing to extraction efficiency (Hagen et al., 1997).

The asymmetric reduction of *o*-chloroacetophenone in analytical scale (1 mL reaction volume) by an *E. coli* whole-cell catalyst co-expressing *Candida tenuis* xylose reductase (*Ct*XR) and a yeast formate dehydrogenase (*Cb*FDH) has recently been reported (Kratzer et al., 2011). The product *S*-1-(2-chlorophenyl)-ethanol is a required key intermediate in the synthesis of attractive cancer therapy candidates, namely polo-like kinase 1 inhibitors (Santamaria et al., 2007). A shortage in chemo- and biocatalysts performing the reduction of *o*-chloroacetophenone in high stereoselectivities and yields renders the scale-up of *S*-1-(2-chlorophenyl)-ethanol production by the above mentioned system a subsequent step in process design: We therefore performed an economic estimation for the production of *S*-1-

(2-chlorophenyl)-ethanol based on previously published data (figure 1). Windows of operation are graphical representations used to visualize biological, reaction and economic constraints governing a process (Blayer et al., 2008; Zhou et al., 2000). High cell concentrations have previously shown to impede product isolation. A maximal catalyst loading of 30 g_{CDW}/L was therefore set as reaction constraint to keep product loss during downstream processing below 15 % (Kratzer et al., 2011). High toxicities of substrate and product limit the applicable substrate concentration. The upper product concentration limit was taken from Gröger et al. (2006), who achieved full conversion of 1 M *o*-chloroacetophenone with 19 g_{CDW}/L of catalyst. The resulting productivity of 8.2 $g_{product}/g_{CDW}$ is, to our knowledge, the highest value found in literature (Gröger et al., 2006) and was therefore used to calculate the maximal cell activity boundary. Product concentration dictates downstream processing costs to a large extent. The economic product concentration limit was calculated based on typical local values for material and working costs (see supplementary material). Using the working conditions from Kratzer et al. (2011), *S*-1-(2-chlorophenyl)-ethanol production cannot be run economically. The aim of this work was to shift the process into the economic boundaries.

Biomass cost is a further main factor influencing economy of the process. Costs per gram cell mass are significantly lowered with increasing culture volume and cell density (see supplementary material). We therefore developed a fed-batch fermentation process aiming at optical densities above 30, which represents a 10-fold scale-up compared to the present method by Kratzer et al. (2011). The used *E. coli* strain was a modified Rosetta 2 (DE3) strain containing two additional plasmids (Mädje et al., 2011).

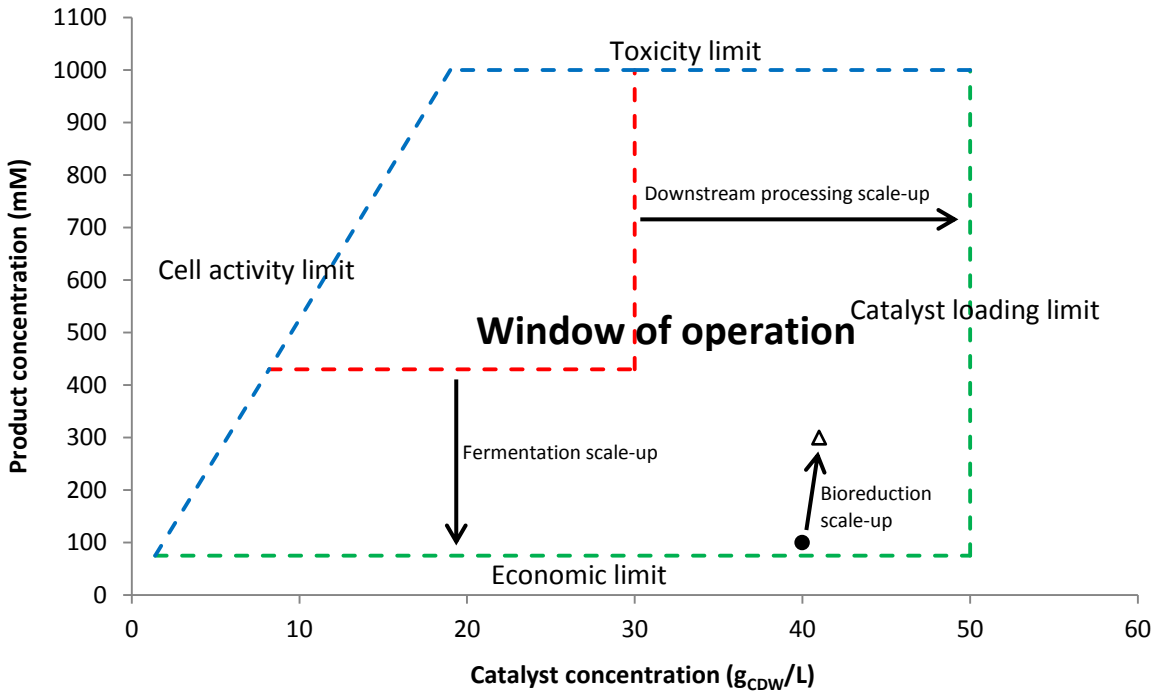

Figure 1: *S*-1-(2-chlorophenyl)-ethanol synthesis process limits as a function of cell and product concentration. The areas within the dashed lines indicate economic operation. Economic boundaries are indicated by red lines in analytical scale (1 mL) (Kratzer et al., 2011) and green lines after scale-up (500 mL). Toxicity and cell activity limits are biological limits displayed by blue lines (Gröger et al., 2006). The economic limit is the lowest product concentration allowing economic *S*-1-(2-chlorophenyl)-ethanol synthesis, based on product price and costs for biomass, chemicals and working time (see supplementary material). The catalyst loading limit defines the maximal cell concentration at which product loss during downstream processing is below 15 %. The process described by Kratzer et al. lies outside economic boundaries (●). Scale-up of the process (Δ) and expanded economic and catalyst loading limits shift the process into the economic area.

Efficient cell growth and co-expression of two genes (*Ct*XR and *Cb*FDH) needs careful optimization of the growth medium with respect to carbon/nitrogen (C/N) ratio. Media development was done in batch experiments with the C/N ratio varying from 4.8 g/g to 14 g/g at an essentially constant mineral salt composition (see supplementary material). Induction temperature and IPTG concentration for batch fermentations were taken from Mädje et al. (2011) (see supplementary material). Specific growth rates, enzyme activities and glucose yield coefficients are summarized in table I. Highest enzyme activities and yield coefficients were obtained with low C/N ratios (M3 medium, C/N = 4.8 g/g). We therefore used the M3 medium in subsequent fed-batch fermentations. However, low glucose concentrations in M3 led to relatively low biomass formation. To maintain cell activities but increase cell densities we kept a constant C/N ratio while increasing concentrations of all media compounds (e.g. $M3^2$; see supplementary material). Hydrolysis-prone ampicillin was substituted by the more stable carbenicillin in fed-batch experiments. Both antibiotics are cleaved by β-lactamase, but carbenicillin displays a doubled half-life as compared to ampicillin in aqueous solutions (Lapidus et al., 1976). Induction conditions were taken from Mädje et al. (2011), except that a 4-fold higher IPTG concentration (1 mM) was used in response to higher cell densities expected in fed-batch fermentations. The nutrient feed was a 15-fold concentrated solution of the fermentation medium (see supplementary material).

Table I: Physiological parameters of *E. coli* cultivations in a batch bioreactor.

Medium	C/N	μ_{max} (h^{-1})	$\mu_{Ind.}$ (h^{-1})[a]	$OD_{Ind.}$[b]	OD_{End}	$Y_{Glc.}$ (g/g)	XR activity (U/g_{CDW})	FDH activity (U/g_{CDW})
M1	14	0.50	0.023	1.6	23	0.21	730	18.1
M2	7.1	0.45	0.082	2.4	18	0.23	974	49.6
M3	4.8	0.24	0.046	2.0	12	0.24	1280	107

[a] Specific growth rate after induction

[b] Optical density at induction

The fed-batch cultivation started with batch fermentation using 3.5 L of $M3^2$ medium supplemented with 40 g/L glucose (see supplementary material). At an optical density of 25, glucose was depleted and the addition of the nutrient feed was started at a flow rate of 0.81 mL/min (figure 2a). At the same time, temperature was lowered to 18 °C and IPTG as well as carbenicillin were added. Due to a 3-fold decreased growth rate upon addition of IPTG, a final optical density of 37 was measured after 42 hrs. Enzyme activities reached a constant level after 36 hrs (24 hrs after induction) and cells were harvested after 60 hrs. Final activities were determined to 1775 and 124 U/g_{CDW} for *Ct*XR and *Cb*FDH, respectively. Activities were 12 % (XR) and 34 % (FDH) lower as reported for shaken flask cultivation in LB medium (Mädje et al., 2011). Discontinuous addition of the nutrient feed led to similar results, however with a further reduction of 14-16 % in enzyme activity (data not shown). A second fed-batch fermentation was started with 1 L initial volume and M3 medium supplemented with 20 g/L glucose; after the initial glucose was depleted, the nutrient solution was continuously supplied. Cells were induced at an optical density of 55 (figure 2b). Induction in the late exponential growth phase led to a higher final cell density that was however compromised by 50 % reduced enzyme activities. High internal enzyme activities and overall enzyme yields in terms of units per liter were obtained with expression induction at a relatively early stage of growth (OD ≈ 25), although limited in final cell densities. Fermentation by-products acetate and formate never exceeded 3 g/L in fed-batch fermentations and were below 1.5 g/L in experiments with early induction. Biomass production costs (per gram) decrease by 90 % at a final OD of 37 (see supplementary material) lowering the economic limit expressed as minimal product concentration from 430 to 75 mM.

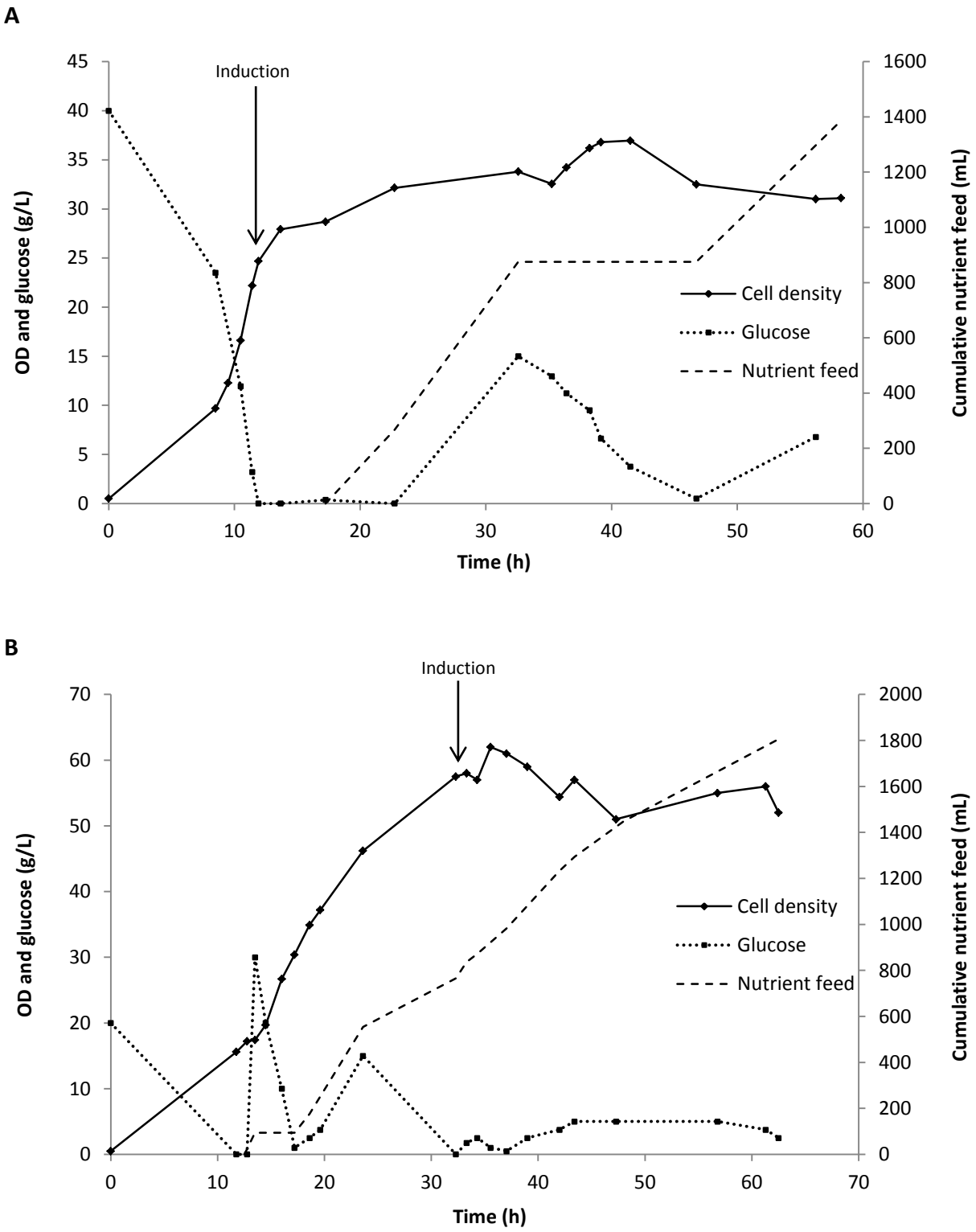

Figure 2: Growth profile, glucose concentration and nutrient feed for fed-batch fermentations. **A:** Early induction with high enzyme activities (XR = 1775 U/g_{CDW}, FDH = 124 U/g_{CDW}) but relatively low final cell density. **B:** Late induction with high final cell density but low XR and FDH activities (XR = 973 U/g_{CDW} and FDH = 62 U/g_{CDW}).

Harvested biomass was freeze-dried to prolong long-term storage capability prior to *o*-chloroacetophenone reduction (Adams, 2007). Kratzer et al. (2011) used a substrate concentration of 100 mM, which is only slightly above the minimal product concentration presuming full conversion (figure 1). *In situ* substrate supply and product removal to circumvent substrate and product toxicities permitted the use of 300 mM substrate (Schmölzer et al., 2011). Enzymatic reduction of *o*-chloroacetophenone by *Ct*XR consumes one mole of H^+ per mole of product formed. Consequently, the pH is shifted from 6.2 to 7.7 by reduction of 300 mM substrate in buffered solution (data not shown). The specific activity of *Ct*XR is pH-dependent and its activity at pH 6.2 is doubled as compared to pH 7.7 (Schmölzer et al., 2011). We therefore controlled the pH by automated addition of 1 M H_3PO_4. pH control was also advantageous for two other reasons. Firstly, reduction progress can be monitored easily by acid consumption; secondly, higher salt concentrations in the aqueous phase lead to improved phase separation during downstream processing (Gu et al., 2004; Legget et al., 1990). Preparative scale of *S*-1-(2-chlorophenyl)-ethanol production required the replacement of the end-over-end rotator used by Kratzer et al. (2011) by a stirred tank reactor. For *in situ* substrate supply and product removal, we used a two-phase system of 20 % (v/v) hexane and 80 % (v/v) buffer (Schmölzer et al., 2011). Efficient mixing of the multi-phase mixture is needed to accelerate mass transfer of the poorly soluble substrate from the hexane phase into the biocatalyst-containing aqueous phase and further into the cell (Kratzer et al., 2011). The used 6-blade disc (“Rushton”) turbine impeller shows good mixing behavior and is used in standard emulsification applications. A stirring speed of 500 rpm turned out as compromise between high shear stress that disrupts labile cells during bioreduction and a sufficient homogeneous emulsion.

Main focus in scale-up of the bioreduction was on full conversion and a productivity ≥1.12 $g_{product}/g_{CDW}$, as obtained in 1 mL scale. Under reaction conditions that prevent catalyst recycling, process costs are especially dependent on productivities. In preparative scale (V = 500 mL), however, a product yield of only 92.6 % with a concomitantly reduced productivity of 0.97 $g_{product}/g_{CDW}$ (-13 %) was obtained. The reduction in yield and productivity is ascribed to the cells' exposure to shear stress caused by stirring. pH control was used in subsequent reduction experiments to compensate for faster catalyst deactivation by maintaining the XR activity at a high level. A constant pH of 6.2 led to a high conversion of 97 % and a productivity of 1.05 $g_{product}/g_{CDW}$, which is 6 % below productivities obtained in 1 mL scale. This and a higher productivity of 8 % as compared to preparative scale without pH control stress the importance of a constant pH during bioreduction (table II). We used the amount of added acid to monitor the reaction progress *in situ*. The experimental error of the acid method is below 6.5 % as compared to HPLC measurements of substrate and product (see supplementary material).

Table II: The effect of scale-up and pH control on productivity and yield of *o*-chloroacetophenone reduction.

Scale (mL)	Conditions	Productivity ($g_{product}/g_{CDW}$)	Yield (%)
1	w/o pH control	1.12	95.0
500	w/o pH control	0.97	92.6
500	with pH control	1.05	97.0

The final product concentration of 291 mM lies above the economic limit of 75 mM; however, the used cell density of 41 g_{CDW}/L is beyond the catalyst loading limit. Optimization of downstream processing was used to keep product loss below the targeted 15 %. Cell fragments and denatured proteins present in the reaction mixture act as emulsifying agents

(Fricker et al., 2010), thereby impeding separation of hexane and water. With the present downstream processing protocol, a cell concentration of 40 g_{CDW}/L leads to significant loss of product through the cell sludge accumulating at the interphase (Kratzer et al., 2011). We therefore used *in situ* extraction with 3 · 400 mL hexane for several reasons: hexane is already present in the reaction mixture, it shows a high extraction efficiency for the product (Schmölzer et al., 2011) and it can be easily removed and recycled by evaporation (bp = 31 °C at 150 mbar). With *in situ* extraction in the stirred tank reactor, a total isolated yield of 86 % was reached, which is ≈20% higher as compared to extractions in small scale (Kratzer et al., 2011) or in a separatory funnel (data not shown). The previously observed product loss in the cell sludge (Kratzer et al., 2011) was also significantly reduced by extraction in the stirred tank reactor (stirring speed: 1000 rpm). Cell concentrations up to 50 g_{CDW}/L did not lead to product losses >15 %, allowing for a new catalyst loading limit in the window of operation. An increase in catalyst loading limit from 30 to 50 g_{CDW}/L (figure 1) shifts the current working conditions into the economic boundaries.

With the method presented, we produced an isolated amount of 20 g product per 500 mL reaction batch, equal to a scale-up of 190-fold as compared to Kratzer et al. (2011). Enzyme yield per liter culture volume was increased 10-fold, i.e. overall enzyme yield obtained from a 5-L-fermentation is sufficient for the production of 60 g *S*-1-(2-chlorophenyl)-ethanol. Summarizing, we illustrated that higher substrate concentrations enabled by an *in situ* extracting phase and the increased upper limit for catalyst loading shifts the biocatalytic process into the economic area of the operational space (figure 1).

Materials and methods

Chemicals and strain

Media components and chemicals used for whole-cell bioreduction were of reagent grade and obtained from standard commercial sources (Sigma Aldrich/Fluka, Gillingham, Dorset, UK; Roth, Karlsruhe, Germany and Merck, Darmstadt, Germany). Hexane for bioreduction and extraction was from Roth (Karlsruhe, Germany) and had a purity of >99 %. Production of the recombinant *E. coli* is described elsewhere (Kratzer et al., 2011; Mädje et al., 2011).

Cell cultivation

Pre-cultures were grown in LB medium as described elsewhere (Mädje et al., 2011). Fermentation studies were performed either in a Biostat CT 6.9-L-fermenter from B. Braun Biotech GmbH or in a 3.6-L-fermenter Labfors III from Infors HT. Bioreactor dimensions can be found in the supplementary material. Both units were assembled and sterilized according to the respective manuals. pH was maintained at 7.0 by the automated addition of 2 M KOH and 1 M H_3PO_4. Dissolved oxygen was kept at 45 % air saturation by an agitation and air flow cascade. Addition of 10 % polypropylene glycol was used for foam control.

Bioreactors were inoculated to an OD of 0.5 with an appropriate amount of preculture. Temperature was 37 °C (batch fermentation experiments) or 25 °C (fed-batch experiments); it was lowered to 18 °C after induction. IPTG and antibiotics (115 mg/L ampicillin or 100 mg/L carbenicillin, 50 mg/L kanamycin, 34 mg/L chloramphenicol) were filter-sterilized prior to addition. An Alitea U 1-M or fermenter-integrated peristaltic pump was used for nutrient feeding with an approximate exponential flow rate to maintain a sufficient glucose concentration during fermentation. The flow rate was calculated according to equa-

tion (1), with the following parameters: $\mu_{glucose}$ = specific growth rate; Δ[Glc] = difference between glucose concentration in feed and reactor set point; $Y_{X/S}$= yield coefficient for biomass from glucose; X_0 and V_0 = values for biomass concentration and medium volume, respectively, at feed start; t = time (Petschacher et al., 2008).

$$F(t) = [\mu_{glucose} / (\Delta[Glc] \cdot Y_{X/S})] \cdot X_0 \cdot V_0 \cdot \exp(\mu_{glucose} \cdot t) \quad (1)$$

Whole-cell bioreduction

Biotransformations were performed in a Labfors 1-L-stirred tank reactor from Infors HT (see supplementary material). The reaction mixture consisted of 400 mL potassium phosphate buffer (100 mM; pH 6.2) and 100 mL hexane. Reaction temperature was 25 °C, stirring speed was 500 rpm and the pH was kept at 6.2 using 2 M KOH and 1 M H_3PO_4. Sodium formate (350 mM), polymyxin B sulphate (36 µM) and NAD^+ (500 µM) were added to the buffer solution. The cells were suspended in the buffer about 1 hr prior to the experiment and reaction was started by the addition of the substrate dissolved in hexane.

Downstream processing

In situ extraction in the stirred tank reactor was done with 3 · 400 mL hexane. After the addition of the solvent, the two phases were mixed at 1000 rpm for 10-20 minutes. Phase separation was done by centrifugation at 5000 rpm for 15-20 minutes in a Sorvall RC-5B centrifuge. The solvent was removed at a rotary evaporator at 40 °C and 100 mbar.

Analytical methods

Analytical methods are described elsewhere (Mädje et al., 2011). HPLC-analysis was performed on a LaChrom HPLC system (Merck-Hitachi) equipped with a Merck L-7490 RI detec-

tor and a thermo-stated column oven. Formate and acetate were analyzed on an Aminex HPX-87H column (Bio Rad Laboratories, Vienna, Austria) using sulphuric acid (5 mM) as eluent at a flow rate of 0.6 mL/min and a temperature of 65°C.

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Figure 1. *S*-1-(2-chlorophenyl)-ethanol synthesis process limits as a function of cell and product concentration. The areas within the dashed lines indicate economic operation. Economic boundaries are indicated by red lines in analytical scale (1 mL) (Kratzer et al., 2011) and green lines after scale-up (500 mL). Toxicity and cell activity limits are biological limits displayed by blue lines (Gröger et al., 2006). The economic limit is the lowest product concentration allowing economic *S*-1-(2-chlorophenyl)-ethanol synthesis, based on product price and costs for biomass, chemicals and working time (see supplementary material). The catalyst loading limit defines the maximal cell concentration at which product loss during downstream processing is below 15 %. The process described by Kratzer et al. lies outside economic boundaries (●). Scale-up of the process (Δ) and expanded economic and catalyst loading limits shift the process into the economic area.

Figure 2. Growth profile, glucose concentration and nutrient feed for fed-batch fermentations. **A:** Early induction with high enzyme activities (XR = 1775 U/g_{CDW}, FDH = 124 U/g_{CDW}) but relatively low final cell density. **B:** Late induction with high final cell density but low XR and FDH activities (XR = 973 U/g_{CDW} and FDH = 62 U/g_{CDW}).

Supplementary material

Window of operation analysis

The economic limit (minimal economic product concentration) was calculated using the energy consumption of the respective devices for bioreduction and downstream processing (stirred tank reactor, rotary evaporator, vacuum pump and centrifuge), working time and costs for chemicals and biomass (water costs are neglected). Calculation is based on a batch reaction with a working volume of one liter.

Costs are divided in structure costs (fixed costs) and product costs (variable costs). Structure costs are independent from product concentration and listed in the table below.

Table S1: Structure costs in *S*-1-(2-chlorophenyl)-ethanol production

	Unit	Unit costs (€)	Amount	Total (€)
Stirred tank reactor	kWh	0.15	29.0	4.35
Hexane	L	30.0	2.6	78.0
Vacuum pump	kWh	0.15	0.32	0.05
Water bath	kWh	0.15	2.64	0.40
Centrifuge	kWh	0.15	4.62	0.69
Working time	h	21.6	8	172.8
Total fixed costs	**-**	**-**	**-**	**256.29**

Product costs are amount-dependent and vary with product concentration. The costs for a product concentration of 300 mM are shown below.

Table S2: Product costs in *S*-1-(2-chlorophenyl)-ethanol production (for information on k_b see below)

	Unit	Unit costs (€)	Amount	Total costs (€)
Biomass	g_{CDW}	k_b	40	$40 \cdot k_b$
o-chloroacetophenone	g	0.9	46.4	41.80
Sodium formate	g	0.02	23.8	0.48
Polymyxin B sulphate	g	24.3	0.05	1.22
NAD^+	g	12.5	0.33	4.15
Total variable costs	**-**	**-**	**-**	**$40 \cdot k_b + 47.65$**
Product concentration		**0.3 M**		
Variable costs		**$(130 \cdot k_b + 159)$ €/M**		

This leads to the overall cost function $\mathbf{K(c) = 256.29\ € + (130 \cdot k_b + 159\ €) \cdot c_{Substrate}}$ (k_b in €, $c_{Substrate}$ in mole/L). Unit costs for biomass (k_b) largely depend on cell cultivation scale. With shaken flask cultivation (OD = 3) (Kratzer et al., 2011), k_b was calculated to 25 €/g_{CDW}. This value decreases to one-tenth (2.5 €/g_{CDW}) after scale-up of cell cultivation (OD = 37).

The price of *S*-1-(2-chlorophenyl)-ethanol was found to be about 4000 €/mole (July 2011). Therefore, with one liter reaction volume, the economic limit before and after fermentation scale-up is **430** and **75 mM,** respectively, as shown in the break even analysis below.

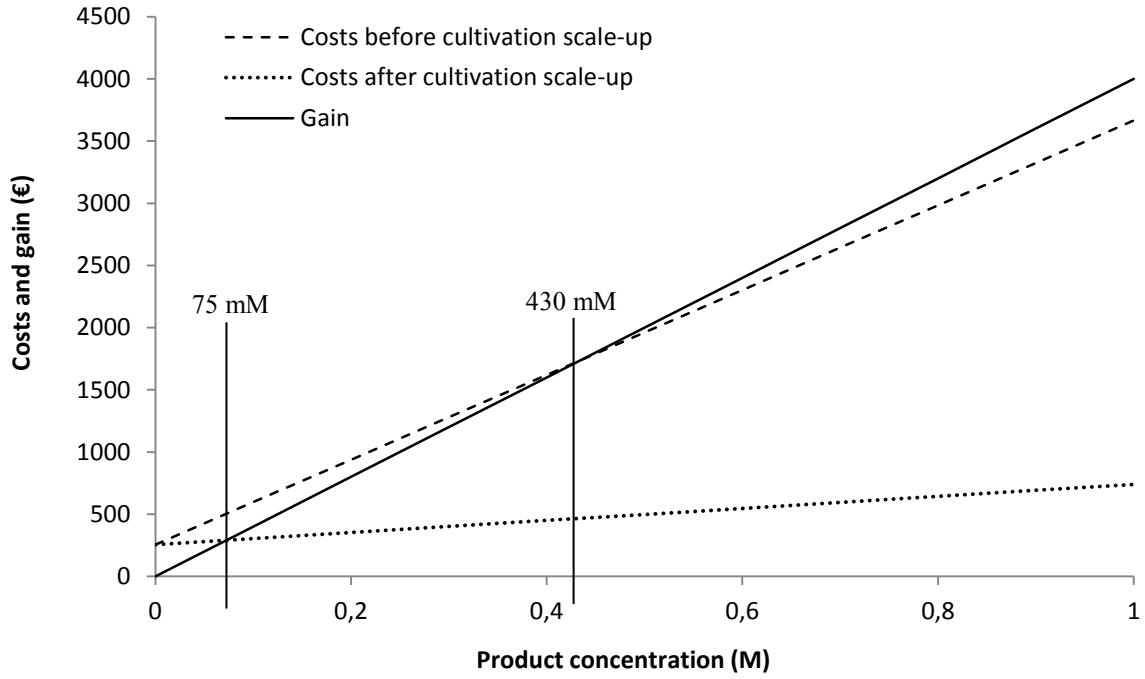

Figure S1: Break even analysis for *S*-1-(2-chlorophenyl)-ethanol production

Induction conditions during media development

For batch fermentations, an induction temperature of 18 °C and IPTG concentrations of 250-450 µM were used.

Composition of growth media

Notation: Example: M3 indicates a medium with both the composition and absolute concentration of all compounds as shown below. $M3^2$ means that each concentration is doubled.

We substituted $MgSO_4 \cdot 7H_2O$ in M1 by $MgCl_2 \cdot 6H_2O$ in later experiments to avoid precipitation of calcium sulfate crystals and added trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) to M2 and M3 to inhibit formation of further insoluble salts (Nagl, 1999).

Medium M1

Part	Component	Concentration (g/L)
A	K_2HPO_4	2.8
	KH_2PO_4	4.0
	NaCl	0.5
	Trace element solution	1.0[a]
	Polypropylene glycol	0.1[a]
B	$(NH_4)_2SO_4$	2.8
	NH_4Cl	2.22
	$MgSO_4 \cdot 7H_2O$	1.3
C	$CaCl_2 \cdot 2H_2O$	0.2
D	Glucose$\cdot H_2O$	44

[a] mL/L

Medium M2

Part	Component	Concentration (g/L)
A	K_2HPO_4	4.0
	KH_2PO_4	4.0
	$Na_3C_6H_5O_7 \cdot 2H_2O$	2.67
	Na_2HPO_4	2.8
B	$(NH_4)_2SO_4$	3.5
	NH_4Cl	3.0
	$MgCl_2 \cdot 6H_2O$	0.83
C	$CaCl_2 \cdot 2H_2O$	0.15
D	Glucose$\cdot H_2O$	30
E	Trace element solution	1.0[a]

[a] mL/L

Medium M3

Part	Component	Concentration (g/L)
A	K_2HPO_4	4.0
	KH_2PO_4	4.0
	$Na_3C_6H_5O_7 \cdot 2H_2O$	2.67
	Na_2HPO_4	2.8
	Polypropylene glycol	0.1[a]
B	$(NH_4)_2SO_4$	3.5
	NH_4Cl	3.0
	$MgCl_2 \cdot 6H_2O$	0.83
C	$CaCl_2 \cdot 2H_2O$	0.15
D	Glucose$\cdot H_2O$	20
E	Trace element solution	1.0[a]

[a] mL/L

Trace element solution

All compounds were dissolved in 5 M HCl.

Component	Concentration (g/L)
$FeSO_4 \cdot 7H_2O$	4
$MnSO_4 \cdot H_2O$	1
$AlCl_3 \cdot 6H_2O$	0.55
$CoCl_2$	0.4
H_3BO_3	0.1
$CuSO_4 \cdot 5H_2O$	0.15
$ZnSO_4 \cdot 7H_2O$	0.2
$Na_2MoO_4 \cdot 2H_2O$	0.2

Composition of nutrient feed

In experiments with an initial glucose concentration of 40 g/L ($M3^2$ medium), nutrient feed 1 was used. In the experiments with 20 g/L glucose (M3), nutrient feed 2 was used. The feed was a 15-fold concentrated solution of the respective growth medium. However, nutrient feed 2 did not contain phosphate to prevent formation of insoluble salts.

Nutrient feed 1

Part	Component	Concentration (g/L)
A	Glucose·H_2O	600
B	K_2HPO_4	120
	KH_2PO_4	120
	$Na_3C_6H_5O_7·2H_2O$	80.1
	Na_2HPO_4	84
C	$(NH_4)_2SO_4$	105
	NH_4Cl	90
	$MgCl_2·6H_2O$	24.9
	$CaCl_2·2H_2O$	4.5
D	Trace element solution	30[a]
	Thiamine	0.06

[a] mL/L

Nutrient feed 2

Part	Component	Concentration (g/L)
A	Glucose·H_2O	300
B	$(NH_4)_2SO_4$	52.5
	NH_4Cl	45
	$MgCl_2·6H_2O$	12.45
C	$CaCl_2·2H_2O$	2.25
D	Trace element solution	15[a]
	Thiamine	0.03

[a] mL/L

In situ monitoring of reaction progress

The addition of H_3PO_4 for pH control was used to monitor the progress of the reaction *in situ*; the resulting diagram is shown below. A comparison of product yield determination via HPLC measurement and acid addition is shown in table S3.

Table S3: Comparison of product yield determination via HPLC measurement and acid addition

Experiment	Product yield (%)		Error (%)
	HPLC measurement	Acid addition	
1	98.1	94.8	3.4
2	97.0	95.2	1.9
3	94.1	100	6.3

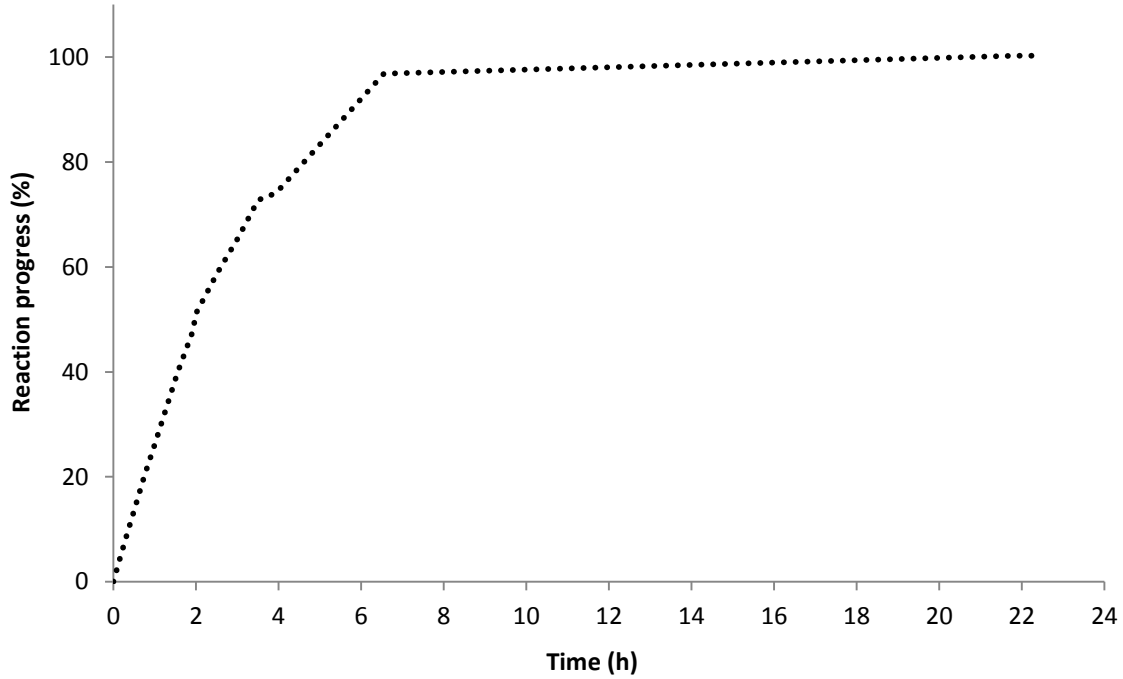

Figure S2: Monitoring of reaction progress via acid addition for pH control

Bioreactor information

H = vessel height; D = vessel diameter; d = stirrer diameter

Biostat CT: Manufacturer: B. Braun Biotech International GmbH
Year of manufacture: 1996
Total volume: 6.9 L
Working volume: 5 L
Stirrer type: twin 6-blade disc turbine
H/D ratio: 2
d/D ratio: 0.4
Control loops: pH (1 M H_3PO_4 and 2 KOH)
pO_2 (agitation and air flow cascade)
Temperature (double jacket vessel)
Anti-foam (10 % polypropylene glycol)

Labfors III: Manufacturer: Infors HT
Year of manufacture: 2008
Total volume: 3.6 L
Working volume: 2 L
Stirrer type: twin 6-blade disc turbine

H/D ratio: 1.6
d/D ratio: 0.4
Control loops: pH (1 M H_3PO_4 and 2 KOH)
pO_2 (agitation and air flow cascade)
Temperature (double jacket vessel)

Labfors: Manufacturer: Infors HT
Year of manufacture: -
Total volume: 1 L
Working volume: 0.5 L
Stirrer type: twin 6-blade disc turbine
H/D ratio: 1.8
d/D ratio: 0.4
Control loops: pH (1 M H_3PO_4 and 2 KOH)
Temperature (double jacket vessel)

Appendix

A Results and discussion

A.1 Batch fermentations

Shaken flask cultivations of *E. coli* Rosetta 2 co-expressing *Ct*XR and *Cb*FDH in LB medium yield enzyme activities of ≤ 2350 and 133 U/g_{CDW} for XR and FDH, respectively. Supplemented LB medium (medium C2) was used in the initial fermentation experiment to investigate reproducibility of growth and enzyme expression in larger scale (figure 1). Enzyme activities decreased by ≈6 % as compared to shaken flasks experiments (table 2). To further reduce process costs, defined mineral media (M1-M3) were used in experiments 2-5 (Hellwig et al., 2005).

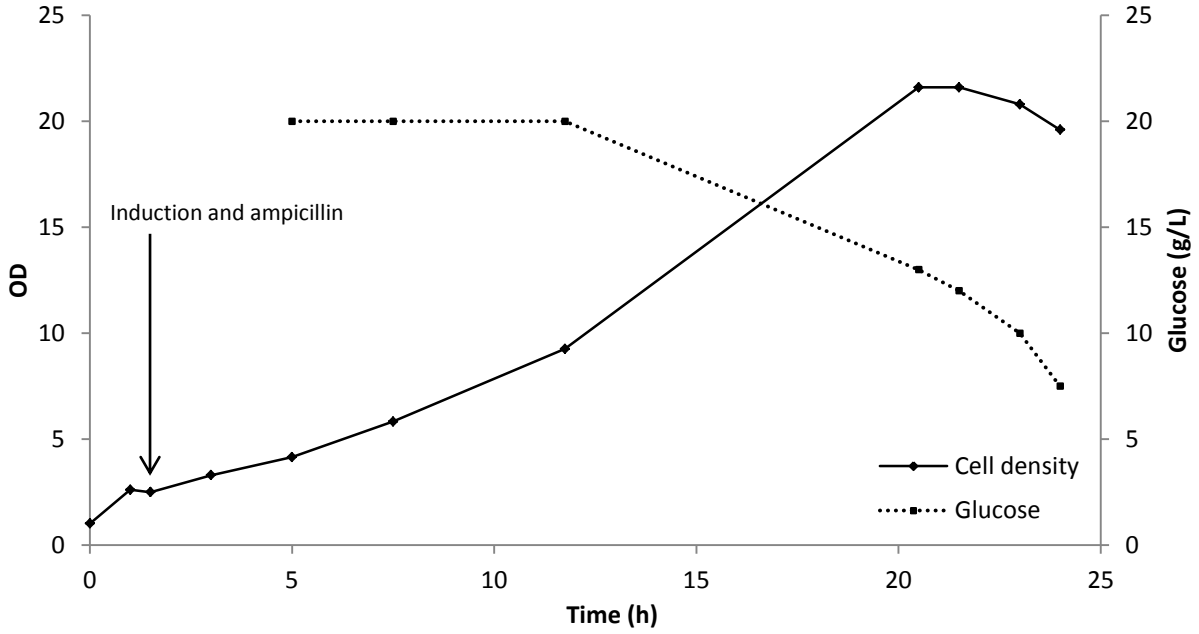

Figure 1: Batch fermentation 1 (complex medium): medium = C2, temperature after induction = 18 °C, maximal OD = 21.6

Table 1: Conditions of batch fermentations (Amp. = ampicillin; Temp. = temperature after induction)

No.	Volume (L)	Medium	Antibiotic	Temp. (°C)	[IPTG] (mM)	OD at induction	Duration (h)
1	5	C2	Amp.	18	0.250	2.50	24
2	5	M1	Amp.	18	0.250	1.60	94
3	5	M1	Amp.	18	0.250	10.3	26
4	2	M2	Amp.	27	0.450	2.42	23
5	2	M3	Amp.	18	0.450	1.98	46

Table 2: Results of batch fermentations

No.	OD_{End}	XR act. (U/g_{CDW})	FDH act. (U/g_{CDW})	μ_{max} (h^{-1})	$\mu_{Ind.}$ (h^{-1})[a]	$Y_{Glucose}$ (g/g)	$[Glucose]_{End}$ (g/L)
1	19.6	2384	125.0	1.650	0.107	-	7.5
2	23.2	729.8	18.10	0.504	0.0226	0.21	0
3	15.4	1206	72.90	0.455	0.0632	0.16	7.5
4	17.8	974.0	49.60	0.453	0.0822	0.23	0
5	12.2	1279	106.7	0.235	0.0462	0.24	0

[a]Specific growth rate after induction

Stability of ampicillin turned out as crucial factor in all fermentation experiments. While kanamycin and chloramphenicol are stable during *E. coli* fermentations (no detectable activity loss after 80 hrs), ampicillin has a half-life of only several hours in aqueous solutions (Peteranderl et al., 1990). In experiments 1 and 2, ampicillin was added at the fermentation start and at the time of induction (see figures1 and 2). In fermentation 2, the sharp increase in μ from 0.0226 h^{-1} to 0.0454 h^{-1} at 45 hrs indicates ampicillin degradation and plasmid loss (figure 2) (Sarrafzadeh et al., 2007). Enzyme activities decreased from 3533 U/g_{CDW} for *Ct*XR and 144.5 U/g_{CDW} for *Cb*FDH to 729.8 U/g_{CDW} and 18.1 U/g_{CDW}. To avoid plasmid loss due to ampicillin degradation, induction and ampicillin addition were done at OD ≈ 10 instead of OD ≈ 2 in experiment 3 (figure 3). However, enzyme activities still were about 50 % lower than in complex medium. Therefore, induction at OD ≈ 2 and further addition of ampicillin after several hours were used in experiments 4 and 5 (see figures 4 and 5). Expression tem-

perature was 27 °C instead of 18 °C in experiment 4, which led to ≈60 % lower enzyme activities as compared to experiment 1 (complex medium).

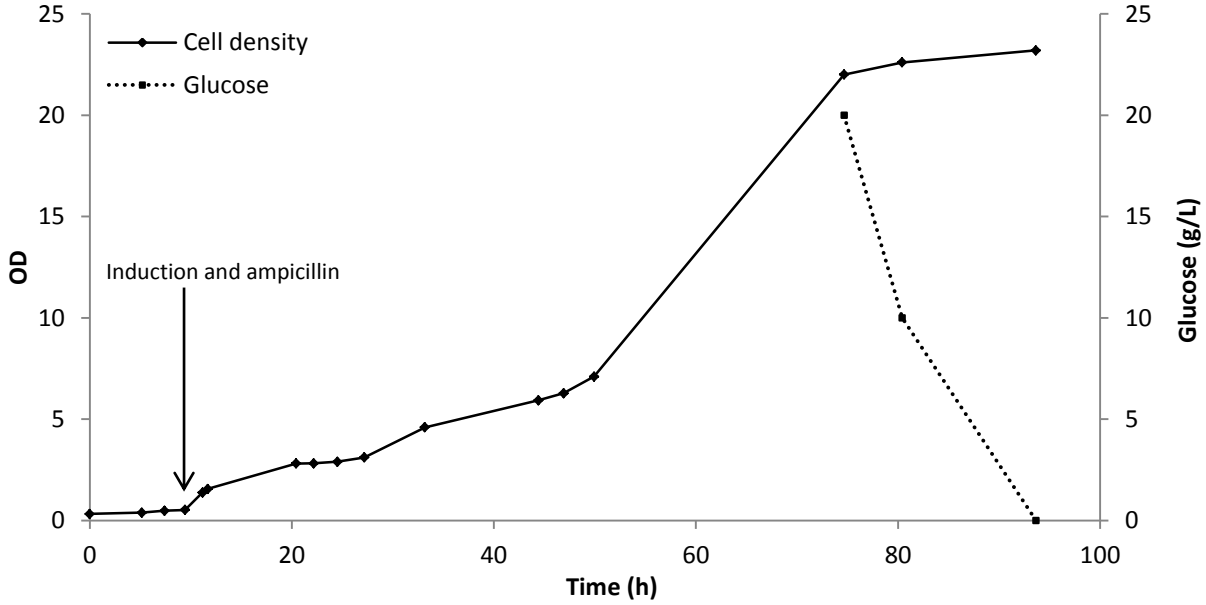

Figure 2: Batch fermentation 2 (mineral salt medium): medium = M1, temperature after induction = 18 °C, maximal OD = 23.2. Medium M1 was used for pre-cultures, which led to extremely slow growth (two and a half days until an OD suitable for induction was reached). Furthermore, due to the low OD of the pre-cultures, the main culture could only be inoculated to an OD of 0.32 instead of 0.50.

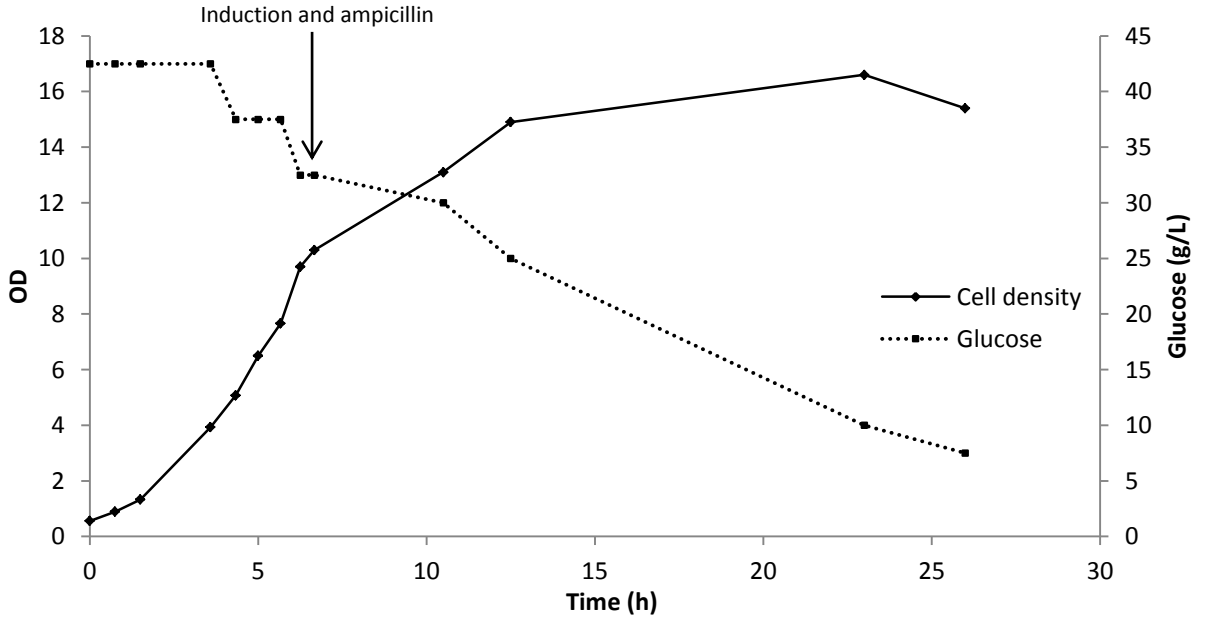

Figure 3: Batch fermentation 3 (mineral salt medium): medium = M1, temperature after induction = 18 °C, maximal OD = 16.6. Growth stopped soon after induction, although glucose was not depleted in the medium. This suggested unfavorable medium composition.

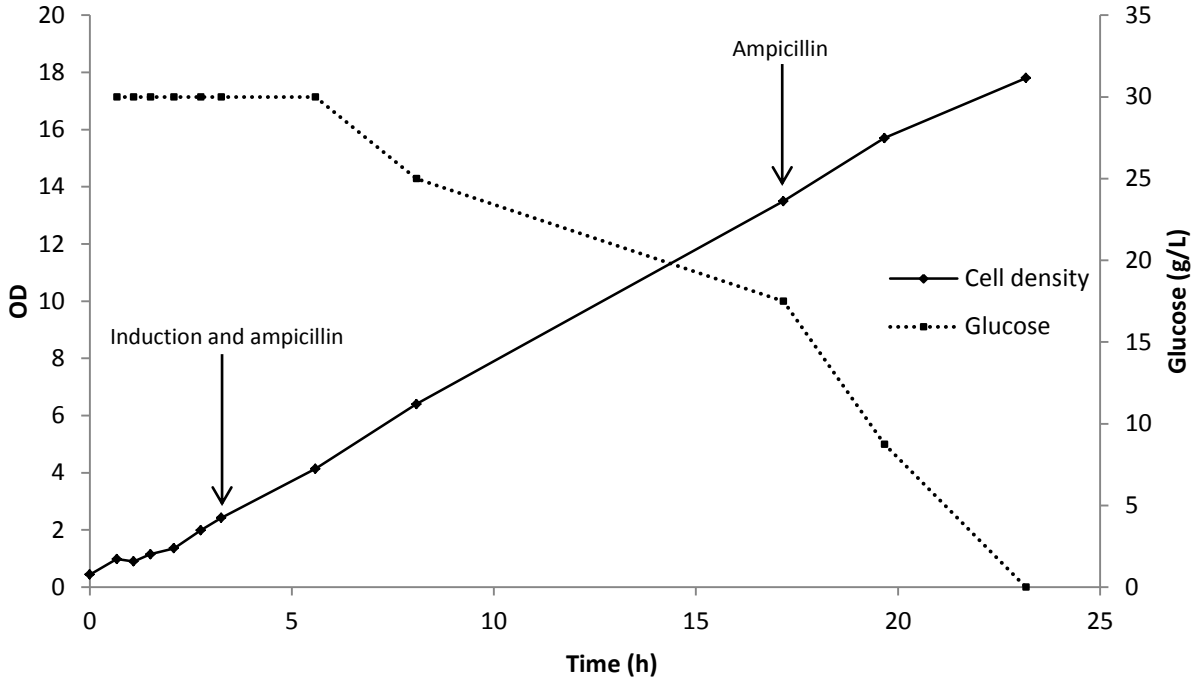

Figure 4: Batch fermentation 4 (mineral salt medium): medium = M2, temperature after induction = 27 °C, maximal OD = 17.8. In this experiment, an additional amount of ampicillin (115 mg/L) was added after 17 hours to ensure a sufficient level of the antibiotic throughout the fermentation.

The conditions for batch fermentation experiment 5 were chosen based on the results obtained in experiments 2-4. Growth medium M3 has the same composition as M2 (experiment 4), however the glucose concentration, i.e. the C/N ratio, was lowered (4.75 g/g instead of 7.13 g/g; see section B.2) and 100 µL/L polypropylene glycol (PPG) were added to the medium. Low PPG concentrations inhibit foam formation, but are not inhibiting for cell growth (Schügerl et al., 1995). IPTG concentration was 450 µM like in experiment 4, but expression temperature was 18 °C.

The results of experiment 5 are shown in figures 5 and 6. The cells showed uniform (exponential) growth and highest levels of enzyme activities (2684 and 182.8 U/g_{CDW} for XR and FDH, respectively) two generation times after induction. Enzyme activities decreased

afterwards to 1279 U/g_{CDW} and 106.7 U/g_{CDW} for XR and FDH, respectively, but were still the highest activities obtained in batch fermentations using mineral medium.

Figures 7 and 8 show the increase in *Ct*XR and *Cb*FDH activities, respectively, from the first to the last experiment with mineral salt medium. A 1.75-fold higher XR and nearly 6-fold higher FDH activity could be achieved. However, XR activity still lies significantly below the value obtained in complex medium (marked in red).

The glucose yield coefficients were notably low (0.23-0.24 g/g) as compared to published values of 0.5-0.55 g/g for $Y_{X/S}$ (Henry et al., 1969; Rinas et al., 2005). Even with a Rosetta strain and protein expression, a value of 0.35 g/g is reported (Sánchez-Ferrer et al., 2007). The low value may be linked to the genetic setup with three plasmids, requiring more carbon for maintenance metabolism.

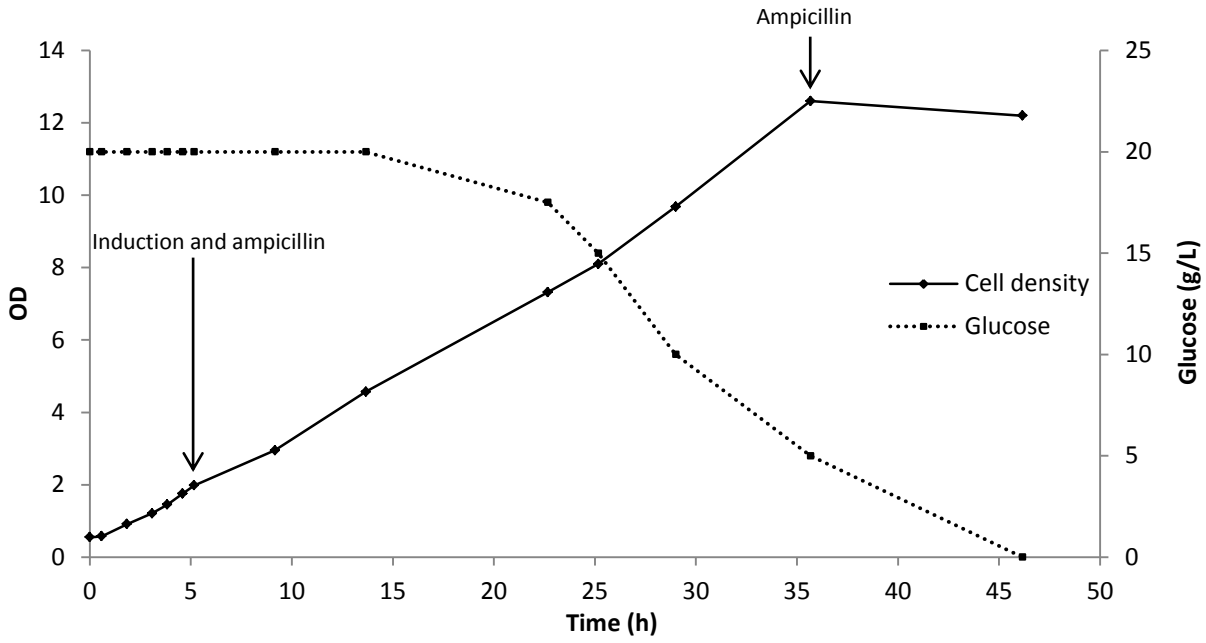

Figure 5: Batch fermentation 5 (mineral salt medium): medium = M3, temperature after induction = 18 °C, maximal OD = 12.6. An additional amount of ampicillin (115 mg/L) was added after 36 hours to ensure a sufficient level of the antibiotic.

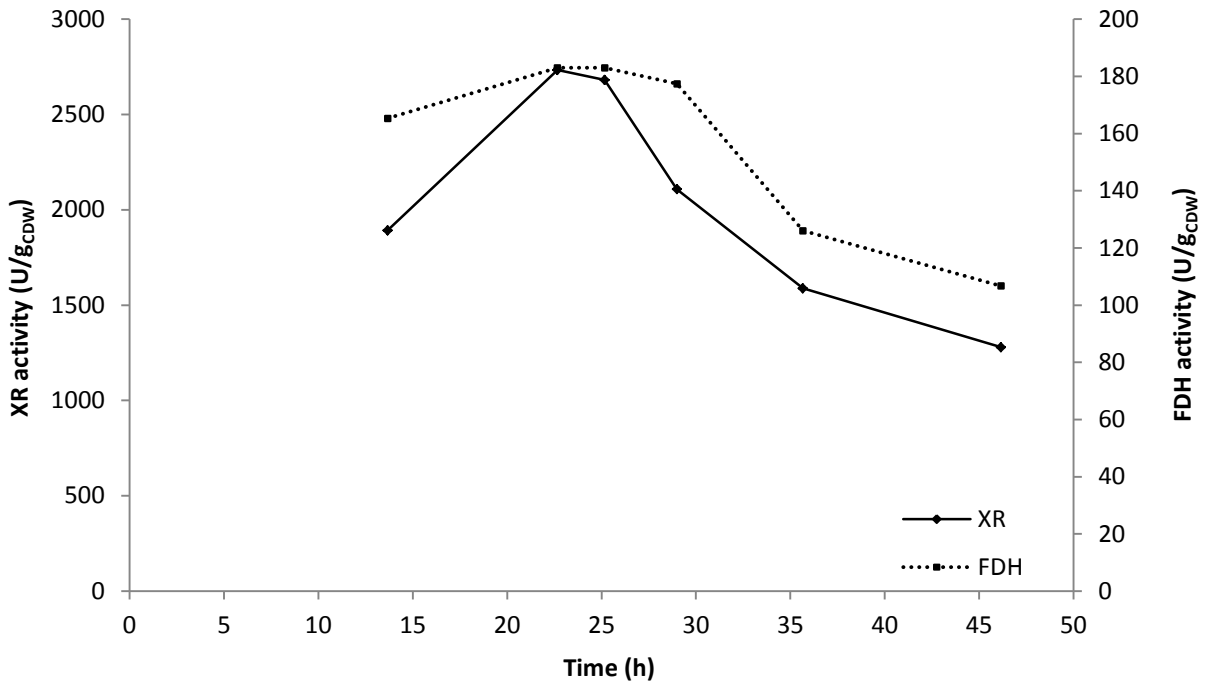

Figure 6: Change of enzyme activities during batch fermentation no. 5. The maximal values were measured already 15-20 h after induction.

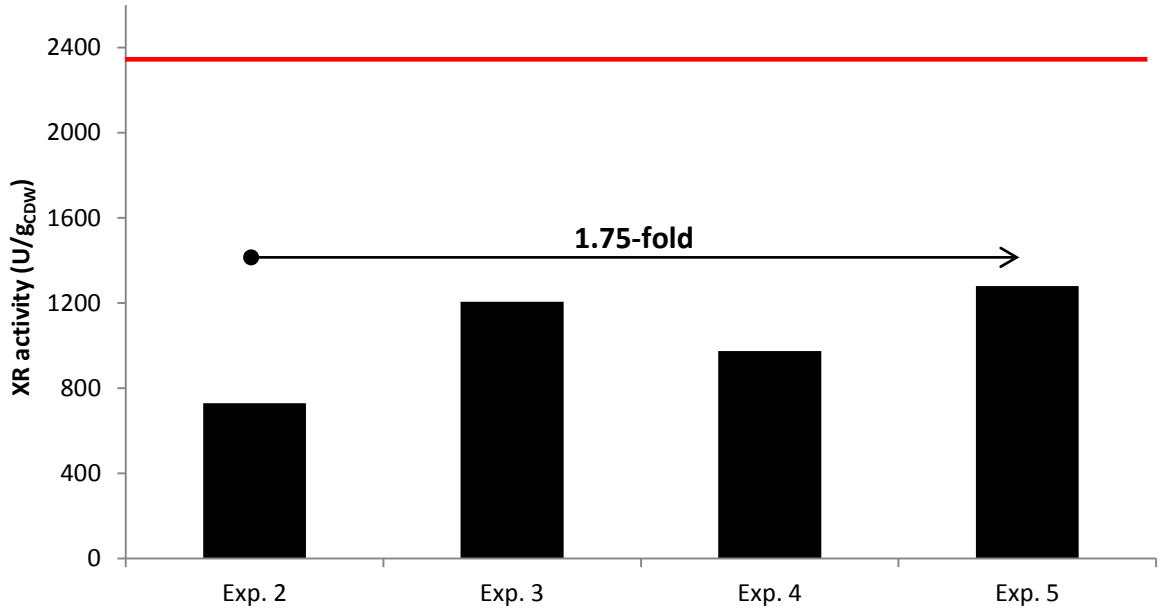

Figure 7: Improvement in XR activity. From the first (Exp. 2) to the last (Exp. 5) batch fermentation experiment with mineral salt medium, the XR activity obtained at the end of the experiment could be improved 1.75-fold (red line: value obtained in complex medium).

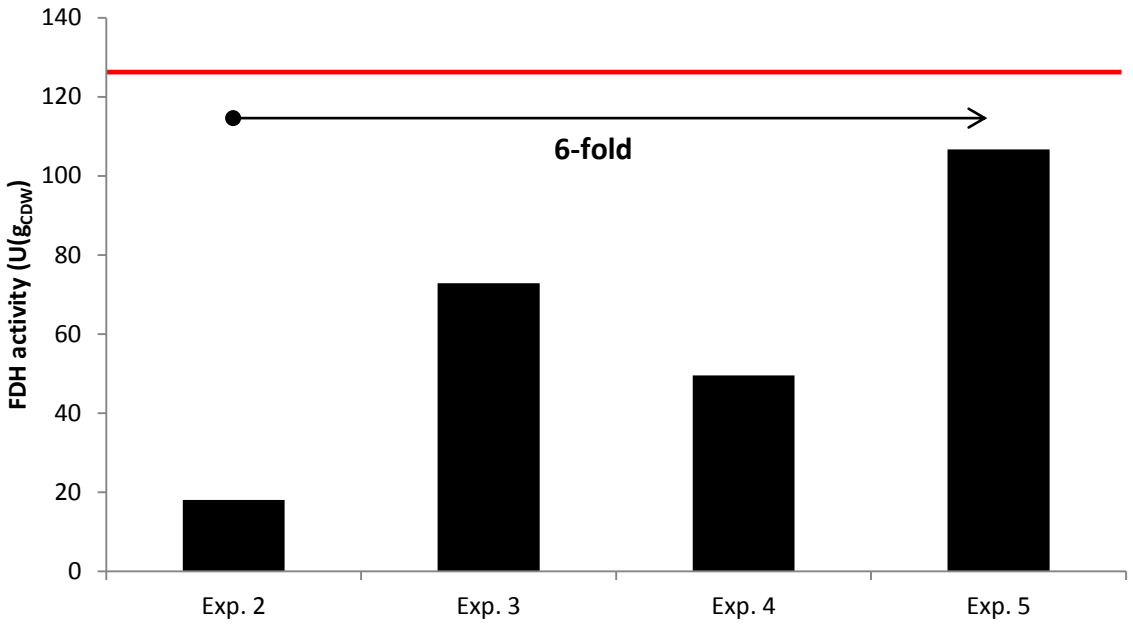

Figure 8: Improvement in FDH activity. FDH activity could be improved 6-fold from the first (Exp. 2) to the last (Exp. 5) experiment with mineral salt medium. With the optimized conditions (Exp. 5), FDH activity is only 15 % below the respective value obtained in complex medium (red line).

A.2 Fed-batch fermentations

Calculation of glucose amount in the nutrient feed

The amount of glucose necessary to achieve the targeted OD was calculated with the following formula (CDW/OD ratio = 0.39):

$$glucose = \frac{OD \cdot 0.39 \cdot volume}{Y_{X/S}} \quad (1)$$

The glucose amount already present in the starting medium was subtracted from this value. Ratios of glucose to all other feed components were adjusted to the used fermentation medium.

A.2.1 Discontinuous nutrient feed experiment

The nutrient feed (750 mL) contained 70 g/L $(NH_4)_2SO_4$, 60 g/L NH_4Cl and 400 g/L glucose. Its amount was calculated to allow an OD of 42 (see above). After a batch fermentation phase with M3 medium, induction and feed started at an OD of 14.

The feed solution was added in four portions of 187.5 mL, as indicated in figure 9. However, growth stopped at an OD of 29.8. We assume that one medium component not included in the nutrient feed (phosphate or one of the trace elements) was depleted.

Figure 9 shows the progress of the experiment. Taking into account that glucose was not fully consumed; an expected glucose yield coefficient of 0.23-0.24 g/g was calculated. Enzyme activities of 1485 U/g_{CDW} for XR and 105.3 U/g_{CDW} for FDH were obtained 24 hours after induction.

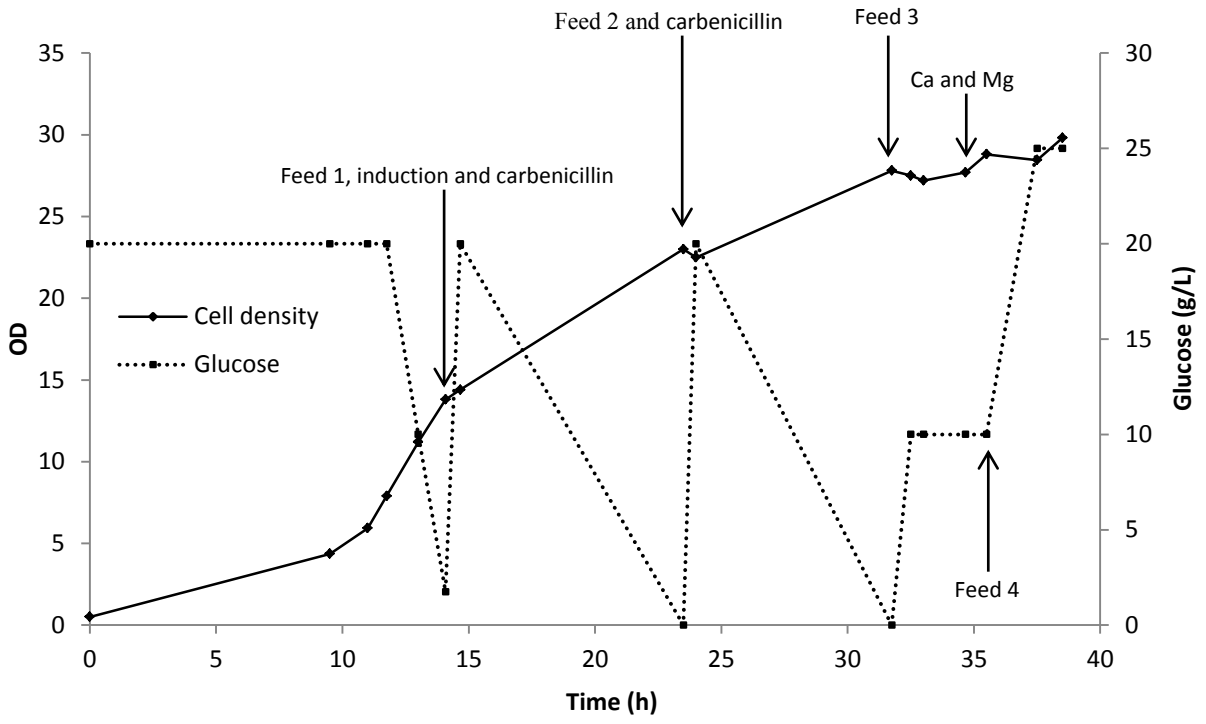

Figure 9: Fed-batch fermentation with discontinuous nutrient feed: medium = M3, temperature after induction = 18 °C, maximal OD = 29.8. In addition to the nutrient feed, after 35 hrs calcium and magnesium were added to the fermentation, as these compounds were not included in the feed.

A.2.2 Continuous nutrient feed experiments

We performed one batch fermentation without induction to check the tolerance of higher glucose and salt concentrations in the M3[2] medium for the experiments with 40 g/L glucose. After inoculation, the cells were grown at 37 °C until glucose was at a concentration of 2.5-5 g/L. The experiment was aborted at this glucose level, as the Labfors III fermenter system was not able to keep the pO_2 value above 40 % air saturation. Nevertheless, a specific growth rate μ of 0.269 h^{-1} and an OD of 23.9 (9.32 g_{CDW}/L) were achieved. These results suggest the M3[2] medium to be well suited for fed-batch experiments.

Table 3 summarizes the conditions and results of the two best fed-batch fermentations with continuous nutrient feed. For details on the procedure see main part.

Table 3: Conditions and results of fed-batch fermentations (carbenicillin was used in all cases; Temp. = temperature after induction)

No.	OD_{End}	Temp. (°C)	[IPTG] (mM)	OD at induction	Duration (h)	XR act. (U/g_{CDW})	FDH act. (U/g_{CDW})
1c	31	18	1.0	24.7	58.3	1775	123.7
2c	52	18	1.0	58.0	62.5	972.9	62.3

In figure 10, the improvement in total enzyme yield (U/L culture broth) for XR and FDH after scale-up is shown. Compared to the previously established shaken flask cultivations (Kratzer et al., 2011), cell density could be improved 18-fold. However, the increase in total enzyme yield was only 10-fold due to the fact that enzyme activities decreased at high optical densities.

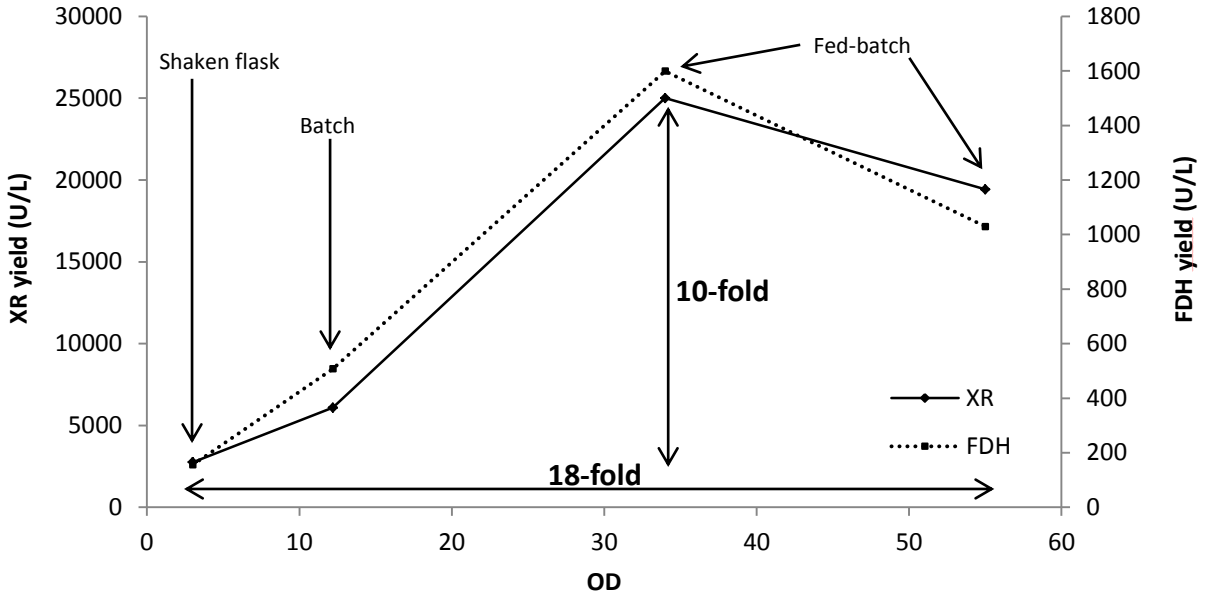

Figure 10: Results of cultivation scale-up. Yield is shown in terms of U/L culture broth and not as total activity obtained from the respective fermentation, as the two bioreactors used had different working volumes.

Several experiments with pure oxygen instead of air for aeration and with a different pump (Knaur Smartline 1000) for feed addition (exponential feed) were also performed. However, technical limitations (pump failure, severe problems with pO_2 control when using pure oxygen) led to very low enzyme activities and/or low cell densities. The results and growth profiles of these experiments are shown in table 4 and figures 13-16, respectively.

Table 4: Conditions and results of fed-batch fermentations (carbenicillin was used in all cases; Temp. = temperature after induction)

No.	OD_{End}	Temp. (°C)	[IPTG] (mM)	OD at induction	Duration (h)	XR act. (U/g_{CDW})	FDH act. (U/g_{CDW})
3c	46	18	1.0	34.8	48	255	20.7
4c	36	18	1.0	27.6	61	1274	65.5
5c	27	18	1.0	26.4	37	0	12.6

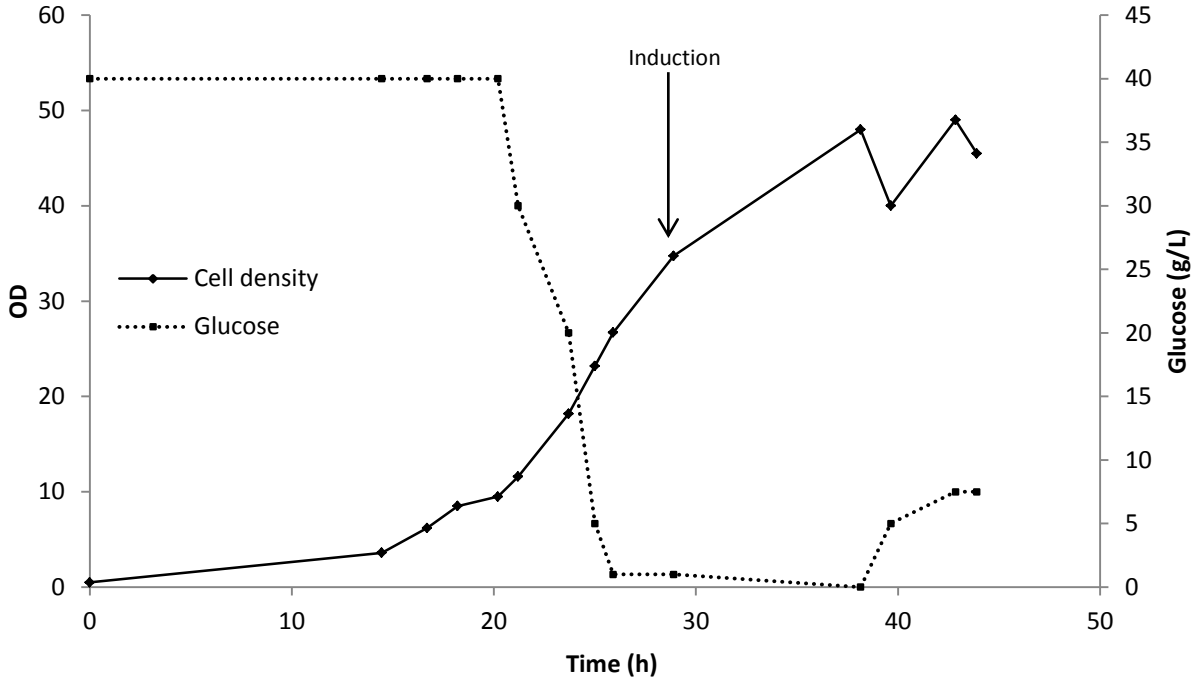

Figure 11: Fermentation 3c: medium = M3[2], temperature after induction = 18°C, maximal OD = 49.

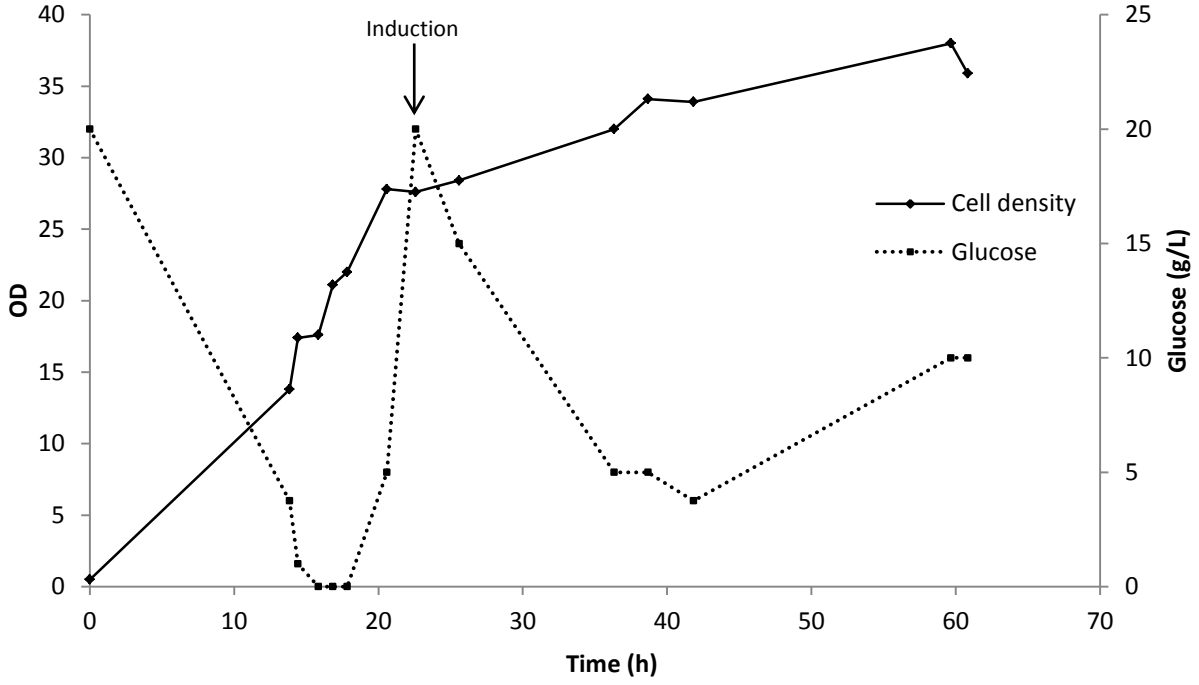

Figure 12: Fermentation 4c: medium = M3, temperature after induction = 18 °C, maximal OD = 38.

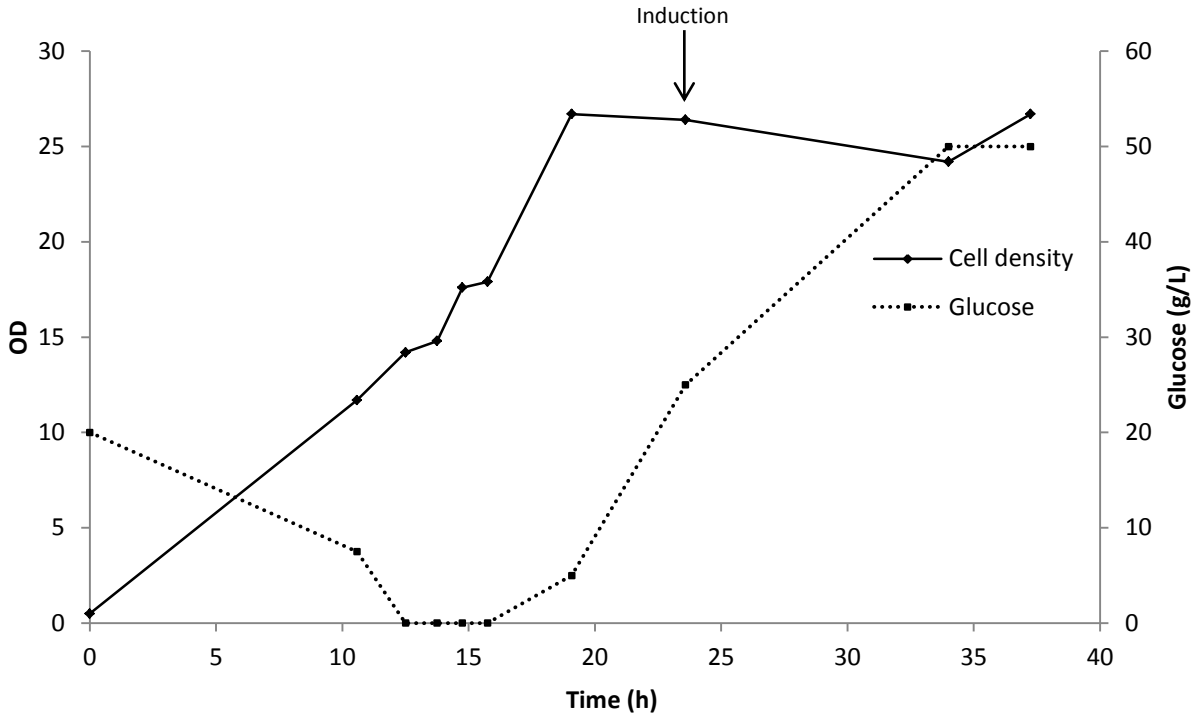

Figure 13: Fermentation 5c: medium = M3, temperature after induction = 18 °C, maximal OD = 26.7. Aeration was made with pure oxygen in this experiment.

A.3 Whole-cell bioreduction

The ratio of cell-dry-weight to OD was previously determined to 0.39 g/L. For cells in freeze-dried state a new ratio (freeze-dried-weight / OD) was measured to enable a simple weigh in of biocatalyst for the bioreductions.

Table 5: Determination of freeze-dried-weight (fdw)/OD ratio

Number	Sample (mg)	Volume (mL)	OD	fdw/OD (g/L)
1	2.1	10	0.638	0.329
2	1.1	10	0.350	0.314
3	2.7	10	0.920	0.293

The average value for the freeze-dried-weight/OD-ratio is therefore **0.312 ± 0.018 g/L**.

The exact procedure for whole-cell bioreduction experiments is described in part B.6 (materials and methods). When stirring started, an immediate emulsion formation of cells, hexane and buffer was observed, which impeded sampling. After a few hours it was visible that the viscosity of the mixture decreased due to cell denaturation caused by the toxic substrate and product, as it was expected from previous work (Schmölzer et al., 2011).

The effect of catalyst concentrations and intracellular enzyme activities on conversions in pH-controlled bioreductions is shown in tables 6 and 7. The results of product yield determination via acid usage are shown in table 7, too. Exclusively *S*-1-(2-chlorophenyl)-ethanol was produced as previously reported (ee >99.9 %) (Kratzer et al., 2011). The HPLC chromatogram of substrate and product is shown in figure 14.

Table 6: Biocatalyst concentrations and enzyme activities used in whole-cell bioreductions

Experiment No.	Cell conc. (g_{CDW}/L)	XR activity (U/g_{CDW})	FDH activity (U/g_{CDW})
1	48.0	1206	72.9
2	52.5	1485	105.3
3	48.0	1775	123.7
4	41.0	1775	123.7

Table 7: Results of whole-cell bioreductions

No.	Product yield (%)		Acid		Duration
	via HPLC	via acid	mL	M	(h)
1	77.8	-	-	-	23.3
2	98.1	94.8	108.6	0.85	24.3
3	97.0	95.2	109.1	0.85	22.0
4	94.1	100	100.2	1.0	22.5

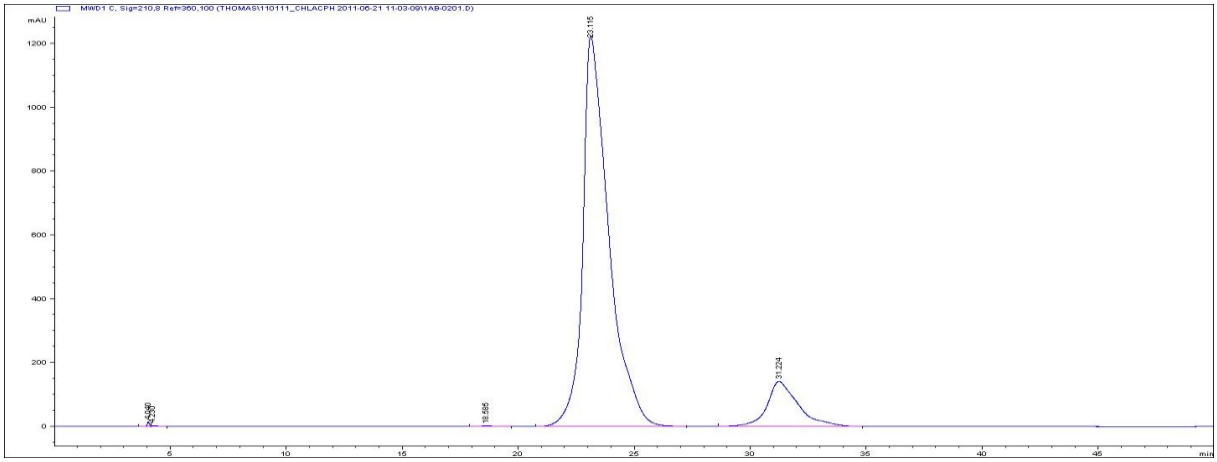

Figure 14: HPLC chromatogram of the product obtained in whole-cell bioreduction (UV detector at 210 nm). The peak at 23.1 min corresponds to *S*-1-(2-chlorophenyl)-ethanol, the peak at 31.2 min to *o*-chloroacetophenone. No peak for *R*-1-(2-chlorophenyl)-ethanol is visible at 25.5 min.

A.4 Downstream processing

The downstream processing procedure is described in detail in section B.7 and figure 15, respectively. Product extraction of the first bioreduction experiment (without pH control) was done in a 1-L-separatory funnel. Subsequent centrifugation resulted in three different phases, a water phase, an intermediate phase consisting of cell sludge and a hexane phase. This procedure (hexane extraction and centrifugation, see figure 15) was repeated two more times, i.e. the reaction mixture was extracted three times with 200 mL hexane. After pooling all hexane phases obtained, the loss of hexane noticed was not explainable by evaporation alone (considering the low bp of 69°C) (Atkins et al., 2006). Therefore it was assumed that significant amounts of hexane (and dissolved product) were bound at the interphase, in the compact cell sludge. Efficient extraction of the product from the cell sludge was not possible in the separatory funnel. The cell fragments were therefore incubated with hexane overnight at 4 °C to improve extraction.

Insufficient extraction of the cell fragments in the separatory funnel led to an isolated product yield of only 48.6 % (11.08 g) despite the high degree of conversion of 92.6 %.

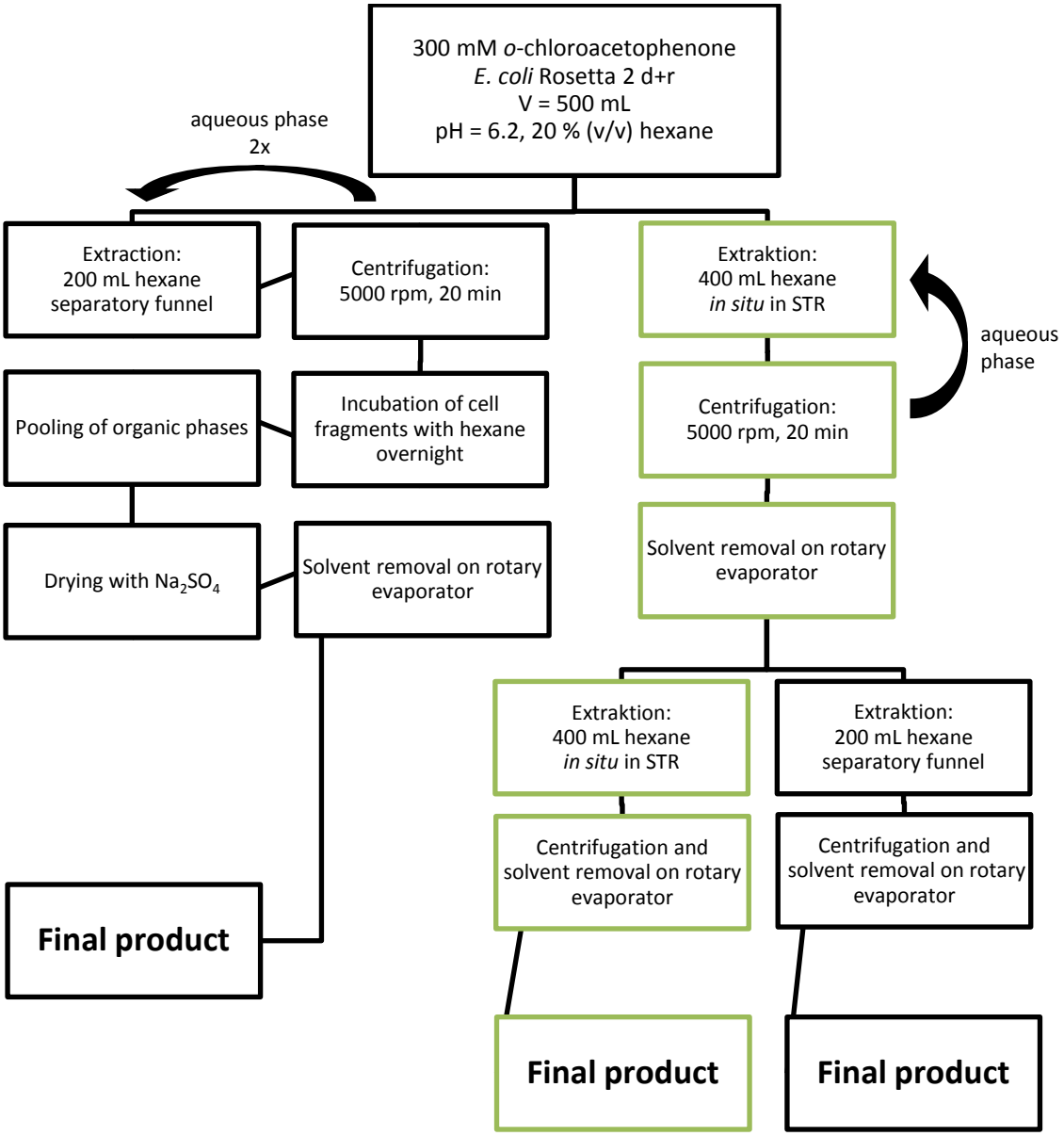

Figure 15: Flow scheme of the different downstream processing ways examined. Repeated steps are indicated with arrows and the optimized way is shown in green. With this variant it was possible to extract up to 96 % of the maximal possible mass.

A change from extraction in a separatory funnel to *in situ* extraction with 3 · 400 mL hexane in a stirred tank reactor (see main part) led to increases in extraction efficiencies from 60 to

96 %. In figure 16, the increase in total isolated mass and isolated product yield is shown. With the optimized downstream processing protocol, product loss during downstream processing is only below 15 % (compared to nearly 50 % in the initial experiment). An isolated product yield of 85.6 % is ≈20 % higher than previously achieved in small scale (67 %) (Kratzer et al, 2011).

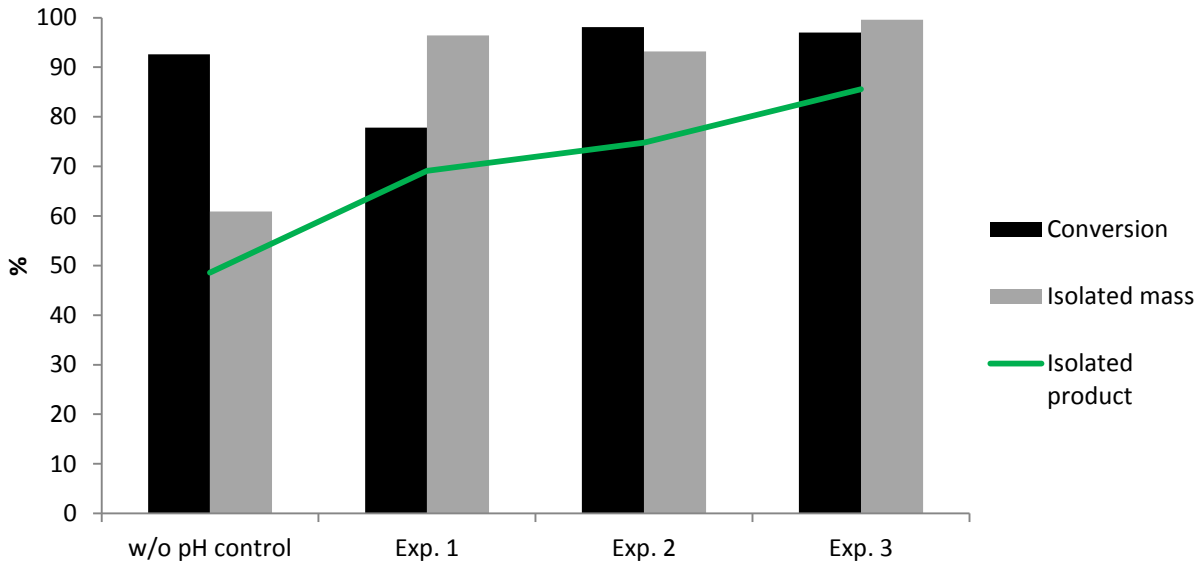

Figure 16: Conversion, isolated mass and isolated product yield (always in percent of maximal possible value) of the different downstream processing experiments. The green line indicates the increase in isolated product yield during optimization. In the isolated mass also the residual substrate and impurities are included.

B Materials and methods

B.1 Chemicals, materials and strain

The substrate *o*-chloroacetophenone was purchased from Sigma Aldrich (Steinheim, Germany) in a purity of >97 %. Racemic 1-(2-chlorophenyl)-ethanol (>96 % pure) was obtained from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). Hexane (>99 %), NADH (sodium salt >98 %) and NAD^+ (>97.5 %) were from Roth (Karlsruhe, Germany). Polymyxin B sulphate was purchased at Sigma Aldrich (Steinheim, Germany). Sodium formate (>95 %) was obtained from Sigma Aldrich (Vienna, Austria) or Merck (Darmstadt, Germany). All other chemicals used in fermentation or analysis were purchased either from Sigma Aldrich/Fluka (Gillingham, Dorset, UK) or Roth (Karlsruhe, Germany) and were of the highest purity available. 15 and 50 mL plastic tubes were purchased at Sarstedt (Wiener Neudorf, Austria).

The 6.9-L-fermenter Biostat CT (year of manufacture: 1996) was produced from B. Braun Biotech International GmbH and had a working volume of 5 L. It was fitted with a twin 6-blade disc ("Rushton") turbine impeller, temperature, pH, pO_2 and automatic foam measurement and control, as well as an *in situ* sterilization function. For sample drawing, a steam sterilizable valve was available.

The 3.6-L-fermenter Labfors III (year of manufacture: 2008) and the 1-L-fermenter Labfors were purchased from Infors HT and had working volumes of 2 L and 0.5 L, respectively. They did not have anti-foam and *in situ* sterilization functions, only temperature, pH and pO_2 control were available. The stirrer was a twin 6-blade disc turbine impeller, like in the Biostat CT.

The microorganisms used were *E. coli* Rosetta 2 (DE3), which carry a pRARE2 plasmid with tRNAs for seven rare codons (Merck, 2011). The strain was modified (*E. coli* Rosetta 2 d+r) and had additional pETDuet_XR_FDH and pRSF plasmids, from which the former carries genes for *Ct*XR and *Cb*FDH, while the latter carries an additional *Cb*FDH gene (Mädje et al., 2011). pETDuet_XR_FDH encodes for an ampicillin resistance, pRSF for a kanamycin resistance and pRARE2 for a chloramphenicol resistance.

B.2 Fermentation media and supplements

Parts A, B, C and D (if existent) were autoclaved separately to prevent Maillard reactions between glucose and nitrogen containing compounds (Kundinger et al., 2008) as well as the formation of insoluble products, e.g. calcium phosphate. The trace element solution was either autoclaved (if contained in part A or B) or filter-sterilized (part E).

Medium C1

Part	Component	Concentration (g/L)
A	Glucose·H_2O	5.5
B	Peptone from casein	10
	Yeast extract	5.0
	NaCl	5.0
	NH_4Cl	1.0
	$MgSO_4 \cdot 7H_2O$	0.25
	Trace element solution	1.0[a]
	Polypropylene glycol	0.1[a]
C	K_2HPO_4	3.0
	KH_2PO_4	6.0

[a] mL/L

Medium C2

Part	Component	Concentration (g/L)
A	Glucose·H_2O	22
B	Peptone from casein	10
	Yeast extract	5.0
	NaCl	5.0
	NH_4Cl	1.0

	$MgSO_4 \cdot 7H_2O$	0.25
B	Trace element solution	1.0[a]
	Polypropylene glycol	0.1[a]
C	K_2HPO_4	3.0
	KH_2PO_4	6.0

[a] mL/L

Medium M4

Part	Component	Concentration (g/L)
A	K_2HPO_4	8.0
	KH_2PO_4	8.0
	$Na_3Citrate \cdot 2H_2O$	2.67
	Na_2HPO_4	5.6
	PPG	0.1[a]
B	$(NH_4)_2SO_4$	3.5
	NH_4Cl	3.0
	$MgCl_2 \cdot 6H_2O$	0.83
C	$CaCl_2 \cdot 2H_2O$	0.15
D	$Glucose \cdot H_2O$	20
E	Trace element solution	1.0[a]

[a] mL/L

Antibiotics and vitamins

E. coli Rosetta 2 d+r carries three plasmids and requires three antibiotics as selection markers in the growth media. All media were therefore supplemented with ampicillin 115 mg/L or carbenicillin 100 mg/L, kanamycin 50 mg/L and chloramphenicol 34 mg/L. Ampicillin/carbenicillin and kanamycin were dissolved in deionized H_2O, while chloramphenicol was dissolved in pure ethanol. Additionally, thiamine was added in a concentration of 2 or 4 mg/L to all fermentations.

B.3 Cultivation of pre-cultures

E. coli pre-cultures were grown in 1000 mL baffled shaken flasks, containing 300 mL of medium C1. All media were supplemented with antibiotics. The cells were cultivated overnight

at 37 °C and at an agitation rate of 130 rpm in a Certomat BS-1 incubator from Sartorius. The number of pre-cultures varied between two and five, depending on the volume of the respective main culture.

All pre-cultures were inoculated under sterile conditions with 30 µL glycerol stock of *E. coli* Rosetta 2 d+r stored at -70 °C.

B.4 Fermentation

For fermentation experiments, either the Biostat CT fermenter system from Braun or the Labfors III fermenter system from Infors was used (see results and discussion). The preparative steps (see below) for the Biostat CT differed from those of the Labfors III only in that way that the former was *in situ* autoclavable.

One part of the respective fermentation medium (part A) was filled into the fermenter and the apparatus was assembled as described in the manual (electrodes, septums, plugs, sparger, cooler, etc.). Afterwards, the Biostat CT was sterilized using the built-in sterilization mode, while the Labfors III was sterilized in an autoclave.

In both cases, the pH electrode was calibrated before sterilization using two buffer solutions (pH = 4.01 and pH = 7.0), while the pO_2 electrode was calibrated just before the fermentation (after sterilization). For the 0 % value, the medium was saturated with N_2 and for the 100 % value with air.

After the preparative steps, the other medium parts (B-D) and the additional components (i.e. the antibiotics, trace element solution and thiamine) were filled into the bioreactor under sterile conditions. The fermentation was started by addition of an appropriate amount of pre-culture, yielding an OD of about 0.5. The conditions at the inoculation time always were as follows:

Temperature: 25 °C or 37 °C
pH value: 6.94-7.00
pO_2 value: 60-70 % air saturation

pH and pO_2 value were automatically held at constant levels (7 and 45 %, respectively) during the fermentation by the fermenter system. For pH control, sterile H_3PO_4 (1 M) and sterile KOH (2 M) were used, which were added via peristaltic pumps. The pO_2 level was controlled via a cascade regulation of stirrer speed and air flow rate. The Biostat CT had an automatic foam control, while in the case of the Labfors III the antifoam solution (polypropylene glycol 10 %) was added manually.

Induction of the enzyme expression was done by addition of IPTG (0.25-1.0 mM). At induction time, in each experiment an additional amount of ampicillin or carbenicillin (yielding 115 mg/L or 100 mg/L, respectively) was added. The exact profile of each fermentation experiment is described in results and discussion.

B.5 Cell harvest and storage

After fermentation, the cells were harvested by centrifugation. For this purpose, a Sorvall RC-5B centrifuge with a FAS-10C rotor at a speed of 5000 rpm was used. Centrifugation times varied between 15 and 20 minutes.

Initially, the cell pellet was stored in a cooling room at 4 °C until use. However, this method had the disadvantage that enzyme activity decreased during storage. It was also difficult to weigh in the appropriate amount of cells for the bioreduction experiments.

Therefore in later experiments the cells were freeze-dried and stored in a freezer at -20 °C. For freeze-drying, the cell pellet was put in a -70 °C freezer at least for one night and afterwards put in the freeze-dryer. The dry cell powder was filled into 50 mL plastic tubes.

B.6 Whole-cell bioreduction

The whole-cell bioreduction experiments were performed in the Labfors stirred tank reactor from Infors HT in a two-phase system (20 % hexane as co-solvent). The total volume was 500 mL, consisting of 400 mL potassium phosphate buffer (100 mM) at pH = 6.2 and 100 mL hexane. The stirred tank reactor was assembled as described in the manual.

The cells (pellet or freeze-dried powder) were suspended in the buffer solution in a concentration of 41 g_{CDW}/L to 52.5 g_{CDW}/L about 1 hr prior to reaction start. Sodium formate (350 mM), polymyxin B sulphate (36 µM) and NAD^+ (500 µM) were also dissolved in the buffer. The reaction was started by addition of 145.5-150 mmole of *o*-chloroacetophenone (22.49-23.19 g) dissolved in 100 mL hexane.

During the reaction, the temperature was held at 25 or 30 °C and the stirring speed was 500 or 1000 rpm. Depending on the experiment, also a pH control with 2 M KOH and 1 M H_3PO_4 was implemented. After 22-24 hours, the experiment was stopped.

B.7 Downstream processing

For product isolation, the reaction mixture was extracted three times with hexane, which was afterwards removed on a rotary evaporator. Different methods for the extraction process were examined.

B.7.1 Extraction in separatory funnel

The emulsion formed during bioreduction did not separate in the separatory funnel, not even after addition of 200 mL hexane. Therefore the phases were separated by centrifugation at 5000 rpm for 20 min. The H_2O phase was extracted again with 200 mL hexane and

centrifuged. As a lot of cellular material had accumulated between the two phases, this cell sludge was incubated with hexane overnight to extract product, which was enclosed in the cell mass.

The organic phases were pooled and dried with Na_2SO_4 for 20 minutes. Afterwards, the hexane was removed at a rotary evaporator (40 °C, 150 mbar); the isolated product was filled in plastic tubes and stored in the freezer at -20 °C.

B.7.2 Extraction in stirred tank reactor

After the bioreduction had finished, 400 mL hexane were added to the reaction mixture in the stirred tank reactor (STR) and mixed for 10-20 minutes at 1000 rpm. The two phases were separated by centrifugation (5000 rpm, 15-20 min) and the H_2O phase (together with the cellular material) was put back in the STR. 400 mL hexane were added, and the mixture was stirred again at 1000 rpm for 20 minutes. After phase separation by centrifugation, the H_2O phase with cellular material was extracted either a third time in the STR with 400 mL hexane at 1000 rpm or with 200 mL hexane in a separatory funnel.

The organic phases were not pooled, but the hexane from each phase was separately removed at a rotary evaporator (40 °C, 100 mbar). No drying of the organic phases was performed. The isolated product was filled in plastic tubes and stored at -20 °C.

B.8 Analytical methods

B.8.1 Activity measurements

The enzyme activity of *Ct*XR was measured in a mixture containing 700 mM xylose and 300 µM NADH, for *Cb*FDH 200 mM sodium formate and 2 mM NAD^+ were used. Xylose and

sodium formate, respectively, were dissolved in potassium phosphate buffer at pH = 6.2, while the cofactors were prepared as 50x stock solutions just before measurement by dissolving an appropriate amount in water.

For the preparation of the cell-free extracts, the cell lysis reagent B-Per (Pierce, Rockford, IL, USA) was used (cell disruption according to the manual).

The measurements were always started by addition of the cofactor. For measurement of the XR activity, the crude extract had to be diluted 1:20 or 1:10.

Measurement solution:	480 µL buffer containing xylose or sodium formate
	10 µL cell-free extract
	10 µL cofactor solution (not for blank)

A Beckmann DU800 spectrophotometer at a wavelength of 340 nm (background wavelength: 250 nm) was used. Measurement time was five minutes for each sample.

B.8.2 Measurement of product concentration and fermentation by-products

The results of the whole-cell bioreduction were determined via high performance liquid chromatography (HPLC). For this purpose, a few milligrams of the isolated product were dissolved in pure ethanol to get a solution with a concentration of about 10 mM product and substrate, respectively.

Two 10 mM standard solutions were prepared, one for *o*-chloroacetophenone and one for 1-(2-chlorophenyl)-ethanol. 0.1 mmole of the respective substance were dissolved in 10 mL ethanol.

An Agilent 1200 Series HPLC system with a Chiralpak AD-RH column from Daicel was used for measurement. Acetonitrile was used as eluent, either acetonitrile 25 % without gradient or acetonitrile containing 0.1 % TFA and water as gradient. The temperature of the column was 40 °C and the detector was a UV detector at 210 nm.

For determination of acetate and formate concentrations during the fermentation experiments with continuous nutrient feed, a LaChrom HPLC system with an Aminex HPX-87H column from Biorad was used. The respective standard solutions were prepared from stock solutions. 5 mM H_2SO_4 was used as eluent, the temperature of the column was 60 °C and the detector was a refractive index detector.

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