Katharina Mädje

# Optimisation of internal cofactor regeneration by host and expression engineering for the whole-cell bioreduction of carbonyl substrates

MASTERARBEIT

zur Erlangung des akademischen Grades
einer Diplomingenieurin
der Studienrichtung Biotechnologie
erreicht an der

**Technischen Universität Graz**

betreut durch:
Univ.-Prof. Dipl.-Ing. Dr. Bernd Nidetzky
Dipl.-Ing. Dr. techn. Regina Kratzer
Institut für Biotechnologie und Bioprozesstechnik

**2010**

# Danksagung

Die Fertigstellung meiner Diplomarbeit markiert nicht nur das Ende meines Diplomstudiums, sondern ist auch das Ergebnis meiner praktischen Arbeit im Labor.

Ich möchte mich daher an dieser Stelle bei allen bedanken, die mich während meines Studiums begleitet und unterstützt haben.

*„Felix, qui potuit rerum cognoscere causas“* (Vergil)

Beim „Ergründen der Dinge“ standen mir Univ.-Prof. Dipl.-Ing. Dr. Bernd Nidetzky und Dipl.-Ing. Dr. Regina Kratzer jederzeit mit Ratschlägen, Anregungen und fachlichen Gesprächen hilfreich zur Seite. Dafür, sowie für die wunderbare Betreuung und Korrektur dieser Arbeit, möchte ich mich ganz herzlich bei Ihnen bedanken.

Ein großes Dankeschön gilt all meinen Kolleginnen und Kollegen für die freundliche Aufnahme am Institut, das angenehme Arbeitsklima und die abwechslungsreichen Kaffeepausen.

Meiner Familie, die mich immer unterstützt hat, widme ich diese Arbeit.

Meine Großeltern haben viel Anteil an meiner Ausbildung genommen und sich stets mit mir gefreut, wenn ich Prüfungen gut bestanden habe.

Meinen Eltern, Ulrike und Scheiberl, die jederzeit für mich da waren und mir Rückhalt gaben, sowie meinem Vater Klaus, danke ich für ihr Verständnis und ihr Interesse an meiner Arbeit, und ihre finanzielle Unterstützung über die Jahre, die mir mein Studium erst ermöglichte.

# EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommene Stellen als solche kenntlich gemacht habe.

Graz, am …… ……..

(Unterschrift)

# Kurzfassung

Die asymmetrische Reduktion von Ketonen, um enantiomeren reine Alkohole zu gewinnen, ist immer mehr auf enzymatisch katalysierte Reaktionen angewiesen. Diese Prozesse verwenden entweder (teilweise) gereinigte Enzyme oder Ganzzellsysteme. Eine Ganzzellbioreduktion benötigt eine gewisse Menge an Cofaktor (NADH oder NADPH), welches während des Umsatzes recycelt werden muss. Stamm- und Expressionsengineering wurden eingesetzt, um einen Katalysator mit einem optimierten Cofaktor Recyclingsystem zu entwickeln. Einzelexpression des Enzyms *Candida boidinii* Formiatdehydrogenase, die wurde unter verschiedenen Kultivierungsbedingungen und mit unterschiedlichen *Escherichia coli* Stämmen durchgeführt wurden, führten zu einer erhöhten Proteinexpression. Die Formiatdehydrogenase Aktivität wurde verdoppelt und ein FDH optimierter Stamm für die Co-Expression mit *Candida tenuis* Xylose Reduktase wurde generiert. Der ursprüngliche Ganzzellkatalysator wurde mit dem optimierten Stamm in einer Reaktion mit D-Xylose als Substrat verglichen. Diese nicht-toxische Reaktion zeigte keinen Unterschied in der Produktausbeute zwischen den beiden Stämmen. Als die gleiche Reaktion mit Rohextrakt durchgeführt wurde, übertraf der optimierte Stamm den ursprünglichen Stamm allerdings in Reaktionsgeschwindigkeit und Produktbildung. Die Überlegenheit des FDH optimierten *E. coli* Stammes war deutlich sichtbar in einer Reaktion mit *o*-Chloroacetophenon als Substrat. Der optimierte Stamm erreichte eine zweimal höhere Produktausbeute. Bedingt durch eine pH Änderung und der Zugabe von Polymyxin B Sulfat, konnte am Ende 95% enatiomeren reiner Alkohol erzielt werden.

**Schlüsselwörter**

Ganzzellreduktion; Katalysator; Keton; Cofaktor Regenerierung; Stammengineering; Expressionsengineering; *Escherichia coli*; *Candida tenuis* Xylose Reduktase, *Candida boidinii* Formiatdehydrogenase

# Abstract

The asymmetric reduction of ketones to generate enantiomerically pure alcohols relies more and more on the usage of enzyme-catalysed reactions. These processes use either a (partially) purified enzyme or a whole-cell system. A whole-cell bioreduction requires a certain amount of cofactor (NADH or NADPH), which has to be recycled during the conversion process. Host and expression engineering were employed to generate a catalyst with an optimised cofactor recycling system. Single-expression of the *Candida boidinii* formate dehydrogenase under various cultivation conditions and with different *Escherichia coli* strains resulted in higher protein expression. The formate dehydrogenase activity was doubled and a FDH optimised strain for the co-expression with *Candida tenuis* xylose reductase was generated. The initial whole-cell catalyst was compared to the optimised strain in a reaction with the native substrate D-xylose. This non-toxic reaction showed no difference in product yield between the two strains. However, when the same reaction was performed with crude extract, the optimised strain exceeded the initial strain in reaction velocity and product formation. The superiority of the FDH optimised *E. coli* strain was clearly visible in a reaction with *o*-chloroacetophenone as substrate. The optimised strain scored a two times higher product yield. Due to a change in pH and the addition of Polymyxin B sulphate, 95% of enantiomerically pure alcohol could be obtained in the end.



**Keywords**

Whole-cell reduction; catalyst; ketone; cofactor regeneration; host engineering; expression engineering; *Escherichia coli*; *Candida tenuis* xylose reductase; *Candida boidinii* formate dehydrogenase

# Contents

# Optimisation of a *Candida boidinii* formate dehydrogenase driven cofactor regeneration system for the efficient production of chiral alcohols

Katharina Mädje[1], Regina Kratzer[1], Katharina Schmölzer[1], Bernd Nidetzky[1,§]

[1]Institute of Biotechnology and Biochemical Engineering, Graz University of Technology (TUG), Petersgasse 12/1, A-8010 Graz, Austria.

[§]Corresponding author

Email addresses:

KM: maedje@student.tugraz.at

RK: regina.kratzer@tugraz.at

KS: katharina.schmoelzer@acib.at

BN: bernd.nidetzky@tugraz.at

# Abstract

## Background

The asymmetric reduction of ketones to generate enantiomerically pure alcohols relies more and more on the usage of enzyme-catalysed reactions. The superiority of whole-cells as compared to isolated enzymes in biocatalytic processes is visible in higher enzyme stability as well as in a simple and low cost catalyst preparation. A priceless advantage of whole-cell systems is the possibility of internal cofactor recycling by the metabolism of a co-substrate. Cofactor regeneration in *Escherichia coli* (*E. coli)*, the prime host for whole-cell biocatalysis, is limited and requires the co-expression of a dehydrogenase. An interesting candidate for cofactor regeneration in a whole-cell reduction system is the enzyme formate dehydrogenase (FDH). The FDH-catalysed oxidation of formate to carbon dioxide is irreversible and its product does not inhibit or inactivate the oxidoreductases involved. However, a rather low FDH expression in whole-cell reduction catalysts complicates its utilisation for cofactor regeneration.

This work aims at increasing initial FDH activity by host and expression engineering of *Candida boidinii* formate dehydrogenase (*Cb*FDH). The optimisation of cofactor regeneration led to an improved whole-cell catalyst co-expressing *Candida tenuis* xylose reductase (*Ct*XR) and *Cb*FDH. The initial whole-cell catalyst was compared to the FDH optimised strain in a reaction with the native substrate D-xylose. The bacterial system was also evaluated for the synthesis of (*S*)-1-(2-chlorophenyl)ethanol under various process conditions.

## Results

The single-expression of a pRSF-1b plasmid carrying *Candida boidinii* formate dehydrogenase in various *Escherichia coli* strains yielded between 402 and 614 $U/g_{CDW}$ of intracellular formate dehydrogenase activity, equivalent to 12 and 19% of soluble *E. coli* proteins. The co-expression of *Ct*XR and *Cb*FDH in *E. coli* BL21 (DE3) resulted in a 5-fold reduced expression of *Cb*FDH activity at high *Ct*XR expression.

We increased the level of co-expressed *Cb*FDH from 85 to 251 $U/g_{CDW}$ by expressing a high gene copy number of *Cb*FDH in *E. coli* Rosetta 2 (DE3). The threefold increase in *Cb*FDH activity caused a decrease in *Ct*XR activity from maximally 1805 $U/g_{CDW}$ to minimally 1140 $U/g_{CDW}$.

Comparison of the initial and the optimised strain in whole-cell reductions of the native substrate D-xylose showed no difference in initial reduction rates (3 $mmol h^{-1} g_{CDW}^{-1}$). However, when using crude extract for the reduction process, the *E. coli* strain for optimised cofactor regeneration surpassed the initial strain in reaction velocity and product formation 2-fold (7.4 → 15.8 $mmol h^{-1} g_{CDW}^{-1}$). After a reaction time of 24 h, a total of 172 mM xylitol (= 69%) could be obtained with crude extract from the initial strain as compared to 224 mM (= 89%) with the optimised strain.

Bioreduction of 100 mM *o*-chloroacetophenone with 40 $g_{CDW}/L$ of the initial strain resulted in 19 mM optically pure (*S*)-1-(2-chlorophenyl)ethanol. A twofold increase in yield could be obtained under equivalent conditions using the cofactor regeneration optimised *E. coli* strain. This result was further augmented up to 95% product yield by cell permeabilisation (Polymyxin B sulphate) and a shift of pH from 7.5 to 6.2. The enatiomeric excess for the production of (*S*)-1-(2-chlorophenyl)ethanol was always >99.9%.

### Conclusions

We used host and expression engineering to increase intracellular *Cb*FDH activity in *E. coli* whole-cell reduction systems based on *Ct*XR. Improved *Cb*FDH expression was reflected by higher xylose reduction rates and yields obtained with the cell-free crude extract of the optimised strain as compared to the initial strain. However, mass transfer covered the higher cofactor recycling capacity when reducing D-xylose with the two whole-cell catalysts.

The amelioration of the optimised strain was more distinct when used for the asymmetric reduction of *o*-chloroacetophenone. We found that the variation of process conditions (pH, external $NAD^+$) and cell-permeabilisation led to higher product yield.

Therefore, the improvement of the intracellular cofactor regeneration machinery requires concomitant reduction of mass transfer over the membrane to take effect.

## Background

Biocatalysis progressively replaces asymmetric organic synthesis in the production of optically active intermediates as part of multi-step pharmaceutical synthesis. Especially secondary alcohols are frequently used as chiral key intermediates. Biocatalytic reductions of ketone substrates depend on oxidoreductases, which can be used as (partially) purified enzymes or as whole-cell reduction system [1-7]. The stability of enzymes under process conditions is a general concern of bioprocesses. The use of reductases in the protecting environment of the cell has been shown to prolong catalyst lifetimes several fold [8]. A whole-cell reduction system holds the additional advantage of a simple, low cost catalyst preparation.

Enzymatic reduction of ketones requires a stoichiometric amount of cofactor such as nicotinamide (NADH or NADPH), which has to be recycled during the conversion process. Whole-cell systems provide internal cofactor regeneration through the oxidation of a co-substrate [8]. This is advantageous since nicotinamide, especially their reduced form, are too expensive to be used as stoichiometric reagents in a large process.

*Escherichia coli* is the prime organism for the development of whole-cell bioreduction systems. Although *E. coli* scores with many advantages such as quick growth and its ability to over-express several keto-reductases to a high level of activity, internal cofactor regeneration is limited [9-12]. To overcome this problem, an intra- or extracellular $NAD(P)^+$-dependent dehydrogenase can be used, which couples the ketone reduction with the oxidation of a co-substrate [8]. Formate dehydrogenase has become quite important for the coenzyme regeneration process and has been well-characterised [13-16]. This $NAD^+$-specific enzyme catalyses the oxidation of formate to carbon dioxide. The reaction is irreversible under standard conditions, it thermodynamically favours regeneration of NADH, and FDH is active in a wide pH spectrum (6.0-9.0) [17-23]. The usage of isolated formate dehydrogenase for NADH recycling on laboratory and even industrial scale is quite common [24]; nevertheless, there are certain problems to consider. Native FDHs have a rather low operational stability because of active cysteine residues [15, 23]. The relatively low specific activity of *Cb*FDH (≤ 10 U/mg) and its rapid inactivation under biotransformation conditions are additional drawbacks, which complicate the application of this enzyme in a whole-cell reduction process [21, 25, 26].

Our researches as well as reports from other groups show that the intracellular activity of *Candida boidinii* formate dehydrogenase is rather low in co-expression strains [8, 18]. Therefore, the first step was to elevate the protein expression in order to enhance intracellular formate dehydrogenase levels. Single-expression of *Cb*FDH (from strain *C. boidinii* ATCC 18810) under various cultivation conditions and with different *Escherichia coli* strains was performed to find optimised parameters for high formate dehydrogenase expression. Thus, we constructed a FDH optimised strain for the co-expression in a whole-cell reduction system with *Candida tenuis* CBS4435 xylose reductase (*Ct*XR).

The native substrate D-xylose was used to test the product formation of xylitol with crude extract of cell-free protein versus whole-cells. We expected no substrate or product inhibition in this non-toxic reaction. The product xylitol, a five-carbon sugar alcohol, is produced as an alternative natural food sweetener and it has anti-cariogenic properties [27].

We have previously shown that *Ct*XR is able to efficiently reduce aromatic ketones into enantiomerically pure alcohols of industrial relevance [28, 29]. The ketone *o*-chloroacetophenone is reduced to (*S*)-1-(2-chlorophenyl)ethanol with absolute enantioselectivity and high activity. The chiral alcohol can act as a vital key intermediate for the PLK1 kinase inhibitor synthesis. This kinase is highly expressed in tumour cells and is consequently a possible approach for the development of a cancer therapy treatment [30-32]. In this study, we employed the synthesis of (*S*)-1-(2-chlorophenyl)ethanol to characterise and determine the catalytic performance of the optimised *E. coli* strain.

## Results and Discussion

### Host and expression engineering of whole-cell catalysts

In the initial strain, *Ct*XR and *Cb*FDH were cloned into the first and second multiple cloning site of pETDuet-1, respectively, with specific activities of 1805 and 85 $U/g_{CDW}$ for XR and FDH [8]. *Candida boidinii* FDH activity was about 5-fold lower when co-expressed with *Candida tenuis* XR (Table 1). Previous work suggests a limitation in cofactor recycling capacity caused by low formate dehydrogenase activity [8]. Therefore, a clear requirement for further optimisation of *E. coli* whole-cell catalysts based on FDH is to balance cofactor regeneration through enhancement of the intracellular level of coenzyme activity. Differential expression levels from duet plasmids might be due to different rates of translation and elongation, codon usage in the ORF, secondary structure in the mRNA, stability of mRNA and of protein in *E. coli* [33].

We employed host and expression engineering to enhance FDH activity in order to optimise cofactor regeneration in a whole-cell catalyst with *Ct*XR. Given that the decay of mRNA controls gene expression, we chose *E. coli* BL21 star (DE3) as host for the single-expression of the pRSF-1b_FDH plasmid. It is a suitable strain for high-level recombinant protein expression and increases mRNA stability. This bacterial strain contains a mutation in the gene encoding RNaseE (*rne* 131), which is one of the major sources of mRNA degradation.

The truncated RNaseE enzyme lacks the ability to degrade mRNA. The average half-life of mRNA in *E. coli* at 37°C ranges from seconds to maximally 20 minutes and the expression rate depends directly on the inherent mRNA stability [34].

Investigation of the genes encoding for *Ct*XR and *Cb*FDH gene revealed that both genes carry several codons for rare tRNAs in *E. coli*. The percentage of specifically rare arginine codons in the two genes (3.6% *Cb*FDH; 3.4% *Ct*XR) was below the average arginine content of a typical *E. coli* protein (5.7 %) [35]. However, *Cb*FDH carries a tandem rare Arg codon double repeat (Arg210, Arg211) (Figure1), which might lead to ribosomal stalling especially under conditions of arginine tRNA shortage caused by high expression of *Ct*XR. Translational errors arising from rare codon bias include mistranslated amino acid substitutions, frame-shift or premature translational termination [35]. Therefore, the pRSF-1b_FDH plasmid was also transformed into Rosetta 2 (DE3), a strain designed to enhance the expression of eukaryotic proteins in *E. coli*. Rosetta strains supply tRNAs for six codons rarely used in *E. coli* (Arg AGG, AGA; Ile ATA; Leu CTA; Pro CCC; Gly GGA) and carries a chloramphenicol resistance.

```
1    atg aag atc gtt tta gtc tta tat gat gct ggt aaa cac gct gcc gat gag gaa
55   aaa tta tac ggt tgt act gaa aac aaa tta ggt att gcc aat tgg ttg aaa gat
109  caa gga cat gaa tta atc acc acg tct gat aaa gaa ggc gga aac agt gtg ttg
163  gat caa cat ATA cca gat gcc gat att atc att aca act cct ttc cat cct gct
217  tat atc act aag gaa aga atc gac aag gct aaa aaa ttg aaa tta gtt gtt gtc
271  gct ggt gtc ggt tct gat cat att gat ttg gat tat atc aac caa acc ggt aag
325  aaa atc tcc gtt ttg gaa gtt acc ggt tct aat gtt gtc tct gtt gca gaa cac
379  gtt gtg atg acc atg ctt gtc ttg gtt aga aat ttt gtt cca gct cac gaa caa
433  atc att aac cac gat tgg gag gtt gct gct atc gct aag gat gct tac gat atc
478  gaa ggt aaa act atc gcc acc att ggt gcc ggt aga att ggt tac aga gtc ttg
541  gaa aga tta gtc cca ttc aat cct aaa gaa tta tta tac tac gat tat caa gct
595  tta cca aaa gat gct gaa gaa aaa gtt ggt gct aga agg gtt gaa aat att gaa
649  gaa ttg gtt gcc caa gct gat ATA gtt aca gtt aat gct cca tta cac gct ggt
703  aca aaa ggt tta att aac aag gaa tta ttg tct aaa ttc aag aaa ggt gct tgg
757  tta gtc aat act gca aga ggt gcc att tgt gtt gcc gaa gat gtt gct gca gct
811  tta gaa tct ggt caa tta aga ggt tat ggt ggt gat gtt tgg ttc cca caa cca
865  gct cca aaa gat cac cca tgg aga gat atg aga aac aaa tat ggt gct ggt aac
919  gcc atg act cct cat tac tct ggt act act tta gat gct caa act aga tac gct
973  caa ggt act aaa aat atc ttg gag tca ttc ttt act ggt aag ttt gat tac aga
1027 cca caa gat atc atc tta tta aac ggt gaa tac gtt act aaa gct tac ggt aaa
1081 cac gat aag aaa taa
```

**Figure 1. Nucleotide sequence of *Cb*FDH**. Number of rare codons in *E. coli* were calculated using the Rare Codon Calculator (http://nih.mbi.ucla.edu/ at the UCLA). ATA = rare Ile codon; aga, agg = rare Arg codons.

FDH activities in the cell-free crude extracts of BL21 (DE3) strain harbouring a high copy pRSF-1b plasmid for single FDH expression was determined to 402 $U/g_{CDW}$. Retransformation of the plasmid into BL21 (DE3) Star as well into Rosetta 2 (DE3) resulted in 10 and 12 % increased FDH activities (Table 1). A reduction in the induction temperature from 25 to 18°C further improved functional FDH expression in different *E. coli* strains. Highest FDH activity was found in the cell-free crude extract of Rosetta 2 (DE3) at an induction temperature of 18°C. The change of cultivation temperature slows down the overall protein expression process thereby decreasing translational mistakes yielding more protein that is active [36].

**Table 1. *Cb*FDH activities of different single- and co-expression *E. coli* strains at various reaction temperatures.**

| ***E coli* strain** | ***Cb*FDH [$U/g_{CDW}$] at 25°C[1]** | ***Cb*FDH [$U/g_{CDW}$] at 18°C[1]** |
|---|---|---|
| BL21r | 402 | 450 |
| BL21r star | 458 | 551 |
| R2r | 445 | 614 |
| BL21d | 85 | 110 |
| BL21d+r | 146 | 186 |
| R2d | 102 | 132 |
| R2d+r | 202 | 251 |

[1] standard errors ≤ 15%.

Table 1 shows that co-expression of *Ct*XR and *Cb*FDH (BL21d) led to 4-5-fold decreased FDH activities in the cell-free crude extracts compared to single FDH expressions.

Increased FDH gene copy numbers by co-transformation of these strains with pRSF-1b_FDH resulted in 40 % higher formate dehydrogenase activity.

Use of the rare codon strain Rosetta 2 (DE3), high FDH gene copy number, and an induction temperature of 18°C yielded 251 $U/g_{CDW}$ FDH in the co-expression strain (Table 1). This is equal to a 3-fold increase in functional FDH expression as compared to the initial co-expression strain (BL21d).

How altered cofactor regeneration activities translate into initial rates and productivities of whole cell reductions were tested with the initial strain (BL21d) and the FDH optimised strain (R2d+r).

## Xylitol production by whole-cells and cell-free crude extracts

We used xylose as substrate in whole-cell reductions for several reasons: xylose is the natural substrate of *Candida tenuis* xylose reductase, it is non-toxic and its reduction product xylitol is used worldwide as non-cariogenic sugar substitute.

Whole-cell reductions of xylose by the initial strain (BL21d) and the FDH optimised strain (R2d+r) resulted in equal reduction rates and final conversions. We obtained initial rates of 3 $mmolh^{-1}g_{CDW}^{-1}$ and a conversion of ~55 % for both strains. The lack of reduction rate acceleration by the FDH optimised strain suggests no limitation in intracellular cofactor regeneration but serious mass transfer limitation of xylose and/or formate import into the cell.

Repetition of the experiment with the same amount of disrupted cells (cell-free crude extract) showed initial rates of 7.4 $mmolh^{-1}g_{CDW}^{-1}$ and 15.8 $mmolh^{-1}g_{CDW}^{-1}$ obtained with BL21d and R2d+r, respectively (Figure 2). Furthermore, the activities of the substrate conversion with crude extract (124 $U/g_{CDW}$ for BL21d, 263 $U/g_{CDW}$ for R2d+r) agree well with the photometric measurements of both strains indicating no limitation due to substrate-product inhibition.

A two-fold higher initial rate and higher conversion obtained with the FDH optimised strain reflects the need for balanced reductase and cofactor regeneration activities in coupled redox-reactions. Furthermore, a hindrance of substrate uptake into the cell is confirmed by higher reduction rates and yields obtained with disrupted cells as compared to intact cells.

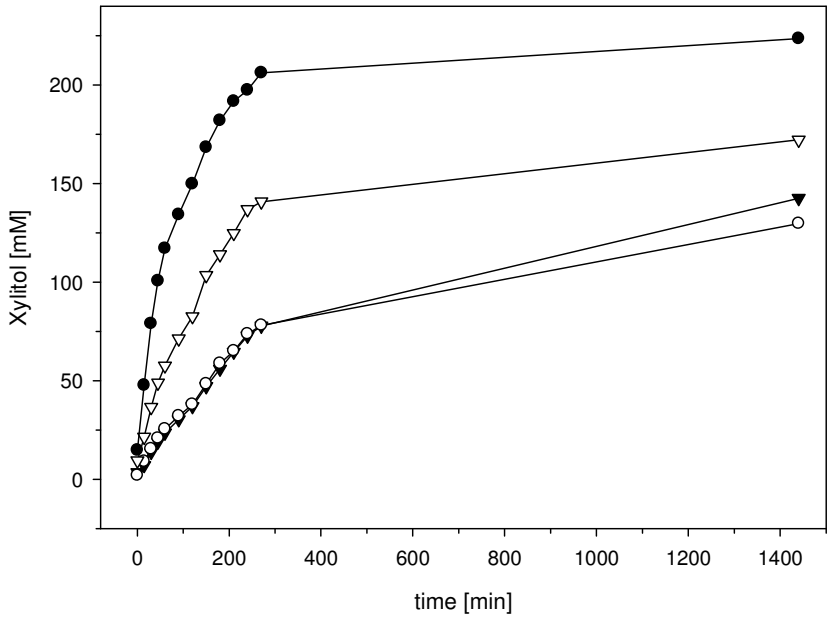


**Figure 2. Whole-cell versus crude extract catalysed reduction of D-xylose by *E. coli* BL21d and R2d+r.** The analytical yield is expressed as concentration of xylitol formed from the initial substrate concentration. Type of *E. coli* strain is indicated by symbols: ● R2d+r crude extract; ▽ BL211d crude extract; ▼ BL21d whole-cell catalyst; ○ R2d+r whole-cell catalyst. Reaction conditions: D-xylose [250 mM], sodium formate [300 mM], cells (10 $g_{CDW}$/L).

## Whole-cell reduction of *o*-chloroacetophenone

We have previously reported xylose reductase from *Candida tenuis* as an unexpected catalyst for synthesis of optically pure (*S*)-1-(2-chlorophenyl)ethanol. 1-(2-chlorophenyl)ethanols are used as chiral key intermediates for the synthesis of PLK1 kinase inhibitors. This kinase is highly expressed in tumour cells and its inhibitors are candidates for the development of new cancer therapy treatments [29-31]. Despite extensive screening a limited number of microbial and plant cells inheriting *o*-chloroacetophenone reductase activity have been identified to date [37-46]. The number of isolated reductases exhibiting useful activity towards *o*-chloroacetophenone is still very small [25, 47-49] and *o*-chloroacetophenone turned out as highly toxic towards isolated xylose reductase [29].

We therefore developed an *E. coli* whole-cell catalyst co-expressing recombinant xylose reductase and FDH for the conversion of *o*-chloroacetophenone to 1-(2-chlorophenyl)ethanol with perfect *S*-stereoselectivity.

We compared the initial strain (BL21d) and the FDH optimised strain (R2d+r) in the reduction of *o*-chloroacetophenone. The usage of the FDH optimised strain under standard conditions (pH 7.5) leads to a higher product yield (35 mM) compared to the initial strain (19 mM) (Table 2). Isolated xylose reductase shows two-fold higher activities at pH 6.2 as compared to 7.5. By lowering the reaction pH from 7.5 to 6.2 we could increase product yields 40% for the initial strain and 80% for the FDH optimised strain (Table 2).

The results of the xylitol production with whole-cells and crude extract had already shown that mass transfer could be an obstacle for a whole-cell catalyst. To improve substrate and co-substrate uptake by cell permeabilisation, the antibiotic Polymyxin B sulphate was added to the reaction mixture. The positive effect was reflected in the enhanced results for both whole-cell catalysts (Table 2). The alcohol production with the initial strain could be improved from 46 mM (pH 7.5) to 78 mM (pH 6.2). The FDH optimised strain was even able to produce 95 mM of enantiomerically pure (*S*)-1-(2-chlorophenyl)ethanol.

**Table 2. (*S*)-1-(2-chlorophenyl)ethanol yield with different whole-cell catalysts.** The product yield is elevated by a lower pH and the addition of Polymyxin B sulphate.

| *E coli* | pH | XR [U/$g_{cdw}$] | FDH [U/$g_{CDW}$] | Substrate [mM] | Yield [mM] [1] | Yield [mM] [1, 2] |
|---|---|---|---|---|---|---|
| BL21d | 7.5 | 1406 | 110 | 100 | 19 | 46 |
| BL21d | 6.2 | 2226 | 48 | 100 | 27 | 78 |
| R2d+r | 7.5 | 1140 | 251 | 100 | 35 | 67 |
| R2d+r | 6.2 | 2024 | 189 | 100 | 63 | 95 |

[1] Determined for a reaction time of 8 h. [2] Additive: 36µM Polymyxin B sulphate and 500 µM $NAD^+$.

## Conclusions

Aim of this study was to increase intracellular FDH activity in an *E. coli* whole-cell bioreduction system based on *Candida tenuis* xylose reductase. By combining an *E. coli* expression host providing rare codon tRNAs with a high *Cb*FDH gene copy number and optimising the cultivation conditions we could increase FDH activity measured in the cell-free crude extract from 85 U/$g_{CDW}$ of the initial strain to 251 U/$g_{CDW}$ of the optimised strain. However, increased co-factor regenerating activity (FDH activity) did not translate into higher xylitol yields in whole-cell bioreductions of D-xylose. In comparison, the same reaction with crude extract of the cell-free enzymes revealed the superiority of the optimised strain. R2d+r is able to surpass the initial strain BL21d in reaction velocity and product yield. This indicates a possible problem with the overall substrate and co-substrate uptake into the cell.

Since *o*-chloroacetophenone is an industrially relevant substrate, we used it for whole-cell reductions to evaluate the initial and the FDH optimised strain. The best results were obtained after lowering of pH and the addition of Polymyxin B sulphate (95%).

# Methods

## Chemicals

NADH (sodium salt; ≥98% pure), $NAD^+$ (free acid; ≥97.5% pure), D-xylose, and ampicillin were purchased at Roth (Karlsruhe, Germany). Sodium formate, Polymyxin B sulphate, kanamycin, chloramphenicol, and *o*-chloroacetophenone were bought from Sigma-Aldrich (Vienna, Austria). B-Per® Reagent was from Pierce (Rockford, Illinois, USA) and 1-(2-chlorophenyl)ethanol from Alfa Aesar (Karlsruhe, Germany). All other chemicals were purchased from either Sigma-Aldrich or Roth, and were of the highest purity available.

## Strains and plasmids

The *Escherichia coli* strains were BL21 (DE3) and Rosetta 2 (DE3) from Novagen (VWR International GmbH, Vienna, Austria), BL21 star (DE3) from Invitrogen (Carlsbad, California, USA). The development of the initial strain *E. coli* BL21 (DE3) harbouring *Ct*XR and *Cb*FDH genes on a pETDuet-1 vector (Novagen) was described elsewhere [8]. This plasmid has two multiple cloning sites each controlled by a T7*lac* promoter and a ribosome binding site for high-level protein expression. In addition, it carries a ColE1 replicon and *bla* gene (ampicillin resistance; Amp). This construct will be termed BL21d from here on.

We chose the pRSF-1b plasmid for the over-expression of *Cb*FDH since its replicon RSF1030 is compatible with the pETDuet-1 vector (ColE1). The *Cb*FDH gene (from strain *Candida boidinii* ATCC 18810) was amplified from the plasmid pETDuet-1 [8] by a PCR using *Pfu* DNA polymerase and primers, which provided PagI (compatible ends to *Nco*I) and AvrII restriction sites.

Forward primer: 5'- GGTGGTTCATGAAGATCGTTTTAG- 3'

Reverse primer: 5'- GTAAACACGATAAGAAATAACCTAGGGGTGGT- 3'

These restriction sites enabled the ligation of the *Cb*FDH gene into the multiple cloning site of pRSF-1b (*Nco*I, *Avr*II), yielding pRSF-1b_FDH. The plasmid was transformed into BL21 (DE3) and the correct integration of the gene verified by sequencing.

Bacterial transformation following a standard protocol was used to obtain four variants of the BL21d strain. For the first variant, we transformed pRSF-1b_FDH with BL21d. This FDH over-expression strain will be called BL21d+r below. The pRSF-1b vector controls the transcription process through a T7*lac* promoter and provides a kanamycin (Kan) resistance. Next, Bl21d and Bl21d+r were transformed into *E. coli* Rosetta 2 (DE3). This bacterial strain provides tRNAs for rare codon usage and has a chloramphenicol (Cam) resistance gene. In the following, these two strains will be referred to as R2d and R2d+r.

The fourth strain was generated by transformation of both plasmids (pETDuet-1_XR_FDH, pRSF-1b_FDH) with *E. coli* BL21 star (DE3), an RNaseE mutant strain for increased mRNA stability, yielding BL21d+r star.

In addition, three different *E. coli* strains for the single-expression of *Candida boidinii* formate dehydrogenase from the high copy number pRSF-1b plasmid were developed using a standard electroporation method.

All bacterial strains, their features, and abbreviations used within this paper are summarised in table 3.

**Table 3. Bacterial expression strains and their abbreviations used within this study.**

| *E. coli* | Plasmid | Enzyme | Antibiotic | Features | Abbreviation |
|---|---|---|---|---|---|
| BL21 (DE3) | pETDuet-1 | *Ct*XR, *Cb*FDH | Amp | high-level protein expression strain | BL21d |
| BL21 (DE3) | pETDuet-1 pRSF-1b | *Ct*XR, *Cb*FDH; *Cb*FDH | Amp, Kan | high-level protein expression strain | BL21d+r |
| Rosetta 2 (DE3) | pETDuet-1 | *Ct*XR, *Cb*FDH | Cam, Amp | tRNAs for rare codons | R2d |
| Rosetta 2 (DE3) | pETDuet-1 pRSF-1b | *Ct*XR, *Cb*FDH; *Cb*FDH | Cam, Amp, Kan | tRNAs for rare codons | R2d+r |
| BL21 star (DE3) | pETDuet-1 pRSF-1b | *Ct*XR, *Cb*FDH; *Cb*FDH | Amp, Kan | Increased mRNA stability | BL21d+r star |
| BL21 (DE3) | pRSF-1b | *Cb*FDH | Kan | high-level protein expression strain | BL21r |
| Rosetta 2 (DE3) | pRSF-1b | *Cb*FDH | Cam, Kan | tRNAs for rare codons | R2r |
| BL21 star (DE3) | pRSF-1b | *Cb*FDH | Kan | Increased mRNA stability | BL21r star |

## Cultivation of strains

All *E. coli* strains were cultivated in 1000 mL baffled shaking flasks using 200 mL LB-medium. In addition, the culture media contained the respective antibiotics for each strain (table 3). The flasks were shaken at 130 rpm and 37°C in a Certomat® BS-1 incubator from Satorius. The temperature for production of recombinant protein was 25°C and alternatively 18°C. Once the optical density reached 1.5 (± 10%), the cells were induced with 250 µM isopropyl-β-D-thiogalactopyranosid (IPTG). After 20 h cultivation, a sample for activity measurements was taken and broken up by lysis with B-Per® reagent. The residual cells were harvested by centrifugation for further use in whole-cell bioreductions.

### Enzyme activity measurements in the cell-free extract

The reductase and dehydrogenase activity was assessed spectrophotometrically at 25°C, monitoring the reduction of NADH and the oxidation of $NAD^+$ were 340 nm over a period of 5 minutes. Typical results ranged from 0.1 to 1.0 $\Delta A$/min. Activity measurements were performed with a Beckman DU-800 spectrophotometer using 100 mM potassium phosphate buffer (PPB; pH 7.5, 6.2).

The standard protocol of the reductase activity used the native substrate D-xylose in a concentration of 700 mM (pH 7.5, 6.2). The reaction was started with NADH in a final concentration of 310 µM.

The standard assay for *Cb*FDH employed 200 mM sodium formate and the reaction started with the addition of 2mM $NAD^+$ (pH 7.5, 6.2).

### Whole-cell bioreduction of D-xylose

Experiments were carried out at 30°C on an end-over-end rotator (SB3 from Stuart) at 30 rpm in 15 mL Sarstedt tubes. Half of the cell material of each strain was diluted to a concentration of 10 $g_{CDW}$/L with 100 mM PPB (pH 7.5). A 10 mL sample was filled into a reaction vial containing the co-substrate sodium formate (300 mM).

Three rounds of mechanical pulping with a French Pressure Cell Press (American Instrument Company, Silver Springs, Maryland, USA) broke the rest of the cells. The crude extract was diluted with 100 mM PPB (pH 7.5) for a crude extract concentration equivalent to 10 $g_{CDW}$/L. After ultra-centrifugation, 10 mL of supernatant was transferred in a Sarstedt tube with co-substrate. Furthermore, 1 mM $NAD^+$ was added to the crude extract solution.

The addition of the substrate D-xylose in a concentration of 250 mM started the biotransformation reaction. In the first 4 ½ hours 200 µL samples were taken in a certain time interval. The reaction stopped by the addition of ethanol in a 1:1 (v/v) ratio after 24 h. Centrifugation separated the biomass and the supernatant containing the product was prepared for high performance liquid chromatography (HPLC) analysis.

## Whole-cell bioreduction of *o*-chloroacetophenone

The substrate conversion experiments were carried out at 30°C on an end-over-end rotator (SB3 from Stuart) at 30 rpm in 2 mL Eppendorf tubes. *E. coli* cells were diluted to a concentration of 40 $g_{CDW}$/L with 100 mM potassium phosphate buffer (pH 7.5, 6.2). The substrate *o*-chloroacetophenone, dissolved in 5% ethanol (by weight), was applied in a concentration of 100 mM. The co-substrate sodium formate was added always with a 50 mM higher concentration than that of the ketone substrate.

For experiments with permeable cells, Polymyxin B sulphate (36 µM) and 500 µM $NAD^+$ was additionally used in the whole-cell sample mix. The total reaction volume of the batch experiments was 1 mL and the biotransformation started with the addition of substrate. The reaction stopped by the addition of ethanol in a 1:1 (v/v) ratio after eight hours. The samples were centrifuged and the supernatant prepared for (HPLC) analysis.

## Analytical methods

Analysis of whole-cell reduction was performed on a LaChrom HPLC system (Merck-Hitachi) equipped with an L-7400 UV-detector, a Merck L-7490 RI detector, and a thermo-stated column oven.

### *D-xylose*

The samples were analysed on an Aminex HPX-87H column (Bio Rad Laboratories, Vienna, Austria) using sulphuric acid (5 mM $H_2SO_4$) as eluent at a flow rate of 0.6 mL/min and a temperature of 65°C. For the matter of xylitol peak identification adequate standards with known concentrations were measured. Reported yields of xylitol product are always from analytical data, because product isolation was beyond the scope of this study.

***o-chloroacetophenone***

The samples were analysed on a reversed phase CHIRALPAK AD-RH column from Daicel (VWR International, Vienna, Austria) at 210 nm absorbance. The eluent was an acetonitrile and water solution (20:80 v/v) at a flow rate of 0.5 mL/min and a column temperature of 40°C. For alcohol peak identification adequate standards with known concentrations were measured. Reported product yields are always from analytical data.

## References


[1] Goldberg K, Schroer K, Lütz S, Liese A: **Biocatalytic ketone reduction- a powerful tool for the production of chiral alcohols- part II: Whole cell reductions.** *Appl Microbiol Biotechnol* 2007, **76**:249-255.

[2] Goldberg K, Schroer K, Lütz S, Liese A: **Biocatalytic ketone reduction- a powerful tool for the production of chiral alcohols- part I: Processes with isolated enzymes.** *Appl Microbiol Biotechnol* 2007, **76**:237-248.

[3] Faber K**: Reduction Reactions.** In *Biotransformations in Organic Chemistry.* 4th edition. Edited by: Faber K. Berlin: Springer; 2000: 177-219.

[4] Brommarius AS, Riebel BR: **Production of enantiomerically pure hydrophobic alcohols: Comparison of different routes and reactor configurations.** In *Biocatalysis.* 1st edition. Edited by: Brommarius AS, Riebel BR. Weinheim: Wiley-VCH; 2004: 456-569.

[5] Daußmann T, Hennemann HG, Rosen TC, Dünkelmann P: **Enzymatic technologies for the synthesis of chiral alcohol derivatives.** *Chem. Ing. Techno.* 2006; **78**: 249–255.

[6] Straathof AJJ, Panke S, Schmid A: **The production of fine chemicals by biotransformations.** *Curr Opin Biotechnol* 2002, **13**: 538-556.

[7] Bertau M: **Novel Developments in Biocatalytic Organic Chemistry.** *Curr Org Chem* 2002, **6**: 987-1014.

[8] Kratzer R, Pukl M, Egger S, Nidetzky B: **Whole-cell bioreduction of aromatic α-keto esters using *Candida tenuis* xylose reductase and *Candida boidinii* formate dehydrogenase co-expressed in *Escherichia coli*.** *Microl Cell Fact* 2008, **7**: 37-48.

[9] Ema T, Yagasaki H, Okita N, Takeda M, Sakai T: **Asymmetric reduction of ketones using recombinant *E. coli* cells that produce a versatile carbonyl reductase with high enantioselectivity and broad substrate specificity.** *Tetrahedron* 2006, **62**: 6143-6149.

[10] Ernst M, Kaup B, Müller M, Bringer-Meyer S, Sahm H: **Enantioselective reduction of carbonyl compounds by whole cell biotransformation, combining a formate dehydrogenase and a (*R*)-specific alcohol dehydrogenase.** *Appl Microbiol Biotechnol* 2005, **66**: 629-634.

[11] Kataoka M, Rohani LPS, Yamamoto K, Wada M, Kawabata H, Kita K, Yanase H, Shimizu S: **Enzymatic production of ethyl (*R*)-4-chloro-3-hydroxybutanoate: Asymmetric reduction of ethyl 4-chloro-3-oxobutanoate by an *Escherichia coli* transformant expressing the aldehyde reductase gene from yeast.** *Appl Microbiol Biotechnol* 1997, **48**: 699-703.

[12] Xu Z, Liu Y, Fang L, Jiang X, Ling K, Cen P: **Construction of a two-strain system for asymmetric reduction of ethyl 4-chloro-3-oxobutanoate to (*S*)-4-chloro-3-hydroxybutanoate ethyl ester.** *Appl Microbiol Biotechnol* 2006, **70**: 40-46.

[13] Popov VO, Lamzin VS: **$NAD^+$-dependent formate dehydrogenase.** *Biochem. J.* 1994, **301**: 625-643.

[14] Labrou NE, Rigden DJ: **Active-site characterization of *Candida boidinii* formate dehydrogenase.** *Biochem. J.* 2001, **354**: 455-463.

[15] Tishkov VI, Popov VO: **Protein engineering of formate dehydrogenase.** *Biomol Eng* 2006, **23**: 89-110.

[16] Tishkov VI, Popov VO: **Catalytic Mechanism and Application of Formate Dehydrogenase.** *Biochem.* 2004, **69**: 1252-1267.

[17] Ernst M, Kaup B, Müller M, Bringer-Meyer S, Sahm H: **Enantioselective reduction of carbonyl compounds by whole cell biotransformation, combining a formate dehydrogenase and a (*R*)-specific alcohol dehydrogenase.** *Appl Microbiol Biotechnol* 2005, **66**: 629-634.

[18] Weckbecker A, Hummel W: **Improved synthesis of chiral alcohols with *Escherichia coli* cells co-expressing pyridine nucleotide transhydrogenase, $NADP^+$-dependent alcohol dehydrogenase and $NAD^+$-dependent formate dehydrogenase.** *Biotechnol Letters* 2004, **26**: 1739-1744.

[19] Kaup B, Bringer-Meyer S, Sahm H: **Metabolic engineering of *Escherichia coli:* Construction of an efficient biocatalyst for *D*-mannitol formation in a whole-cell biotransformation.** *Appl Microbiol Biotechnol* 2004, **64**: 333-339.

[20] Bräutigam S, Bringer-Meyer S, Weuster-Botz D: **Asymmetric whole cell biotransformation in biphasic ionic liquid/water- systems by use of recombinant *Escherichia coli* with intracellular cofactor regeneration.** *Tetrahedron: Asymm* 2007, **18**: 1883-1887.

[21] Yamamoto H, Mitsuhashi K, Kimoto N, Kobayashi Y, Esaki N: **Robust NADH-regenerator: Improved α–haloketone-resistant formate dehydrogenase.** *Appl Microbiol Biotechnol* 2005, **67**: 33-39.

[22] Mesentsev AV, Lamzin VS, Tishkov VI, Ustinnikova TB, Popov VO: **Effect of pH on kinetic parameters of $NAD^+$-dependent formate dehydrogenase.** *Biochem J* 1997, **321**: 475-480.

[23] Neuhauser W, Steininger M, Haltrich D, Kulbe KD, Nidetzky B: **A pH-controlled fed-batch process can overcome inhibition by formate in NADH-dependent enzymatic reductions using formate dehydrogenase-catalysed coenzyme regeneration.** *Biotechnol Bioeng* 1998, **60**: 277-282.

[24] Bommarius AS, Schwarm M, Stingl K, Kottenhahn M, Huthmacher K, Drauz K: **Synthesis and use of enantiometrically pure tert-leucine.** *Tetrahedron-Asymmetry* 1995, **6**: 2851–2888.

[25] Gröger H, Chamouleau F, Orologas N, Rollmann C, Drauz K, Hummel W, Weckbecker A, Mayer O: **Enantioselective reduction of ketones with 'designer cells' at high substrate concentrations: Highly efficient access to functionalized optically active alcohols.** *Angew Chem Int Ed* 2006, **45**: 5677-5681.

[26] Slusarczyk H, Felber S, Kula MR, Pohl M: **Stabilisation of $NAD^+$-dependent formate dehydrogenase from *Candida boidinii* by site-directed mutagenesis of cysteine residues.** *Eur J Biochem* 2000, **267**: 1280-1289.

[27] Nidetzky B, Neuhauser W, Haltrich D, Kulbe KD: **Continuous Enzymatic Production of Xylitol with Simultaneous Coenzyme Regeneration in a Charged Membrane Reactor.** *Biotech. Bioeng.* 1996, **52**: 387-396.

[28] Kratzer R, Nidetzky B: **Identification of *Candida tenuis* xylose reductase as highly selective biocatalyst for the synthesis of chiral α-hydroxy esters and improvement of its efficiency by protein engineering.** *Chem Comm* 2007, **10**: 1047-1049.

[29] Kratzer R, Pukl M, Egger S, Vogl M, Brecker L, Nidetzky B: **Enzyme identification and development of a whole-cell biotransformation for asymmetric reduction of *o*-chloroacetophenone.** *Biotech. Bioeng. (in press)*.

[30] Santamaria A, Neef R, Eberspächer U, Eis K, Husemann M, Mumberg D, Prechtl S, Schulze V, Siemeister G, Wortmann L, Barr FA, Nigg EA: **Use of the novel Plk1 inhibitor ZK-thiazolidinone to elucidate functions of Plk1 in early and late stages of mitosis.** *Mol Biol Cell* 2007, **18**: 4024-4036.

[31] Sato Y, Onozaki Y, Sugimoto T, Kurihara H, Kamijo K, Kadowaki C, Tsujino T, Watanabe A, Otsuki S, Mitsuya M, Iida M, Haze K, Machida T, Nakatsuru Y, Komatani H, Kotani H, Iwasawa Y: **Imidazopyridine derivatives as potent and selective Polo-like kinase (PLK) inhibitors.** *Bioorg Med Chem Lett* 2009, **19:** 4673-4678.

[32] Rheault TR, Donaldson KH, Badiang-Alberti JG, Davis-Ward RG, Andrews CW, Bambal R, Jackson JR, Cheung M: **Heteroaryl-linked 5-(1*H*-benzimidazol-1-yl)-2-thiophenecarboxamides: Potent inhibitors of polo-like kinase 1 (PLK1) with improved drug-like properties.** *Bioorg Medic Chem Lett* 2010, **20**: 4587-4592.

[33] Sørensen HP, Mortensen KK: **Advanced genetic strategies for recombinant protein expression in *Escherichia coli*.** *Journ. Biotechn.* 2005, **115**: 113-128.

[34] Régnier P, Arraiano CM: **Degradation of mRNA in bacteria: emergence of ubiquitous features.** *BioEss.* 2000, **22**: 235-244.

[35] Kane JF: **Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coil*.** *Curr. Opin. Biot.* 1995, **6**: 494-500.

[36] Chalmers JJ, Kim E, Telford JN, Wong EY, Tacon WC, Shuler ML, Wilson DB: **Effects of temperature on *Escherichia coli* overproducing beta-lactamase or human epidermal growth factor.** *Appl Environ Microbiol* 1990, **56**: 104-111.

[37] Andrade LH, Comasseto JV, Rodrigues DF, Pellizari VH, Porto ALM: **Enantioselective reduction of *ortho*-substituted acetophenones by bacterial strains isolated from medium enriched with biphenyl or diesel fuel.** *J Mol Catal B: Enzym* 2005, **33**: 73-79.

[38] Andrade LH, Piovan L, Pasquini MD: **Improving the enantioselective bioreduction of aromatic ketones mediated by *Aspergillus terreus* and *Rhizopus oryzae*: The role of glycerol as a co-solvent.** *Tetrahedron: Asymm* 2009, **20**: 1521-1525.

[39] Kagohara E, Pellizari VH, Comasseto JV, Andrade LH, Porto ALM: **Biotransformations of substituted phenylethanols and acetophenones by environmental bacteria.** *Food Technol Biotech* 2008, **46**: 381-387.

[40] Kurbanoglu EB, Zilbeyaz K, Kurbanoglu NI, Kilic H: **Enantioselective reduction of substituted acetophenones by *Aspergillus niger*.** *Tetrahedron: Asymm* 2007a, **18**: 1159-1162.

[41] Kurbanoglu EB, Zilbeyaz K, Kurbanoglu NI, Kilic H: **Asymmetric reduction of acetophenone analogues by *Alternaria alternata* using ram horn peptone.** *Tetrahedron: Asymm* 2007b, **18**: 2332-2335.

[42] Kurbanoglu EB, Zilbeyaz K, Ozdal M, Taskin M, Kurbanoglu NI: **Asymmetric reduction of substituted acetophenones using once immobilized *Rhodotorula glutinis* cells.** *Bioresource Technol* 2010, **101**: 3825-3829.

[43] Nakamura K, Matsuda T: **Asymmetric reduction of ketones by the acetone powder of *Geotrichum candidum*.** *J Org Chem* 1998, **63**: 8957–8964.

[44] Ou Z, Wu J, Yang L, Cen P: **Asymmetric reduction of chloroacetophenone to produce chiral alcohols with microorganisms.** *Korean J Chem Eng* 2008, **25**: 124-128.

[45] Salvi NA, Chattopadhyay S: **Asymmetric reduction of halo-substituted arylalkanones with *Rhizopus arrhizus*.** *Tetrahedron: Asymm* 2008, **19**: 1992-1997.

[46] Xie Q, Wu J, Xu G, Yang L: **Asymmetric reduction of *o*-chloroacetophenone with *Candida pseudotropicalis* 104.** *Biotechnol Prog* 2006, **5**: 1301-1304.

[47] Itoh N, Mizuguchi N, Mabuchi M: **Production of chiral alcohols by enantioselective reduction with NADH-dependent phenylacetaldehyde reductase from *Corynebacterium* strain, ST-10.** *J Mol Catal B Enzym* 1999, **6**: 41–50.

[48] Yang Y, Zhu D, Piegata TJ, Hua L: **Enzymatic ketone reduction: Mapping the substrate profile of a short-chain alcohol dehydrogenase (YMR226c) from *Saccharomyces cerevisiae*.** *Tetrahedron: Asymm* 2007, **18**: 1799-1803.

[49] Zhu D, Rios BE, Rozzell JD, Hua L: **Evaluation of substituent effects on activity and enantioselectivity in the enzymatic reduction of aryl ketones.** *Tetrahedron: Asymm* 2005, **16**: 1541-1546.

## Figures

**Figure 1. Nucleotide sequence of *Cb*FDH.**

Number of rare codons in *E. coli* were calculated using the Rare Codon Calculator (http://nih.mbi.ucla.edu/ at the UCLA). ATA = rare Ile codon; aga, agg = rare Arg codons.

**Figure 2. Whole-cell versus crude extract catalysed reduction of D-xylose by *E. coli* BL21d and R2d+r.**

The analytical yield is expressed as concentration of xylitol formed from the initial substrate concentration. Type of *E. coli* strain is indicated by symbols: ● R2d+r crude extract; ▽ BL211d crude extract; ▼ BL21d whole-cell catalyst; ○ R2d+r whole-cell catalyst. Reaction conditions: D-xylose [250 mM], sodium formate [300 mM], cells (10 $g_{CDW}$/L).

## Tables

**Table 1. *Cb*FDH activities in different single- and co-expression *E. coli* strains at various reaction temperatures.**

**Table 2. (*S*)-1-(2-chlorophenyl)ethanol yield with different whole-cell catalysts.**

The product yield is elevated by a lower pH and the addition of Polymyxin B sulphate.

**Table 3. Bacterial expression strains and their abbreviations used in this study.**

# Appendix

## Introduction

The asymmetric synthesis of chiral building blocks for the production of optically pure pharmaceuticals has become an important field of research. Especially secondary alcohols are frequently used as chiral key intermediates, and their preparation by asymmetric reduction of a precursor ketone is generally preferred to resolution of a racemic mixture of alcohols. Biocatalytic reductions of ketone substrates depend on oxidoreductases, which are used as (partially) purified enzymes or as whole-cell reduction system [1-7].

A general concern of bioprocesses is the stability of enzymes under process conditions. The whole-cell bioreduction system provides a stable cellular environment for the enzyme and the use of reductases in the protecting environment of the cell has been shown to prolong catalyst lifetimes several fold [8]. A whole-cell reduction system holds the additional advantage of a simple, low cost catalyst preparation.

Enzymatic reduction of ketones requires a stoichiometric amount of coenzyme (NADH or NADPH), which has to be recycled during the conversion process. Whole-cell systems provide internal coenzyme regeneration through the oxidation of a co-substrate [8]. This is very welcome since the nicotinamide coenzymes, especially their reduced form, are too expensive to be used as stoichiometric reagents in a large process.

*Escherichia coli* (*E. coli*) is the prime organism for the development of whole-cell bioreduction systems. Although *E. coli* scores with many advantages such as quick growth and its ability to over-express several keto-reductases to a high level of activity, internal cofactor regeneration is limited [9-12]. To overcome this problem, an intra- or extracellular $NAD(P)^+$-dependent dehydrogenase can be used, which couples the ketone reduction with the oxidation of a co-substrate [8].

An ideal co-substrate for the recycling system should be quickly taken up by the cell and should yield no by-product upon oxidation. The enzyme formate dehydrogenase (FDH) meets these criteria perfectly. FDH is $NAD^+$-specific and catalyses the oxidation of formate to carbon dioxide [10, 13-16]. The usage of a formate dehydrogenase for the coenzyme regeneration process has several advantages. $CO_2$ evaporation is irreversible under standard conditions and thermodynamically favours regeneration of NADH. Formate is a cheap substrate and the reaction product carbon dioxide is removed easily. The main disadvantage of native FDHs is the rather low operational stability especially at elevated temperatures because of active cysteine residues. The chemical modification or oxidation of active cysteine residues leads to rapid inactivation of FDHs. For instance, during xylose reduction to xylitol, *Candida boidinii* formate dehydrogenase (*Cb*FDH) was quickly deactivated by a pH shift during the formate oxidation and inhibited by the formate anion [17].

Another drawback of native formate dehydrogenases is the high stereoselectivity towards $NAD^+$, which disqualifies the enzyme for an application in a NADPH regeneration process. Although there have been several publications on the use of isolated formate dehydrogenase for NADH regeneration, the relatively low specific activity of *Cb*FDH (≤ 10 U/mg) and its instability complicate the application of this enzyme in whole-cell reduction process [16, 18, 19].

To compensate for the current lack of highly efficient whole-cell catalysts based on FDH, I developed a whole-cell bioreduction system with improved cofactor regeneration capacity. The expression of formate dehydrogenase from *Candida boidinii* ATCC 18819 was several fold increased by host and expression engineering in the present study. *Cb*FDH was co-expressed with xylose reductase from *Candida tenuis* CBS4435 (*Ct*XR; AKR2B5) in various *E. coli* strains (Figure 1). *Ct*XR is a member of the aldo-keto reductase (AKR) subfamily of mainly NAD(P)(H)-dependent reductases. AKRs are found in a wide range of different organisms and cell types, and serve a number of physiological functions [20].

*Ct*XR reduces D-xylose to the corresponding polyol xylitol by using NADPH or NADH [21, 22]. In addition, it is able to reduce several ketone and aldehyde substrates, whose non-reacting parts can have a wide range of chemical structures [21, 23, 24]. Therefore, whole-cell reduction systems based on xylose reduction can be used for the production of several optically active alcohols [25, 26]

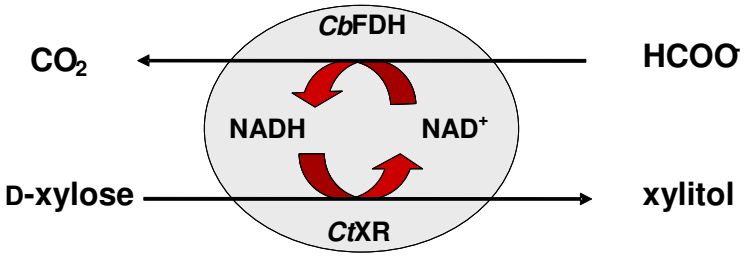


**Figure 1. Xylose reduction and cofactor regeneration by co-expression of *Ct*XR and *Cb*FDH.**

An example for the application of biocatalysts is the inhibition of a cancer related enzyme: The serine-threonine kinase PLK1 (polo-like kinase 1) is known to be a key regulator of mitosis and of cell cleavage in eukaryotes. This kinase is highly expressed in tumour cells and is therefore a possible approach for the development of a cancer therapy treatment.

So far, several *in vivo* studies have confirmed that a competitive inhibition of the PLK1 kinase shows anti-tumour efficiency [27, 28]. Thiophenebenzimdazole and –imidazopyridine are considered the most successful PLK1 suppressors. The asymmetric reduction of *o*-chloroacetophenone is a preferred synthetic route for the production of (*S*)-1-(2-chlorophenyl)ethanol, which acts as a vital key intermediate in the PLK1 kinase inhibitor synthesis [28, 29]. Although *o*-chloroacetophenone is not a native substrate for reductases previous studies showed high activity and perfect *S*-enantioselectivity (ee >99.9%) for NADH-dependent ketone reductions with the enzyme *Ct*XR.

I utilised the improved whole-cell catalyst in order to test the reduction of ketones such as *o*-chloroacetophenone, whose purpose of application is described above. With the intention of further improving substrate conversion, I also conducted experimental set-ups where Polymyxin B sulphate was added to make the cell membrane permeable and to ease the substrate uptake into the *E. coli* bacterium. Other substrates in my studies were ethyl 4-cyanobenzoylformate and ethyl 4-chlorobenzoylformate, which are both less toxic for the whole-cell catalyst compared to *o*-chloroacetophenone. Furthermore, I varied the type of reaction vial in order to find out, if the size of the sample vessel influences the amount of product formation when the volume is constant.

# Materials and Methods

## Chemicals and media

All media were autoclaved at 121°C for 20 minutes. All thermolabile stock-solutions (antibiotics, IPTG) were filter sterilised.

LB-medium (Low Salt Luria Bertani): 10 g/L soy peptone, 5 g/L yeast extract, and 5 g/L NaCl were dissolved in dd$H_2O$ and autoclaved. Antibiotics were added after sterilisation.

SOC medium (Super Optimal Broth with catabolite repression; 250 mL): For solution A 0.865 g glucose was dissolved in 50 mL dd$H_2O$ (3.46 g/L glucose). For solution B 5 g peptone (20 g/L), 0.145 g NaCl (0.58 g/L), 1.25 g yeast extract (5 g/L), 0.045 g KCl (0.18 g/L), and 0.625 g $MgSO_4$ (2.46 g/L) were dissolved in 200 mL dd$H_2O$.

Solutions A and B were autoclaved separately, pooled after sterilisation, and 1 mL sterile aliquots stored at -20°C.

LB-Agar: 10 g/L soy peptone, 5 g/L yeast extract, 5 g/L NaCl, and 15 g/L agar were dissolved in dd$H_2O$ and autoclaved. Antibiotics were added after sterilisation.

Potassium phosphate buffer (PPB): For 100 mM PPB (pH 7.5; 1 L) 0.0286 mol of $KH_2PO_4$ and 0.0713 mol of $K_2HPO_4$ were dissolved in dd$H_2O$, the pH adjusted, and the solution autoclaved.

For 100 mM PPB (pH 6.2; 1 L) 0.09 mol of $KH_2PO_4$ and 0.0099 mol of $K_2HPO_4$ were dissolved in dd$H_2O$, the pH adjusted, and the solution autoclaved.

HPLC-standards: 5 mM ethyl 4-cyanomandelate in EtOH; 5 mM *o*-chloroacetophenone in EtOH; 5 mM (*S*)-1-(2-chlorophenyl)ethanol in EtOH; 10 mM ethyl 4-chloromandelate in EtOH.

Antibiotics: 115 mg/mL ampicillin; 50 mg/mL kanamycin; 34 mg/mL chloramphenicol

IPTG stock: 1 M isopropyl-β-D-thiogalactopyranosid

## Strains and plasmids

All *Escherichia coli* strains were stored as glycerol-stocks (60% glycerol) at -70°C.

**Table 1. Bacterial strains and their abbreviations used in this study.**

*Ct*XR and *Cb*FDH co-expression strains. *Cb*FDH single-expression strains.

| ***E. coli*** | **Plasmid** | **Enzyme** | **Antibiotic** | **Features** | **Abbreviation** |
|---|---|---|---|---|---|
| BL21 (DE3) | pET11a<br>pRSF-1b | *Ct*XR,<br>*Cb*FDH | Amp/ Kan | Two-plasmid system | BL21XR+FDH |
| BL21 (DE3) | pETDuet-1 | *Ct*XR, *Cb*FDH | Amp | high-level protein expression strain | BL21d |
| BL21 (DE3) | pETDuet-1<br>pRSF-1b | *Ct*XR, *Cb*FDH;<br>*Cb*FDH | Amp/ Kan | high-level protein expression strain | BL21d+r |
| Rosetta 2 (DE3) | pETDuet-1 | *Ct*XR, *Cb*FDH | Cam/ Amp | tRNAs for rare codons | R2d |
| Rosetta 2 (DE3) | pETDuet-1<br>pRSF-1b | *Ct*XR, *Cb*FDH;<br>*Cb*FDH | Cam/ Amp/ Kan | tRNAs for rare codons | R2d+r |
| BL21 star (DE3) | pETDuet-1<br>pRSF-1b | *Ct*XR, *Cb*FDH;<br>*Cb*FDH | Amp/ Kan | Increased mRNA stability | BL21d+r star |
| BL21 (DE3) | pRSF-1b | *Cb*FDH | Kan | high-level protein expression strain | BL21r |
| Rosetta 2 (DE3) | pRSF-1b | *Cb*FDH | Cam/ Kan | tRNAs for rare codons | R2r |
| BL21 star (DE3) | pRSF-1b | *Cb*FDH | Kan | Increased mRNA stability | BL21r star |

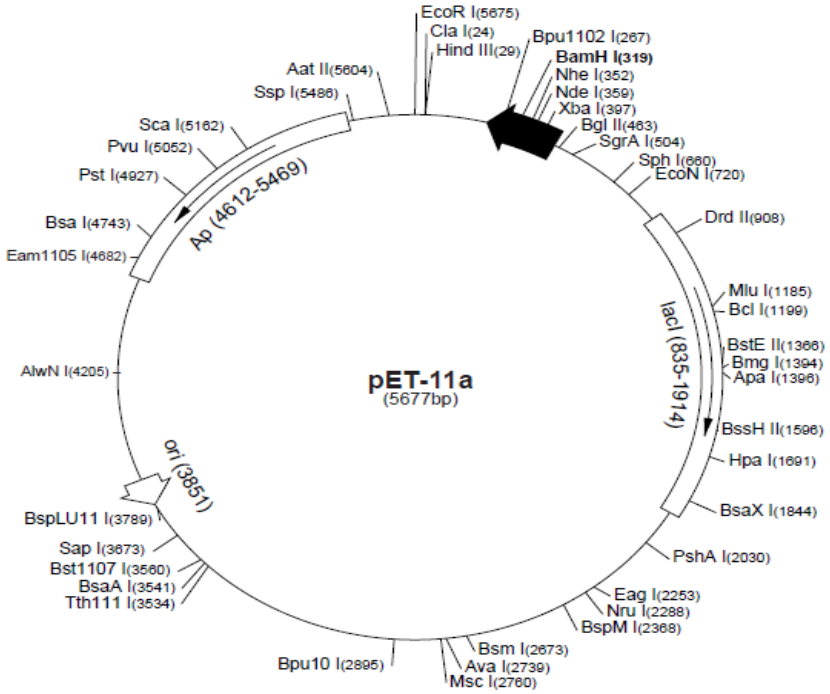


**Figure 2. pET-11a vector map.**

The pET-11a vector carries an N-terminal T7•Tag® sequence, a *Bam*HI cloning site, and encodes for an ampicillin resistance [30].

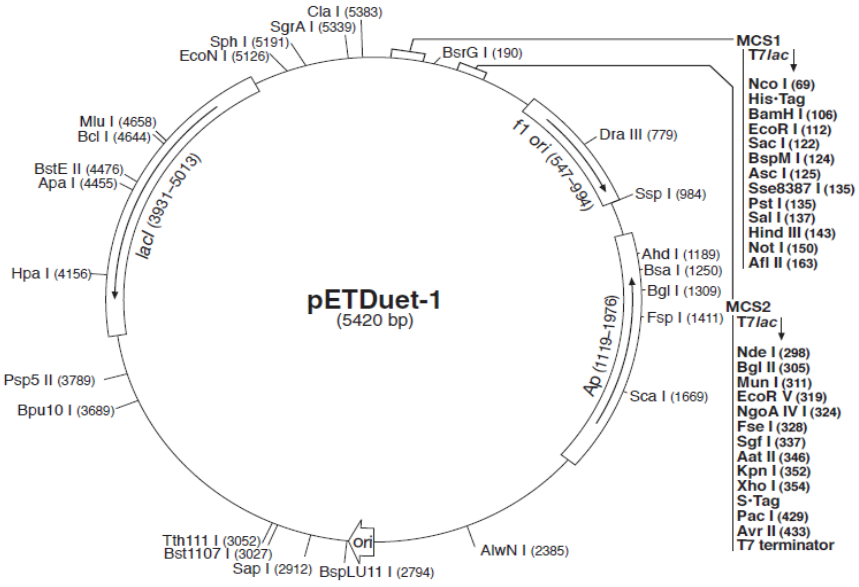


**Figure 3. pETDuet-1 vector map.**

pETDuet™-1 is designed for the co-expression of two target genes. It therefore contains two multiple cloning sites (MCS), which are each preceded by a T7 promoter/*lac* operator and a ribosome binding site (rbs). The vector also carries the pBR322-derived ColE1 replicon, *lac*I gene and ampicillin resistance gene [31].

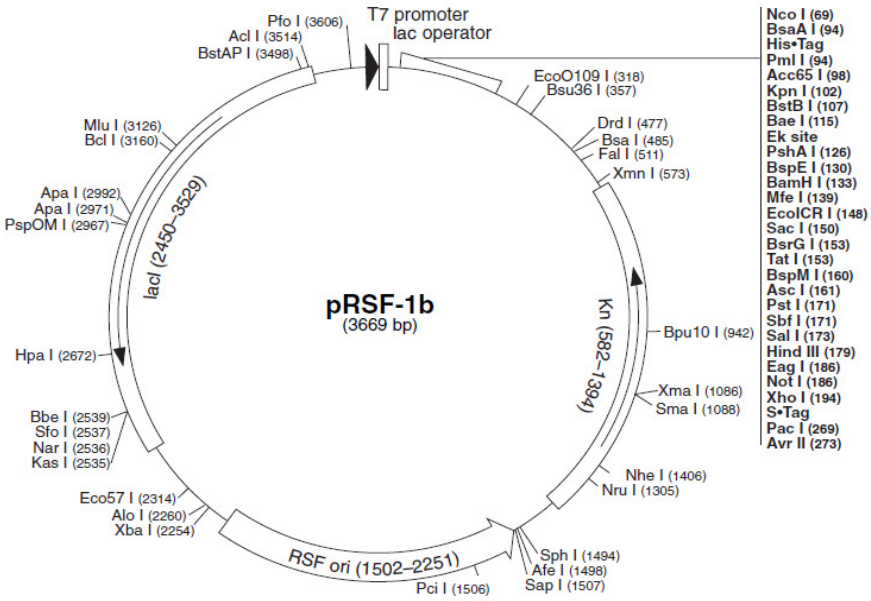


**Figure 4. pRSF-1b vector map.**

pRSF-1b carries a T7 promoter and *lac* operator to control transcription, a replication origin derived from RSF1030, and kanamycin antibiotic resistance [32].

## Kits and Protocols

### Promega Wizard® Plus SV Minipreps

An *E. coli* colony was streaked out evenly with a sterile toothpick on a selective media plate, which was then incubated overnight at 37°C. Cell material from a quarter to half of an agar plate was abraded with a sterile toothpick. The DNA purification system from Promega (Madison, Wisconsin, USA) was used as described in the manual. The DNA was eluted with 50 µL of distilled water.

### Pierce® BCA Protein Assay Kit

For the determination of total protein concentration the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific Corp., Waltham, Massachusetts, USA) was employed. A purified sample or standard (25 µL) was mixed with BCA™ reagent (500 µL) in a cuvette. These were then covered with aluminum and incubated for 30 minutes at 37°C. The absorption was determined at 562 nm with a Beckman DU-800 spectrophotometer.

### Protocol for the preparation of electro-competent cells

Autoclave: 6x 200 mL LB-medium in 1 L shaking flasks, 2x 50 mL LB-medium in 300 mL shaking flasks, 4x 400 mL centrifuge beakers, 2 L $H_2O$, 1 L glycerol (10% in $H_2O$), Eppendorf tubes, and pipette tips.

Keeping everything sterile is crucial, as strains do not carry an antibiotic resistance gene.

Protocol: Inoculate two pre-cultures (PC; 50 mL) and incubate them over night at 37°C, 140 rpm. Prepare glycerol stocks from PCs. Inoculate main-cultures (MC; 6x 200 mL) with 4 mL of PC and incubate them at 37°C, 140 rpm. Check $OD_{600}$ every hour and harvest cells at $OD_{600}$ 0.5 - 0.8 (after approx. 2.5 h). Put the MC shaking flasks on ice in the cooling room (4°C) until they are cooled down (ca. 20 min). Pour the culture medium into the sterile, cooled centrifuge beakers and centrifuge cells (5,000 rpm, 4°C, 30 min).

Resuspend pellets in 10 mL of sterile, cold $H_2O$. Merge the pellets of two centrifuge beakers (→ 3 beakers) and fill the beaker up to 2/3 of its volume with cold, sterile $H_2O$ (first washing step). Centrifuge (5,000 rpm, 4°C, 30 min). Resuspend pellets in 10 mL cold, sterile glycerol (10%; second washing step). Merge all pellets in one beaker; then fill the beaker up to 2/3 of its volume with cold, sterile glycerol (10%), and centrifuge (5,000 rpm, 4°C, 30 min). Resuspend pellet in ca. 30 mL cold, sterile glycerol (10%; third washing step) and transfer everything into a sterile falcon (50 mL). Centrifuge (5,000 rpm, 4°C, 30 min). Resuspend pellet in 4 mL cold, sterile glycerol (10%) and make aliquots of 100 µL. Quick-freeze them using liquid nitrogen and store the electro-competent cells at -70°C.

Make a transformation with a small plasmid afterwards to check the quality of the electro-competent cells.

**Transformation protocol for electro-competent cells**

Thaw electro-competent cells (80 µL) on ice and add 2 µL DNA (60-100 ng/µL). Transfer the mix into a cooled electroporation cuvette. Make sure that the cells reach the bottom by slightly tapping the cuvette on the table. Dry the contacts carefully and place the electroporation cuvette in the Micro Pulser (Bio-Rad, Vienna, Austria). For *E. coli* transformations use the “Ec.2”- program. Pulse the cells once and add quickly 480 µL SOC medium. Transfer the transformation sample into an Eppendorf tube and incubate them on a thermomixer (37°C, 600 rpm, 4 h). Plate an adequate amount of cells on LB-agar plates containing the respective antibiotics.

**Cultivation of strains**

Inoculate the PC (50 mL LB-medium; 50 µL antibiotic) with 30 µL of bacterial glycerol-stock or a single bacterial colony from an agar plate. Conditions: 37°C, 130 rpm, ON. Measure $OD_{600}$. Inoculate the MC (200 mL LB-medium; 200 µL antibiotic) with 5 mL of PC. Conditions: 37°C, 130 rpm, ca. 2 ½ h. Measure $OD_{600}$. At $OD_{600}$ ≥1.3 the cultures are induced with 250 µM IPTG (50 µL of 1 M stock) and 200 µL Amp is added if strain contains ampicillin resistance. Conditions: 25°C/ 18 °C, 130 rpm, 20 h. Optionally, 10 mM $MgSO_4$ (*E. coli* stabilisation) or a drop of PPG (antifoam) is added to cultures

**Enzyme activity measurements in the cell-free extract**

Take a 1 mL culture sample and break the cells open by lysis with 1 mL B-Per® reagent. Activity measurement in the cell-free extract (CE): *Ct*XR (700 mM D-xylose + 310 µM NADH); *Cb*FDH (200 mM sodium formate + 2 mM $NAD^+$); 100 mM PPB (pH 7.5, 6.2) is used as blank and for dilutions if needed.

**Whole-cell bioreduction**

Harvest the cell cultures and centrifuge for 15 min at 5,000 rpm. Resuspend the pellet in PPB to a dilution of 40 $g_{CDW}$/L (check $OD_{600}$). Add 1 mL cells (40 $g_{CDW}$/L) to the Co-substrate (HCOONa). After the Co-substrate (Co-Su) is dissolved (ca. 10 min; pivot carefully), transfer cells into 2 mL Eppendorf tubes or cryogenic vials containing carbonyl substrate (+63 µL EtOH) and put samples on end-over-end rotator. For permeabilisation experiments, Polymyxin B sulphate (36 µM, 180 µM) and $NAD^+$ (50 µM, 2.5 mM) are added as well. Conditions: 30°C, 30 rpm. Stop the reaction by adding 1 mL EtOH (sample should be well-mixed). Centrifugation (13,000 rpm, 10 min) and storage of supernatant in -20°C freezer for further analysis.

## Solubility test of chiral alcohols

For the solubility test, various concentrations of chiral alcohols were diluted in 10 mL PPB (100 mM, pH 7.5).

Ethyl-4-caynomandelate (E4CM): MW 205.21 g/mol

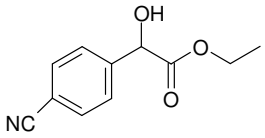


**Table 2. Weighted samples for ethyl 4-cyanomandelate at different concentrations.**

| E4CM [mM] | Weighted sample [g] | Additive |
|---|---|---|
| 10 | 0.0205 | 5% EtOH |
| 20 | 0.041 | 5% EtOH |
| 30 | 0.0615 | 5% EtOH |
| 50 | 0.0101 | 5% EtOH |

1-(2-chlorophenyl)ethanol (CPE): MW 156.61 g/mol

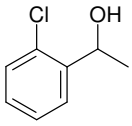


**Table 3. Weighted samples for 1-(2-chlorophenyl)ethanol at different concentrations.**

| CPE [mM] | Weighted sample [g] | Additive |
|---|---|---|
| 10 | 0.0156 | 5% EtOH |
| 20 | 0.0312 | 5% EtOH |
| 30 | 0.0468 | 5% EtOH |
| 40 | 0.0624 | 5% EtOH |
| 50 | 0.078 | 5% EtOH |
| 60 | 0.0936 | 5% EtOH |
| 70 | 0.1092 | 5% EtOH |

# Results

## Protein concentration measurements

The co-expression strain BL21d+r was cultivated under various induction temperatures. We furthermore tried to stabilise the FDH by media supplementation with the substrate sodium formate. The reference strain was cultivated without the inductor IPTG. A lower cultivation temperature affects the concentration of soluble protein positively (see Table 4).

**Table 4. Results of the BCA Protein Assay.**

BL21d+r was cultivated under various conditions prior to cell disruption by B-Per® and subsequently, the protein concentration in the cell-free crude extract was determined. The pellet was resuspended in the lysis reagent to reveal the amount of remaining protein after extraction.

| Strain | Sample | Cultivation conditions | Protein [mg/mL] |
|---|---|---|---|
| BL21d+r | supernatant | 25°C, no IPTG | 2.808 |
| BL21d+r | supernatant | 25°C, 250 µM IPTG | 2.172 |
| BL21d+r | supernatant | 18°C, 250 µM IPTG | 3.624 |
| BL21d+r | supernatant | 25°C, 250 µM IPTG, 10 mM HCOONa | 3.328 |
| BL21d+r | pellet | 25°C, no IPTG | 0.745 |
| BL21d+r | pellet | 25°C, 250 µM IPTG | 0.436 |
| BL21d+r | pellet | 18°C, 250 µM IPTG | 0.394 |
| BL21d+r | pellet | 25°C, 250 µM IPTG, 10 mM HCOONa | 0.331 |

## Activity measurements

During my work, I constructed five different *Escherichia coli* strains for the co-expression of *Candida tenuis* xylose reductase and *Candida boidinii* formate dehydrogenase (BL21 (DE3) pETDuet-11a_XR + pRSF-1b_FDH; BL21 (DE3) pETDuet-1_XR_FDH + pRSF-1b_FDH; BL21 star (DE3) pETDuet-1_XR_FDH + pRSF-1b_FDH; Rosetta 2 (DE3) pETDuet-1_XR_FDH; Rosetta 2 (DE3) pETDuet-1_XR_FDH + pRSF-1b_FDH).

Three strains were generated for the single-expression of *Cb*FDH (BL21 (DE3) pRSF-1b_FDH; BL21 star (DE3) pRSF-1b_FDH; Rosetta 2 (DE3) pRSF-1b_FDH).

Since *Cb*FDH expression is repressed by the co-expression with *Ct*XR, the first strategy to enhance FDH activity was to slow down protein expression by lowering the incubation temperature from 25°C to 18°. The idea was that cooler cultivation conditions would slow down the XR enzyme production and therefore give the formate dehydrogenase more time for expression before rare building blocks are consumed.

To over-express *Candida boidinii* formate dehydrogenase in the cell, *E. coli* strains harbouring pET11-a_XR or pETDuet-1_XR_FDH were co-transformed with a high copy number plasmid coding for *Cb*FDH (pRSF-1b_FDH). However, loss of FDH activity in the co-expression strains was still several fold reduced as compared to strains solely expressing FDH. In order to support formate dehydrogenase expression, I re-transformed the plasmids into different *Escherichia coli* strains.

*E. coli* BL21 star (DE3) was picked for its ability to increase mRNA stability and Rosetta 2 (DE3) for providing tRNAs for rare codons. The best results were obtained with R2d+r when cultivated at 18°C.

Table 5 shows *Ct*XR and *Cb*FDH activities determined in the cell-free crude extracts. The column “features” displays different conditions for culture cultivation and used additives.

**Table 5. Activity measurements of five *E. coli* strains under various cultivation conditions.**

| *E. coli* | Antibiotic | *Ct*XR [U/$g_{CDW}$] | *Cb*FDH [U/$g_{CDW}$] | Features |
|---|---|---|---|---|
| BL21XR+FDH | Amp/ Kan | 1770 | 83 | 250 µM IPTG; 25°C |
| BL21d | Amp | 2887 | 145 | 250 µM IPTG; 18°C; PPB (pH 6.2); 10 mM $MgSO_4$ |
| BL21d | Amp | 1822 | 110 | 250 µM IPTG; 18°C; 10 mM $MgSO_4$ |
| BL21d | Amp | 2596 | 133 | 250 µM IPTG; 18°C; PPB (pH 6.2) |
| BL21d | Amp | 2226 | 48 | 250 µM IPTG; 25°C; PPB (pH 6.2) |
| BL21d+r | Amp/ Kan | 878 | 105 | no IPTG; 25°C |
| BL21d+r | Amp/ Kan | 2322 | 153 | 250 µM IPTG; 25°C; 10 mM HCOONa |
| BL21d+r | Amp/ Kan | 1292 | 167 | 1 mM IPTG; 18°C |
| BL21d+r | Amp/ Kan | 1300 | 140 | 1 mM IPTG; 18°C; 10 mM HCOONa |
| BL21d+r | Amp/ Kan | 1953 | 172 | 250 µM IPTG; 25°C |
| BL21d+r | Amp/ Kan | 2077 | 188 | 250 µM IPTG; 18°C |
| BL21d+r | Amp/ Kan | 1345 | 141 | 250 µM IPTG; 18°C; PPB (pH 6.2) |
| BL21d+r | Amp/ Kan | 1940 | 113 | 250 µM IPTG; 25°C; PPB (pH 6.2) |
| R2d | Cam/ Amp | 1800 | 112 | 250 µM IPTG; 18°C |
| R2d | Cam/ Amp | 1464 | 120 | 250 µM IPTG; 18°C; PPB (pH 6.2) |
| R2d | Cam/ Amp | 1756 | 62 | 250 µM IPTG; 25°C; PPB (pH 6.2) |
| R2d+r | Cam/ Amp/ Kan | 1501 | 109 | 250 µM IPTG; 25°C; 100 ml MC |
| R2d+r | Cam/ Amp/ Kan | 2813 | 190 | 250 µM IPTG; 25°C; PPG |
| R2d+r | Cam/ Amp/ Kan | 1875 | 195 | 250 µM IPTG; 18°C; PPB (pH 6.2); 10 mM $MgSO_4$ |
| R2d+r | Cam/ Amp/ Kan | 1325 | 226 | 250 µM IPTG; 25°C |
| R2d+r | Cam/ Amp/ Kan | 1135 | 320 | 250 µM IPTG; 18°C |
| R2d+r | Cam/ Amp/ Kan | 1900 | 225 | 250 µM IPTG; 18°C; 10 mM $MgSO_4$ |
| R2d+r | Cam/ Amp/ Kan | 1735 | 203 | 250 µM IPTG; 18°C; PPB (pH 6.2) |
| R2d+r | Cam/ Amp/ Kan | 1880 | 100 | 250 µM IPTG; 25°C; PPB (pH 6.2) |

## Whole-cell bioreduction

Ethyl 4-cyanobenzoylformate

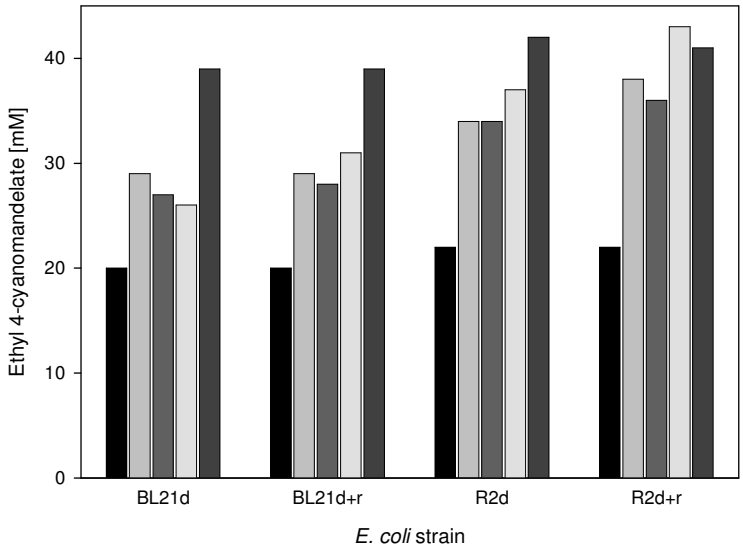


**Figure 5. WCR of ethyl 4-cyanobenzoylformate, variation of substrate concentration, growth medium, and reaction tube.**

The product is ethyl 4-cyanomandelate [mM]. Reaction time 5 h. Reaction pH 7.5.

*Left to right:* Substrate 50 mM 4CN, 2 mL reaction volume in a 2 mL Eppendorf tube. Substrate 50 mM 4CN, 1 mL reaction volume in cryogenic vial. Substrate 50 mM 4CN, 1 mL reaction volume in cryogenic vial, 10 mM $MgSO_4$ added to growth medium. Substrate 100 mM 4CN, 1 mL reaction volume in cryogenic vial. Substrate 100 mM, 2 mL reaction volume in a 2 mL Eppendorf tube.

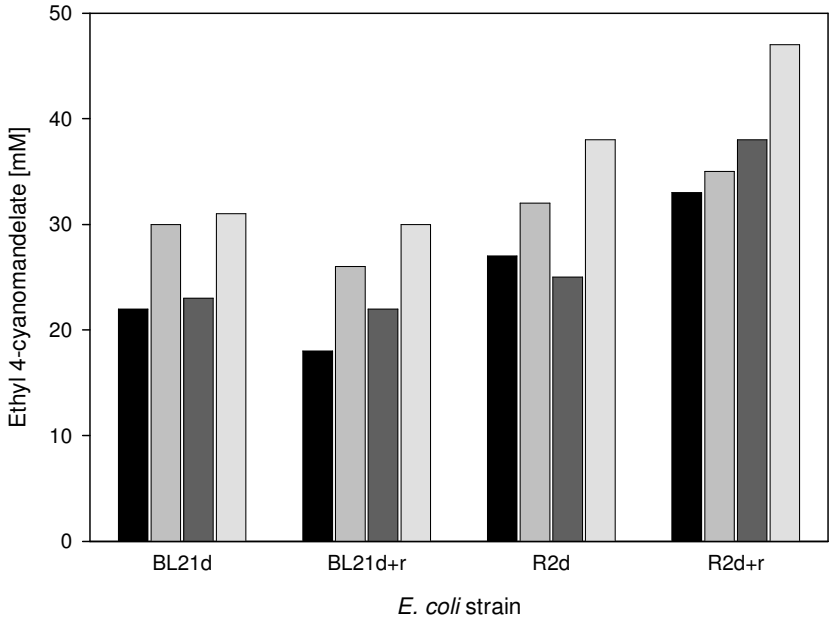


**Figure 6. WCR of ethyl 4-cyanobenzoylformate, variation of substrate concentration and reaction tube.**

The product is ethyl 4-cyanomandelate [mM]. Reaction time 4 ½ h. Reaction pH 7.5.

*Left to right:* Substrate 50 mM 4CN, 1 mL reaction volume in a 2 mL Eppendorf tube. Substrate 50 mM 4CN, 1 mL reaction volume in cryogenic vial. Substrate 100 mM 4CN, 1 mL reaction volume in a 2 mL Eppendorf tube. Substrate 100 mM, 1 mL reaction volume in cryogenic vial.

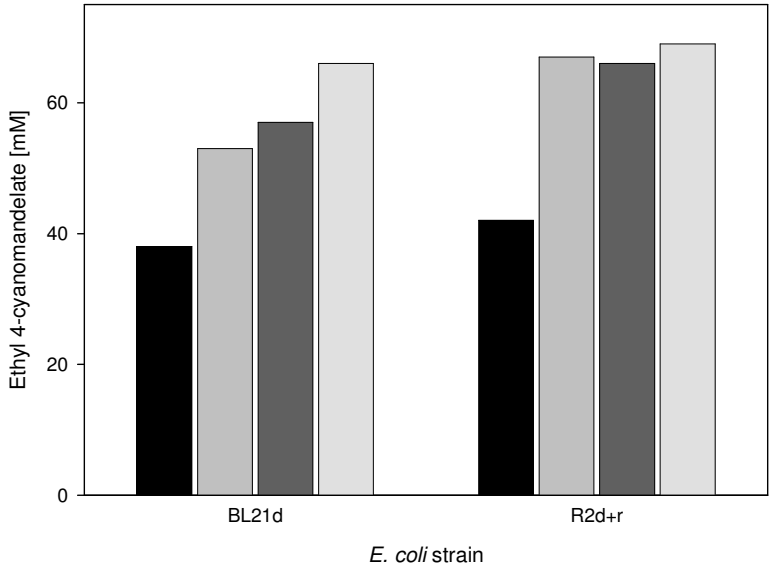


**Figure 7. WCR of ethyl 4-cyanobenzoylformate, variation of substrate concentration, growth medium, and additives.**

The product is ethyl 4-cyanomandelate [mM]. Reaction time 8 h. Reaction pH 6.2. Reaction volume 1 mL in a 2 mL Eppendorf tube.

*Left to right:* Substrate 100 mM 4CN. Substrate 100 mM 4CN, 36 µM PM, 500 µM $NAD^+$. Substrate 200 mM 4CN, 36 µM PM, 500 µM $NAD^+$. Substrate 200 mM 4CN, 180 µM PM, 500 µM $NAD^+$, 10 mM $MgSO_4$ added to growth medium.

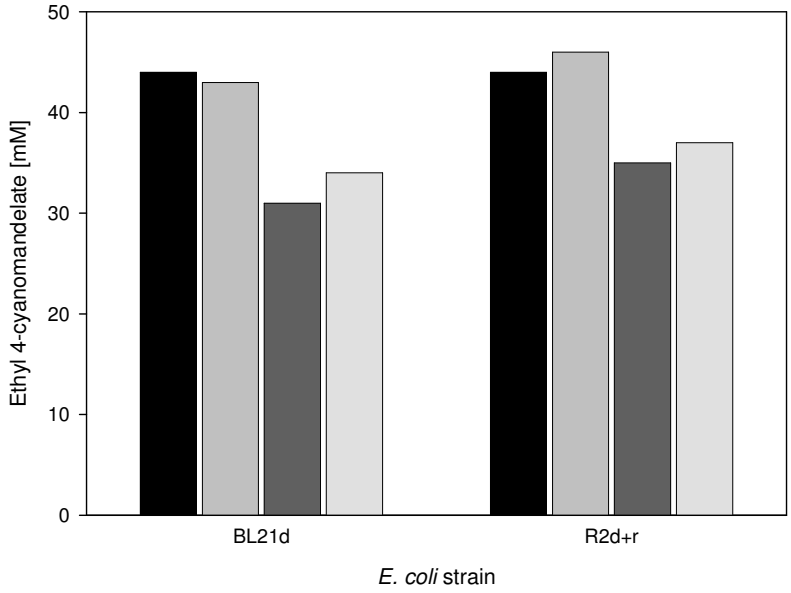


**Figure 8. WCR of ethyl 4-cyanobenzoylformate, variation of pH, growth medium, and reaction tube.**

The product is ethyl 4-cyanomandelate [mM]. Reaction volume 1 mL. Reaction time 8 h.

*Left to right:* Substrate 50 mM 4CN, pH 6.5. Substrate 50 mM 4CN, cryogenic vial, pH 6.5. Substrate 50 mM 4CN, 10 mM $MgSO_4$ added in growth medium, pH 7.5. Substrate 50 mM 4CN, cryogenic vial, 10 mM $MgSO_4$ added to growth medium, pH 7.5.

*o*-chloroacetophenone

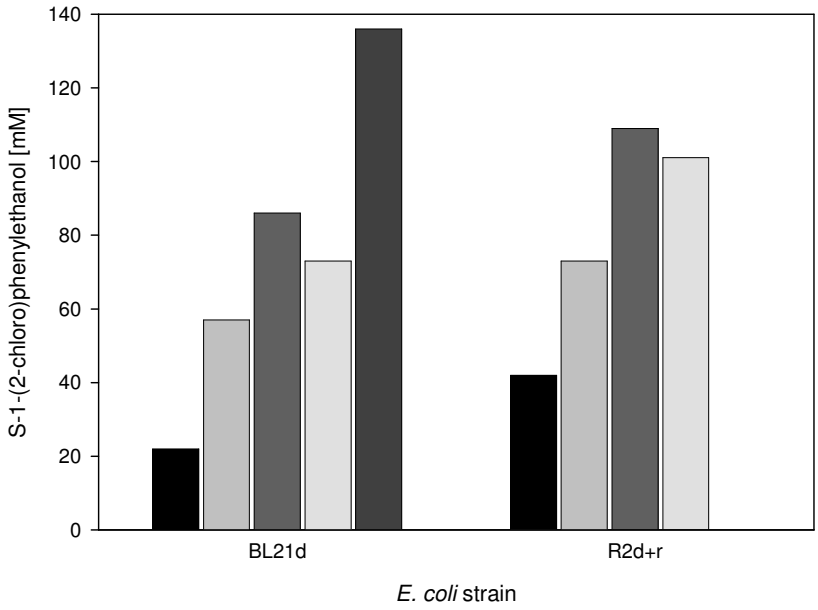


**Figure 9. WCR of *o*-chloroacetophenone, variation of substrate concentration, growth medium, and additives.**

The product is (*S*)-1-(2-chlorophenyl)ethanol [mM]. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction time 8 h. Reaction pH 6.2.

*Left to right:* Substrate 100 mM oChl. Substrate 100 mM oChl, 36 µM PM, 500 µM $NAD^+$. Substrate 200 mM oChl, 36 µM PM, 500 µM $NAD^+$. Substrate 200 mM oChl, 180 µM PM, 500 µM $NAD^+$. Substrate 200 mM oChl, 180 µM PM, 2.5 mM $NAD^+$, 10 mM $MgSO_4$ added to growth medium.

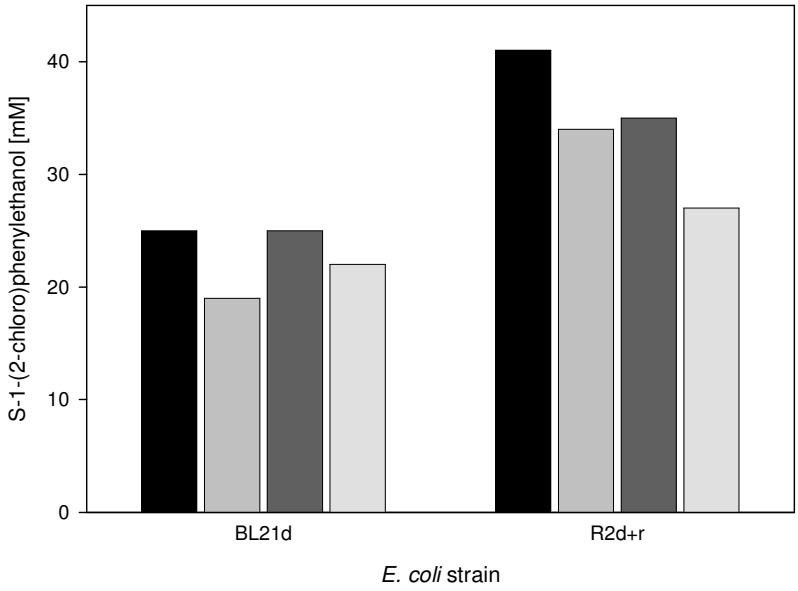


**Figure 10. WCR of *o*-chloroacetophenone, variation of pH, growth medium, and reaction tube.**

The product is (*S*)-1-(2-chlorophenyl)ethanol [mM]. Reaction volume 1 mL. Reaction time 8 h.

*Left to right:* Substrate 50 mM oChl, pH 6.5. Substrate 50 mM oChl, pH 6.5, cryogenic vial. Substrate 50 mM oChl, pH 7.5, 10 mM $MgSO_4$ added to growth medium. Substrate 50 mM oChl, pH 7.5, cryogenic vial, 10 mM $MgSO_4$ added to growth medium.

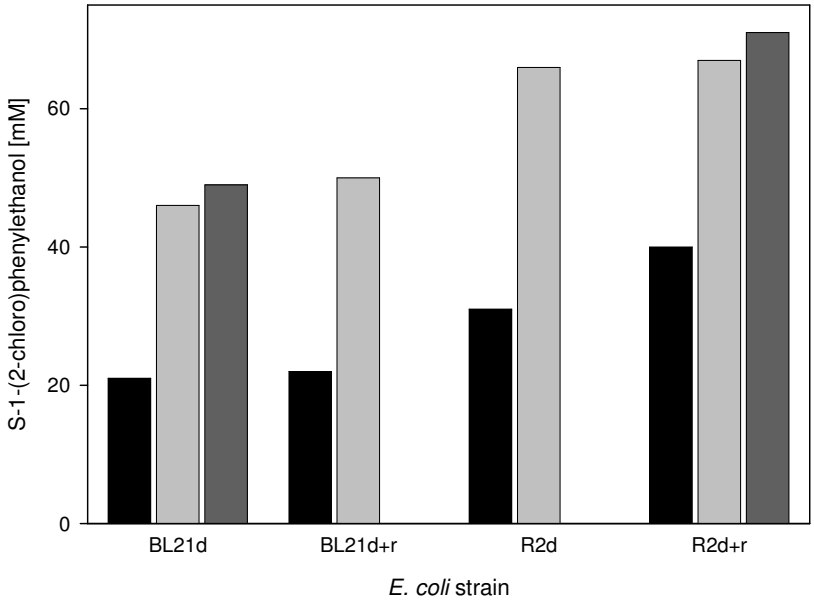


**Figure 11. WCR of *o*-chloroacetophenone, variation of additives.**

The product is (*S*)-1-(2-chlorophenyl)ethanol [mM]. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction time 8 h. Reaction pH 7.5.

*Left to right:* Substrate 100 mM oChl. Substrate 100 mM oChl, 36 µM PM, 500 µM $NAD^+$. Substrate 100 mM oChl, 180 µM PM, 500 µM $NAD^+$;

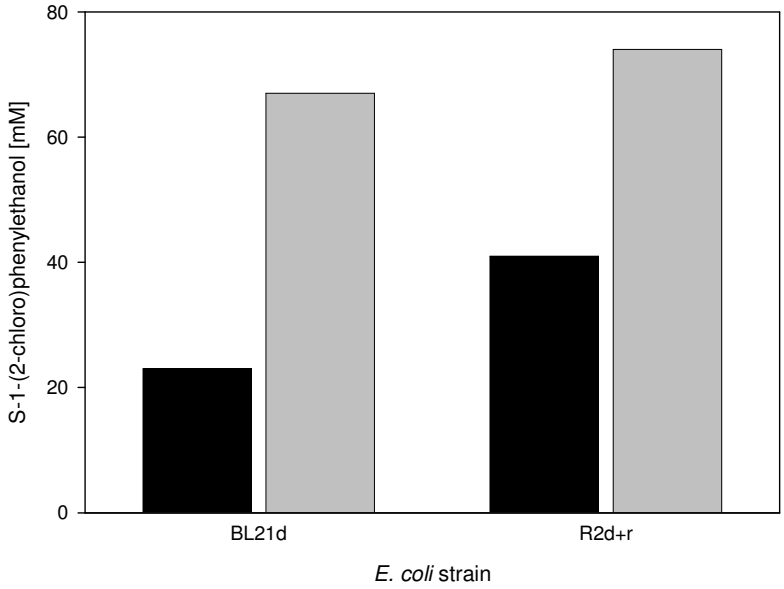


**Figure 12. WCR of *o*-chloroacetophenone, variation of additives.**

The product is (*S*)-1-(2-chlorophenyl)ethanol [mM]. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction time 8 h. Reaction pH 6.2.

*Left to right:* Substrate 100 mM oChl. Substrate 100 mM oChl, 36 µM PM, 500 µM $NAD^+$.

**Table 6. WCR of ethyl 4-cyanobenzoylformate, variation of reaction tube, additives, and growth medium.**

The product is ethyl 4-cynaomandelate. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction pH 7.5. §[1]reaction tube was a cryogenic vial. §[2] no addition of 5% EtOH to the substrate. §[3] PPG was added to MC.

| *E. coli* | Substrate [mM][1] | Co-Su [mM][2] | Feature | Incubation | Yield [mM] | Yield [%] |
|---|---|---|---|---|---|---|
| BL21d | 100 | 150 | | 3 ½ h | 21 | 21 |
| BL21d | 100 | 150 | | 8 ½ h | 23 | 23 |
| BL21d | 100 | 150 | | 8 ½ h | 22 | 22 |
| BL21d | 50 | 100 | | 20 ½ h | 23 | 46 |
| BL21d | 50 | 100 | §[1] | 20 ½ h | 21 | 42 |
| BL21d+r | 100 | 150 | | 8 ½ h | 33 | 33 |
| BL21d+r | 100 | 150 | | 8 ½ h | 33 | 33 |
| BL21d+r | 50 | 100 | | 20 ½ h | 21 | 42 |
| BL21d+r | 50 | 100 | §[1] | 20 ½ h | 24 | 48 |
| R2d | 100 | 150 | | 8 ½ h | 25 | 25 |
| R2d | 100 | 150 | | 8 ½ h | 23 | 23 |
| R2d | 50 | 100 | | 20 ½ h | 25 | 50 |
| R2d | 50 | 100 | §[1] | 20 ½ h | 27 | 54 |
| R2d+r | 50 | 100 | | 20 ½ h | 24 | 47 |
| R2d+r | 50 | 100 | §[1] | 20 ½ h | 21 | 43 |
| R2d+r | 100 | 150 | | 8 ½ h | 30 | 30 |
| R2d+r | 100 | 150 | | 8 ½ h | 30 | 30 |
| R2d+r | 100 | 150 | | 2 ½ h | 30 | 30 |
| R2d+r | 100 | 150 | | 2 ½ h | 30 | 30 |
| R2d+r | 250 | 300 | | 2 ½ h | 24 | 10 |
| R2d+r | 250 | 300 | | 2 ½ h | 24 | 10 |
| R2d+r | 100 | 150 | | 3 ½ h | 32 | 32 |
| R2d+r | 250 | 300 | | 3 ½ h | 33 | 13 |
| R2d+r | 500 | 550 | | 3 ½ h | 26 | 5 |
| R2d+r | 500 | 550 | §[2] | 3 ½ h | 35 | 7 |
| R2d+r | 100 | 150 | | 3 ½ h | 31 | 31 |
| R2d+r | 500 | 550 | | 3 ½ h | 26 | 5 |
| R2d+r | 500 | 550 | §[2] | 3 ½ h | 57 | 11 |
| R2d+r | 250 | 300 | | 3 ½ h | 35 | 14 |
| R2d+r | 100 | 150 | §[3] | 4 h | 50 | 50 |
| R2d+r | 250 | 300 | §[3] | 4 h | 19 | 8 |

[1] + 5%ETOH; [2] HCOONa

**Table 7. WCR of ethyl 4-chlorobenzoylformate.**

The product is ethyl 4-chloromandelate. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction pH 7.5.

| *E. coli* | Substrate [mM][1] | Co-Su [mM][2] | Incubation | Yield [mM] | Yield [%] |
|---|---|---|---|---|---|
| R2d+r | 100 | 150 | 2 ½ h | 21 | 21 |
| R2d+r | 100 | 150 | 2 ½ h | 21 | 21 |
| R2d+r | 250 | 300 | 2 ½ h | 19 | 8 |
| R2d+r | 250 | 300 | 2 ½ h | 18 | 7 |

[1] + 5%ETOH; [2] HCOONa

**Table 8. WCR of *o*-chloroacetophenone.**

The product is (*S*)-1-(2-chlorophenyl)ethanol. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction pH 7.5.

| *E. coli* | Substrate [mM][1] | Co-Su [mM][2] | Incubation | Yield [mM] | Yield [%] |
|---|---|---|---|---|---|
| BL21d | 100 | 150 | 3 ½ h | 13 | 13 |
| BL21d | 100 | 150 | 3 ½ h | 8 | 8 |
| BL21d | 100 | 150 | 3 ½ h | 9 | 9 |
| BL21d | 250 | 300 | 3 ½ h | 7 | 3 |
| BL21d | 50 | 100 | 2 ½ h | 13 | 27 |
| BL21d | 100 | 150 | 2 ½ h | 16 | 16 |
| BL21d | 250 | 300 | 3 ½ h | 3 | 1 |
| BL21d+r | 50 | 100 | 2 ½ h | 12 | 24 |
| BL21d+r | 100 | 150 | 2 ½ h | 14 | 14 |
| BL21d+r | 100 | 150 | 3 ½ h | 12 | 12 |
| BL21d+r | 100 | 150 | 3 ½ h | 13 | 13 |
| BL21d+r | 250 | 300 | 3 ½ h | 4 | 2 |
| BL21d+r | 250 | 300 | 3 ½ h | 4 | 2 |
| R2d | 100 | 150 | 3 ½ h | 16 | 16 |
| R2d | 100 | 150 | 3 ½ h | 16 | 16 |
| R2d | 250 | 300 | 3 ½ h | 14 | 5 |
| R2d | 250 | 300 | 3 ½ h | 13 | 5 |
| R2d | 50 | 100 | 2 ½ h | 18 | 36 |
| R2d | 100 | 150 | 2 ½ h | 23 | 23 |
| R2d+r | 50 | 100 | 2 ½ h | 20 | 39 |
| R2d+r | 100 | 150 | 2 ½ h | 23 | 23 |

[1] + 5%ETOH; [2] HCOONa

**Table 9. WCR of *o*-chloroacetophenone, variation of additives and growth medium.**

The product is (*S*)-1-(2-chlorophenyl)ethanol. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction pH 7.5. §[1] no addition of 5% EtOH to the substrate. §[2] PPG was added to MC.

| ***E. coli*** | **Substrate [mM][1]** | **Co-Su [mM][2]** | **Feature** | **Incubation** | **Yield [mM]** | **Yield [%]** |
|---|---|---|---|---|---|---|
| R2d+r | 100 | 150 | | 4 h | 30 | 30 |
| R2d+r | 250 | 300 | | 4 h | 73 | 29 |
| R2d+r | 100 | 150 | | 3 ½ h | 29 | 29 |
| R2d+r | 250 | 300 | | 3 ½ h | 18 | 7 |
| R2d+r | 250 | 300 | | 3 ½ h | 16 | 6 |
| R2d+r | 500 | 550 | | 3 ½ h | 11 | 2 |
| R2d+r | 500 | 550 | | 3 ½ h | 20 | 4 |
| R2d+r | 500 | 550 | §[1] | 3 ½ h | 10 | 2 |
| R2d+r | 500 | 550 | §[1] | 3 ½ h | 7 | 1 |
| R2d+r | 100 | 150 | | 3 ½ h | 18 | 18 |
| R2d+r | 100 | 150 | | 3 ½ h | 17 | 17 |
| R2d+r | 100 | 150 | §[2] | 3 ½ h | 12 | 12 |
| R2d+r | 100 | 150 | §[2] | 3 ½ h | 11 | 11 |
| R2d+r | 100 | 150 | | 3 ½ h | 26 | 26 |
| R2d+r | 250 | 300 | | 3 ½ h | 16 | 6 |
| R2d+r | 250 | 300 | | 3 ½ h | 13 | 5 |
| R2d+r | 250 | 300 | §[2] | 3 ½ h | 9 | 4 |
| R2d+r | 250 | 300 | §[2] | 3 ½ h | 9 | 4 |

[1] + 5%ETOH; [2] HCOONa

### Results of the solubility test

Ethyl 4-cyanomandelate, the corresponding alcohol of the substrate ethyl 4-cyanobenzoylformate, is only soluble up to a concentration of 10 mM. Whereas the solubility of 1-(2-chlorophenyl)ethanol, which is the product of a reduction reaction with the substrate *o*-chloroacetophenone, is good up to of 50 mM.

Insoluble are less toxic for the cells and therefore the enzyme than soluble products. For that reason, the less soluble ethyl 4-cyanobenzoylformate is better suited for a whole-cell reduction in a fed batch reaction compared to *o*-chloroacetophenone.

## Abbreviations

| | |
|---|---|
| 4CN | ethyl 4-cyanobenzoylformate |
| Amp | ampicillin |
| BL21d | *E. coli* BL21 (DE3) pETDuet-1_*Ct*XR_*Cb*FDH |
| BL21d+r star | *E. coli* BL21 star (DE3) pETDuet-1_*Ct*XR_*Cb*FDH + pRSF-1b_*Cb*FDH |
| BL21d+r | *E. coli* BL21 (DE3) pETDuet-1_*Ct*XR_*Cb*FDH + pRSF-1b_*Cb*FDH |
| BL21r star | *E. coli* BL21 star (DE3) pRSF-1b_*Cb*FDH |
| BL21r | *E. coli* BL21 (DE3) pRSF-1b_*Cb*FDH |
| BL21XR+FDH | *E. coli* BL21 (DE3) pET11a_*Ct*XR + pRSF-1b_*Cb*FDH |
| Cam | chloramphenicol |
| *Cb*FDH | *Candida boidinii* formate dehydrogenase |
| CDW | cell dry weight |
| CE | cell-free extract |
| Co-Su | co-substrate |
| CPE | 1-(2-chlorophenyl)ethanol |
| *Ct*XR | *Candida tenuis* xylose reductase |
| dd$H_2O$ | double distilled water |
| DNA | deoxyribonucleic acid |
| *E. coli* | *Escherichia coli* |
| E4CM | ethyl 4-cynomandelate |
| E4CN | ethyl 4-cyanobenzoylformate |
| EtOH | ethanol |
| FDH | formate dehydrogenase |
| HCOONa | sodium formate |
| HPLC | high performance liquid chromatography |
| IPTG | Isopropyl-β-D-thiogalactopyranosid |

| | |
|---|---|
| $K_2HPO_4$ | dipotassium phosphate |
| Kan | kanamycin |
| KCl | potassium chloride |
| $KH_2PO_4$ | monopotassium phosphate |
| LB | Luria-Bertani Broth medium |
| MC | main-culture |
| MCS | multiple cloning site |
| $MgSO_4$ | magnesium sulphate |
| mRNA | messenger ribonucleic acid |
| MW | molecular weight |
| NaCl | sodium chloride |
| NAD(H) | nicotinamide adenine dinucleotide |
| NADP(H) | nicotinamide adenine dinucleotide phosphate |
| oChl | *o*-chloroacetophenone |
| OD | optical density |
| ON | over night |
| PC | pre-culture |
| PCR | polymerase chain reaction |
| PM | Polymyxin B sulphate |
| PPB | potassium phosphate buffer |
| PPG | polypropylene glycol; antifoam |
| R2d | *E. coli* Rosetta 2 (DE3) pETDuet-1_*Ct*XR_*Cb*FDH |
| R2d+r | *E. coli* Rosetta 2 (DE3) pETDuet-1_*Ct*XR_*Cb*FDH + pRSF-1b_*Cb*FDH |
| R2r | *E. coli* Rosetta 2 (DE3) pRSF-1b_*Cb*FDH |
| rbs | ribosome binding site |
| rpm | rounds per minute |
| SOC | Super Optimal Broth |

| | |
|---|---|
| tRNA | transfer ribonucleic acid |
| U | Units |
| WCR | whole cell reduction |
| XR | xylose reductase |

## References


[1] Goldberg K, Schroer K, Lütz S, Liese A: **Biocatalytic ketone reduction- a powerful tool for the production of chiral alcohols- part II: Whole cell reductions.** *Appl Microbiol Biotechnol* 2007, **76**:249-255.

[2] Goldberg K, Schroer K, Lütz S, Liese A: **Biocatalytic ketone reduction- a powerful tool for the production of chiral alcohols- part I: Processes with isolated enzymes.** *Appl Microbiol Biotechnol* 2007, **76**:237-248.

[3] Faber K**: Reduction Reactions.** In *Biotransformations in Organic Chemistry.* 4th edition. Edited by: Faber K. Berlin: Springer; 2000: 177-219.

[4] Brommarius AS, Riebel BR: **Production of enantiomerically pure hydrophobic alcohols: Comparison of different routes and reactor configurations.** In *Biocatalysis.* 1st edition. Edited by: Brommarius AS, Riebel BR. Weinheim: Wiley-VCH; 2004: 456-569.

[5] Daußmann T, Hennemann HG, Rosen TC, Dünkelmann P: **Enzymatic technologies for the synthesis of chiral alcohol derivatives.** *Chem. Ing. Techno.* 2006; **78**: 249–255.

[6] Straathof AJJ, Panke S, Schmid A: **The production of fine chemicals by biotransformations.** *Curr Opin Biotechnol* 2002, **13**: 538-556.

[7] Bertau M: **Novel Developments in Biocatalytic Organic Chemistry.** *Curr Org Chem* 2002, **6**: 987-1014.

[8] Kratzer R, Pukl M, Egger S, Nidetzky B: **Whole-cell bioreduction of aromatic α-keto esters using *Candida tenuis* xylose reductase and *Candida boidinii* formate dehydrogenase co-expressed in *Escherichia coli*.** *Microl Cell Fact* 2008, **7**: 37-48.

[9] Ema T, Yagasaki H, Okita N, Takeda M, Sakai T: **Asymmetric reduction of ketones using recombinant *E. coli* cells that produce a versatile carbonyl reductase with high enantioselectivity and broad substrate specificity.** *Tetrahedron* 2006, **62**: 6143-6149.

[10] Ernst M, Kaup B, Müller M, Bringer-Meyer S, Sahm H: **Enantioselective reduction of carbonyl compounds by whole cell biotransformation, combining a formate dehydrogenase and a (*R*)-specific alcohol dehydrogenase.** *Appl Microbiol Biotechnol* 2005, **66**: 629-634.

[11] Kataoka M, Rohani LPS, Yamamoto K, Wada M, Kawabata H, Kita K, Yanase H, Shimizu S: **Enzymatic production of ethyl (*R*)-4-chloro-3-hydroxybutanoate: Asymmetric reduction of ethyl 4-chloro-3-oxobutanoate by an *Escherichia coli* transformant expressing the aldehyde reductase gene from yeast.** *Appl Microbiol Biotechnol* 1997, **48**: 699-703.

[12] Xu Z, Liu Y, Fang L, Jiang X, Ling K, Cen P: **Construction of a two-strain system for asymmetric reduction of ethyl 4-chloro-3-oxobutanoate to (*S*)-4-chloro-3-hydroxybutanoate ethyl ester.** *Appl Microbiol Biotechnol* 2006, **70**: 40-46.

[13] Weckbecker A, Hummel W: **Improved synthesis of chiral alcohols with *Escherichia coli* cells co-expressing pyridine nucleotide transhydrogenase, $NADP^+$-dependent alcohol dehydrogenase and $NAD^+$-dependent formate dehydrogenase.** *Biotechnol Letters* 2004, **26**: 1739-1744.

[14] Kaup B, Bringer-Meyer S, Sahm H: **Metabolic engineering of *Escherichia coli:* Construction of an efficient biocatalyst for D-mannitol formation in a whole-cell biotransformation.** *Appl Microbiol Biotechnol* 2004, **64**: 333-339.

[15] Bräutigam S, Bringer-Meyer S, Weuster-Botz D: **Asymmetric whole cell biotransformation in biphasic ionic liquid/water- systems by use of recombinant *Escherichia coli* with intracellular cofactor regeneration.** *Tetrahedron: Asymm* 2007, **18**: 1883-1887.

[16] Yamamoto H, Mitsuhashi K, Kimoto N, Kobayashi Y, Esaki N: **Robust NADH-regenerator: Improved α–haloketone-resistant formate dehydrogenase.** *Appl Microbiol Biotechnol* 2005, **67**: 33-39.

[17] Neuhauser W, Steininger M, Haltrich D, Kulbe KD, Nidetzky B: **A pH-controlled fed-batch process can overcome inhibition by formate in NADH-dependent enzymatic reductions using formate dehydrogenase-catalysed coenzyme regeneration.** *Biotechnol Bioeng* 1998, **60**: 277-282.

[18] Gröger H, Chamouleau F, Orologas N, Rollmann C, Drauz K, Hummel W, Weckbecker A, Mayer O: **Enantioselective reduction of ketones with 'designer cells' at high substrate concentrations: Highly efficient access to functionalized optically active alcohols.** *Angew Chem Int Ed* 2006, **45**: 5677-5681.

[19] Slusarczyk H, Felber S, Kula MR, Pohl M: **Stabilisation of $NAD^+$-dependent formate dehydrogenase from *Candida boidinii* by site-directed mutagenesis of cysteine residues.** *Eur J Biochem* 2000, **267**: 1280-1289.

[20] Jez JM, Bennett MJ, Schlegel BP, Lewis M, Penning TM: **Comparative anatomy of the aldo-keto reductase superfamily.** *Biochem J* 1997, **326**: 625-636.

[21] Neuhauser W, Haltrich D, Kulbe KD, Nidetzky B: **Noncovalent enzyme-substrate interactions in the catalytic mechanism of yeast aldose reductase.** *Biochem* 1998, **37**: 1116-1123.

[22] Neuhauser W, Haltrich D, Kulbe KD, Nidetzky B: **NAD(P)H-dependent aldose reductase from the xylose-assimilating yeast *Candida tenuis*. Isolation, characterisation and biochemical properties of the enzyme.** *Biochem J* 1997, **326**: 683-692.

[23] Nidetzky B, Mayr P, Hadwiger P, Stütz AE: **Binding energy and specifity in the catalytic mechanism of yeast aldose reductases.** *Biochem J* 1999, **344**: 101-107.

[24] Mayr P, Nidetzky B: **Catalytic reaction profile for NADH-dependent reduction of aromatic aldehydes by xylose reductase from *Candida tenuis*.** *Biochem J* 2002, **366**: 889-899.

[25] Kataoka M, Kita K, Wada M, Yasohara Y, Hasegawa J, Shimizu S: **Novel bioreduction system for the production of chiral alcohols.** *Appl Microbiol Biotechnol* 2003, **62**: 437-445.

[26] Kratzer R, Nidetzky B: **Identification of *Candida tenuis* xylose reductase as highly selective biocatalyst for the synthesis of chiral α-hydroxy esters and improvement of its efficiency by protein engineering.** *Chem Comm* 2007, **10**: 1047-1049.

[27] Santamaria A, Neef R, Eberspächer U, Eis K, Husemann M, Mumberg D, Prechtl S, Schulze V, Siemeister G, Wortmann L, Barr FA, Nigg EA: **Use of the novel Plk1 inhibitor ZK-thiazolidinone to elucidate functions of Plk1 in early and late stages of mitosis.** *Mol Biol Cell* 2007, **18**: 4024-4036.

[28] Sato Y, Onozaki Y, Sugimoto T, Kurihara H, Kamijo K, Kadowaki C, Tsujino T, Watanabe A, Otsuki S, Mitsuya M, Iida M, Haze K, Machida T, Nakatsuru Y, Komatani H, Kotani H, Iwasawa Y: **Imidazopyridine derivatives as potent and selective Polo-like kinase (PLK) inhibitors.** *Bioorg Med Chem Lett* 2009, **19:** 4673-4678.

[29] Rheault TR, Donaldson KH, Badiang-Alberti JG, Davis-Ward RG, Andrews CW, Bambal R, Jackson JR, Cheung M: **Heteroaryl-linked 5-(1*H*-benzimidazol-1-yl)-2-thiophenecarboxamides: Potent inhibitors of polo-like kinase 1 (PLK1) with improved drug-like properties.** *Bioorg Medic Chem Lett* 2010, **20**: 4587-4592.

[30] ©Merck KGaA: **pET-11a-d vectors.**
https://wasatch.biochem.utah.edu/chris/links/pET11a.pdf (found 10-10-05).

[31] ©Merck KGaA: **Duet vectors.**
http://www.merck-chemicals.com/life-science-research/vector-table-novagen-duet-vector-table/c_wXub.s1O3asAAAEhEmsLdcXH (found 10-10-05).

[32] ©Merck KGaA: **pCDF and pRSF vectors.**
http://www.merck-chemicals.de/life-science-research/vector-table-novagen-pcdf-and-prsf-vector-table/c_r6Sb.s1O7X0AAAEhGpsLdcaY (found 10-10-05).

## Figures

**Figure 2. Xylose reduction and cofactor regeneration by co-expression of *Ct*XR and *Cb*FDH.**

**Figure 2. pET-11a vector map.**

The pET-11a vector carries an N-terminal T7•Tag® sequence, a *Bam*HI cloning site, and encodes for an ampicillin resistance [30].

**Figure 3. pETDuet-1 vector map.**

pETDuet™-1 is designed for the co-expression of two target genes. It therefore contains two multiple cloning sites (MCS), which are each preceded by a T7 promoter/*lac* operator and a ribosome binding site (rbs). The vector also carries the pBR322-derived ColE1 replicon, *lac*I gene and ampicillin resistance gene [31].

**Figure 4. pRSF-1b vector map.**

pRSF-1b carries a T7 promoter and *lac* operator to control transcription, a replication origin derived from RSF1030, and kanamycin antibiotic resistance [32].

**Figure 5. WCR of ethyl 4-cyanobenzoylformate, variation of substrate concentration, growth medium, and reaction tube.**

The product is ethyl 4-cyanomandelate [mM]. Reaction time 5 h. Reaction pH 7.5.

*Left to right:* Substrate 50 mM 4CN, 2 mL reaction volume in a 2 mL Eppendorf tube. Substrate 50 mM 4CN, 1 mL reaction volume in cryogenic vial. Substrate 50 mM 4CN, 1 mL reaction volume in cryogenic vial, 10 mM $MgSO_4$ added to growth medium. Substrate 100 mM 4CN, 1 mL reaction volume in cryogenic vial. Substrate 100 mM, 2 mL reaction volume in a 2 mL Eppendorf tube.

**Figure 6. WCR of ethyl 4-cyanobenzoylformate, variation of substrate concentration and reaction tube.**

The product is ethyl 4-cyanomandelate [mM]. Reaction time 4 ½ h. Reaction pH 7.5.

*Left to right:* Substrate 50 mM 4CN, 1 mL reaction volume in a 2 mL Eppendorf tube. Substrate 50 mM 4CN, 1 mL reaction volume in cryogenic vial. Substrate 100 mM 4CN, 1 mL reaction volume in a 2 mL Eppendorf tube. Substrate 100 mM, 1 mL reaction volume in cryogenic vial.

**Figure 7. WCR of ethyl 4-cyanobenzoylformate, variation of substrate concentration, growth medium, and additives.**

The product is ethyl 4-cyanomandelate [mM]. Reaction time 8 h. Reaction pH 6.2. Reaction volume 1 mL in a 2 mL Eppendorf tube.

*Left to right:* Substrate 100 mM 4CN. Substrate 100 mM 4CN, 36 µM PM, 500 µM $NAD^+$. Substrate 200 mM 4CN, 36 µM PM, 500 µM $NAD^+$. Substrate 200 mM 4CN, 180 µM PM, 500 µM $NAD^+$, 10 mM $MgSO_4$ added to growth medium.

**Figure 8. WCR of ethyl 4-cyanobenzoylformate, variation of pH, growth medium, and reaction tube.**

The product is ethyl 4-cyanomandelate [mM]. Reaction volume 1 mL. Reaction time 8 h.

*Left to right:* Substrate 50 mM 4CN, pH 6.5. Substrate 50 mM 4CN, cryogenic vial, pH 6.5. Substrate 50 mM 4CN, 10 mM $MgSO_4$ added in growth medium, pH 7.5. Substrate 50 mM 4CN, cryogenic vial, 10 mM $MgSO_4$ added to growth medium, pH 7.5.

**Figure 9. WCR of *o*-chloroacetophenone, variation of substrate concentration, growth medium, and additives.**

The product is (*S*)-1-(2-chlorophenyl)ethanol [mM]. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction time 8 h. Reaction pH 6.2.

*Left to right:* Substrate 100 mM oChl. Substrate 100 mM oChl, 36 µM PM, 500 µM $NAD^+$. Substrate 200 mM oChl, 36 µM PM, 500 µM $NAD^+$. Substrate 200 mM oChl, 180 µM PM, 500 µM $NAD^+$. Substrate 200 mM oChl, 180 µM PM, 2.5 mM $NAD^+$, 10 mM $MgSO_4$ added to growth medium.

**Figure 10. WCR of *o*-chloroacetophenone, variation of pH, growth medium, and reaction tube.**

The product is (*S*)-1-(2-chlorophenyl)ethanol [mM]. Reaction volume 1 mL. Reaction time 8 h.

*Left to right:* Substrate 50 mM oChl, pH 6.5. Substrate 50 mM oChl, pH 6.5, cryogenic vial. Substrate 50 mM oChl, pH 7.5, 10 mM $MgSO_4$ added to growth medium. Substrate 50 mM oChl, pH 7.5, cryogenic vial, 10 mM $MgSO_4$ added to growth medium.

**Figure 11. WCR of *o*-chloroacetophenone, variation of additives.**

The product is (*S*)-1-(2-chlorophenyl)ethanol [mM]. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction time 8 h. Reaction pH 7.5.

*Left to right:* Substrate 100 mM oChl. Substrate 100 mM oChl, 36 µM PM, 500 µM $NAD^+$. Substrate 100 mM oChl, 180 µM PM, 500 µM $NAD^+$;

**Figure 12. WCR of *o*-chloroacetophenone, variation of additives.**

The product is (*S*)-1-(2-chlorophenyl)ethanol [mM]. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction time 8 h. Reaction pH 6.2.

*Left to right:* Substrate 100 mM oChl. Substrate 100 mM oChl, 36 µM PM, 500 µM $NAD^+$.

## Tables

**Table 1. Bacterial strains and their abbreviations used in this study.**

*Ct*XR and *Cb*FDH co-expression strains. *Cb*FDH single-expression strains.

**Table 2. Weighted samples for ethyl 4-cyanomandelate at different concentrations.**

**Table 3. Weighted samples for 1-(2-chlorophenyl)ethanol at different concentrations.**

**Table 4. Results of the BCA Protein Assay.**

BL21d+r was cultivated under various conditions prior to cell disruption by B-Per® and subsequently, the protein concentration in the cell-free crude extract was determined. The pellet was resuspended in the lysis reagent to reveal the amount of remaining protein after extraction.

**Table 5. Activity measurements of five *E. coli* strains under various cultivation conditions.**

**Table 6. WCR of ethyl 4-cyanobenzoylformate, variation of reaction tube, additives, and growth medium.**

The product is ethyl 4-cynaomandelate. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction pH 7.5. §[1]reaction tube was a cryogenic vial. §[2] no addition of 5% EtOH to the substrate. §[3] PPG was added to MC.

**Table 7. WCR of ethyl 4-chlorobenzoylformate.**

The product is ethyl 4-chloromandelate. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction pH 7.5.

**Table 8. WCR of *o*-chloroacetophenone.**

The product is (*S*)-1-(2-chlorophenyl)ethanol. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction pH 7.5.

**Table 9. WCR of *o*-chloroacetophenone, variation of additives and growth medium.**

The product is (*S*)-1-(2-chlorophenyl)ethanol. Reaction volume 1 mL in a 2 mL Eppendorf tube. Reaction pH 7.5. §[1] no addition of 5% EtOH to the substrate. §[2] PPG was added to MC.