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Design and Application of a Whole-cell Catalyst for the Asymmetric Amination of Ketones

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Dedicated to my Dad for awakening my curiosity in science from childhood on;

and to my Mum for always being there.

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Okay wow, acknowledgment has become longer than expected.

Making it short: having all of you in my life means everything to me. THANK YOU!!!

"THERE IS A TIDE IN THE AFFAIRS OF MEN.
WHICH, TAKEN AT THE FLOOD, LEADS ON TO FORTUNE;
OMITTED, ALL THE VOYAGE OF THEIR LIFE
IS BOUND IN SHALLOWS AND IN MISERIES.
ON SUCH A FULL SEA ARE WE NOW AFLOAT,
AND WE MUST TAKE THE CURRENT WHEN IT SERVES,
OR LOSE OUR VENTURES."

William Shakespeare – *"Julius Caesar"*

ABSTRACT

A single whole-cell biocatalyst using recombinant *Escherichia coli* as a host has been successfully constructed and used for the asymmetric amination of a prochiral ketone to the corresponding amine requiring three enzymes.

The aim was to enable a flexible co-expression system consisting of three modules, namely module 1 bearing different ω-transaminases, module 2 providing an alanine dehydrogenase and module 3 offering various enzymes for nicotinamide cofactor regeneration. The genes encoding the ω-transaminases originating from *Arthrobacter sp*. and *Chromobacterium violaceum* as well as the enzymes for cofactor recycling (either formate dehydrogenase from *Komagataella pastoris*, glucose dehydrogenase from *Bacillus megaterium* or phosphite dehydrogenase from *Pseudomonas stutzeri*) were cloned into two different plasmids, whereas the gene encoding the alanine dehydrogenase from *Bacillus subtilis* was integrated into the *E. coli* genome. Due to this modular assembly of the whole-cell catalyst, which is illustrated in Figure 1, a high degree of flexibility was achieved.

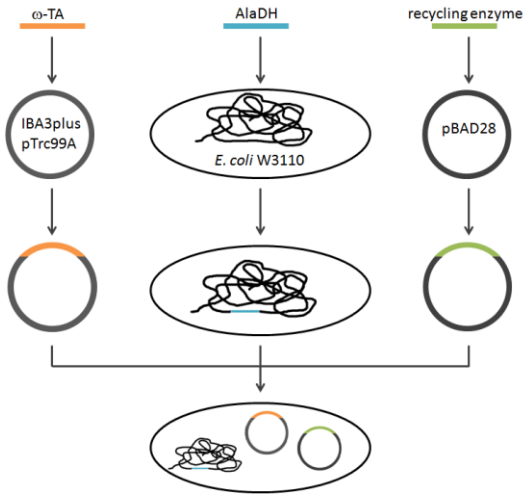

Figure 1 Construction of the recombinant *E. coli* whole-cell catalyst

The reductive amination of 4-phenyl-2-butanone to 4-phenyl-2-butylamine was used as model reaction in order to investigate the applicability of the recombinant *E. coli* biocatalyst. The resting cells used in the above mentioned biotransformation exhibited a catalytic activity of 0.302 ± 0.02 U/g for the ω-TA/AlaDH/FDH, 0.218 ± 0.02 U/g for the ω-TA/AlaDH/GDH and 0.992 ± 0.07 U/g for the ω-TA/AlaDH/PtDH system, respectively. Furthermore, the performance of different catalyst preparations (lyophilized whole cells, cell-free extract and lyophilized cell-free extract) was compared, in particular with respect to efficiency and stability. With the ω-TA/AlaDH/GDH system, used as lyophilized cell-free extract, a maximum conversion of 37 % amine could be achieved within 24 hours, using 25 mM substrate concentration. The best results though were obtained applying lyophilized cells bearing the ω-TA/AlaDH/FDH system or the ω-TA/AlaDH/PtDH system, respectively, yielding up to 56 % of the amine. However, this reaction outcome could be further increased to 77 % using a ten-fold loading of the biocatalyst.

All in all, the use of a recombinant whole-cell catalyst co-expressing the desired enzymes for the asymmetric reductive amination of ketones has several advantages compared to the use of isolated enzymes.

In particular the access to the biocatalyst is simplified and it is economically more attractive and greener. The results obtained making the described approach a promising method for a more efficient use of ω-transaminases.

KURZFASSUNG

Mithilfe eines rekombinanten *E. coli* Wirts erfolgte die Konstruktion eines Ganzzell-Katalysators für die asymmetrische reduktive Aminierung von Ketonen zu den korrespondierenden Aminen unter Verwendung von drei Enzymen.

Das Ziel der Arbeit war die Etablierung eines flexiblen Co-Expressionssystems bestehend aus drei Modulen. Während Modul 1 verschiedene ω-Trans-aminasen bereitstellte, beinhaltete Modul 2 eine Alanindehydrogenase und Modul 3 diverse Enzyme für die Regenerierung des Nicotinamid-Cofaktors. Sowohl die Gene kodierend für die ω-Transaminasen aus *Arthrobacter sp.* and *Chromobacterium violaceum*, als auch jene für die Cofaktor regenerierenden Enzyme (Formiatdehydrogenase aus *Komagataella pastoris*, Glukosedehydrogenase aus *Bacillus megaterium*, oder Phosphitdehydrogenase aus *Pseudomonas stutzeri*) wurden in zwei verschiedene Plasmide kloniert, während das Gen kodierend für die Alanindehydrogenase aus *Bacillus subtilis* in das *E. coli* Genom integriert wurde. Basierend auf diesem modularen Aufbau des Ganzzell-Katalysators, welcher in Abbildung 1 veranschaulicht ist, konnte ein hoher Grad an Flexibilität sichergestellt werden.

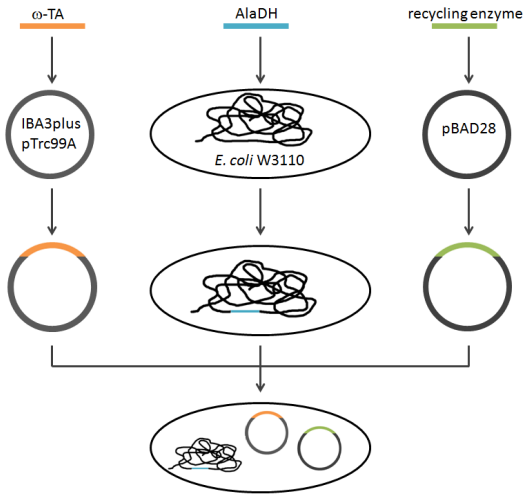

Abbildung 1 Konstruktion eines rekombinanten *E. coli* Ganzzellkatalysators

Die Anwendbarkeit des rekombinanten *E. coli* Ganzzellkatalysators wurde anhand der reduktiven Aminierung von 4-Phenyl-2-butanon zu 4-Phenyl-2-butylamin untersucht. Unter Verwendung von ganzen Zellen des Katalysators wurde in der oben genannten Modellreaktion eine katalytische Aktivität von 0.302 ± 0.02 U/g für das ω-TA/AlaDH/FDH System, 0.218 ± 0.02 U/g für das ω-TA/AlaDH/GDH und 0.992 ± 0.07 U/g für das ω-TA/AlaDH/PtDH System erzielt. Des Weiteren wurden verschiedenen Katalysatorpräparationen (lyophilisierte Zellen, zellfreies Extrakt und lyophilisiertes zellfreies Extrakt) hinsichtlich ihrer Effizienz und Stabilität untersucht und miteinander verglichen. Mit dem katalytischen System ω-TA/AlaDH/GDH in Form von lyophilisiertem zellfreiem Extrakt konnte innerhalb von 24 Stunden bei einer Substratkonzentration von 25 mM ein maximaler Umsatz von 37 % zum Amin erreicht werden.

Die besten Ergebnisse wurden mit den lyophilisierten Zellen des ω-TA/AlaDH/FDH- bzw. ω-TA/AlaDH/PtDH Systems erzielt, resultierend in bis zu 56 % des Amins. Dieser Umsatz konnte durch den Einsatz einer 10-fachen Biokatalysatormenge weiter auf 77 % erhöht werden.

Zusammengefasst zeigt sich die Verwendung eines rekombinanten Ganzzell-Katalysators, der die benötigten Enzyme für die reduktive Aminierung von Ketonen co-exprimiert, sehr vorteilhaft im Gegensatz zum Einsatz von isolierten Enzymen. Im Besonderen ist der Zugang zum Biokatalysator vereinfacht und es handelt sich um eine wirtschaftlich betrachtet attraktivere und grünere Alternative. Die erhaltenen Ergebnisse machen die beschriebene Herangehensweise zu einer vielversprechenden Methode für eine effizientere Nutzung von ω-Transaminasen.

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1 INTRODUCTION

Enzymes are catalyzing the majority of biochemical reactions in living cells. However, these chiral catalysts are not only active on their natural target molecules, but they also accept numerous non-natural substrates with high specificities. Therefore, they offer an enormous potential for the transformation of organic compounds into highly valuable products.[1] Nowadays more than 3000 types of enzymes are known and hundreds of them are commercially available. They have been classified into six categories according to the type of reaction they are catalyzing (Table 1.1).[2] This growing number of investigated enzymes as well as their ability to catalyze reactions equivalent to almost every type of chemical reaction has led to the increasing application of such enzymatic transformations in asymmetric synthesis.[2-8]

Table 1.1 Enzyme classes and their corresponding catalyzed reaction[2]

Enzyme class	Catalyzed reaction
1. Oxidoreductases	Oxidation and reduction reactions
	(e.g. reductases, oxidases, monooxygenases, dehydrogenases)
2. Transferases	Translocation of functional groups like acyl, phosphoryl, glycosyl or amino groups
	(e.g. transaminases, acetyltransferases, transketolases)
3. Hydrolases	Hydrolysis and formation of esters, amides, epoxides, nitriles
	(e.g. esterases, amidases, lipases, proteases, glycosidases, nitrilases)
4. Lyases	Addition or elimination reactions of small molecules on multiple bonds
	(e.g. carboxy-lyases, decarboxlases, dehydratases)
5. Isomerases	Isomerization reactions
	(e.g. racemases, epimerases, isomerases, tautomerases, mutases)
6. Ligases	Formation or cleavage of C-O, C-S, C-N, C-C bonds under simultaneous triphosphate cleavage
	(e.g. carboxylases)

In general, enzymes are very efficient catalysts accelerating reactions by a factor of 10^8-10^{10} compared to the corresponding non-catalyzed reaction, which is more than chemical catalysts are capable of. Enzyme-catalyzed reactions can be performed in water at ambient temperatures, neutral pH and atmospheric pressure. Therefore, the use of harsh reaction conditions is avoided, which often leads to side reactions like isomerization, racemization, epimerization and rearrangement of the compounds. Furthermore, from an environmental point of view, enzymes offer a safer and greener process with respect to organic solvents and work-up procedures. Additionally, they often reduce the number of process steps required for the synthesis of the target molecule.

It is for all the reasons presented above and due to the high stereo-, regio-, and enantio-selectivity of enzymes, that their application is often even preferred over traditional synthetic methods.[2, 9-10]

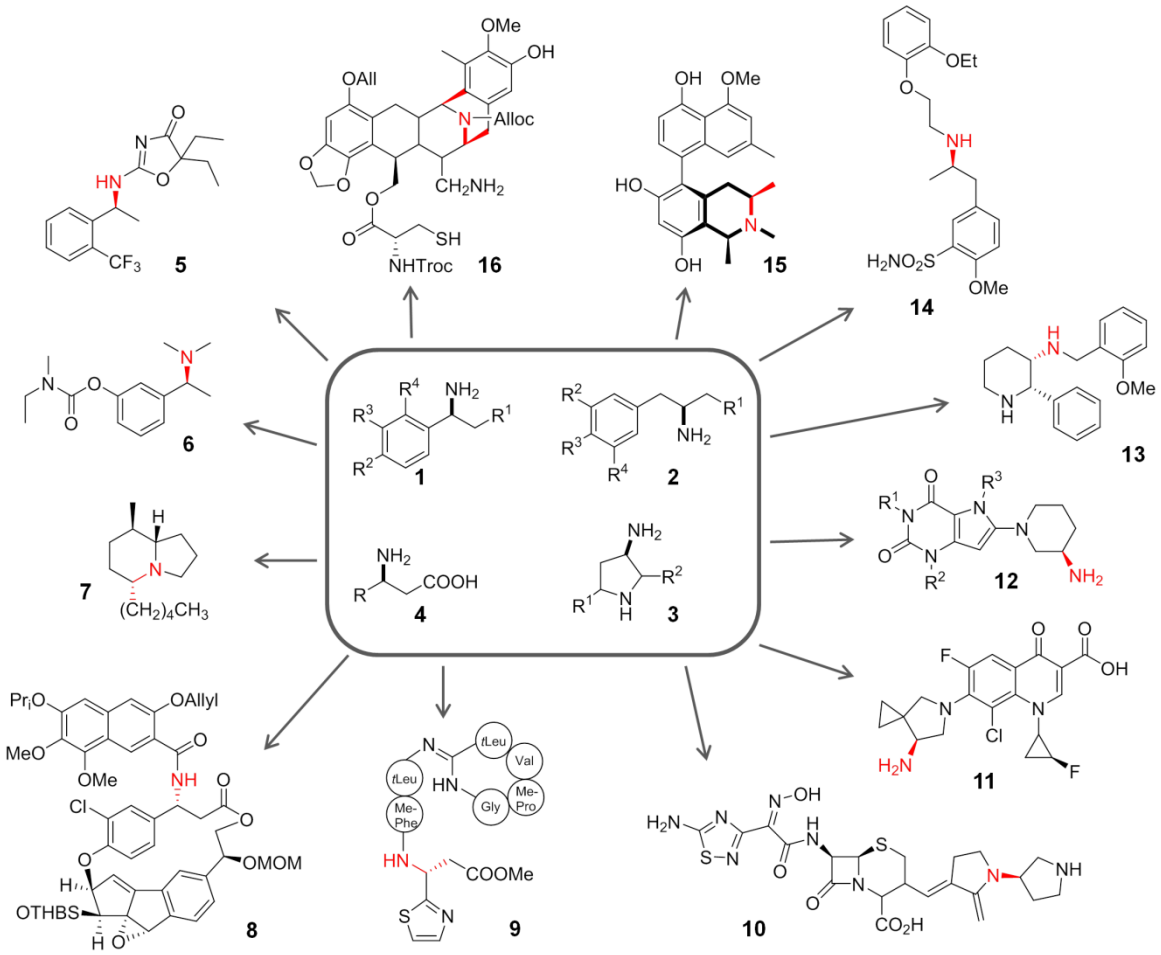
However, there are also some limitations when applying enzymes as biocatalysts due to their biological and chemical properties. Some enzymes are rather unstable in an isolated state, thus for an optimal use the factors influencing its stability need to be known.[8] Furthermore, they are susceptible to inhibition by the substrate, the product or any other component of the reaction mixture. Some enzymes need to be bound to their natural cofactors, which serve as molecular shuttles of redox equivalents such as flavin and nictoninamide cofactors or as storage for chemical energy like it is the case for ATP. Unfortunately, these compounds are often unstable and too expensive to be used in stoichiometric amounts.[2] As a consequence, a number of cofactor-regeneration systems have been established in order to overcome this limitation, enabling the use of cofactor-dependent enzymes in organic synthesis.[11] Additionally, the use of various methods for protein design like directed evolution enables the tailoring of enzymes with respect to their properties, activity and selectivity and allows to adapt the catalyst to the desired reaction conditions. Moreover, microbial cells and enzymes can be immobilized and reused for many cycles, making biocatalytic processes still economically efficient.[9]

Consequently, biocatalysis has become an important tool for creating optically pure molecules, which are essential building blocks for the manufacture of fine chemicals, pharmaceuticals, agrochemicals and food ingredients. Moreover, the emergence of new scientific developments in molecular biology and the heterologous expression in genetically optimized hosts enabled the production of enzymes from microbes in the amount required by industry, leading to the increased application of enzyme catalysis in synthetic industrial processes. A considerable amount of chiral compounds produced on large industrial scale are already synthesized with the use of enzymes, leading to greener and economically competitive processes.[12-14]

Generally, enzymatic syntheses of optically active compounds can proceed according to two different ways. In resolution-based strategies a racemate is resolved, leading only to a maximum yield of 50 %. Although this strategy is versatile and can often be used to rapidly prepare a required chiral intermediate, alternatives are preferred. However, more attention has been paid to the development of asymmetric processes, in which either new chiral centers are introduced into achiral starting materials or, alternatively, racemic mixtures are converted to enantiomerically pure compounds with yields up to 100 % (dynamic kinetic resolution).[15]

1.1 Preparation of optically active amines

Optically active amines are of increasing importance in the pharmaceutical, agrochemical and chemical industry, as they are highly valuable products and key intermediates for various pharmaceutically active compounds and agrochemicals (Figure 1.1).[16]



Product	Physiological effect / application
5 oxazolone derivative	Inhibitor of 11β-hydroxysteroid dehydrogenase type I; treatment of obesity/diabetes
6 rivastigmine	Treatment of Alzheimer's disease
7 (-)-indolizidine 209B	Mixture of highly cytotoxic alkaloids, isolated from poisonous frog *Dendrobatidae*
8 protected kedarcidine aglycon	Antitumor antibiotic
9 bottromycin A_2	Antibacterial
10 vinylpyrrolidinone cephalosporine	Antibacterial
11 sitafloxacine	Antibacterial
12 xanthine derivative	Inhibition of dipeptidylpeptidase IV; treatment of diabetes mellitus
13 3-aminopiperidine derivative	Neurokinine 1 receptor antagonist; treatment of nausea
14 Flomax (tamsulosin)	Prostate drug
15 korupensamine D	Naphthylisoquinoline alkaloid; treatment of malaria parasite Plasmodium falciparum
16 precursor of ecteinascidin 734	Antitumor antibiotic

Figure 1.1 Optically active amines found as structural motifs or used as building blocks in the preparation of bioactive compounds.[16] Compounds **1-4** resemble possible starting materials for the bioactive compounds **5-16**

Indeed, around 40 % of all optically active drugs are built up from chiral amines and they are frequently used as resolving agents to obtain chiral carboxylic acids. Therefore, the preparation of enantiomerically pure amines, both *via* chemical and enzymatic routes, has become a fairly demanded research field in asymmetric synthesis.[16-17]

Classical chemical approaches to these building blocks are amongst others the resolution of racemates through crystallization with chiral carboxylic acids, organo-metal catalyzed reduction of C=N bonds, amidocarbonylation catalyzed by transition metals and asymmetric hydrogenation of acetamides and imines.[16, 18-19] The most frequently used enzymatic methods for the production of optically active amines rely upon the kinetic resolution of racemic starting materials by enantioselective hydrolysis or amidation, catalyzed for example by lipases, proteases and acylases.[20] Some of them have also been performed successfully on an industrial scale.[3, 13] In certain cases the unreactive enantiomer can be racemized by the addition of a racemization catalyst, resulting in a dynamic kinetic resolution process.[21-24] Alternative approaches based on deracemization have also been developed, in which an enantioselective amine oxidase was combined with a non-selective chemical reduction of the imine intermediate, giving yields greater than 50 %.[25] However, recently the focus has been turned to the asymmetric synthesis of chiral amines starting from the corresponding prochiral ketones using transaminases.[13]

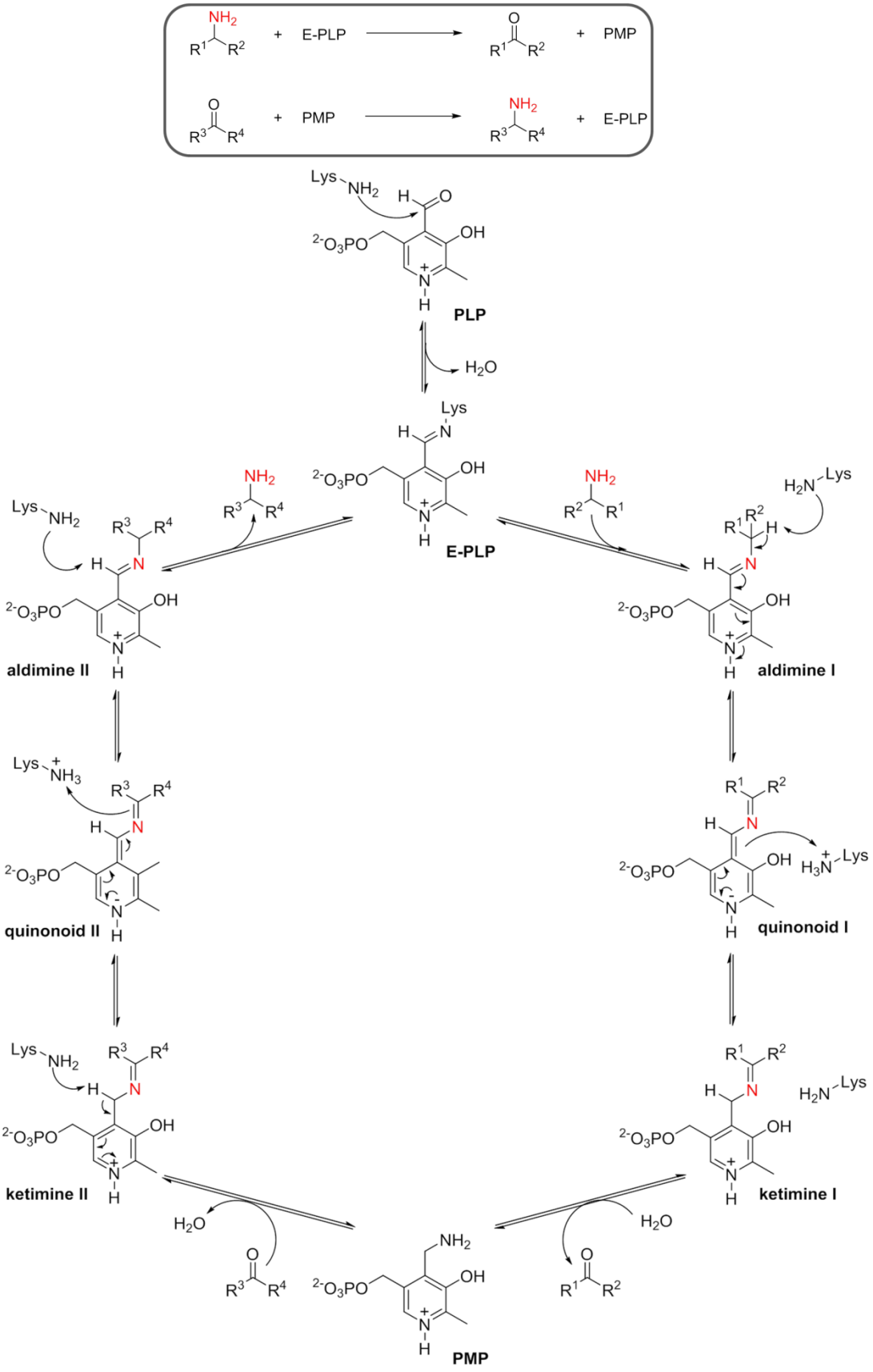
1.1.1 ω-Transaminases

Transaminases (TAs, [EC 2.6.1.X]), also known as amino transferases, are pyridoxal-5'-phosphat (PLP) dependent enzymes which catalyze the amination of ketones by transfer of an amino group from a donor substrate to the carbonyl moiety of the acceptor compound.[2, 18] In general there are two types of transaminases, namely α-TAs and ω-TAs. α-TAs require the presence of a carboxylic acid group in the α-position to the keto or in β-position to the amine functionality and thus are only able to form α-amino acids. On the contrary, ω-TAs are more useful, as they are known to catalyze the amination of keto acids, aldehydes and ketones independent of an adjacent carboxylic acid group.[26]

1.1.2 Mechanism of the PLP-dependent ω-TA catalyzed transamination

As already mentioned all transaminases require PLP as cofactor to act as a shuttle for the transfer of the amine group. The cofactor is fully regenerated within the transamination reaction and hence needs no external regeneration system.[27] PLP represents an activated form of vitamin B_6 and is ubiquitous in nature. It is present in microorganisms and higher organisms, where it plays an important role in several reactions, most prominent transaminations and decarboxylations.

The PLP-dependent transamination mechanism consists of two half-reactions involving two substrates yielding in two products and is often referred to as "Ping-Pong mechanism" (Scheme 1.1). Thereby PLP is used as carrier to transport amines and electrons between the acceptor and the donor. PLP is covalently bound in the active site of the transaminase, where it forms a Schiff base linkage to a Lys-residue (E-PLP). As soon as an amine donor enters the active site, the linkage is replaced by the amine group of the donor resulting in an aldimine (aldimine I). Subsequent proton rearrangements and a hydrolysis reactions leads to the generation of pyridoxamine-5'-phosphate (PMP) and the corresponding oxidized donor, which leaves the active site. Then, the amine acceptor enters the catalytic cycle starting the second half-reaction. Here the amino group is transferred from PMP to the amine acceptor and a ketimine (ketimine II) is formed, which again undergoes rearrangement to the corresponding aldimine (aldimine II). The latter releases the formed amine by regenerating E-PLP for the next catalytic cycle. It is presumed, that the enantiospecificity is a result from an unfavored coordination of one enantiomer in a catalytically productive conformation.[18, 26, 28]

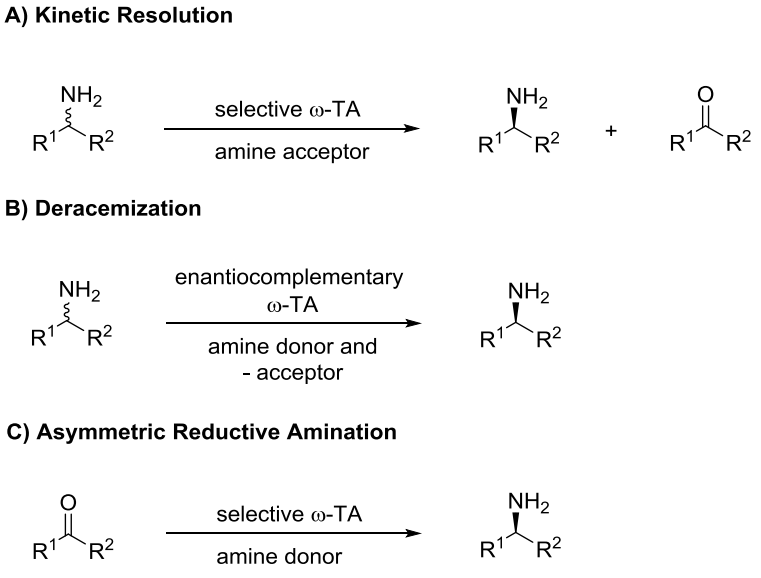


Scheme 1.1 Detailed mechanism of PLP-dependent transamination reaction. The reaction consists of two main parts: 1) the oxidative deamination of an amine donor and 2) the reductive amination of an amine acceptor. E-PLP = pyridoxal-5'-phosphate linked to the enzyme; PMP = pyridoxamine-5'-phosphate

1.1.3 Application of ω-TAs

Although ω-TAs were already discovered around 50 years ago, the first groundbreaking work with respect to their use in organic synthesis was carried out in the late 1980s by the American company Celgene. They successfully used ω-TAs for the enantioselective deamination of racemic amines via kinetic resolution for the first time.[2, 29] In the subsequent decades, ω-TAs have been extensively studied and by now more than 30 enzymes from different microbial origin have been characterized, showing similar substrate specifities and exhibiting both (*S*)- and (*R*)-selectivity. As most ω-TAs offer excellent stereoselectivity, they offer the presently unique possibility to prepare optically active amines directly from the prochirale ketone with a theoretically quantitative yield.[16, 18]

Generally, ω-transaminases can be employed for the preparation of optically active amines in three different ways (Scheme 1.2). One approach is the kinetic resolution of racemic amines with a theoretical yield of ≤50 %. In order to convert a racemate into a single enantiomer with 100 % theoretical yield a deracemization reaction can be performed, either in terms of a dynamic kinetic resolution or by using two enantiocomplementary ω-TAs in a two-step one-pot process. The third approach is the asymmetric reductive amination, which has been of increasing importance in the last years since it facilitates the production of chiral amines with yields up to 100 % starting from prochiral ketones.[18, 30]

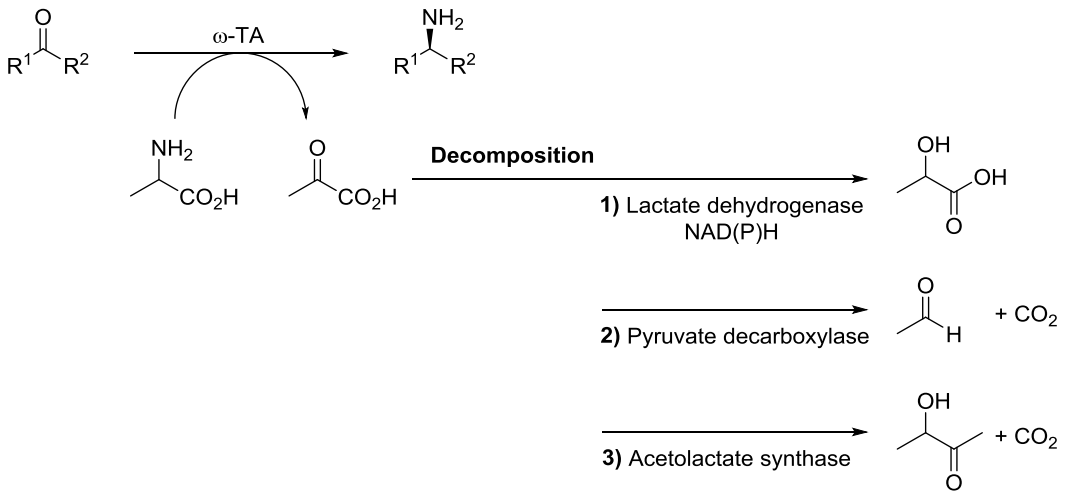


Scheme 1.2 ω-TA catalyzed reactions for the preparation of enantio-enriched amines. (A) Kinetic resolution and **(B)** deracemization employing the racemic amine or **(C)** asymmetric reductive amination starting from the prochiral ketone.

A major limitation in the asymmetric synthesis starting from prochiral ketones is the unfavorable thermodynamic equilibrium, especially when using alanine as amine donor. In this case the equilibrium generally lies on the side of the starting materials. Furthermore efficient conversions are prevented due to the co-product inhibition of TAs by pyruvate. Thus, these issues have to be circumvented either by using a large excess of amine donor and/or by removal of the co-product.[18, 31]

1.1.4 Strategies for shifting the equilibrium by co-product removal

For the efficient use of ω-TAs in the asymmetric reductive amination of ketones the thermodynamic barrier needs to be overcome in order to shift the equilibrium toward product formation. For this reason and besides to eliminate the inhibition issue the removal of the co-product, which is generated in the deamination of the amine donor, is crucial. When 2-propylamine or 2-butylamine is used as amine donor, volatile ketones like acetone and butanone, respectively are formed as the co-product, which can be easily removed under reduced pressure. In this approach only a single enzyme and an excess of amine donor is required. However, most transamination reactions rely on alanine as the amine donor and thus require the removal of pyruvate to drive the reaction to completion. For this purpose several well working strategies have been developed, which are based on the enzymatic degradation of the co-product pyruvate (Scheme 1.3).[18, 26, 30]

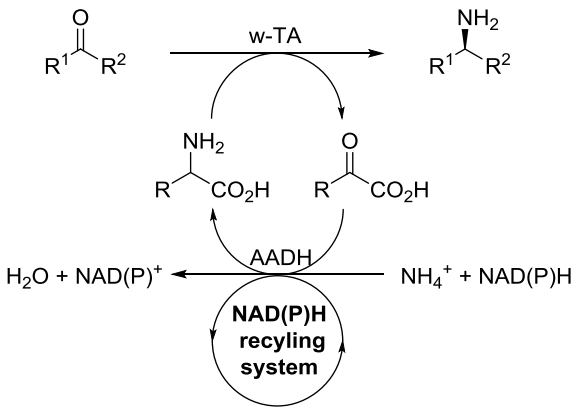


Scheme 1.3 Enzymatic strategies for the removal of the co-product pyruvate to shift the reaction equilibrium in ω-TA-catalyzed asymmetric synthesis

The decarboxylation of pyruvate using pyruvate decarboxylase (PDC) yields acetaldehyde and CO_2 and represents a simple process for co-product removal.[32] A highly related system is the use of an acetolactate synthase, which converts two molecules of pyruvate into acetolactate.[33] The latter decomposes to acetoin and CO_2.

These methods require two enzymes and a significant excess of amine donor. However, they do not require expensive cofactor and due to the CO_2 formation the reaction equilibrium is irreversibly shifted to the product side. An often applied method employs lactate dehydrogenase (LDH) for the reduction of pyruvate, requiring reducing equivalents of NAD(P)H.[34-35] Since stoichiometric amounts of the cofactor are consumed in the course of this reaction, an additional enzyme is needed for its regeneration.

Besides the degradation of pyruvate there is also the possibility of recycling the amine donor with the use of an alanine dehydrogenase (AlaDH, [EC 1.4.1.1]), which is a NAD^+ dependent amino acid dehydrogenase.[36-37] Generally, it catalyzes the reversible oxidative deamination of L-alanine to pyruvate. The enzymological and kinetic properties of AlaDH have been investigated and the enzymes have been cloned from numerous bacteria. Most commonly known are the ones from *Bacillus* species, such as *Bacillus subtilis*. However, in transamination reactions the AlaDH is used for catalyzing the backward reductive amination of pyruvate under the consumption of ammonia and NAD(P)H leading to regenerated alanine (Scheme 1.4). Therefore, again an efficient cofactor recycling system for the resultant $NAD(P)^+$ is necessary. [16, 18, 30]



Scheme 1.4 Concept for the asymmetric synthesis catalyzed by ω-TA with concomitant recycling of the amine donor using an amino acid dehydrogenase (AADH)

A further limitation of this approach is that so far only an L-AlaDH has been applied, recycling exclusively L-alanine. The use of (*R*)-selective ω-TAs would require D-alanine as amine donor, though. In this case the system works like the one using LDH for pyruvate removal, albeit some (*R*)-selective ω-TAs were noticed to accept also L-alanine.[31] A possible approach addressing this issue would be the use of an alanine racemase. Therefore, racemization of L-alanine to the stereocomplementary D-alanine would facilitate a more efficient use of (*R*)-selective ω-TAs.

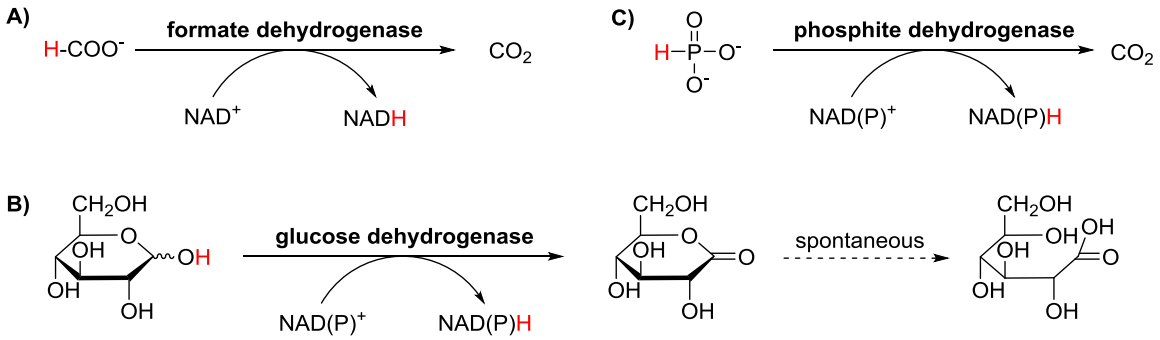
In order to address the issue of required cofactor regeration several well-established techniques for nicotinamide cofactor recycling have been developed and the most widely used ones are summarized in the following chapter 1.2.

1.2 Regeneration of nicotinamide cofactors

The use of ω-TAs in combination with an AlaDH requires the addition of nicotinamide cofactors (Scheme 1.4), which serve as carrier of redox equivalents. These cofactors are not permanently bound to the enzyme and thus act as co-substrates that must be used in stoichiometric amounts. However, since they are too expensive to be added as stoichiometric agents for preparative applications, they have to be regenerated *in situ*. Therefore, considerable research efforts have been put into the establishment of reliable regeneration systems in the past.[2, 11, 38]

A useful and efficient cofactor regeneration method should fulfill several requirements. The enzymes and reagents must be easily available, inexpensive and stable under the used reaction conditions. The energetics of the regeneration step should be favorable with respect to both kinetic and thermodynamic considerations. Furthermore, the reagents or co-products of the recycling step should not interfere with product isolation and have to be compatible with the reaction system. An important parameter representing the efficiency of a regeneration method is the 'total turnover number' (TTN), which is defined as the total number of moles of product formed per mole of cofactor during the entire lifetime. TTNs of 10^3 to 10^5 are considered to be sufficient to make a process economically feasible. Highly effective cofactor regeneration methods have already been developed, both chemical and enzymatic ones. Chemical and electrochemical strategies frequently are not able to achieve high TTNs and are often incompatible with the components of the enzymatic reaction.

Therefore, enzymatic methods are currently preferred for cofactor regeneration and have already been used in combination with numerous oxidoreductases and transferases for different applications.[39] Traditional enzymatic methods comprise the coupled-substrate approach and the coupled-enzyme approach. In the former strategy a single enzyme and a co-substrate are needed, whereas an additional recycling enzyme with its specific co-substrate is used together with the enzyme catalyzing the reaction of interest in the latter.[40] The following part focuses on the enzyme-coupled regeneration of nicotinamide cofactors, using either formate dehydrogenase, glucose dehydrogenase or phosphit dehydrogenase (Scheme 1.5).



Scheme 1.5 Methods for the enzymatic regeneration of reduced nicotinamide cofactors using either A) formate dehydrogenase, B) glucose dehydrogenase or C) phosphite dehydrogenase.

1.2.1 Formate dehydrogenase

A commonly applied method for recycling NADH uses formate dehydrogenase (FDH [EC 1.2.1.2]), which catalyzes the preferred oxidation of formate to carbon dioxide with the concomitant reduction of NAD^+ to NADH (Scheme 1.5). The enzyme has been characterized from different methanol-utilizing microorganisms such as *Candida boidinii*, *Pseudomonas sp.*, *Candida methanolica*, *Mycobacterium vaccae* or *Komagataella pastoris*.[40] This method is advantageous since the reaction utilizes a cheap and stable auxiliary substrate, namely (ammonium) formate, which is beyond that innocuous to the enzyme. The co-product, CO_2, is also harmless to enzymes and is easily removed from the reaction, driving the equilibrium toward product formation. Moreover, FDH is commercially available, reasonably stable, if protected from autooxidation and it can be easily immobilized. However, the application of FDH is limited to the regeneration of NADH due to its high cofactor specificity. This drawback can be circumvented though by the use of genetically engineered enzymes, which also accept $NADP^+$.[41] Therefore, the only disadvantage of this system is the low specific activity of FDH (3 U/mg). Nevertheless, the FDH/formate system is the most convenient and economical most attractive method for regeneration of NADH, with TTNs up to 6×10^5.[2, 42]

1.2.2 Glucose dehydrogenase

Another widely used method for recycling both NADH and NADPH makes use of a glucose dehydrogenase (GDH, [E.C. 1.1.1.47]), which catalyzes the $NAD(P)^+$-dependent oxidation of D-glucose to D-glucono-δ-lactone (Scheme 1.5). Since the formed lactone undergoes spontaneous hydrolysis to gluconic acid, the reaction is irreversible providing a favorable equilibrium. Several bacterial sources for GDHs do exist; the purified enzymes from *Bacillus cereus*, *Bacillus subtilis* and *Bacillus megaterium* have been characterized and are the most common ones.[43]

The enzyme offers several advantages that explain its favored use in regeneration: it is highly stable and it accepts both NAD^+ and $NADP^+$ equally well with high specific activity of up to 550 U/mg.[40] Additionally, the auxiliary substrate, glucose, is readily available, cheap, stable and even enhances the stability of many enzymes. However, there are also some drawbacks. Like FDH, GDH is not cheap and the polar co-product gluconate may complicate the reaction work-up. For processes, in which the gluconate does not complicate the isolation of the product, this method is quite a convenient way to regenerate NAD(P)H especially for laboratory use with TTNs up to 10^7.[2, 42, 44]

1.2.3 Phosphite dehydrogenase

More recently, phosphite dehydrogenase (PtDH, [EC 1.20.1.1]) has become of increasing importance as an alternative for NAD(P)H regeneration. The enzyme has lately been isolated from *Pseudomonas stutzeri* and happens to be the first protein that catalyzes a redox reaction on inorganic phosphorous compounds[45-46], namely the quasi irreversible oxidation of phosphite to phosphate by simultaneously reducing NAD^+ to NADH (Scheme 1.5). It also accepts $NADP^+$ as the oxidant, albeit far less effectively. However, by means of rational design and directed evolution an engineered thermostable mutant was created, that can successfully reduce both nicotinamide cofactors with high efficiency and, moreover, showed a considerably increased thermal stability compared to the wild-type PtDH. The use of PtDH for cofactor regeneration is quite advantageous due to the highly favorable reaction equilibrium and the inexpensive substrate phosphite. Furthermore, both phosphite and the product phosphate are innocuous to the enzymes and act as buffer. Altogether the above mentioned facts and TTNs up to 5×10^4 make the PtDH an efficient and potential alternative as an NAD(P)H-regeneration system.[40, 47-48]

1.3 Whole-cell biocatalysts

The history of using whole-cells as biocatalysts is a long and alternating one. In 1858, Louis Pasteur reported for the first time the microbial resolution of racemic tartaric acid to the enantiopure (-)-form using the fungi *Penicillium glaucum*. Brown *et al.* described in 1886 the selective oxidation of mannitol to fructose mediated by *Bacterium xylinum* and in 1921 Neuberg and Hirsch already discovered a biocatalytic whole-cell approach for C-C-bond formation. They realized the condensation of benzaldehyde with acetaldehyde in the presence of the yeast *Penicillium cerevisiae* producing optically active 1-hydroxy-1-phenyl-2-propanone.[10, 49]

However, the achievements in whole-cell biotransformations were not restricted to fundamental research, but whole-cells have also been applied in a number of early industrial biocatalytic reactions. Indeed, some of the first large-scale enzyme processes were catalyzed by whole-cells. For example the hydrolysis of penicillin and the production of glucose-fructose syrup starting from starch, both replacing classical chemical processes and representing greener and highly sustainable alternatives.[8] Therefore, whole-cell biocatalysis has become well-known over the last centuries and has even attracted increasing interest for the production of chiral compounds in the last years.

In whole-cell biocatalytic reactions one can generally distinguish between two possible procedures. In a growing-cell biotransformation organisms are cultivated in a growth medium and after the biomass has reached an appropriate substantial level, the substrate is added and the product formation proceeds in a fermentative way. Alternatively, the biomass is harvested by centrifugation or filtration after a proper period of growth, then washed and resuspended in a buffer of choice. The resultant cell suspension is subsequently combined with the substrate, representing a resting-cell biotransformation. In each case, the necessary enzymes for the transformation are provided in its 'natural' state inside the cells. Therefore, the substrate has to cross the cell membrane of the organism in order to be converted and formed products must cross the same barrier back into the surrounding medium after transformation.[1]

The industrial application of living or resting microorganisms for whole-cell biotransformation is quite favorable compared to the use of isolated enzymes. The major advantage is that the addition of a cofactor is not necessary and the cell metabolism facilitates the *in vivo* regeneration of the redox equivalents. Metabolic intermediates are available in the intracellular environment and their supply can be regulated for example by feeding certain nutrients like glucose. Furthermore, whole-cell biocatalysts are usually more stable due to the presence of the natural cell surrounding protecting the enzymes and there is no need for expensive enzyme isolation and purification, hence whole-cell catalysts can be much more inexpensively and readily prepared. [50-53] Therefore, the use of wild-type cells in asymmetric synthesis has already been reported numerous times. [54-55]

However, wild-type microorganisms from natural environments often show decreased stereoselectivities due to the presence of multiple competing enzymes or due to a low stereoselectivity of the enzyme for the non-natural substrate. Moreover, the yields are quite limited since the produced compounds can be metabolized by the cell. Due to these above mentioned reasons and the rapid advances and investigations in molecular biology and genetics, the use of unmodified wild-type microorganisms is constantly declining.[2]

An alternative approach addressing these issues is the application of so-called “designer-cells”, which are recombinant microbial cells overexpressing the required biocatalysts simultaneously. For this purpose only the intracellular amount of cofactor is used and unwanted side reactions are reduced due to the high amount of recombinant enzyme produced.[56] In the last decade, a number of applications of such “tailor-made” biocatalysts in asymmetric synthesis has been published, e.g. in the stereoselective reduction of ketones, α-hydroxy-ketones, α-halo-ketones, α-keto esters or in the reductive amination of ketones and α-keto esters.[50, 56-60]

For biotransformations using recombinant whole-cells the first crucial parameter to consider is the choice of the production host. An ideal host is susceptible to genetic manipulation, grows fast in simple media and reaches high levels of enzyme production. Furthermore, it should be recalcitrant to a wide range of substrates and products and be also compatible with two-phase systems and subsequent downstream processing procedures. If the biotransformation is intended to be coupled with the host’s cellular metabolism, the ideal organism enables easy export of products and should not have any endogenous catalytic activity toward potential reaction components. Based on the ease of genetic manipulation and the high levels of heterologous protein expression, *E. coli* is a valuable host for whole-cell biotransformations.[61]

Further requirements for the successful construction of an efficient “tailor-made” whole-cell catalyst are a good co-expression of the genes leading to comparable enzyme activities in the cell.[56] In general, there are different strategies available to generate such a recombinant whole-cell catalyst. In an indirect approach each of the various enzymes is at first produced in a different *E. coli* strain and in the subsequent biotransformation, cells from each strain are combined in different amounts according to the varying enzyme activities. While this concept facilitates an easy adjustment of enzyme activities, disadvantages could be the membrane barrier and diffusion problems as well as the requirement of multiple fermentations. Alternatively, the use of recombinant DNA technology enables the simultaneous production of all enzymes in a single strain. For this purpose one can choose between a one- or a two-plasmid strategy by cloning the genes of interest into one or two plasmids, that have compatible origins of replication and independent antibiotic selection important for maintenance within the cell. Using this approach there are several ways to adapt the activities of the different enzymes toward the substrate for an optimal reaction. On the one hand the genes can be expressed with different promoters, e.g. the gene with the lowest activity is expressed under the control of the strongest promoter and *vice versa*. On the other hand the genes can be introduced into plasmids with different copy numbers, so that the enzymes are produced at different levels according to the given plasmid copy number.[62-63]

Another possibility for the construction of a recombinant whole-cell catalyst would be the combination of the one- or two-plasmid strategy with the integration of one gene of interest into the genome of the used microbial strain.

A comparison of different co-expression strategies for the construction of whole-cell biocatalysts has been reported by Josef Altenbuchner and co-workers.[63] They developed a catalytic system for the enzymatic production of L-amino acids from hydantoins by co-expressing a L-hydantoinase, a L-*N*-carbamoylase and a hydantoin racemase from *Arthrobacter aurescens* using various approaches. The enzymatic activity within the cells was adjusted by employing vectors with different copy numbers for protein expression and in one approach the gene encoding the hydantoinase was integrated into the *E. coli* chromosome. Different strategies for co-expression were also used by Manfred T. Reetz and Rubén Agudo for the construction of designer cells catalyzing an enzymatic cascade reaction.[64] They combined a cytochrome P450 enzyme from *Bacillus megaterium* with the enoate reductase YqjM belonging to the Old Yellow Enzyme family. For this purpose, the co-expression was either realized by using two compatible plasmids or by combining the one-plasmid strategy with a chromosomal integration. Recent and most important publications dealing with the construction of whole-cell catalysts based on either a one- or a two-plasmid strategy or on genomic integration are summarized in Table 1.2.

Table 1.2 Representative applications of designer cells using different strategies for co-expression

Co-expression strategy	Product	Co-expressed genes	Used plasmid(s)	Ref.
1 plasmid	Chiral alcohols	Alcohol dehydrogenase (ADH) from *Rhodococcus erythropolis*; GDH from *Bacillus subtilis*	pNO14c	[57]
	Chiral amino acids	Amino acid dehydrogenase originating from various sources; FDH from *Candida boidinii*	pMcFDH	[65]
	Chiral diols	Diketoreductase from *Acinetobacter baylyi*; GDH from *Bacillus megaterium*	pETDuet-1	[66]
	L-Glyceraldehyde	Glycerol dehydrogenase from *Gluconobacter oxydans*; GDH from *Bacillus subtilis*	pETDuet-1	[60]
	Chiral α-hydroxy-ketones and -diols	Butanediol dehydrogenase from *Bacillus subtilis*; NADH oxidase from *Lactobacillus brevis*	pETDuet-1	[67]
	L-Isoleucine	Transaminase C from *E. coli*; AlaDH from *Bacillus subtilis*	pTrc99A	[50]
	Xylitol	Xylose reductase from *Candida tenius*; FDH from *Candida boidinii*	pETDuet-1	[68]

1 Plasmid and chromosomal integration	L-Amino acids	Hydantoinase (hyuH), *N*-carbamoylase and hydantoin racemase from *Arthrobacter aurescens*	pBR322 and integration of hyuH into genome	[63]
	Chiral oxocyclohexane carboxylates	Cytochrome P450 enzyme from *Bacillus megaterium*; YqjM enoate reductase	pRSFDuet-1 and integration of P450-BM into genome	[64]
2 plasmids	Chiral alcohols	ADH from *Lactobacillus kefir*; FDH from *Candida boidinii*; pyridine nucleotide transhydrogenase from *E. coli*	pACYCDuet-1 and pETDuet-1	[69]
	L-Amino acids	Hydantoinase, *N*-carbamoylase and hydantoin racemase from *Arthrobacter aurescens*	pBR322 and pACYC184	[63]
	Chiral hydroxyl acid derivatives	ADH from *Lactobacillus brevis*; FDH from *Mycobacterium vaccae*	pBtacLB and pBBR1MCS2	[70]
	Chiral β-hydroxy nitriles	ADH from *Lactobacillus kefir*; halohydrin dehalogenase from *Agrobacterium radiobacter*	pET30a and pACYCDuet-1	[71]
	L-*tert*-Leucine; L-Neopentylglycine	Leucine dehydrogenase from *Bacillus cereus*; FDH from *Candida boidinii*	pAM10-1 and pAM3-25	[56, 72]
	D-Mannitol	Mannitol dehydrogenase from *Leuconostoc pseudomesenteroides*; FDH from *Mycobacterium vaccae*, glucose facilitator protein from *Zymomonas mobilis*	pET24 and pZY507	[73]
	Chiral oxocyclohexane carboxylates	Cytochrome P450 enzyme from *Bacillus megaterium*; YqjM enoate reductase	pRSFDuet-1 and pACYC	[64]

The use of such recombinant whole-cell catalysts offers a considerable economical impact compared to the application of isolated enzymes (Figure 1.2). Major disadvantages of using isolated enzymes are the requirement of a number of separate fermentations, followed by an elaborate down-stream processing consisting of cell disruption, clarification and concentration for all fermentations runs. In contrast, the whole-cell approach only requires one fermentation run, with a subsequent simple cell separation serving as concentration step.[72]

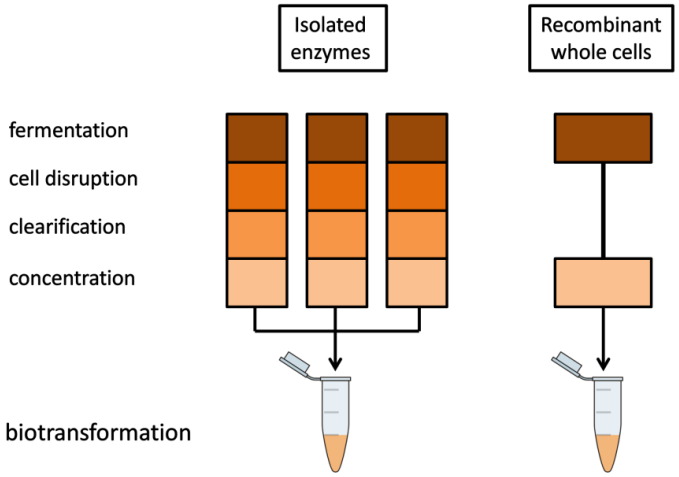


Figure 1.2 Comparison of the process economy of the concepts: isolated enzymes vs. whole cells

Therefore, the use of recombinant whole-cells offers significant advantages with respect to a greener and cost-attractive biocatalyst access, which is reflected in the growing number of processes applying such biocatalysts.[65, 67-70, 72-74]

2 RESEARCH OBJECTIVE

The main goal of this thesis was to develop a recombinant *E. coli* whole-cell catalyst co-expressing all required enzymes for the asymmetric reductive amination of ketones. This was enabled by a flexible co-expression approach, combining a two-plasmid strategy with the integration of one gene into the genome of the used *E. coli* host. Therefore, a modular and highly flexible assembly of the whole-cell catalyst was facilitated with module 1 bearing different ω-transaminases, module 2 providing an alanine dehydrogenase and module 3 offering various enzymes for nicotinamide cofactor regeneration (Figure 2.1).

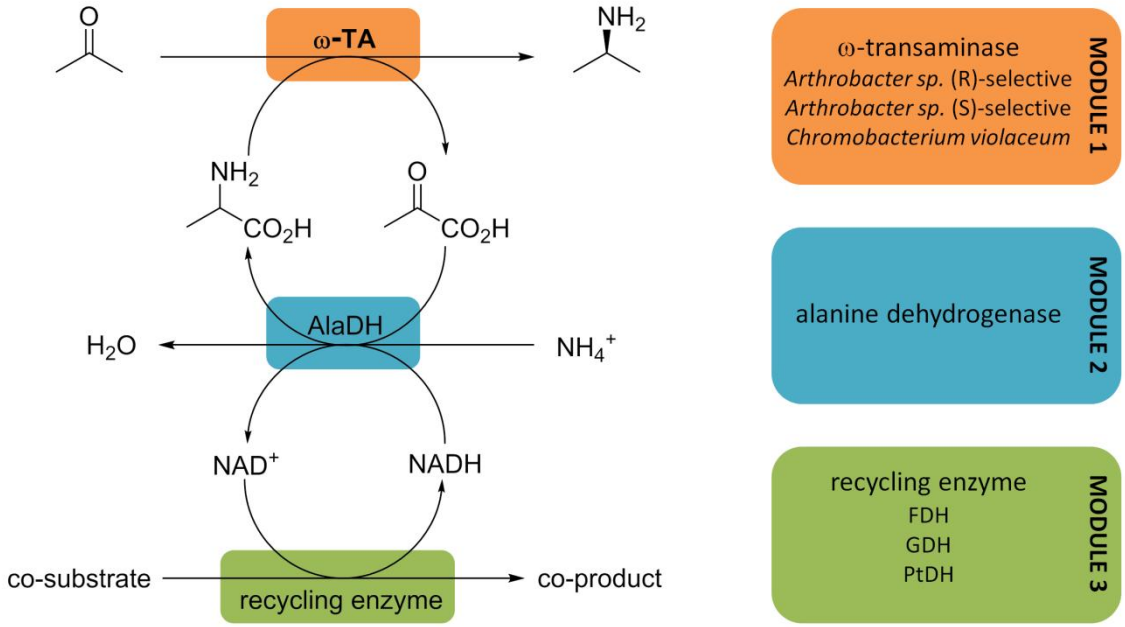


Figure 2.1 Modular flexibility for the asymmetric reductive amination employing the recombinant *E. coli* whole-cell biocatalyst. Module 1 provides different ω-TAs, module 2 consists of AlaDH and module 3 offers different enzymes for cofactor regeneration.

Three different ω-transaminases were selected for catalyzing the desired reductive amination, namely two (*S*)-selective ones from *Arthrobacter sp.* (ArS) and *Chromobacterium violaceum* (CV) and one (*R*)-selective ω-TA from *Arthrobacter sp.* (ArR). The genes encoding these enzymes were cloned into the pTrc99A vector under the control of the trc-promoter. Additionally they were cloned into the pASK-IBA3plus vector under the control of the tet-promoter. Using the latter for co-expression should facilitate the induction of the ω-TA protein production independently from the other enzymes. The genes encoding the enzymes for cofactor regeneration were cloned into a different vector, pBAD28, under the control of the ara-promoter.

For cofactor recycling one could choose between the formate dehydrogenase (FDH) from *Komagataella pastoris* GS115, the glucose dehydrogenase (GDH) from *Bacillus megaterium* and the phosphite dehydrogenase (PtDH) from *Pseudomonas stutzeri*. The gene for the alanine dehydrogenase (AlaDH) from *Bacillus subtilis*, which represented the fixed component of the enzymatic system, was integrated into the *E. coli* genome.

With the constructed whole-cell catalyst in hand, optimization of co-expression of the three enzymes was desired. In order to validate the production of active proteins and an ideal ratio of enzyme activities, photometric activity assays were performed. Furthermore, the applicability of the whole-cell catalyst for the synthesis of optically active amines should be investigated. For this purpose, the asymmetric reductive amination of 4-phenyl-2-butanone was chosen as model reaction and different catalyst preparations were employed and compared.

3 MATERIALS AND METHODS

3.1 General material

All chemicals, solvents, antibiotics and the components for the preparation of lysogeny broth (LB) medium were obtained from Sigma Aldrich or Roth and used as received unless stated otherwise. The used T4 DNA ligase, the restriction enzymes, the alkaline phosphatase and the corresponding buffers were purchased from Thermo Scientific. Centrifugation was performed in a Heraeus Biofuge pico at 13000 rpm or in a Heraeus Biofuge primo at 4000 rpm. Cell disruption *via* sonication was carried out with a Branson Digital Sonifier®. Measurement of DNA concentration, OD_{600}, and protein content was performed with an Eppendorf Bio Photometer Plus. All biotransformations were performed in an Eppendorf Thermomixer comfort. Overexpressions were carried out in a HT Infors Unitron AJ 260 incubator.

3.2 Strains and Plasmids

Table 3.1 Bacterial strains used in this work

Strain	Description	Source / reference
***E. coli* TOP10**	F´(proAB, lacIq, LacZΔM15, Tn10(tet-r)), mcrA,Δ(mrr-hsdRMS-mcrBC), Φ80ΔLacZΔM15, ΔlacX74, deoR, recA1, araD139(ara, leu), 7697, galU, galK, λ-, rpsL(streptomycin-r), endA1, nupG	Invitrogen
***E. coli* W3110 FCC 535**	$F^- \lambda^-$ IN(*rrn*D-*rrn*E)1 *rph*-1	-[75]
***E. coli* W1 FCC 536**	*E. coli* W3110; *Δ(araD-araB)*567::P_{trc}-*ald*, gene for alanine dehydrogenase (*ald*) from *Bacillus subtilis* D196A/L197R integrated into ara locus in the choromosome and expressed from P_{trc} promoter	Bielefeld
***E. coli* W1_FDH**	*E. coli* W1; carrying pBAD28 vector with gene encoding formate dehydrogenase (*fdh*) from *Komagataella pastoris* GS115	Bielefeld
***E. coli* W1_GDH**	*E. coli* W1; carrying pBAD28 vector with gene encoding glucose dehydrogenase (*gdh*) from *Bacillus megaterium*	Bielefeld
***E. coli* W1_PtDH**	*E. coli* W1; carrying pBAD28 vector with gene encoding phosphite dehydrogenase (*ptdh*) from *Pseudomonas stutzeri*	Bielefeld
***E. coli* W2 FCC 537**	W3110; *Δ(araD-araB)*567::P_{T5}-*ald*, *ald* expressed from strong bacteriophage P_{T5} promoter	Bielefeld

***E. coli* W2_FDH**	*E. coli* W2; carrying pBAD28 vector with gene encoding formate dehydrogenase (*fdh*) from *Komagataella pastoris* GS115	Bielefeld
***E. coli* W2_GDH**	*E. coli* W2; carrying pBAD28 vector with gene encoding glucose dehydrogenase (*gdh*) from *Bacillus megaterium*	Bielefeld
***E. coli* W2_PtDH**	*E. coli* W2; carrying pBAD28 vector with gene encoding phosphite dehydrogenase (*ptdh*) from *Pseudomonas stutzeri*	Bielefeld

Table 3.2 Plasmids used in this work

Plasmid	pEG	Description	Source / reference
pET21a-ArR	23	pET21a carrying (*R*)-selective ω-transaminase from *Arthrobacter sp.*	-[76]
pET21a-ArS	22	pET21a carrying (*S*)-selective ω-transaminase from *Arthrobacter sp.*	-
pET21a-CV	20	pET21a carrying (*S*)-selective ω-transaminase from *Chromobacterium violaceum*	-[20]
pTrc99A	-	P_{trc}, amp^R, pBR322*ori*, *rrnB* T1, *rrnB* T2, lac^{Iq}, bla, template for P_{trc} promoter	-[77]
pTrc99A-ArR	230	pTrc99A carrying (*R*)-selective ω-transaminase from *Arthrobacter sp.*	This work
pTrc99A-ArS	231	pTrc99A carrying (*S*)-selective ω-transaminase from *Arthrobacter sp.*	This work
pTrc99A-CV	232	pTrc99A carrying (*S*)-selective ω-transaminase from *Chromobacterium violaceum*	This work
pASK-IBA3plus	-	Amp^R, P_{Tet}, ColE1*ori*, F1*ori*,	-
pASK-IBA3plus-ArR	224	pASK-IBA3plus carrying (*R*)-selective ω-transaminase from *Arthrobacter sp.*	This work
pASK-IBA3plus-ArS	225	pASK-IBA3plus carrying (*S*)-selective ω-transaminase from *Arthrobacter sp.*	This work
pASK-IBA3plus-CV	226	pASK-IBA3plus carrying (*S*)-selective ω-transaminase from *Chromobacterium violaceum*	This work
pBAD28	-	P_{ara}, amp^R, cam^R, p15A*ori*, *rrnB* T1, *rrnB* T2, bla	-[78]
pBAD28*Δbla*	-	pBAD28 with deletion of β-lactamase gene	Bielefeld
pBAD28*Δbla-fdh*	227	pBAD28*Δbla* carrying formate dehydrogenase (*fdh*) from *Komagataella pastoris* GS115	Bielefeld
pBAD28*Δbla-gdh*	228	pBAD28*Δbla* carrying glucose dehydrogenase (*gdh*) from *Bacillus megaterium*	Bielefeld
pBAD28*Δbla-ptdh*	229	pBAD28*Δbla* carrying phosphite dehydrogenase (*ptdh*) from *Pseudomonas stutzeri*	Bielefeld

3.3 Oligonucleotides

The used oligonucleotides were purchased in purified and lyophilized form from Eurofins MWG Operon. The received amount was dissolved in dH_2O to a final concentration of 100 pmol/µL and stored at -20 °C.

Table 3.3 Oligonucleotides used in this work

Nr.	Name	Sequence 5'→3'	Tm [°C]	GC content [%]
1	ArS_Fw	ATATAGAATTCATGGGTCTGACCGTGCAG	65.3	44.8
2	ArS_Rev	TATAGTCGACTTAGCTCTGCTGCCATTC	65.1	46.4
3	ArR_Fw	ATATAGAATTCATGACCAGCGAAATTGTT	59.6	31
4	ArR_Rev	TATAGTCGACTTATTAATACTGAACCGG	60.7	35.7
5	CV_Fw	TATATGAATTCATGCAGAAACAGCGTACC	62.4	37.9
6	CV_Rev	TATAGTCGACTTAGGCCAGACCACGTG	66.5	51.9
7	IBA357_Fw	GAGTTATTTTACCACTCCCT	53.3	40
8	IBA357_Rev	CGCAGTAGCGGTAAACG	57.9	58.8
9	pTrc99a_Fw	CAATGCTTCTGGCGTCAGGCAG	65.6	59.1
10	pTrc99a_Rev	GATTTAATCTGTATCAGG	45.8	33.3
11	pBAD28_Fw	CGGCGTCACACTTTGCTATG	59.4	55
12	pBAD28_Rev	TTATCAGACCGCTTCTGCG	56.7	52.6

3.4 Microbiological methods

3.4.1 Growth media and conditions for the cultivation of *E. coli*

The cultivation of *E. coli* TOP10 and *E. coli* W3110 cells was performed at 37 °C using LB-medium, which was prepared as follows: sodium chloride (10 g/L), yeast extract (5 g/L) and tryptone (10 g/L) were dissolved in deionized water and autoclaved at 121 °C prior to use. For the preparation of agar plates bacteriological agar (15 g/L) was added.

Over-night cultures (ONC) were prepared by supplementing 10 mL LB-medium with the required antibiotic according to Table 3.4. After inoculation with a single colony of transformed cells the cultures were incubated over night at 37 °C and 120 rpm.

Table 3.4 Used antibiotic concentrations for the cultivation of *E. coli*

Antibiotic	Final concentration [µg/mL]		
	ONC	Agar plates	Main cultures
Amp (100 mg/mL)	100	100	100
Cam (50 mg/mL)	25	25	25
Kan (50 mg/mL)	25	25	-

3.4.2 Protein expression

Main cultures (60 mL-1000 mL) supplemented with the required antibiotic (Table 3.4) were inoculated with the appropriate volume of ONC to a start-OD_{600} of 0.05 and incubated at 37 °C and 120 rpm until an OD_{600} of approximately 0.5-0.6 was reached. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 0.25-0.5 mM), L-arabinose (0.2-0.002%) and anhydrotetracycline (AHTC, 200 ng/mL), if required. Shaking was continued over night at 120 rpm and 20 °C, 25 °C, 30 °C, 37 °C, respectively. The cells were then harvested by centrifugation (4000 rpm, 2683 g, 4 °C, 2x15 minutes) and the resulting cell pellet was either applied directly in whole cell biotransformations or stored at -20 °C until further use. By lyophilizing the whole-cell biocatalyst, its storage at 4 °C was possible for several weeks without any significant loss of enzyme activity. For this purpose the cell pellet was resuspended in a minimum amount of sodium phosphate buffer (NaP_i, 50 mM, pH 8, 0.5 mM PLP), frozen in liquid nitrogen and lyophilized over night.

3.4.3 Cell disruption

For cell disruption the cells were resuspended in NaP_i buffer (50 mM, pH 8) yielding a 15 wt% cell solution. Cells were disrupted by sonication using following settings: duration 15 s, 0.1 s sonicate, 0.4 s pause, 40% amplitude. Sonication was performed 2-3 times with short breaks in between and the cells were constantly cooled with ice. Afterwards the cell extract was centrifuged for 10 minutes with 13000 rpm (16060 g). The supernatant was transferred into a fresh tube and kept at 4 °C for further activity assays. Otherwise, the supernatant was frozen in liquid nitrogen and lyophilized over night yielding in lyophilized cell-free extract. The remaining cell debits were resuspended (15 vol%) in urea (6 M) for SDS-analysis.

3.4.4 Preparation of competent *E. coli* cells

The preparation of competent *E. coli* cells was carried out based on the rubidium chloride method of Hanahan.[79] For this purpose LB-medium (50 mL) supplemented with $MgSO_4$ (1 mL of a 1 M solution) and $MgCl_2$ (1 mL of a 1 M solution) was inoculated with an ONC to a start-OD_{600} of 0.05 and incubated at 37 °C and 120 rpm. After an OD_{600} of 0.7 was reached, the cells were centrifuged at 4 °C for 15 minutes at 5000 rpm (4193 g). The following steps were exclusively performed on ice. The supernatant was removed and the cell pellet was resuspended in ice-cold TMF-buffer (5 mL, 100 mM $CaCl_2$, 50 mM $RbCl_2$, 40 mM $MnCl_2$, 20 vol% glycerol. Aliquots of 250 µL were frozen in liquid nitrogen and stored at -80 °C.

3.4.5 Transformation of competent *E. coli* cells

For the transformation of DNA into *E. coli* TOP10 and *E. coli* W3110 cells, the competent cells (250 µL) were mixed with plasmid solution (approximately 100-150 ng DNA) and incubated for 30-60 minutes on ice. In case of transforming a ligation reaction 5 µL (approx. 300 ng DNA) were used. After the mentioned incubation time a heat shock was performed for 1 minute at 42 °C, followed by an incubation on ice for 2 minutes and the addition of LB-medium (750 µL). The transformation samples were then shaken at 37 °C and 650 rpm in a thermomixer using incubation times according to the antibiotic(s) encoded by the plasmid (Table 3.5). Afterwards the cells were spread on LB-Agar containing the necessary antibiotic corresponding to the plasmid encoded resistance.

Table 3.5 Incubation times at 37 °C and 650 rpm for expression of the plasmid encoded antibiotic resistance

	Incubation time [min]
Amp	30
Kan	60
Cam	120

3.5 Molecularbiological methods

3.5.1 Preparation of plasmid DNA

The plasmid DNA was isolated from the respective ONCs using the QIAprep Spin Miniprep Kit according to the manual provided by the supplier. Afterwards the concentration of double stranded DNA in solution was determined using an Eppendorf BioPhotometer Plus.

3.5.2 Amplification of DNA via polymerase chain reaction

For the general amplification of DNA a polymerase chain reaction (PRC) was performed. The basic components for the PCR reaction are listed in Table 3.6.

Table 3.6 Basic reaction components for PCR with a total volume of 50 µL

Component	Total volume of 50 µL	Final concentration
dH_2O	Add to 50 µL	
5x Phusion HF buffer	10 µL	1x
10 mM dNTP's	1 µL	200 µM
Primer A (forward)	2.5 µL	0.5 µM
Primer B (reverse)	2.5 µL	0.5 µM
Template DNA	1 µL	< 250 ng
Phusion DNA Polymerase (2 U/µL)	0.5 µL	1 U/50 µL PCR

For the amplification the temperature program given in Table 3.7 was used. The PCR was performed in the Mastercycler® gradient thermocycler from Eppendorf. The annealing temperature depends on the primer length and on the GC content. Concerning the extrension time the amplicon length and complexity has to be considered. Generally, 25-35 cycles yields sufficient product.

Table 3.7 General PCR program

PCR Steps	Temperature [°C]	Time [s]	Cycles
Initial denaturation	98	30	1
Denaturation	98	10	30
Primer annealing	X	15	
Extension	72	45	
Final extension	72	300	1
Hold	4	hold	1

In Table 3.8 the primer pairs with the corresponding annealing temperature for all standard PCR reactions performed during this work are listed.

Table 3.8 PCR conditions for all standard PCRs

PCR product	Template	Primer pairs	Annealing temperature [°C]
ArR	pET21a-ArR	ArR_Fw ArR_Rev	57.8
ArS	pET21a-ArS	ArS_Fw ArS_Rev	63.1
CV	pET21a-CV	CV_Fw CV_Rev	60.5

In order to purify the PCR reaction products the QIAquick PCR Purification Kit from Qiagen was carried out according to the manual provided by the supplier.

3.5.3 Restriction digest

The PCR products and the vectors were digested with the restriction enzymes *EcoR*I and *Sal*I. By using these two restriction enzymes sticky ends were produced with matching regions to overlap during the ligation process. The components for the digestion of the plasmids were mixed as described in Table 3.9 and incubated for 20 min at 37 °C. The components for the digestion of the PCR products were mixed and incubated as described in Table 3.10. Since *EcoR*I offers a higher star activity it was added as soon as the digest with *Sal*I have lasted for 1.5 hours and was then prolonged for another 30 minutes. The reactions were stopped by thermal inactivation of the enzyme at 80 °C for 5 minutes.

Table 3.9 Digestion of the plasmids

Restriction component	Volume [µL]
Plasmid DNA (approx. 3 µg)	50.5
FastDigest Green Buffer (10x)	6
FastDigest *EcoR*I	1.5
FastDigest *Sal*I	2
Total volume	60

In order to purify the digested products the QIAquick PCR Purification Kit from Qiagen was performed according to the manual provided by the supplier. Otherwise, the samples were purified *via* preparative agarose gel followed by the QIAquick Gel Extraction Kit.

Table 3.10 Digestion of PCR products

Restriction component	Volume [µL]	
DNA	10	Incubation for 1.5 h at 37 °C
FastDigest Green Buffer (10x)	5	
FastDigest *Sal*I	4	
dH_2O	31	
FastDigest *Eco*RI	4	Incubation for 0.5 h at 37 °C
FastDigest Green Buffer (10x)	1	
dH_2O	5	
Total volume	60	

The procedure of restriction digest was further used for validating correct ligation. For this purpose ONCs of the respective *E. coli* cells transformed with ligated plasmid were prepared. Afterwards the DNA was isolated and digested with the restriction enzymes *Eco*RI and *Sal*I by incubating following components (Table 3.11) for 10 minutes at 37 °C. The control cut was subsequently analyzed on agarose gel.

Table 3.11 Restriction control conditions

Component	Volume [µL]
Plasmid DNA	3
FastDigest Green Buffer (10x)	1
FastDigest *Eco*RI	0.5
FastDigest *Sal*I	0.5
Total volume	10

3.5.4 Dephosphorylation

Dephosphorylation of the linear vector using an alkaline phosphatase was performed in order to prevent recircularization during ligation and thus to increase the efficiency of the ligation. The basic components for the dephosphorylation are listed in Table 3.12.

Table 3.12 Dephosphorylation of linear vectors

Dephosphorylation component	Volume [µL]
Plasmid DNA (approx. 250 ng)	17
FastAP Buffer (10x)	2
FastAP	1
Total volume	20

After the mixture was incubated at 37 °C for 10 minutes the reaction was stopped by thermal inactivation of the alkaline phosphatase at 75 °C for 5 minutes.

3.5.1 Agarose gel electrophoresis

To separate nucleic acids according to their size for analytical and preparative purpose, agarose gel electrophoresis was performed. For the preparation of the agarose gels 1 wt% agarose was dissolved in 1xTAE buffer (40 mM Tris-acetate and 1 mM EDTA, Invitrogen) by microwave treatment. After cooling Invitrogen SYBR® Safe DNA Gel Stain solution was added (1:10000) and the mixture was transferred into the casting tray together with a comb, which was removed after polymerization. The samples were mixed with 5x Loading Dye (100 mM EDTA, 43% glycerol, 0.05% bromophenol blue) and then loaded onto the gel. As DNA standard GeneRuler™ DNA ladder mix (5 µL, see Figure 3.1) from Thermo Scientific was applied. Electrophoretic separation was performed at a voltage of 130 V for 20-30 min. For detection the Vilber Lourmat Gel Imaging System was used. After performing a preparative gel the particular bands of the gel were excised and the DNA was isolated by QIAquick Gel Extraction Kit according to the manual provided by the supplier.

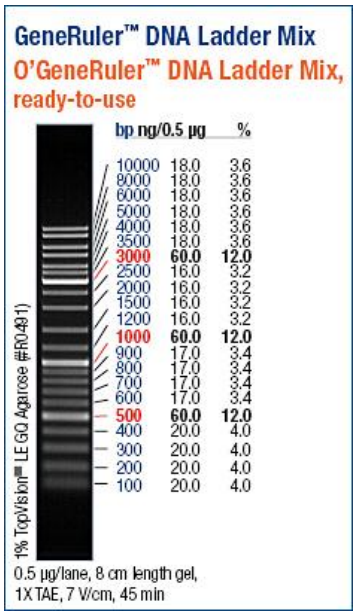


Figure 3.1 Reference bands and mass distribution of the GeneRuler™ DNA Ladder Mix

3.5.2 Ligation

For the ligation approximately 50-60 ng of digested vector and a 3-5 molar excess of insert DNA were incubated with T4 DNA Ligase for 20 minutes at room temperature. Afterwards the samples were stored over night at 4 °C and then directly used for transformation in *E. coli* TOP10 cells. Additionally, one ligation control without insert was performed for each backbone vector. The components for the ligation reaction are listed in Table 3.13.

Table 3.13 Basic reaction components for ligation

Component	Volume [µL]
Plasmid DNA	1
Insert DNA	4
Puffer (10x)	2
dH_2O	12
T4 DNA Ligase (5 weiss U/µL)	1
Total volume	20

3.5.3 Sequencing

Sequencing was performed by LGC Genomics GmbH in order to confirm the correct sequence of the cloning products. For this purpose plasmid DNA was isolated and a total volume of 10 µL containing approximately 100 ng/µL DNA was prepared and sent together with 4 µL of the respective primer (5 pmol/L) for sequencing.

3.6 Biochemical methods

3.6.1 Determination of protein concentration

The protein concentration in the cell extract was determined by the Bio-Rad Protein Assay based on the method by Bradford.[80] The binding of Coomassie Brilliant Blue G-250 to protein present in the solution leads to a shift in the absorption maxiumum of the dye into the blue region and thus the increase of absorption at 595 nm can be measured. For this purpose the Biorad Bradford reagent (490 µL , 1:5 dilution) was mixed with the supernatant (10 µL; 1:100 dilution) and incubated at room temperature. After at least 5 minutes incubation the absorption of the sample was measured at 595 nm. The dye solution with 10 µL of sodium-phosphate buffer (50 mM, pH 8) served as reference sample. The calculation of the corresponding protein content was carried out based on a calibration curve with bovine serum albumine.

3.6.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins can be separated on SDS-PAGE in an electrical field according to their molecular weight due to complexation of the polypeptide with the negatively charged detergent sodium dodecyl sulfate (SDS).[81] The separating gel (10%) was prepared by mixing acrylamide (5 mL, 30 wt%), Tris-HCl buffer (5.625 mL, 1 M, pH 8.8), distilled water (4.093 mL), SDS stock solution in dH_2O (150 µL, 10 wt%), *N,N,N',N'*-tetramethylethylenediamine (TEMED, 12 µL) and freshly prepared ammonium persulfate (APS) stock solution in dH_2O (120 µL, 10 wt%) in order to initiate the polymerization. Then the mixture was filled in a gel caster and covered with isopropanol, which was again completely removed after occurred polymerization (0.5-1 h).

The stacking gel was prepared by adding TEMED (5 µL) to a solution of acrylamide (0.833 mL, 30 wt%), Tris-HCl buffer (0.625 mL, 1 M, pH 6.8), distilled water (3.462 mL), SDS (50 µL, 10 wt%) and freshly prepared APS (25 µL, 10 wt%) stock solution in dH_2O. The mixture was filled upon the separating gel layer and a comb was placed in order to create wells, which was removed after polymerization (0.5-1 h).

For preparation of the samples in general the amount corresponding to 15 µg of protein was mixed with a 2-fold Laemmli buffer (1 mL 1M Tris/HCl buffer, pH 6.8; 400 mg SDS, 300 mg DTT, 20 mg bromophenol blue, 2 mL glycerol). After incubation at 95 °C for 10 minutes the samples were loaded onto the gel together with BioRad Precision Plus Protein All Blue Standard (5 µL, see Figure 3.2) as reference. The gel was run for 50 minutes at 180 V in a SDS running buffer (3.75 g/L Tris, 18 g/L glycine, 1.25 g/L SDS).

The gel was then incubated for 1-4 hours in a colloidal Coomassie "blue silver" staining solution[82] (0.6 mg Coomassie Blue G-250, 50 g ammonium sulfate, 58.82 mL 85% orthophosphoric acid, 100 mL ethanol, 500 mL dH_2O) followed by decolorization in distilled water over night.

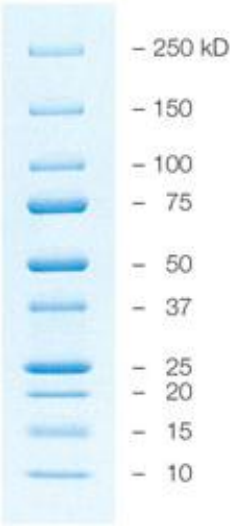
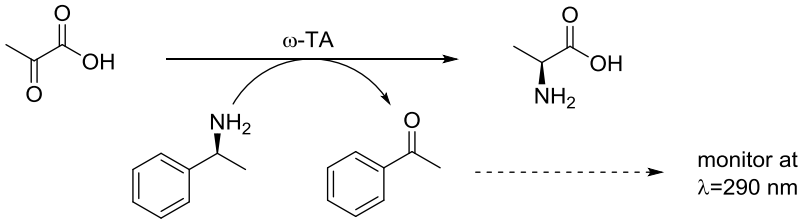


Figure 3.2 BioRad Precision Plus All Blue Protein Standard

3.6.3 Determination of enzyme activities in cell-free extracts

Activity assay for the CV-ω-TA

The determination of the enzyme activity of the CV-ω-TA was performed *via* an indirect photometric assay based on the reductive amination of pyruvate to L-alanine catalyzed by the ω-TA using (*S*)-methylbenzylamine as amine donor (Scheme 3.1). The increase of acetophenone over time was monitored photometrically at 290 nm.



Scheme 3.1 Representative figure for the oxidative deamination catalyzed by ω-transaminase

For this purpose a substrate solution (990 µL) containing PLP (0.1 mM), (*S*)-methylbenzylamine (10 mM) and sodium pyruvate (5 mM) in NaP_i buffer (50 mM, pH 8) was mixed with the cell-free extract (10 µL) in a semi-micro UV-cuvette. Immediately after the addition the increase of acetophenone over time (initial rate $\Delta c/\Delta t$) was measured at 290 nm.

Using the Lambert-Beer law the volumetric enzyme activity could be calculated according to Formula 3.1. The molar extinction coefficient of acetophenone was determined *via* calibration at 290 nm (Figure 3.3) and has a value of 0.8304 $mL \cdot mmol^{-1} \cdot cm^{-1}$ at pH 8. The specific activity, that is the activity per mg protein in solution, was calculated according to

Formula 3.2.

Formula 3.1 Lambert-Beer law for the determination of the volumetric enzyme activity

$$\frac{\Delta c}{\Delta t} = \frac{\Delta E}{\Delta t} \times \frac{v_{total}}{v_{sample}} \times \frac{1}{\varepsilon \times d}$$

$\Delta c/\Delta t$...change of concentration per time unit [mmol/min·mL]

$\Delta E/\Delta t$...change of absorption per time unit [min^{-1}]

ε...molar extinction coefficient [mL/mmol·cm]

d...layer thickness [cm]

v_{total}/v_{sample}...dilution factor

Formula 3.2 Calculation of the specific activity

$$specific\ activity\ \left[\frac{U}{mg}\right] = \frac{volumetric\ activity\ [\frac{U}{mL}]}{protein\ content\ [\frac{mg}{mL}]}$$

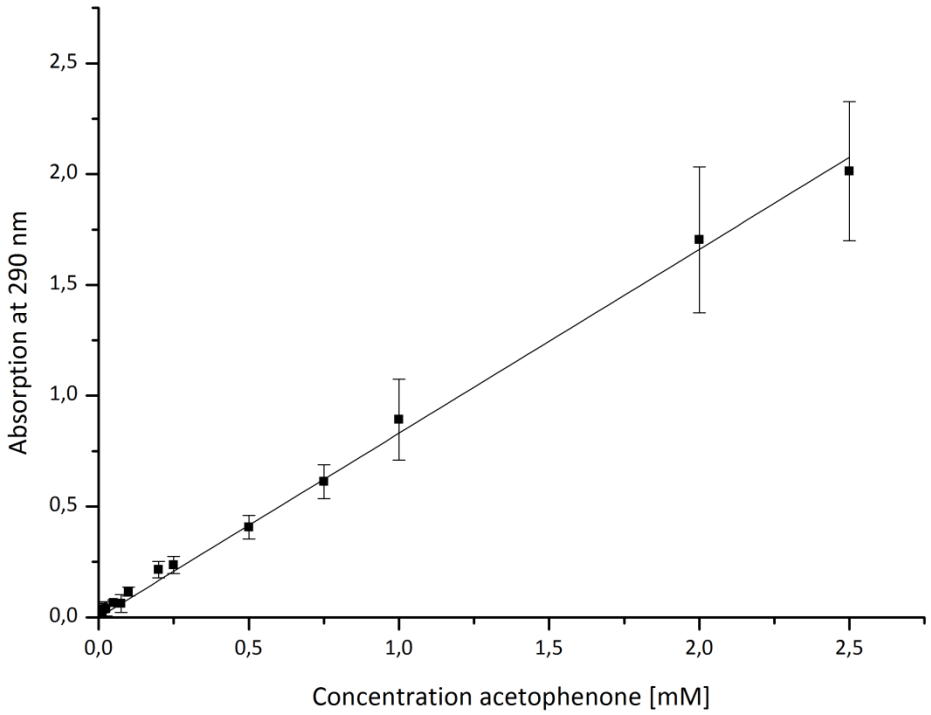
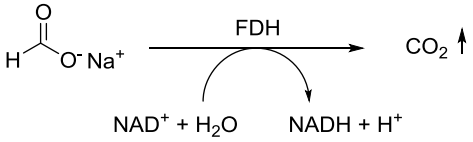


Figure 3.3 Calibration curve of acetophenone at 290 nm resulting in the molar extinction coefficient

Activity assay for the AlaDH and the cofactor regenerating enzymes

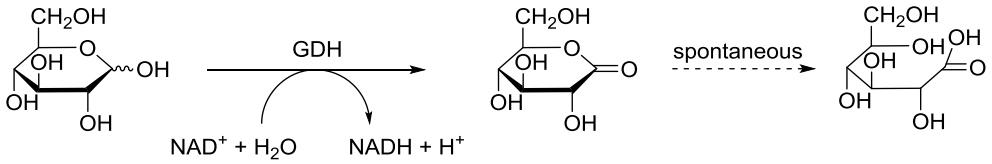
The determination of the enzyme activities of formate dehydrogenase (FDH), glucose dehydrogenase (GDH), phosphite dehydrogenase (PtDH) and alanine dehydrogenase (AlaDH) was performed by an indirect photometric assay based on a redox reaction catalyzed by the particular enzyme using NAD^+/NADH as oxidizing/reducing agent. As the cofactor NADH has its absorption maximum at a wavelength of 340 nm (ε = 6.2 $mL \cdot mmol^{-1} \cdot cm^{-1}$), its increase or decrease, respectively can be measured photometrically.

The assay for the determination of the FDH activity was based on the redox reaction illustrated in Scheme 3.2. Formate was oxidized to carbon dioxide catalyzed by FDH using NAD^+ as oxidant, which in turn was reduced to NADH.



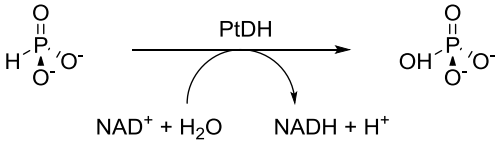
Scheme 3.2 Representative figure for the oxidation of formate to carbon dioxide catalyzed by formate dehydrogenase

The determination of the enzyme activity of GDH was based on the redox reaction shown in Scheme 3.3. Glucose was oxidized to gluconolactone catalyzed by GDH using NAD^+ as oxidant, which in turn was reduced to NADH. The formed gluconolactone undergoes spontaneous hydrolysis to gluconic acid afterwards.



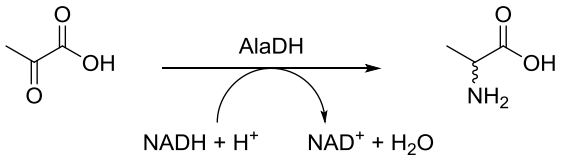
Scheme 3.3 Representative figure for the oxidation of glucose to gluconolactone catalyzed by glucose dehydrogenase.

The activity assay for the PtDH uses the redox reaction illustrated in Scheme 3.4, where phosphite (III) is oxidized to phosphate (V) catalyzed by PhoDH using NAD^+ as oxidant, which in turn is reduced to NADH.



Scheme 3.4 Representative figure for the oxidation of phosphite to phosphate catalyzed by phoshite dehydrogenase

On the other hand for the determination of the activity of AlaDH the reductive amination of pyruvat to alanine was monitored, which can be seen in Scheme 3.5. NADH was used as reducing agent, which in turn was oxidized to NAD^+.



Scheme 3.5 Representative figure for the reductive amination of pyruvate to alanine catalyzed by alanine dehydrogenase

The substrate solution (970 µL) containing either ammonium formate (100 mM), D-glucose (100 mM), sodium phosphite (100 mM) or sodium pyruvate (10 mM) in NaP_i buffer (50 mM, pH 8) was mixed with a NAD^+ (100 M) or NADH (12.5 mM) stock solution, respectively. After the addition of cell-free extract (10 µL) the increase/decrease of NADH over time (initial rate $\Delta c/\Delta t$) was determined immediately at 340 nm.

3.6.1 Determination of initial rates of the whole-cell catalyst

The activity of the recombinant *E. coli* whole-cell system was assayed for the reductive amination of 4-phenyl-2-butanone to the corresponding amine over 1-2 hours. Freshly harvested cells (50 mg) were resuspended in NaP_i buffer (500 µL, 50 mM, pH 8, 2 mM PLP) and the substrate solution (500 µL) was added containing L-alanine (250 mM, 5 eq), NAD^+ (2 mM), the particular co-substrate (300 mM, 6 eq) depending on the cofactor-recycling enzyme and ammonium acetate (150 mM, 3 eq) dealing as nitrogen donor for the reaction systems using GDH and PtDH. 4-Phenyl-2-butanone (3.8 µL, 25 mM) was added and the reaction was incubated at 30 °C and 800 rpm. After 5, 10, 20, 40, 60 and 120 minutes, respectively the reaction was stopped with aqueous NaOH solution (20 µL, 10 M) and extracted with EtOAc (2 x 400 µL). The combined organic layers were dried over Na_2SO_4 and conversions were analyzed by GC on an achiral column (see chapter 3.8). The enzyme activity was determined by plotting the amount of converted substrate (µmol) against the time, as 1 U is defined as the amount of enzyme that catalyzes the conversion of 1 micromole of substrate per minute.

3.7 Biotransformations

The biotransformations were performed in NaP_i buffer (50 mM, pH 8) with L-alanine as amine donor and a substrate concentration of 25 mM. The particular co-substrate was applied according to the used cofactor-recycling enzyme. Along with the FDH catalyzed recycling system ammonium formate was used, while for the GDH system glucose and for the PtDH system sodium phosphite was required. For the GDH and the PtDH system additionally ammonium acetate was needed as nitrogen donor.

3.7.1 Whole-cell biotransformations

Freshly harvested cells (50 mg) of the recombinant *E. coli* were resuspended in NaP_i buffer (500 µL, 50 mM, pH 8, 2 mM PLP) in an Eppendorf tube (1.5 mL). A substrate stock (500 µL) was added containing L-alanine (250 mM, 5 eq), NAD^+ (2 mM), the particular co-substrate (300 mM, 6 eq) depending on the cofactor-recycling enzyme and ammonium acetate (150 mM, 3 eq) in case of the GDH and PtDH recycling system. Finally the biotransformation was started by the addition of the substrate 4-phenyl-2-butanone (3.8 µL, 25 mM) and the mixture was shaken at 30 °C and 800 rpm for up to 48 hours using an orbital shaker. After 4 h, 24 h and 48 h, respectively 200 µL of each sample were withdrawn and the reaction was quenched with aqueous NaOH solution (20 µL, 10 M). After extraction with EtOAc (2 x 400 µL) the combined organic layers were dried over Na_2SO_4, and conversions were analyzed by GC on an achiral column (see Table 3.14).

3.7.2 Biotransformation with cell-free extract

The cell-free extract obtained after cell disruption (~350 µL, corresponding to 50 mg wet cells, see chapter 3.4.3) was added to NaP_i buffer (150 µL, 50 mM, pH 8, 6.6 mM PLP) in an Eppendorf tube (1.5 mL). Then the substrate stock solution in NaP_i buffer (500 µL) was added containing L-alanine (250 mM, 5 eq), NAD^+ (2 mM), the co-substrate (300 mM, 6 eq.) corresponding to the cofactor-recycling system and ammonium acetate (150 mM, 3 eq)) in case of the GDH and PtDH recycling system. After starting the reaction with the addition of the substrate 4-phenyl-2-butanone (3.8 µL, 25 mM) the samples were shaken at 30 °C and 800 rpm for up to 48 hours using an orbital shaker. After 4 h, 24 h and 48 h 200 µL of each sample were withdrawn and the reaction was quenched with aqueous NaOH solution (20 µL, 10 M). Then the mixture was extracted with EtOAc (2 x 400 µL), the combined organic layers were dried over Na_2SO_4 and conversions were analyzed by GC on an achiral column (see Table 3.14).

3.7.3 Biotransformation with lyophilized cells and lyophilized cell-free extract

The lyophilized cells (corresponding to 50 mg wet cells) or lyophilized cell-free extract (corresponding to 350 µL lysate), respectively of the recombinant *E. coli* were rehydrated in NaP_i buffer (500 µL, 50 mM, pH 8, 2 mM PLP) for 15 minutes at 30 °C and 120 rpm. Then NaP_i buffer (500 µL) was added containing L-alanine (250 mM, 5 eq), NAD^+ (2 mM), the required co-substrate (300 mM, 6 eq) depending on the cofactor-recycling system and ammonium acetate (150 mM, 3 eq) in case of the GDH and PtDH recycling system. Finally the biotransformation was started by the addition of the substrate 4-phenyl-2-butanone (3.8 µL, 25 mM) and the samples were incubated at 30 °C and 800 rpm for up to 48 hours using an orbital shaker. After 4 h, 24 h and 48 h, respectively 200 µL of each sample were withdrawn, aqueous NaOH solution (20 µL, 10 M) was added and the mixture was extracted with EtOAc (2 x 400 µL). The combined organic layers were dried over Na_2SO_4 and conversions were analyzed by GC on an achiral column (Table 3.14).

3.7.4 Derivatization for determination of the *enantiomeric excess*

For the determination of the *enantiomeric excess* (*ee*) the regarding samples were derivatized by incubating them with pyridine (2 eq) and acetic anhydride (5 eq) for 2 hours at 40 °C and 800 rpm. The reaction was quenched with aqueous $NaHCO_3$ (250 µL) and extracted with EtOAc (500 µL). The combined organic layers were dried over Na_2SO_4 and subsequently analyzed on GC using a chiral column (see Table 3.15).

3.8 Analytical methods

3.8.1 Determination of conversion

GC-analysis for determination of conversions was carried out on an Agilent 7890 A GC system equipped with a flame ionization detector (FID) using H_2 as carrier gas. The conversion of 4-phenyl-2-butanone to the corresponding 4-phenyl-2-butylamine was determined on an achiral stationary phase using a 14% cyanopropylphenyl phase capillary column (J&W Scientific DB-1701; 30 m x 250 µm x 0.25 µm) with an injection and detection temperature of 250 °C.

Table 3.14 GC-Method and retention times for the determination of conversion

Method parameters	
Injector temperature	250 °C
H_2 flow	1.5 mL/min
Injection pressure	0.796 bar
Temperature program	120 °C, 10 °C/min to 180 °C, 60 °C/min to 280 °C, hold 2 min
t_{ret} (amine)	3.30 min
t_{ret} (ketone)	3.65 min

3.8.2 Determination of the *enantiomeric excess*

GC-analysis for determination of *ee* was carried out on an Agilent 7890 A GC system equipped with a flame ionization detector (FID) using H_2 as carrier gas. The conversion of 4-phenyl-2-butanone to the corresponding 4-phenyl-2-butylamine was determined on a chiral stationary phase using a β-cyclodextrin capillary column (CP-ChiraSil-DEX CB; 30 m x 250 µm x 0.25 µm) with an injection and detection temperature of 250 °C.

Table 3.15 GC-Method and retention times for the determination of *enantiomeric excess*

Method parameters	
Injector temperature	200 °C
H_2 flow	2 mL/min
Injection pressure	0.739 bar
Temperature program	120 °C, 5 °C/min to 180 °C, hold 2 min
t_{ret} (*S*)-amine	11.7 min
t_{ret} (*R*)-amine	11.9 min

4 RESULTS

4.1 Cloning of ω-TA constructs

In a first step different ω-transaminases (ω-TA), namely two (*S*)-selective ω-TAs from *Arthrobacter sp.* (ArS) and *Chromobacterium violaceum* (CV) and one (*R*)-selective ω-TA from *Arthrobacter sp.* (ArR) were selected as catalysts for the reductive amination. In order to facilitate co-expression, the genes encoding these enzymes were cloned into the pTrc99A vector under the control of the trc-promoter. Additionally, they were cloned into the pASK-IBA3plus vector under the control of the tet-promoter. For this purpose the genes encoding the ω-TAs were at first amplified from pET21 constructs via PCR. In order to check the received constructs an analytical agarose gel was performed (Figure 4.1), confirming the successful amplification of the ω-TA genes.

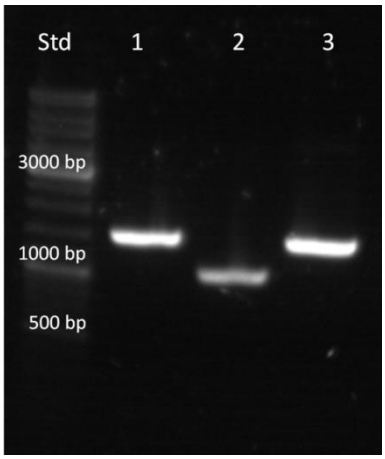


Figure 4.1 Agarose-gel of the amplified PCR-products. Std: GeneRuler™ DNA Ladder Mix; Lane 1: ArS (1431 bp); lane 2: ArR (981 bp); lane 3: CV (1380 bp)

For ligation of the ω-TA genes into the vectors, both the PCR products and the two vectors were digested with the restriction enzymes *EcoR*I and *Sal*I. By using these two restriction enzymes sticky ends were produced with matching regions to overlap during the ligation process. In order to reduce the amount of religated vector and thus to increase the efficiency of the ligation, the vectors were additionally dephosphorylated.

After ligation of the three PCR products (ArS, ArR, CV) into both cloning vectors, pTrc99A and pASK-IBA3plus, the correct integration was confirmed by restriction analysis using *EcoR*I and *Sal*I. Agarose gels of the results of the restriction analysis are shown in Figure 4.2, indicating that the cloning of ArR, ArS and CV both into pTrc99A and pASK-IBA3plus was successful.

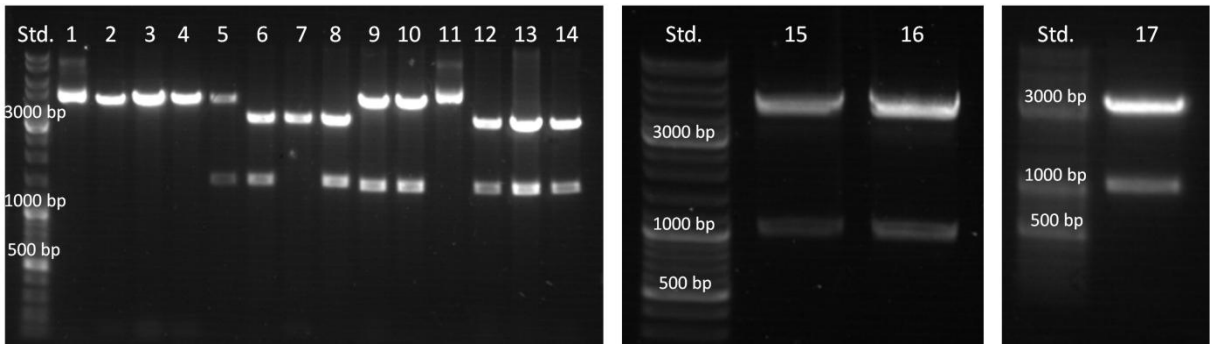


Figure 4.2 Agarose gel after restriction control cut. Lane 1-5: pTrc-ArS, lane 6-8: IBA-ArS, lane 9-11: pTrc-CV**,** lane 12-14: IBA-CV, Std.: GeneRuler^{TM} DNA Ladder Mix, lane 15-16: pTrc-ArR, lane 17: IBA-ArR

In case of the positive clones two fragments with different size were obtained, namely the one of the ω-TA (ArR: 981 bp; ArS: 1431 bp; CV: 1380 bp) and the one of the vector backbone (pTrc99A: 4176 bp; pASK-IBA3plus: 3247 bp). One positive result could be obtained for pTrc99A-ArS (lane 5), whereas two positive clones were received for pASK-IBA3plus-ArS (lane 6 and 8). For pASK-IBA3plus-ArR (lane 17) one correct construct and for pTrc99A-ArR (lane 15 and 16) two correct ones were obtained. Moreover, two positive clones were found for pTrc99A-CV (lane 9 and 10) and three correct constructs were obtained for pASK-IBA3plus-CV (lane 12-14). All positive clones were sent for sequencing and the obtained sequences were aligned with the gene sequences of ArR, CV and ArS using ClustalW, resulting in 100 % sequence identity for all positive clones. Hence, the correct sequence of the genes could be confirmed.

In summary, the cloning of the selected ω-TAs into suitable vectors could be successfully accomplished representing the base for the subsequent assembly of the whole-cell catalyst.

4.2 Construction of recombinant *E. coli* cells co-expressing ω-TA, AlaDH and a cofactor regenerating enzyme

For the construction of an efficient whole-cell biocatalyst the three required genes had to be co-expressed in a single recombinant *E. coli* strain. Therefore a recombinant *E. coli* strain with the gene encoding the AlaDH integrated into the genome and two plasmids containing a ω-TA and a cofactor recycling enzyme, respectively was prepared.

For regeneration of the amine donor alanine the gene *ald* from *Bacillus subtilis* encoding an alanine dehydrogenase (AlaDH) was integrated into the ara locus of the *E. coli* geome (strain W3110). The transcription of the gene was either controlled by a trc- or a bacteriophage t_5-promoter.

For cofactor regeneration three different enzymes were selected, namely the formate dehydrogenase (FDH) from *Komagataella pastoris*,[83] the glucose dehydrogenase (GDH) from *Bacillus megaterium*[84] and the phosphite dehydrogenase (PtDH) from *Pseudomonas stutzeri*.[85] The genes encoding these enzymes were cloned into the pBAD28 vector under the control of the ara-promoter. While the cloning of the selected ω-TAs has been accomplished in the course of this work (chapter 4.1), both desired *E. coli* strains harbouring the AlaDH as well as the plasmids containing the enzymes for cofactor regeneration were constructed by the group of Prof. Volker Wendisch at the University of Bielefeld. In Figure 4.3 the concept of the construction of the whole-cell biocatalyst is illustrated.

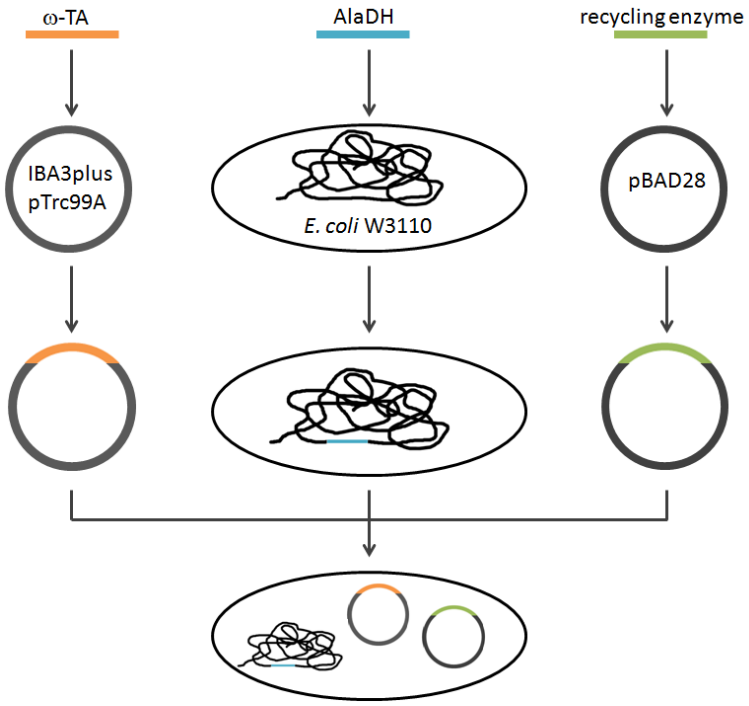
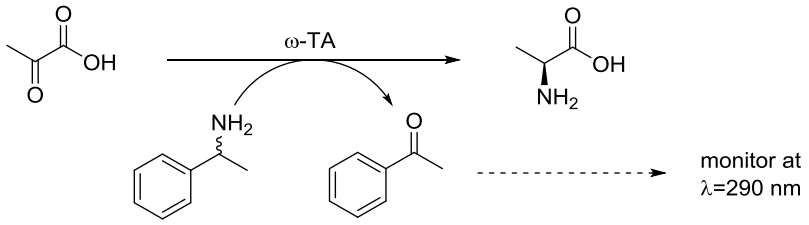


Figure 4.3 Construction of the recombinant *E. coli* whole-cell catalyst. The ω-TA genes were cloned either into a pTrc99A or in a pASK-IBA3plus vector. The gene sequences for the cofactor regenerating enzymes FDH, GDH and PtDH were cloned into pBAD28. The gene encoding AlaDH was inserted into the genome of the used *E. coli* strain, facilitating a flexible catalyst set-up by offering different combinations of ω-TA and recycling system.

To assemble the desired catalyst, competent cells of the *E. coli* strain bearing the AlaDH and the cofactor regenerating enzyme were prepared leading to the *E. coli* strains W1_FDH/GDH/PtDH and W2_FDH/GDH/PtDH, respectively (Table 3.1). Afterwards these strains were successfully transformed with the plasmids pASK-IBA3plus-CV or pTrc99A-CV (Table 3.2) encoding the CV-ω-TA, thus introducing the last module of the aimed system into the *E. coli* whole-cell catalyst (Figure 4.3.) After the assembly has been successfully accomplished, the whole-cell catalyst was then ready for characterization concerning the functional co-expression of the enzymes as well as the application of the catalyst in the asymmetric reductive amination.

4.3 Development of a ω-TA activity assay

In order to be able to investigate the enzyme activities of the expressed ω-TAs in the cell-free extracts, a simple photometric-based method was developed. Therefore, the amination of pyruvate was performed using (*R*)- or (*S*)-methylbenzylamine as amine donor (Scheme 4.1). For the determination of the ω-TA activity the formation of acetophenone was followed phometrically.



Scheme 4.1 Reductive amination of pyruvate to alanine catalyzed by ω-TA using (*R*)- or (*S*)-methylbenzylamine as amine donor. The increase of the acetophenone concentration was monitored at a wavelength of 290 nm.

4.3.1 Absorption spectra

At first absorption spectra of all involved components were measured, in order to ascertain the most suitable wavelength for determining the ω-TA activity based on the acetophenone formation. The spectra can be seen in Figure 4.4. Based on the results from the absorption spectra it seemed most suitable for the ω-TA assay to follow the increase of acetophenone at its absorption maximum (290 nm), since at this wavelength none of the other components interfered with the desired absorbtion of acetophenone.

4.3.1 Evaluation of the molar extinction coefficient of acetophenone

As a next step the molar extinction coefficient of acetophenone was determined under the standard reaction conditions (chapter 3.6.3) *via* a calibration and the obtained curve is shown in Figure 3.3. The molar extinction coefficient is represented by the slope of the regression line and offers a value of 0.8304 abs*mM*cm^{-1} at a pH of 8.

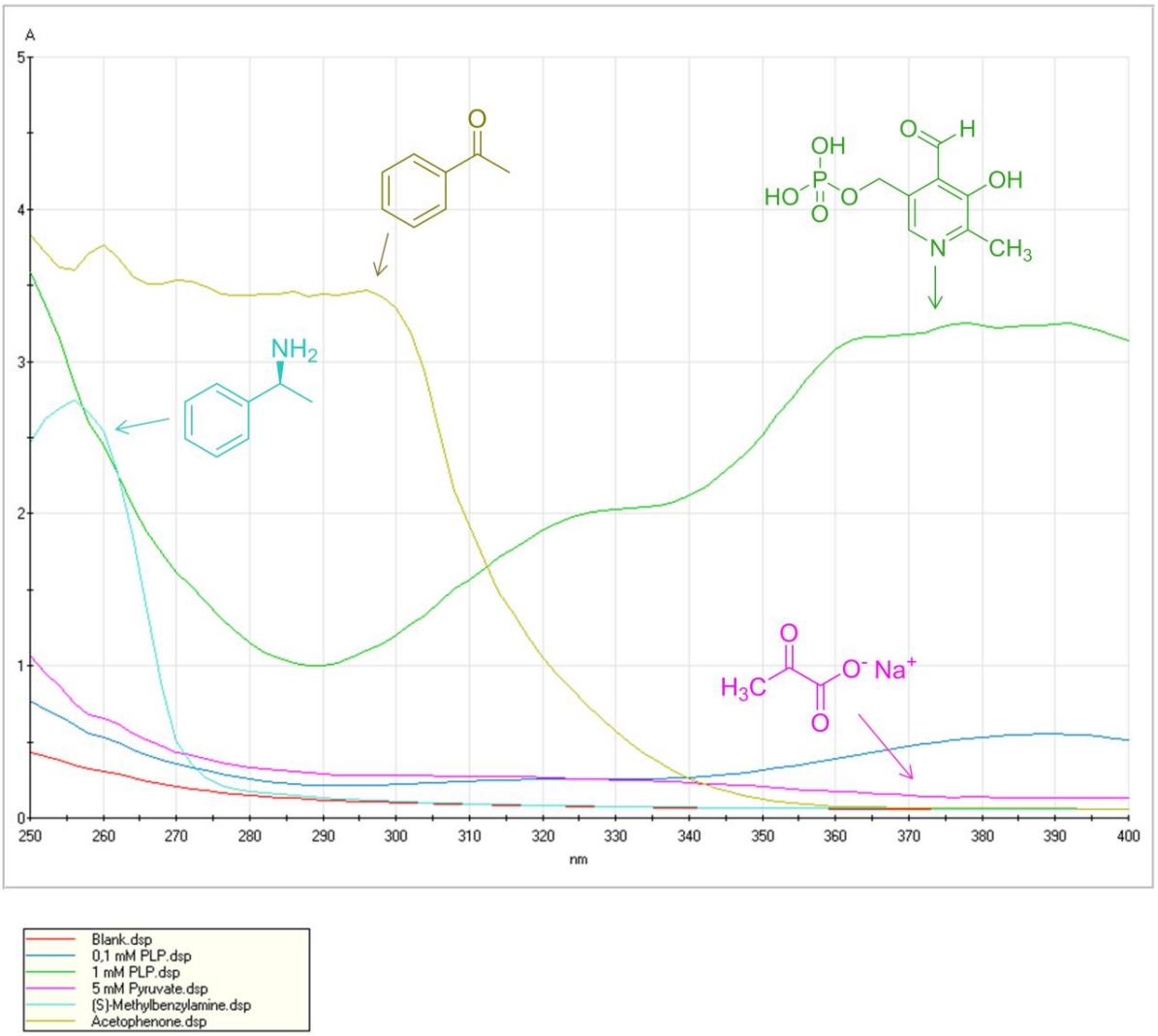


Figure 4.4 Absorption spectra of all components involved in the reductive amination of pyruvate catalyzed by a ω-TA using (*S*)- or (*R*)-methylbenzylamine as amine donor.

4.4 Co-expression

Holding the CV-AlaDH-FDH/GDH/PtDH-whole cell catalyst fulfilling the desired flexibility in hand, the next aim was to facilitate a functional co-expression of all required genes. To cope with this task, parameters such as the expression level and the ideal ratio of the activities of the three co-expressed enzymes are crucial. Besides the regulation of the enzyme expression on molecular level, variation of growth conditions is another possibility to influence the expression levels of the involved enzymes. Therefore, expression parameters were optimized for each system in order to achieve maximum enzyme activities of CV-ω-TA, AlaDH and cofactor regenerating enzyme. In the following studies primarily parameters with respect to growth temperature, time period and induction were further investigated and optimized.

4.4.1 Co-expression of ω-TA, AlaDH and FDH (catalyst A)

E. coli W1_FDH competent cells were transformed with pASK-IBA3plus-CV and pTrc99A-CV, respectively and the co-expression of the three enzymes was investigated at different temperatures and incubation times (Table 4.1).

Table 4.1 Expression conditions for the co-expression of ω-TA, AlaDH and FDH with respect to temperature optimization

Condition	Plasmid encoding CV	Expression parameters
A1	pASK-IBA3plus	37 °C, 4h [a]
A2	pASK-IBA3plus	37 °C, 24h [a]
A3	pASK-IBA3plus	30 °C, 24 [a]
A4	pASK-IBA3plus	20 °C, 24h [a]
A5	pTrc99A	37 °C, 4h [b]
A6	pTrc99A	37 °C, 24h [b]
A7	pTrc99A	30 °C, 24h [b]
A8	pTrc99A	20 °C, 24h [b]
A9	pASK-IBA3plus	25 °C, 24h [a]
A10	pTrc99A	25 °C, 24h [b]

[a] Induction was performed with 0.25 mM IPTG, 0.02 % L-arabinose and 200 ng/mL AHTC; [b] Induction was performed with 0.25 mM IPTG, 0.02 % L-arabinose

The heterologous expression of all enzymes was initially visualized by analyzing the cell cultures *via* SDS-PAGE. As can be seen in Figure 4.5 no notably strong protein bands were apparent, thus it was not obvious, if the protein production took place sufficiently.

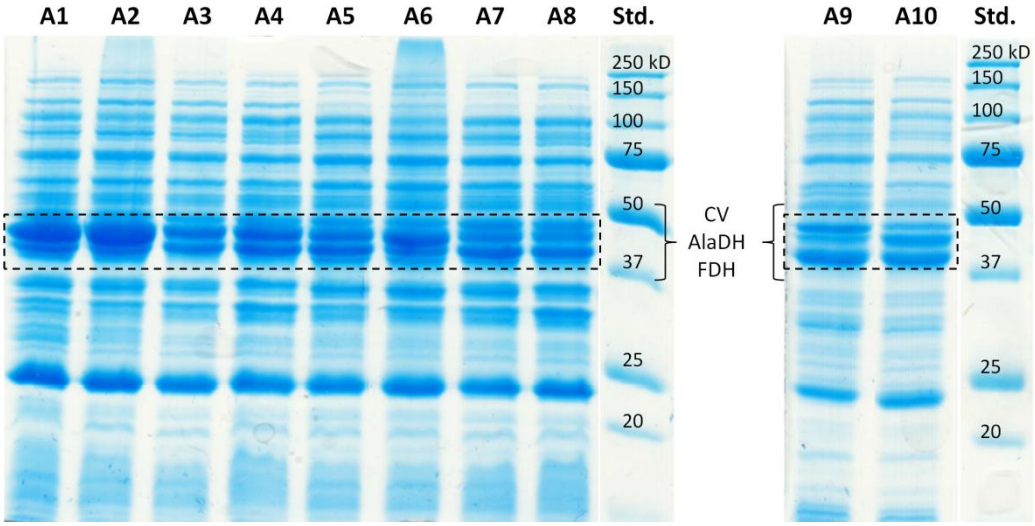


Figure 4.5 Co-expression of ω-TA, AlaDH and FDH in *E. coli* W1_FDH at different temperatures with 0.25 mM IPTG, 0.02 % L-arabinose and 200 ng/mL AHTC if required. (A1) IBA-CV, 37 °C, 4h; **(A2)** IBA-CV, 37 °C, 24h; **(A3)** IBA-CV, 30 °C, 24h; **(A4)** IBA-CV, 20 °C, 24h; **(A5)** pTrc-CV, 37 °C, 4h; **(A6)** pTrc-CV, 37 °C, 24h; **(A7)** pTrc-CV, 30 °C, 24h; **(A8)** pTrc-CV, 20 °C, 24h; **(A9)** IBA-CV, 25 °C; **(A10)** pTrc-CV, 25 °C; **Std**: BioRad Precision Plus Protein All Blue Standard

Furthermore, since the three proteins are quite similar with respect to their size it was difficult to clearly assign a particular band to the respective enzyme. The AlaDH (44 kDA) and the FDH (42 kDA) should be represented by the bands located between 37 and 50 kDA (dashed box). Also CV, which has a size of 55 kDA, should probably constitute one of these bands. However, compared to all the other bands, the assumed protein bands didn't contribute significantly to the total amount of protein. Actually, another well-defined band between 25 and 20 kDA at the bottom of the gel was visible, which probably represents background protein expressed by *E. coli* or a degradation product by diverse proteases.

In addition to the verification by SDS-Page the functional expression of the enzymes was determined by measuring the activities in the cell-free extracts. In order to get an idea, which expression conditions are favorable, at first just activity assays for AlaDH and FDH were performed, since these two enzymes responsible for the shifting of the equilibrium and the regeneration of the cofactor should be present with a slight excess. The results are summarized in Table 4.2.

Table 4.2 AlaDH and FDH activities for the temperature optimization *

	Activity of AlaDH [mU/mg][a]	**Activity of FDH [mU/mg][a]**
A1	314	6
A2	250	2
A3	257	59
A4	338	70
A5	564	11
A6	130	14
A7	350	90
A8	384	72
A9	360	133
A10	492	141

Expression conditions are according to **Table 4.1.** [a]Activities are given in mU/ mg total protein

The AlaDH activities were predominantly located in the range of 200-300 mU/mg total protein. The highest values were obtained for the expression performed at 37 °C for 4 hours (condition A5) and at 25 °C for 24 hours (condition A10). In terms of the FDH quite low activity levels were achieved throughout the different expression conditions, hardly ever exceeding 100 mU/mg total protein. However, the expression at 25 °C (conditions A9-A10) resulted in more active enzyme, offering activities higher than 100 mU.

Therefore, with respect to FDH as well as AlaDH activities, the most promising results for this system could be obtained when expressed at 25 °C for 24 hours in combination with the CV expressed from the pTrc99A vector (condition A10). Using this condition an AlaDH-activity of 492 mU/mg protein and a FDH-activity 141 mU/mg protein could be achieved.

In a second optimization study the influence of different conditions concerning the induction was investigated (Table 4.3). In order to further increase the FDH activity the best conditions identified in the previous experiment were used (25 °C for 24 hours). Moreover, only the *E. coli* W1_FDH cells transformed with pTrc99A-CV were used as expression hosts, since this combination provided the best results.

Table 4.3 Expression conditions for the co-expression of ω-TA, AlaDH and FDH in terms of an induction study

Condition	Induction with IPTG	Induction with L-arabinose
B1	0.25 mM	0.2 %
B2	0.25 mM	0.02 %
B3	0.25 mM	0.002 %
B4	0.25 mM after 3 hours	0.2 %
B5	0.25 mM after 3 hours	0.02 %
B6	0.25 mM after 3 hours	0.002 %
B7	0.5 mM	0.2 %
B8	0.5 mM	0.02 %
B9	0.5 mM	0.002 %

The induction of the ara-promoter controlling FDH expression was carried out with the addition of L-arabinose to a final concentration of 0.2, 0.02 and 0.002 %, respectively. Simultaneously the protein production of the CV under the control of the trc-promoter was induced by the addition of 0.25 and 0.5 mM IPTG, respectively. Moreover, it was investigated if inducing the enzyme expression of the ω-TA with 0.25 mM IPTG three hours after induction of the FDH expression had a positive effect on the enzyme activity. The functional expression of all enzymes was again initially analyzed *via* SDS-PAGE.

In the SDS gel picture in Figure 4.6 the results of the protein expression at different induction conditions are shown. This time the protein band at about 37 kDA could be assigned to the expressed FDH, while no particular bands could be assigned to the other proteins. However, the bands below the 50 kDA band (dashed box) were most likely corresponding to AlaDH and CV. In general the protein bands did not contribute significantly to the total amount of protein though. Again the well-defined band between 25 and 20 kDA at the bottom of the gel probably originating from *E. coli* background was visible.

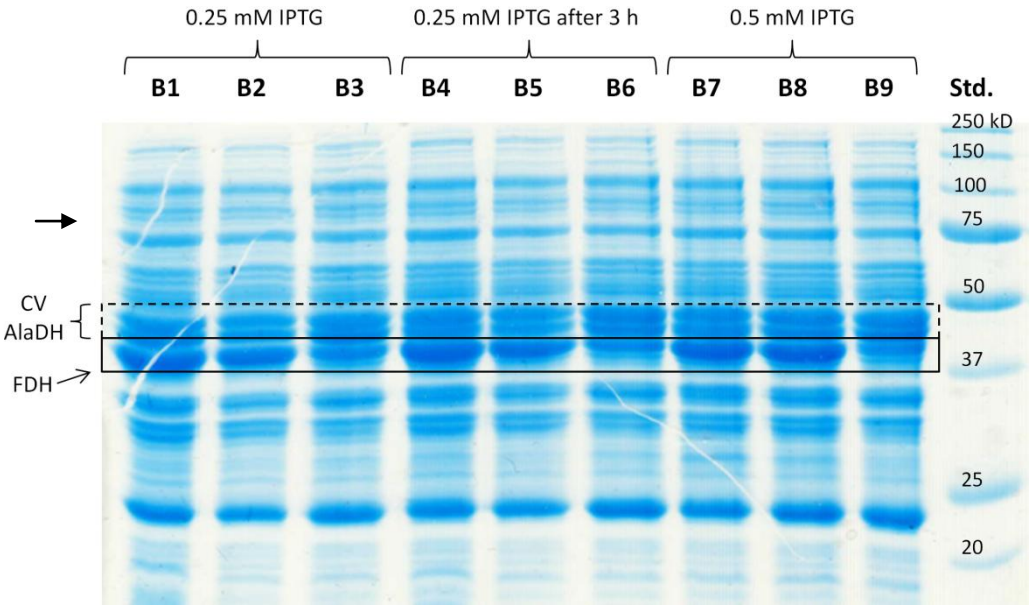


Figure 4.6 Co-expression of ω-TA (pTrc-CV), AlaDH and FDH in *E. coli* W1_FDH at 25 °C with different modes of induction. (B1), **(B4)**, **(B7)** 0.2 % L-arabinose; **(B2)**, **(B5)**, **(B8)** 0.02 % L-arabinose; **(B3)**, **(B6)**, **(B9)** 0.002 % L-arabinose; **(B1-3)** 0.25 mM IPTG simultaneously with L-arabinose induction; **(B4-6)** 0.25 mM IPTG 3 h after L-arabinose induction; **(B7-9)** 0.5 mM IPTG simultaneously with L-arabinose induction; **Std**: BioRad Precision Plus Protein All Blue Standard

For a further quantification of protein expression the enzyme activities of the cell-free extracts were assayed and the results are summarized in Table 4.4. Additional to the AlaDH- and the FDH-assay, this time also the ω-TA activity was determined.

The highest activities for the CV-ω-TA could be obtained when inducing with 0.5 mM IPTG simultaneously with induction of the ara-promoter (condition B7-B9). Additionally, the concentration of L-arabinose for the induction of the ara-promoter seemed to influence the functional expression of the ω-TA to a certain extent, as with an inducer concentration of 0.002 % of L-arabinose the ω-TA showed higher activities (condition B3, B6 and B9). The protein expression of AlaDH on the other hand was positively influenced by a lower IPTG concentration (condition B1-B3) and higher concentration of L-arabinose (condition B1, B4, B7).

The highest activities for FDH were obtained by inducing with 0.2 % L-arabinose (conditions B1, B4, B7), which also correlated with the considerably stronger protein bands visible at approximately 37 kDA in the SDS-gel picture (Figure 4.6). In general, a low concentration of IPTG (0.25 mM) had a beneficial effect on the production of active FDH (conditions B1-B3). Accordingly, the heterologous expression of FDH went along with the one of AlaDH in terms of induction conditions, whereas the expression of CV behaved contrariwise.

Table 4.4 ω-TA, AlaDH and FDH activities and relative ratios for the induction study

	Activity of ω-TA [mU/mg][a]	Activity of AlaDH [mU/mg][a]	Activity of FDH [mU/mg][a]	Ratio ω-TA : AlaDH : FDH
B1	116	413	209	1 : 3.5 : 1.8
B2	134	341	182	1 : 2.5 : 1.4
B3	251	246	81	1 : 1 : 0.3
B4	114	365	172	1 : 3.2 : 1.5
B5	203	317	150	1 : 1.6 : 0.7
B6	305	144	54	1 : 0.5 : 0.2
B7	177	390	167	1 : 2.2 : 1.1
B8	249	327	128	1 : 1.3 : 0.5
B9	385	160	31	1 : 0.4 : 0.1

Expression conditions are according to **Table 4.3.** [a]Activities are given in mU/ mg total protein

Considering all parameters and obtained enzyme activities the protein expression induced simultaneously with 0.25 mM IPTG and 0,02 % L-arabinose at 25 °C (condition B2) gave the best overall results. Using these conditions a relative ratio for ω-TA : AlaDH : FDH of 1 : 2.5 : 1.4 could be obtained. Thus this approach was chosen to be the standard co-expression strategy in *E. coli* W1_FDH cells and was used for all further experiments.

4.4.2 Co-expression of ω-TA, AlaDH and GDH (catalyst B)

E. coli W1_GDH and W2_GDH competent cells were transformed with pASK-IBA3plus-CV and pTrc99A-CV, respectively. The co-expression of the three enzymes was investigated at different temperatures and incubation times (see Table 4.5).

Table 4.5 Expression conditions for the co-expression of ω-TA, AlaDH and GDH

Condition	Expression strain	Plasmid encoding CV	Expression parameters
1	W1_GDH	pASK-IBA3plus	25 °C, 24h [a]
2	W1_GDH	pTrc99A	25 °C, 24h [b]
3	W2_GDH	pASK-IBA3plus	25 °C, 24h [a]
4	W2_GDH	pTrc99A	25 °C, 24h [b]
5	W1_GDH	pASK-IBA3plus	37 °C, 4h [a]
6	W1_GDH	pTrc99A	37 °C, 4h [b]
7	W2_GDH	pASK-IBA3plus	37 °C, 4h [a]
8	W2_GDH	pTrc99A	37 °C, 4h [b]

[a] Induction was performed with 0.25 mM IPTG, 0.02 % L-arabinose and 200 ng/mL AHTC; [b] Induction was performed with 0.25 mM IPTG, 0.02 % L-arabinose

The heterologous co-expression of all enzymes was examined *via* SDS-PAGE and the gel picture is shown in Figure 4.6.

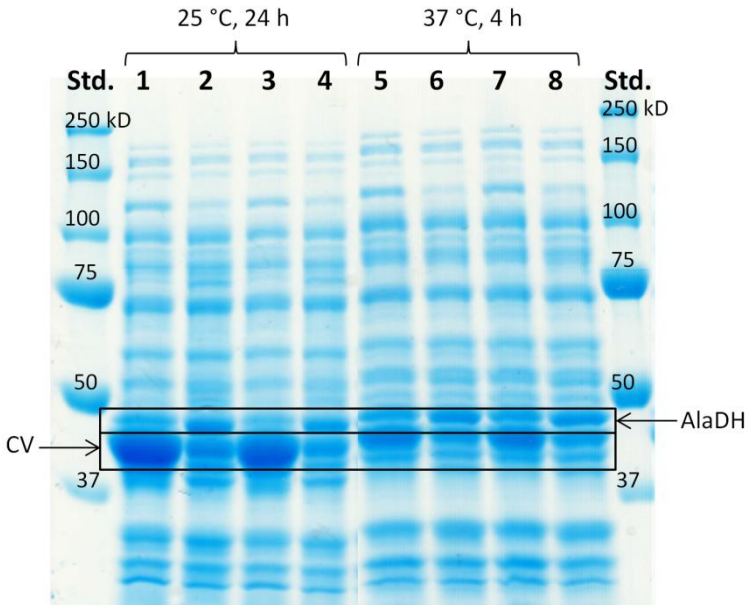


Figure 4.7 Co-expression of ω-TA, AlaDH and GDH in *E. coli* W1_GDH and in *E. coli* W2_GDH at different temperatures with 0.25 mM IPTG, 0.02 % L-arabinose and 200 ng/mL AHTC if required. (1) IBA-CV in W1_GDH; **(2)** pTrc-CV in W1_GDH; **(3)** IBA-CV in W2_GDH; **(4)** pTrc-CV in W2_GDH; **(5)** IBA-CV in W1_GDH; **(6)** pTrc-CV in W1_GDH; **(7)** IBA-CV in W2_GDH; **(8)** pTrc-CV in W2_GDH; **Std**: BioRad Precision Plus Protein All Blue Standard

In general a stronger expression of the CV-ω-TA was achieved from the pASK3-IBA3plus vector, especially at 25 °C (condition 1 and 3), regardless of the used *E. coli* strain. The bands located between 37 and 50 kDA corresponded to the expressed CV and obviously contributed a notable part to the total amount of protein using the above mentioned conditions. In contrast, expression at 37 °C for 4 hours seemed to negatively influence the CV resulting in considerably weaker protein bands. The narrow bands above the ones from CV could be assigned to the produced AlaDH, whereas no protein bands corresponding to the expressed GDH (31 kDA) could be visualized on the SDS-gel.

In order to verify the amount of actually active protein produced, the cell-free extracts were employed in photometric activity assays. The received activities for the three co-expressed enzymes and the corresponding relative ratios are summarized in Table 4.6

The results of the activity assays correlated quite well with the observed protein bands in the SGS-gel picture. According to that the highest activities for the ω-TA of approximately 400 mU/mg total protein were obtained with the expression from the pASK3-IBA3plus construct at 25 °C (conditions 1 and 3). Protein production at 37 °C over 4 h generally resulted in lower ω-TA activities (conditions 5-8).

Table 4.6 ω-TA, AlaDH and GDH activities and relative ratios for the co-expression experiment

	Activity of ω-TA [mU/mg][a]	Activity of AlaDH [mU/mg][a]	Activity of GDH [mU/mg][a]	Ratio ω-TA : AlaDH : GDH
1	448	235	4910	1 : 0.5 : 11
2	193	375	11904	1 : 1.9 : 62
3	426	282	4753	1 : 0.7 : 11
4	131	379	11619	1 : 2.9 : 89
5	173	210	7504	1 : 1.2 : 43
6	51	356	8986	1 : 7 : 176
7	151	219	4673	1 : 1.5 : 31
8	57	375	7370	1 : 6.6 : 129

Expression conditions are according to **Table 4.5.** [a]Activities are given in mU/ mg total protein

Equal levels of AlaDH were expressed, no matter what conditions were used, leading to activities in the range of 200-400 mU/mg total protein. This fact indicates that the type of promoter which controls transcription of the *ald* gene from the *E. coli* genome did not seem to have a great effect neither on the amount of expressed protein nor on the enzyme activity. Even though no protein expression of GDH could be observed on the SDS-gel, by means of the photometric assay instead quite respectable activities could be detected. This could be due to the fact that the GDH in general has a high specific activity of up to 550 U/mg pure protein.[86] The highest GDH activities in the course of this work (11 U/mg total crude protein) were obtained at 25 °C using both *E. coli* strains in combination with pTrc99A-CV.

It has to be mentioned that the enzyme activities of AlaDH and GDH were slightly decreased, when the pASK-IBA3plus construct was used for CV co-expression instead of the pTrc99A construct. This might be due to the fact that the addition of AHTC together with IPTG and L-arabinose for induction led to a higher stress potential for the cells resulting in decreased functional protein expression. However, this effect could not be observed for the expression of CV itself though, since the CV production was far more successful from the pASK-IBA3plus construct for unknown reasons.

In conclusion, the co-expression in *E. coli* W1_GDH cells transformed with pTrc99A-CV at 25 °C for 24 hours (condition 2) resulted in a relative ratio for ω-TA : AlaDH : GDH of 1 : 1.9 : 62. Therefore, this system combined with the mentioned conditions was chosen to be the standard co-expression strategy for this whole-cell catalyst and was used for all further experiments.

4.4.1 Co-expression of ω-TA, AlaDH and PtDH (catalyst C)

As the third catalytic system, the catalyst co-expressing the ω-TA and AlaDH together with a PtDH was characterized using *E. coli* W1_PtDH competent cells transformed with pASK-IBA3plus-CV as well as pTrc99A-CV. The heterologous expression of the three enzymes was again investigated at different temperatures and incubation times (see Table 4.7).

Table 4.7 Expression conditions for the co-expression of ω-TA, AlaDH and PtDH

Condition	Plasmid encoding CV	Expression parameters
1	pASK-IBA3plus	37 °C, 4h [a]
2	pTrc99A	37 °C, 4h [b]
3	pASK-IBA3plus	37 °C, 24h [a]
4	pTrc99A	37 °C, 24h [b]
5	pASK-IBA3plus	37 °C, 48h [a]
6	pTrc99A	37 °C, 48h [b]
7	pASK-IBA3plus	25 °C, 24h [a]
8	pTrc99A	25 °C, 24h [b]

[a] Induction was performed with 0.25 mM IPTG, 0.02 % L-arabinose and 200 ng/mL AHTC;
[b] Induction was performed with 0.25 mM IPTG, 0.02 % L-arabinose

The protein expression of all enzymes was initially visualized by SDS-PAGE analysis. As can be seen on the SDS-gel picture in Figure 4.8 the most successful expression of the CV-ω-TA was achieved in combination with the pASK3-IBA3plus vector at 37 °C (condition 1, 3 and 5).

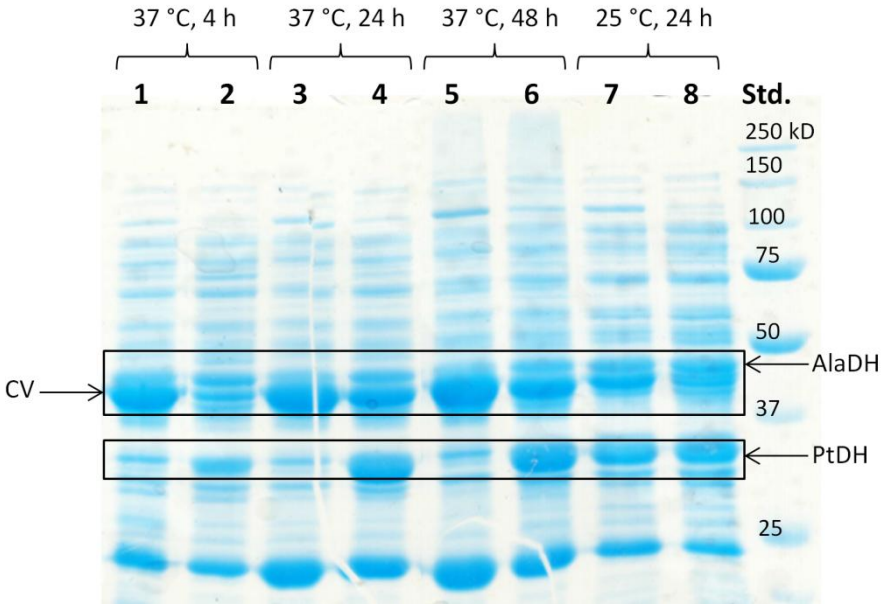


Figure 4.8 Co-expression of ω-TA, AlaDH and PtDH in *E. coli* W1_PtDH at different temperatures with 0.25 mM IPTG, 0.02 % L-arabinose and 200 ng/mL AHTC if required. (1),(3),(5),(7) pASK-IBAplus-CV; **(2),(4),(6),(8)** pTrc99A-CV; **Std**: BioRad Precision Plus Protein All Blue Standard

Here the protein bands at about 37 kDA contributed significantly to the total amount of protein. But also the CV expression from the pTrc99A vector at 37 °C (condition 2, 4 and 6) seemed to continuously increase with proceeding incubation time, according to the stronger protein bands. The AlaDH protein bands (44 kDA) were nicely visible close above the ones for CV and indicated that the AlaDH production was quite comparable, independent of the used expression conditions. The bands below the 37 kDA could be definitely assigned to the expressed PtDH (31 kDA) and were strongest using condition 4 and 6.

The results obtained from the SDS-Page could be further verified by the photometric determination of the enzyme activities, which are summarized together with the received activity ratios in Table 4.8. Corresponding to the observed protein bands the CV-expressions from pASK3-IBA3plus construct at 37 °C were most successful. Interestingly, the highest CV activity (499 mU/mg total protein) was already obtained after 4 hours and decreased with proceeding expression time. The activities determined for AlaDH were in the range of 350 - 700 mU/mg total protein for all expression samples, except for the one which was performed over 48 hours. The strongest expression of functional AlaDH (698 mU/mg total protein) was achieved equally with the highest CV production after incubation at 37 °C for 4 hours (condition 1).

Table 4.8 ω-TA, AlaDH and PtDH activities and relative ratios for the co-expression experiment

	Activity of ω-TA [mU/mg][a]	**Activity of AlaDH [mU/mg][a]**	**Activity of PtDH [mU/mg][a]**	**Ratio ω-TA : AlaDH : PtDH**
1	499	583	0	1 : 1 : 0
2	67	698	91	1 : 10 : 1.4
3	250	409	1	1 : 1.6 : 0
4	161	356	583	1 : 2.2 : 3.6
5	102	108	3	1 : 1.1 : 0
6	21	47	246	1 : 2.2 : 12
7	189	417	293	1 : 2.2 : 1.6
8	28	585	231	1 : 21 : 8.3

Expression conditions are according to **Table 4.7.** [a]Activities are given in mU/ mg total protein

In general, co-expression of PtDH was more successful in combination with the ω-TA encoded by the pTrc99A plasmid and longer expression times, at least concerning the expression at 37 °C (conditions 1-6) Thus, the enzyme activity of 91 mU/mg total protein after 4 hours (condition 2) could be increased to 583 mU/mg total protein (condition 4), when prolonging expression to 24 hours.

However, expression for a period of 48 hours at this temperature seemed to negatively influence the production of active protein, as the activity decreased to 246 mU/mg total protein. This might be due to the fact that some of the protein again had been degraded during the long incubation at 37 °C. In combination with the pASK3-IBA3plus-CV vector the desired co-expression of PtDH did not work at all, except for the expression performed at 25 °C (condition 7). Here the obtained activity was in the range of the corresponding one received from co-expression with the pTrc99A-CV vector (condition 8). Based on these results it can be deduced that the protein production of PtDH seemed to be more sensitive toward the additional induction with AHTC than the before mentioned co-expression in chapter 4.4.1 and 4.4.2.

In summary, the most promising activities with a suitable relative ratio of 1 : 2.2 : 3.6 for ω-TA : AlaDH : PtDH were obtained when employing *E. coli* W1_PtDH cells transformed with pTrc99A-CV in the co-expression at 37 °C for 24 hours (condition 4). Hence, this approach was chosen to be the standard co-expression strategy for the whole-cell system applying a PtDH and was used for all further experiments.

After the co-expression of all enzymes has been successfully accomplished, the constructed whole-cell biocatalysts were ready for further characterization and for the application in the desired model reaction. The final set-ups and the corresponding optimized expression parameters for each catalytic system are summarized in Table 4.9. The received whole-cell biocatalysts were entitled catalyst A (CV-AlaDH-FDH), catalyst B (CV-AlaDH-GDH) and catalyst C (CV-AlaDH-PtDH). These denotations were henceforward used throughout the thesis.

Table 4.9 Final whole-cell catalysts and the corresponding optimized expression parameters

	***E. coli* strain**	**Plasmid encoding recycling enzyme**	**Plasmid encoding CV-ω-TA**	**Expression conditions***
Catalyst A	*E. coli* W1	pBAD28-FDH	pTrc99A-CV	25 °C, 24 h
Catalyst B	*E. coli* W1	pBAD28-GDH	pTrc99A-CV	25 °C, 24 h
Catalyst C	*E. coli* W1	pBAD28-PtDH	pTrc99A-CV	37 °C, 24 h

*Induction was performed with 0.25 mM IPTG and 0.02 % L-arabinose

4.5 Activities of whole-cell catalysts

After optimizing the expression of the three catalytic systems, where only individual activities of the enzymes have been assayed, next the overall performance of the whole-cell biocatalysts was comparatively studied. Therefore, the activity of each system was determined in the reductive amination of 4-phenyl-2-butanone using the standard conditions (see chapter 3.6.1) and initial rates were determined measuring the production of the corresponding 4-phenyl-2-butylamine over 60 or 120 minutes, respectively. Figure 4.9 shows the time courses of the reactions used for the determination of initial rates of the three whole-cell catalysts. The slope which represents the converted substrate in µmol per minute has been used for the calculation of the activity of each system in Units per g wet cells.

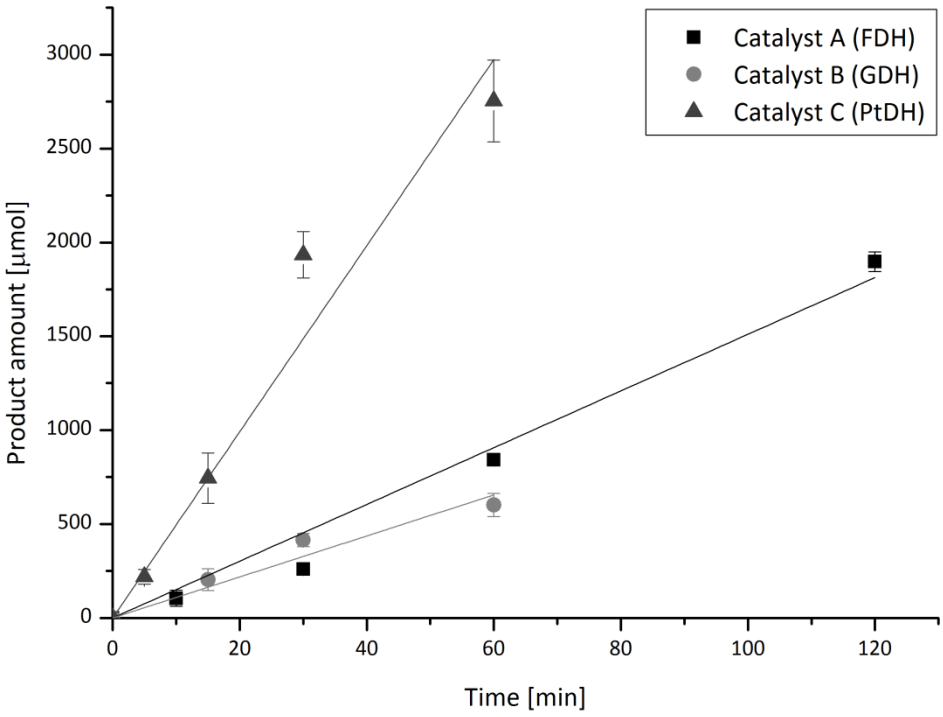
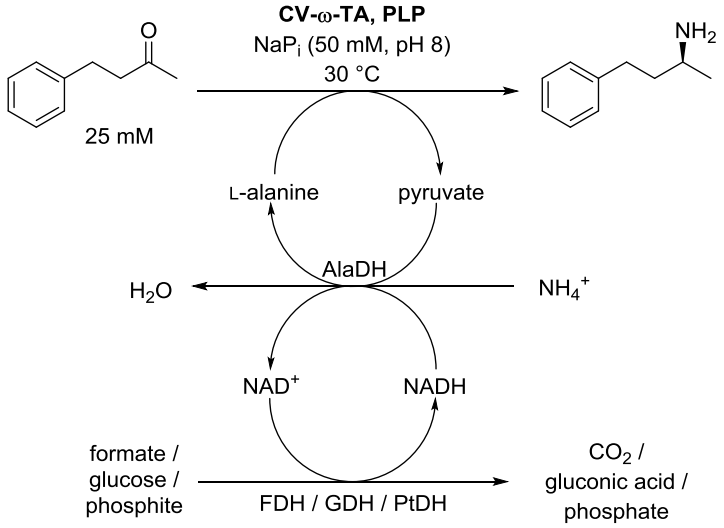


Figure 4.9 Determination of the initial rates of the whole-cell catalyst in the reductive amination of 25 mM 4-phenyl-2-butanone using either the FDH system, the GDH system or the PtDH system for cofactor regeneration. Typical time curves are shown for the production of 4-phenyl-2-butylamine over a time period of 60 or 120 minutes, respectively using 50 mg whole-cell catalyst per mL.

For the catalyst A co-expressing the CV-ω-TA and AlaDH together with a FDH an activity of 0.302 ± 0.02 U/g (Figure 4.9) was obtained, whereas catalyst B applying a GDH for cofactor regeneration showed an activity of 0.218 ± 0.02 U/g. The best result was obtained for the catalyst C which combines the CV-ω-TA and AlaDH with a PtDH, giving an activity of 0.992 ± 0.07 U/g.

4.6 Reductive amination using a whole-cell biocatalyst

In order to investigate the potential application of the recombinant whole-cell biocatalysts in the synthesis of chiral amines the reductive amination of 4-phenyl-2-butanone to 4-phenyl-2-butylamine was chosen as model reaction (Scheme 4.2). The biotransformation was carried out with all three different systems, either applying a FDH, a GDH or a PtDH for the recycling of the cofactor NADH. Besides the use of resting cells, the catalyst was also applied in form of lyophilized whole-cells, cell-free extract and lyophilized cell-free extract in order to study differences of these preparations in terms of efficiency and stability. For a better comparability the amount of used lyophilized cells, lysate and lyophilized lysate always correlated to 50 mg of wet cells.



Scheme 4.2 Reductive amination of 4-phenyl-2-butanone using a recombinant *E. coli* whole-cell catalyst co-expressing a CV-ω-TA, an AlaDH and an enzyme for cofactor regeneration (FDH, GDH or PtDH). Reaction conditions: 30 °C, 50 mM NaP_i buffer (pH 8), 1 mM PLP, 1 mM NAD^+, 25 mM ketone, 125 mM L-alanine, 150 mM cosubstrate and 75 mM ammonium acetate when using the GDH and PtDH recycling system.

4.6.1 Reductive amination with catalyst A

The reductive amination with catalyst A using FDH for cofactor regeneration was carried out applying the reaction conditions as given in chapter 3.7. The production of 4-phenyl-2-butylamine was monitored over 48 hours and is illustrated in Figure 4.10.

Using resting cells of the catalyst (50 mg/mL) 35 % of the desired amine were obtained after 48 hours. When the reaction was performed with a two-fold catalyst loading (100 mg), just a slight increase of the conversion to 45 % was observed. However, by applying the biocatalyst in form of lyophilized whole-cells significantly improved conversion levels (52 %) were reached. Moreover, the determination of the *enantiomeric excess* (*ee*) showed that the (*S*)-amine was afforded with perfect optical purity (98 %).

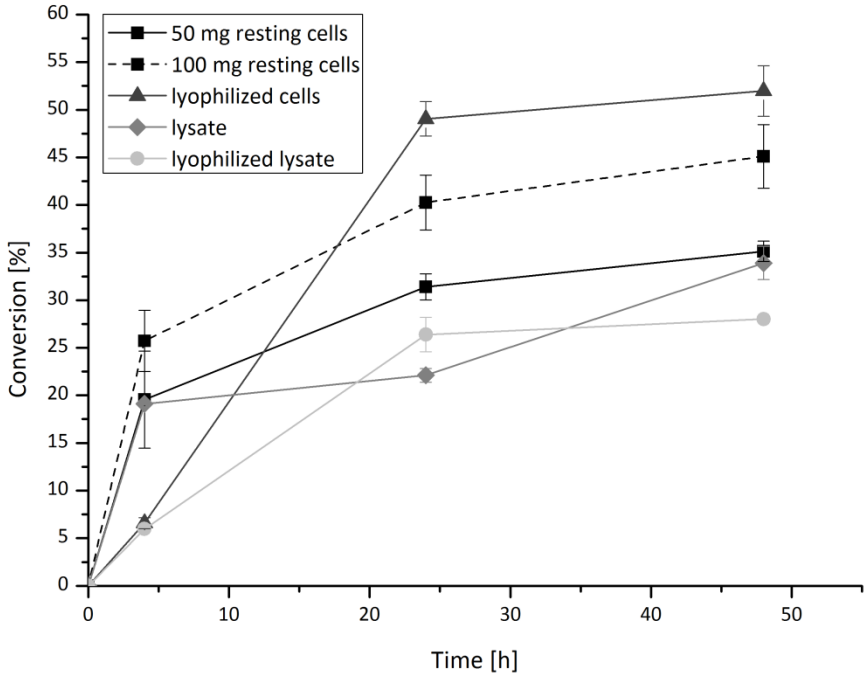


Figure 4.10 Reductive amination of 4-phenyl-2-butanone using catalyst A (CV, AlaDH and FDH). The biocatalyst was either applied as resting cells, as lyophilized cells, as cell-free extract or as lyophilized cell-free extract, respectively and the reaction was performed at 30 °C for 48 hours.

Especially within the first 24 hours the reaction rate seemed to be accelerated, which can be explained by the fact that due to lyophilization the cells have been permeabilized and hence the substrate had a better access to the enzymes in the cell. After 24 hours the amount of amine formed did not further increase particularly, which might indicate that limitations such as reduced enzyme activities due to inactivation or inhibition occurred. Performing the biotransformation either with cell-free extract or lyophilized cell-free extract, respectively did not show a beneficial effect on the outcome of the reaction. With the lysate the conversion was in the same range as with resting cells, since 33 % of the amine were obtained. The lyophilization of the lysate seemed to reduce the overall enzyme activity to some extent, as just 26 % of the ketone were converted. This phenomenon of slight activity loss when lyophilizing is commonly known though.[87-88]

In order to further optimize the conversion, the influence of a higher catalyst loading was investigated. Since the results obtained with lyophilized whole-cells were most promising, this catalyst preparation was also applied in the optimization study. In the previous experiment 10 mg of lyophilized cells (correlating to 50 mg of wet cells) were employed in the reaction. This time the biotransformation was performed with an up to 10-times higher catalyst loading by employing 20, 50 and accordingly 100 mg of the lyophilized cells. The obtained results are illustrated in Figure 4.11.

In theory, by doubling the amount of whole-cell catalyst the amount of formed product should be duplicated as well. Unfortunately, the study showed that the amount of produced amine did not increase in the expected way by just using a higher biocatalyst loading. With 100 mg of lyophilized cells and thus a 10-fold amount a maximum conversion of 77 %, being just a 1.5-fold enhancement, could be obtained.

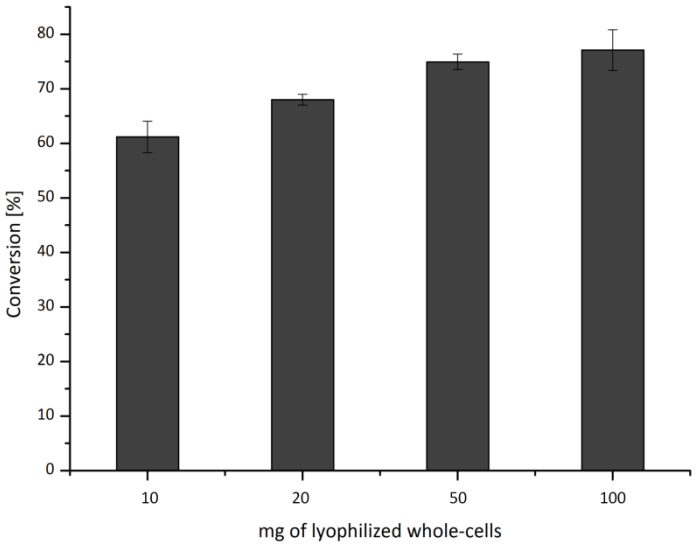


Figure 4.11 Results for the optimization of the reductive amination of 4-phenyl-2-butanone using catalyst A (CV, AlaDH and FDH). The reaction was performed with different amounts of the lyophilized whole-cell preparation at 30 °C for 24 hours.

4.6.2 Reductive amination with catalyst B

The potential of catalyst B, using GDH for cofactor regeneration, was investigated in the desired reductive amination according to the standard reaction conditions (chapter 3.7). The results for the biotransformation monitored over 48 hours are shown in Figure 4.12.

When comparing the reaction progress for 20 mg, 50 mg and 100 mg of applied whole-cells, respectively it became obvious that in the first 4 hours the reaction rate was accelerated corresponding to the higher catalyst loading. Hence, doubling of the catalyst amount also led to the 2-fold amount of produced amine. However, in the following course of the reaction this correlation between catalyst amount and reaction rate decreased significantly. This again indicated the emergence of limitations, most likely an inactivation of one or more enzymes.

When 50 mg of the whole-cell catalyst were applied in the biotransformation 25 % of the amine were obtained, which was further increased by using a two-fold amount of wet cells (giving 32 %) or the lyophilized whole-cell preparation (giving 33 %). However, the observed results for the latter were considerably better since the applied amount correlated to only 50 mg of wet cells. The highest conversion was achieved applying the cell-free extract in its lyophilized form, leading to 37 % of the (*S*)-amine with a perfect *ee* of 99 %.

Due to the cell disruption the substrate might have had an easier access to the enzymes resulting in better and faster conversions. Moreover, it can be assumed that with whole-cells the glucose uptake is insufficient leading to a limited amount of co-substrate.

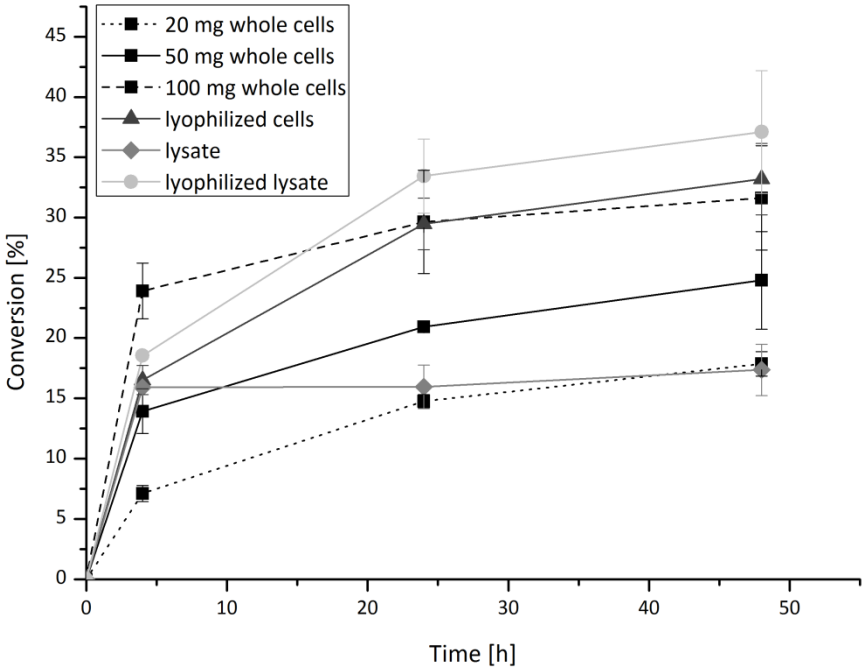


Figure 4.12 Reductive amination of 4-phenyl-2-butanone using catalyst B (CV, AlaDH and GDH). The biocatalyst was either applied as resting cells, as lyophilized cells, as cell-free extract or as lyophilized cell-free extract respectively and the reaction was performed at 30 °C for 48 hours.

Surprisingly, the cell-free extract itself yielded a poor conversion of only 17 % which was in the range of the values received with half the amount of whole cells (20 mg). Probably the enzymes were not stable enough when used in form of a cell-free extract, whereas the lyophilization seemed to prevent a significant loss of enzyme activity.

4.6.3 Reductive amination with catalyst C

By using catalyst C the reductive amination was accomplished with concomitant cofactor regeneration catalyzed by PtDH. The biotransformations were performed according to the standard conditions given in chapter 3.7 and monitored for 48 hours (Figure 4.12).

With catalyst C already 33 % of amine were obtained applying 20 mg resting cells/mL, which could be increased to 48 % with 50 mg and even 56 % when 100 mg wet cells were used. However, it was obvious that there were some limitations or the enzymes lost part of their activity over time since a two-fold loading of biocatalyst did not lead to a duplicate amine production, which already could be observed previously in combination with the FDH and GDH system in chapter 4.6.1 and 4.6.2.

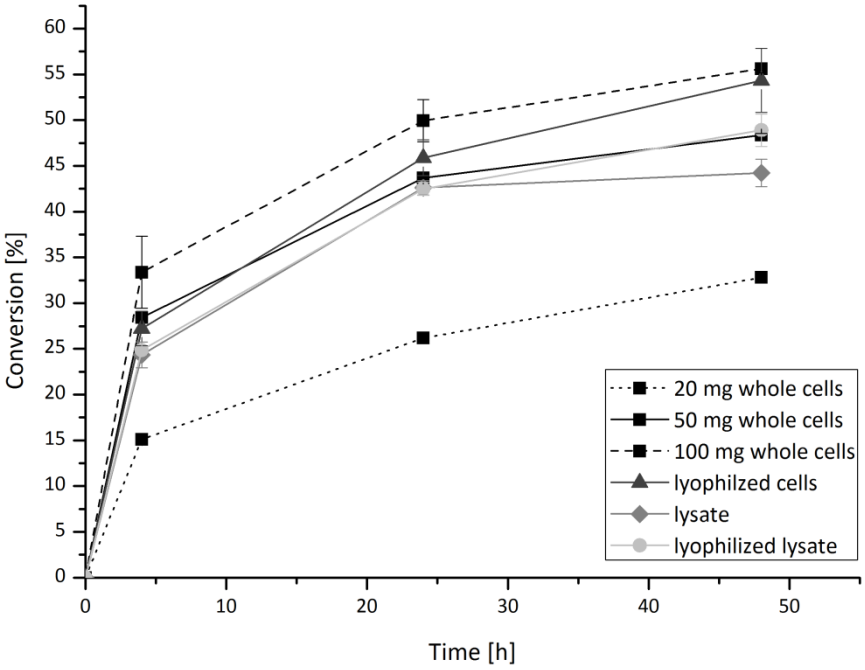


Figure 4.13 Reductive amination of 4-phenyl-2-butanone using catalyst C (CV, AlaDH and PtDH). The biocatalyst was either applied as resting cells, as lyophilized cells, as cell-free extract or as lyophilized cell-free extract respectively and the reaction was performed at 30 °C for 48 hours.

The biotransformations performed with the lysate and the lyophilized lysate produced 44 % and 49 % of the amine, respectively and thus were similarly successful as the reaction using wet whole-cells. Generally, it can be pointed out that the co-expression system using a PtDH for cofactor regeneration seemed to be less influenced by the type of used cell-preparation. However, lyophilization of the whole-cells definitely had a positive effect on the outcome of the reaction, which might be due to the better accessibility of the subtrates into the cell caused by permeabilization. Therefore, the best results were received with this lyophilized whole-cell preparation yielding 54 % of the amine which was even comparable to the conversion achieved with the twofold amount of wet cells. Furthermore, the *ee* was determined showing that the (*S*)-amine was obtained with high optical purity (92 % *ee*).

5 DISCUSSION

"Tailor-made" whole-cell biocatalysts have already been applied successfully in numerous biotransformations, especially for the preparation of industrially interesting compounds like chiral alcohols and amino acids. These syntheses predominantly start from oxidized precursors and thus require cofactors like NADH and NADPH. Therefore, many existing approaches utilize recombinant *E. coli* cells co-expressing the required oxidoreductase simultaneously with an enzyme for cofactor regeneration and have already been reported both for the enantioselective reduction and the asymmetric amination of various carbonyl compounds.[56-58, 60, 67-70, 72-74] Less effort has been dedicated so far to the use of aminotransferases in such recombinant whole-cell biocatalysts, in particular using ω-transaminases. Galkin *et al.* achieved the production of optically active amino acids from α-keto acids by means of recombinant *E. coli* cells overexpressing a D-amino acid aminotransferase, an alanine racemase, an alanine dehydrogenase and a formate dehydrogenase.[65] In the course of this work the simultaneous expression of all enzymes was accomplished through a one-plasmid strategy. The principal strategies for co-expression have already been discussed in chapter 1.3. More recently the transaminase C (alanine-valine transaminase) was coupled with an AlaDH in a recombinant *E. coli* catalyst using a two-plasmid strategy by Lorenz and co-workers.[50] With this whole-cell biocatalyst they facilitated the reductive amination of 2-keto-3-methylvalerate to L-isoleucine.

In the present thesis the successful construction and characterization of a recombinant whole-cell catalyst is presented, employing for the first time a ω-TA in combination with an AlaDH and an enzyme for cofactor regeneration. Furthermore, the successful application of the constructed whole-cell catalyst in the asymmetric reductive amination of 4-phenyl-2-butanone was shown. Two different plasmids were used to co-express the ω-TA and the cofactor regenerating enzyme in a single *E. coli* cell. Moreover, the gene encoding the AlaDH was introduced into the genome of *E. coli* facilitating the simultaneous co-expression of the required proteins (Figure 4.3). The main reason for choosing this co-expression strategy was the high flexibility that goes in hand with this approach. By providing the genes for the ω-TA as well as for the cofactor regenerating enzyme on two different plasmids, it was possible to easily use various combinations of the enzymes. In case of the ω-TA it was possible to initially select between ArR, ArS and CV, whereas for the regeneration of the nicotinamide cofactor three different enzymes were available, namely FDG, GDH and PtDH. For the efficient assembly of the desired whole-cell catalyst it was crucial that the two selected plasmids were compatible. Therefore, suitable origins of replications and independent antibiotic selection for maintenance were required (see chapter 2).

Moreover, by using two plasmids, one for ω-TA and one for the cofactor regenerating enzyme, respectively enabled the independent expression of the two proteins and thus the regulation of the enzyme activities. The pTrc99A vector used for ω-TA expression is a high copy-number plasmid offering strong regulation through the trc-promoter and hence facilitates sufficient protein production.[77] The pBAD28 vector though is a low copy-number plasmid and was selected for the protein production of the cofactor regenerating enzymes. However, it has been reported that genes cloned under the tight control of the araC-P_{BAD} promoter system are efficiently expressed with high levels, even though the promoter is not as strong as the trc-promoter.[78] Since the AlaDH was always needed in the desired reductive amination reaction, the gene encoding this enzyme was integrated into the genome of the used *E. coli* strain. This means that the AlaDH gene was only present as a single copy. Nevertheless, a high-level expression of the enzyme was still possible due to the trc-promoter controlling transcription. In general, the trc-promoter is strong and allows the accumulation of up to 15-30 % of total cell protein.[89-90] Therefore, in theory an appropriate co-expression level and thus a suitable ratio of the three enzyme activities should be assured. In Table 5.1 the advantages and disadvantages of the different co-expression approaches are summarized.

Table 5.1 Pros and Cons of the different co-expression approaches compared to the applied strategy

	Pros	Cons
One-plasmid strategy	• Easy set-up (just one antibiotic needed implying less stress)	• Difficult regulation of enzyme activities • No flexibility
Two-plasmid strategy	• Easy adaption of enzyme activities by different copy numbers • Independent expression of each gene	• Compatibility of plasmids necessary • Limited flexibility with more than three enzymes
Two-plasmids combined with genomic integration	• Easy adaption of enzyme activities by different copy numbers • Independent expression of each gene • High level of flexibility	• Compatibility of plasmids necessary • Genome present in just one copy

By means of this co-expression strategy the construction of a highly flexible whole-cell biocatalyst was enabled. After the successful assembly of the different modules (ω-TA, AlaDH and FDH/GDH/PtDH), the co-expression of the three genes using the three different systems was performed. The enzyme activities measured in the cell-free extracts were low, if compared to activities obtained from individual protein expressions.[43, 91] This might be due to an increased metabolic burden caused by the co-expression of three different enzymes, which leads to a limited capacity of the protein biosynthesis machinery.[63]

However, by optimization of expression parameters the production of the enzymes was adjusted in a manner, that appropriate activity ratios could be obtained representing an important parameter in terms of an efficient application of the catalyst.[56, 72] The required enzymes were obtained with activities ranging from 100-200 mU/mg total protein for the CV-ω-TA and 300-400 mU/mg total protein for AlaDH. Rather varying activity levels were received for the cofactor regenerating enzymes. While the FDH offered an activity less than 200 mU/mg total protein, almost 600 mU/ mg total protein were reached for PtDH and even up to 12 U/mg total protein for GDH (Figure 5.1). Therefore, in all cases the activities of the enzymes required for shifting the reaction equilibrium towards the product side (AlaDH and FDH/GDH/PtDH) were higher relative to the ω-TA activity, theoretically facilitating an efficient transamination reaction.

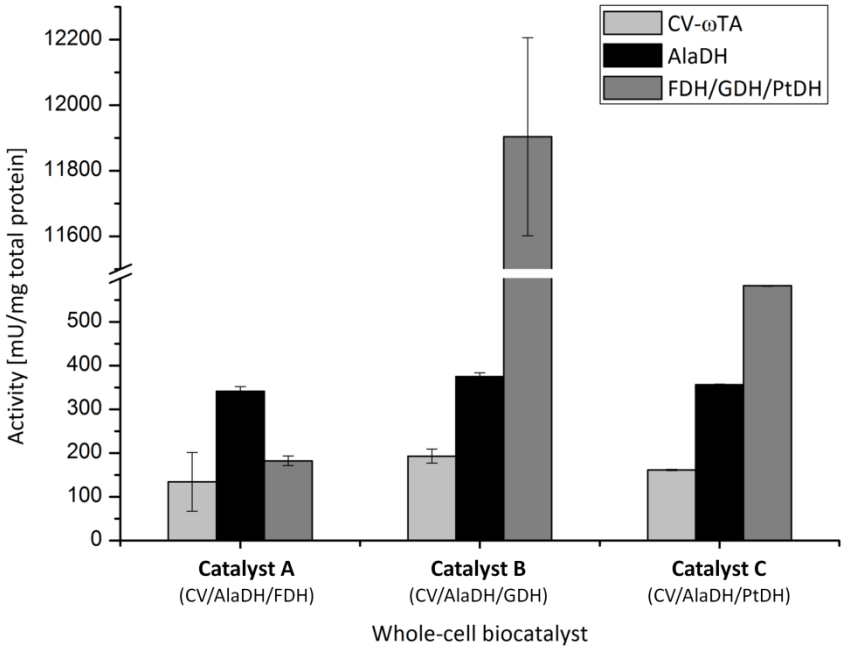


Figure 5.1 Comparison of the obtained enzyme activities for each optimized catalytic system. Catalyst A: CV/AlaDH/FDH; catalyst B: CV/AlaDH/GDH; catalyst C: CV/AlaDH/PtDH

As model reaction for the whole-cell catalyzed reductive amination the transformation of 4-phenyl-2-butanone was chosen and different preparations of the whole-cell catalyst were comparatively studied (resting cells, lyophilized cells, cell-free extract and lyophilized cell-free extract). The obtained results are illustrated in Figure 5.2. In general, it has to be mentioned that in the course of the whole-cell biotranformations it turned out that the intracellular coenzyme level was too low to perform the reactions satisfyingly without the addition of coenzymes. The conversions were much higher only when NAD^+ was present in the reaction mixture. According to literature the NAD^+ level of *E. coli* is in the range of 5.3 nmol/mg dry weight under standard growth conditions.[92]

Thus, the application of whole-cell biocatalysts without additional coenzyme may be possible, but it has been reported previously that by adding coenzymes the efficiency of the reaction is significantly enhanced.[60, 69] Moreover, the degradation of the coenzymes in the cell could be a further limiting parameter.[93] Therefore, the biotransformations were always carried out in the presence of 1 mM NAD^+.

The highest conversions to the desired amine (54 %) were obtained with catalyst C, correlating well with the fact that this system also provided the highest overall activity of 0.992 U/g whole cells (see chapter 4.5). Similar results were achieved with the other cell preparations of catalyst C. In contrast, with catalyst A and catalyst B bigger differences concerning the employed cell preparation were observed. Indeed, for catalyst A the positive effect of lyophilization became apparent, since considerably higher amine amounts were obtained (52 %), almost comparable to the maximum conversion obtained with catalyst C.

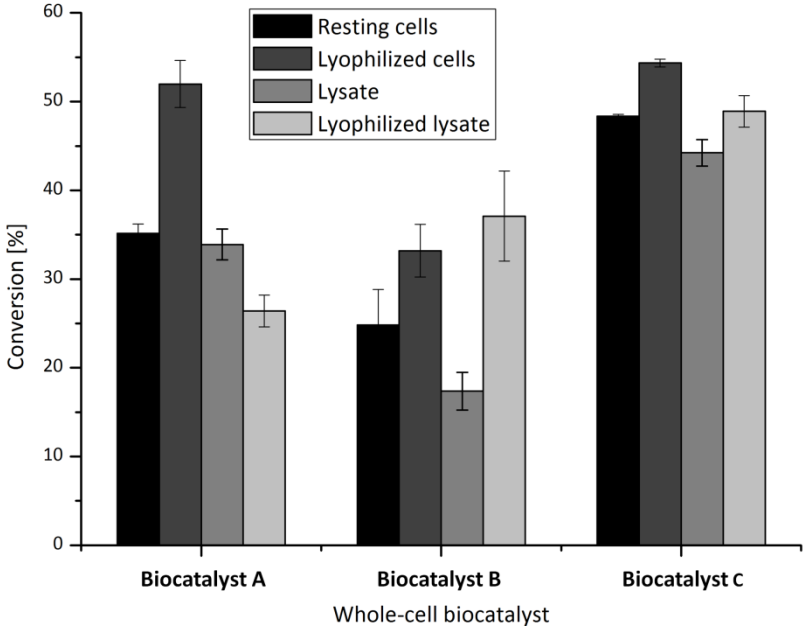


Figure 5.2 Comparison of the obtained conversions for each catalytic system in the reductive amination of 4-phenyl-2-butanone. The catalyst was applied in different preparations. Catalyst A: CV/AlaDH/FDH; catalyst B: CV/AlaDH/GDH; catalyst C: CV/AlaDH/PtDH

The positive influence of lyophilization can be explained by the permeabilization of the cell membrane, enabling a better access of the reaction components to the enzymes. However, regarding Catalyst B the lyophilization of the cells could not improve the reaction outcome. Here, the highest conversion was obtained when using the lyophilized lysate. It can be assumed, that this is due to the reduced ability of *E. coli* to import glucose, which is a major drawback of GDH-catalyzed cofactor regeneration.[70, 94] Hence, a sufficient permeabilization of the cell essentially for the glucose transfer is required.

In general, the product formation with catalyst B was considerably lower compared to the other catalytic systems. A further issue in this context could also be the acidification caused by the co-product gluconic acid, presumably leading to some loss of enzyme activity. Especially, using a whole-cell preparation of the catalyst might have an even greater effect on the outcome of the reaction, since the gluconic acid could accumulate only in the cell and would not be directly transferred to the reaction medium. This fact would come along with a local acidification in the cell, further enhancing the inactivation of the enzymes. Thus, also for this reason the use of catalyst B as lyophilized lysate seemed to be more favorable.

In order to further increase the amine production by the recombinant *E. coli* whole-cell catalyst, the effect of a higher catalyst loading using catalyst A in form of lyophilized cells was investigated. Surprisingly, with a ten-fold amount of the catalyst the conversion to the amine could only be increased by a factor of 1.5. This indicated the occurrence of limitations such as the loss of enzyme activity, caused by inactivation or inhibition over the time. A too low activity level of AlaDH or the cofactor regenerating enzyme, for example, would lead to an unfavorable thermodynamic equilibrium of the reaction since the shifting to the product side would become insufficient. Nevertheless, a recombinant *E. coli* whole-cell catalyst has been successfully developed and applied in the asymmetric reductive amination of 4-phenyl-2-butanone. From an industrial point of view this whole-cell concept would represent a quite promising and cost-efficient alternative to the separate use of isolated enzymes. Major advantages of the described approach are the decrease of fermentations required as well as the avoidance of costly and laborious isolation and purification steps (Table 5.2).

Table 5.2 Comparison of two biocatalytic approaches for the asymmetric reductive amination: isolated enzymes *vs.* recombinant whole-cell catalyst

	Isolated enzyme approach	**Whole-cell catalyst approach**
Fermentation	3	1
Catalyst preparation	Cell disruption, purification, concentration	Cell separation
Reaction efficiency	≤100 % conversion in 24 h	Max. 77 % conversion in 48 h

However, the only drawback is that so far a maximum amine yield of only 77 % could be obtained after 48 hours, whereas with isolated enzymes full conversion was achieved already after 24 hours.[95]

In conclusion, the described whole-cell approach is a promising alternative to the well established ω-TA catalyzed reaction, not only making the catalyst preparation easier but also facilitating a more efficient and greener process for the production of chiral amines.

6 OUTLOOK

Since the construction of the whole-cell biocatalyst has already been successfully accomplished in the course of this work, in future studies much attention will be dedicated to its application in order to make it more efficient. The observed limitations in the reductive amination of 4-phenyl-2-butanone catalyzed by the whole-cell biocatalyst are not fully understood yet. Therefore, it has to be clarified if deactivation or inhibition of the enzymes takes place. However, so far it is not clear which enzyme is the limiting parameter in the reaction. As soon as these limitations are identified, the next task would be to overcome them. Additionally, a further optimization of the co-expression resulting in higher enzyme activities might lead to a more efficient whole-cell biocatalyst.

Different preparations of the whole-cell catalyst have already been tested and comparatively studied. Here it became obvious that permeabilization of the cell membrane had a positive effect on the outcome of the reaction. It has been reported that the presence of an organic phase is expected to significantly influence the cell-membrane permeability.[57] Therefore, it would be interesting if the addition of an organic co-solvent would lead to a further increase of the conversion.

Another important task would be the investigation of the substrate scope for the whole-cell biocatalyst. Since up to now only 4-phenyl-2-butanone has been tested, the application of the catalyst could be extended to some other substrates which are known to be well accepted by ω-TAs.[20]

Since so far only the CV-ω-TA has been investigated in this catalytic whole-cell system, employing also the other (*R*)- and (*S*)-selective ω-TAs originating from *Athrobacter sp.* would be of great interest. Therefore, the assembly of the biocatalysts, the expression optimization as well as the application in a model reaction will be necessary.

Another approach for increasing the flexibility of the whole-cell catalytic system would be to additionally co-express an alanine racemase. Therefore, the *in-situ* racemization of the amine donor L-alanine to the stereocomplemetary D-alanine would enable a more efficient use of the whole-cell biocatalyst employing (R)-selective ω-TAs.

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8 ABBREVIATIONS

AADH	amino acid dehydrogenase
ADH	alcohol dehydrogenase
AHTC	anhydrotetracyclin
AlaDH	alanine dehydrogenase
Amp	ampicillin
APS	ammonium persulfate
ArR	*Arthrobacter species* (*R*)-selective
ArS	*Arthrobacter species* (*S*)-selective
ATP	adenosine tripohosphate
$CaCl_2$	calcium chloride
Cam	chloramphenicol
CV	*Chromobacterium violaceum*
dH_2O	deionized water
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotides
EC	enzyme commission number
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ee	*enantiomeric excess*
eq	equivalents
EtOAC	ethyl acetate
FCC	Fab-crew code
FDH	formate dehydrogenase
FID	flame ionization detector
g	gravitational force
GC	gas chromatography
GDH	glucose dehydrogenase
H_2	Hydrogen
HCl	hydrochloric acid
IPTG	isopropyl-*β*-D-thiogalactopyranoside
Kan	kanamycine
kDA	kilo dalton
LB	lysogeny broth
LDH	lactate dehydrogenase

$MgCl_2$	magnesium(II) chloride
$MgSO_4$	mangnesium(II) sulfate
$MnCl_2$	manganese(II) chloride
$NaHCO_3$	sodium hydrogencarbonate
NaOH	sodium hydroxide
Na_2SO4	sodium sulfate
$NADH/NAD^+$	nicotinamide adenine dinucleotide
$NADPH/NADP^+$	nicotinamide adenine dinucleotide phosphate
NaP_i	sodium phosphate
OD_{600}	optical density at λ= 600 nm
ONC	over-night culture
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pEG	plasmide Elk Group
PLP	pyridoxal-5'-phosphate
PMP	pyridoxamine-5'-phosphate
PtDH	phosphite dehydrogenase
SDS	sodium dodecyl sulphate
$RbCl_2$	rubidium(II) chloride
rpm	rounds per minute
TAE	Tris-acetate-EDTA
TEMED	*N*,*N*,*N*',*N*'-tetramethylethylendiamine
t_{ret}	retention time
Tris	tris(hydroxymethyl)aminomethane
U	enzyme activity [1 U= 1 µmol/min]
vol%	percentage per volume
wt%	percentage per weight
α-TA	α-transaminase
λ	wavelength [nm]
ω-TA	ω-transaminase

9 APPENDIX

9.1 Amino acid sequences

9.1.1 (*R*)-selective ω-TA from *Arthrobacter sp.*

MTSEIVYTHDTGLDYITYSDYELDPANPLAGGAAWIEGAFVPPSEARISIFDQGYLHSDVTYTVFHVWNG
NAFRLDDHIERLFSNAESMRIIPPLTQDEVKEIALELVAKTELREAFVSVSITRGYSSTPGERDITKHRPQ
VYMYAVPYQWIVPFDRIRDGVHAMVAQSVRRTPRSSIDPQVKNFQWGDLIRAVQETHDRGFEAPLLLD
GDGLLAEGSGFNVVVIKDGVVRSPGRAALPGITRKTVLEIAESLGHEAILADITLAELLDADEVLGCTTAG
GVWPFVSVDGNPISDGVPGPITQSIIRRYWELNVESSSLLTPVQY

9.1.2 (*S*)-selective ω-TA from *Arthrobacter sp.*

MGLTVQKINWEQVKEWDRKYLMRTFSTQNEYQPVPIESTEGDYLITPGGTRLLDFFNQLCCVNLGQKN
QKVNAAIKEALDRYGFVWDTYATDYKAKAAKIIIEDILGDEDWPGKVRFVSTGSEAVETALNIARLYTN
RPLVVTREHDYHGWTGGAATVTRLRSFRSGLVGENSESFSAQIPGSSCSSAVLMAPSSNTFQDSNGNYL
KDENGELLSVKYTRRMIENYGPEQVAAVITEVSQGVGSTMPPYEYVPQIRKMTKELGVLWISDEVLTGF
GRTGKWFGYQHYGVQPDIITMGKGLSSSSLPAGAVVVSKEIAAFMDKHRWESVSTYAGHPVAMAAVCA
NLEVMMEENLVEQAKNSGEYIRSKLELLQEKHKSIGNFDGYGLLWIVDIVNAKTKTPYVKLDRNFRHG
MNPNQIPTQIIMEKALEKGVLIGGAMPNTMRIGASLNVSRGDIDKAMDALDYALDYLESGEWQQS

9.1.3 (*S*)-selective ω-TA from *Chromobacterium violaceum*

QKQRTTSQWRELDAAHHLHPFTDTASLNQAGARVMTRGEGVYLWDSEGNKIIDGMAGLWCVNVGYG
RKDFAEAARRQMEELPFYNTFFKTTHPAVVELSSLLAEVTPAGFDRVFYTNSGSESVDTMIRMVRRYW
DVQGKPEKKTLIGRWNGYHGSTIGGASLGGMKYMHEQGDLPIPGMAHIEQPWWYKHGKDMTPDEFG
VVAARWLEEKILEIGADKVAAFVGEPIQGAGGVIVPPATYWPEIERICRKYDVLLVADEVICGFGRTGEW
FGHQHFGFQPDLFTAAKGLSSGYLPIGAVFVGKRVAEGLIAGGDFNHGFTYSGHPVCAAVAHANVAALR
DEGIVQRVKDDIGPYMQKRWRETFSRFEHVDDVRGVGMVQAFTLVKNKAKRELFPDFGEIGTLCRDIFF
RNNLIMRACGDHIVSAPPLVMTRAEVDEMLAVAERCLEEFEQTLKARGLA

9.1.4 AlaDH from *Bacillus subtilis*

MFFYFLNQTKNFPKYQATLKISHIQEETDMIIGVPKEIKNNENRVALTPGGVSQLISNGHRVLVETGAGL
GSGFENEAYESAGAEIIADPKQVWDAEMVMKVKEPLPEEYVYFRKGLVLFTYLHLAAEPELAQALKDKG
VTAIAYETVSEGRTLPLLTPMSEVAGRMAAQIGAQFLEKPKGGKGILLAGVPGVSRGKVTIIGGGVVGTN
AAKMAVGLGADVTIIDLNADRLRQLDDIFGHQIKTLISNPVNIADAVAEADLLICAVLIPGAKAPTLVTEE

MVKQMKPGSVIVDVAIDQGGIVETVDHITTHDQPTYEKHGVVHYAVANMPGAVPRTSTIALTNVTVPY
ALQIANKGAVKALADNTALRAGLNTANGHVTYEAVARDLGYEYVPAEKALQDESSVAGA

9.1.5 FDH from *Komagataella pastoris*

MKIVLVLYSAGKHAADEPKLYGCIENELGIRQWLEKGGHELVTTSDKEGENSELEKHIPDADVIISTPFH
PAYITKERIQKAKKLKLLVVAGVGSDHIDLDYIEQNGLDISVLEVTGSNVVSVAEHVVMTILNLVRNFVPA
HEQIVNHGWDVAAIAKDAYDIEGKTIATIGAGRIGYRVLERLVAFNPKELLYYDYQGLPKEAEEKVGARR
VDTVEELVAQADVVTVNAPLHAGTKGLVNKELLSKFKKGAWLVNTARGAICNAQDVADAVASGQLRG
YGGDVWFPQPAPKDHPWRDMRNKYGYGNAMTPHYSGTTLDAQVRYAEGTKNILNSFLTKKFDYRPQ
DVILLNGKYKTKAYGNDKKVA

9.1.6 GDH from *Bacillus megaterium*

MYKDLEGKVVVITGSSTGLGKSMAIRFATEKAKVVVNYRSKEDEANSVLEEIKKVGGEAIAVKGDVTVES
DVINLVQSAIKEFGKLDVMINNAGLENPVSSHEMSLSDWNKVIDTNLTGAFLGSREAIKYFVENDIKGTV
INMSSVHEKIPWPLFVHYAASKGGMKLMTETLALEYAPKGIRVNNIGPGAINTPINAEKFADPEQRADV
ESMIPMGYIGEPEEIAAVAAWLASSEASYVTGITLFADGGMTQYPSFQAGRG

9.1.7 PtDH from *Pseudomonas stutzeri*

MLPKLVITHRVHEEILQLLAPHCELITNQTDSTLTREEILRRCRDAQAMMAFMPDRVDADFLQACPELR
VIGCALKGFDNFDVDACTARGVWLTFVPDLLTVPTAELAIGLAVGLGRHLRAADAFVRSGKFRGWQPR
FYGTGLDNATVGFLGMGAIGLAMADRLQGWGATLQYHARKALDTQTEQRLGLRQVACSELFASSDFIL
LALPLNADTLHLVNAELLALVRPGALLVNPCRGSVVDEAAVLAALERGQLGGYAADVFEMEDWARADR
PQQIDPALLAHPNTLFTPHIGSAVRAVRLEIERCAAQNILQALAGERPINAVNRLPKANPAAD

9.2 Nucleotide sequences

9.2.1 (*R*)-selective ω-TA from *Arthrobacter sp.*

caattcATGACCAGCGAAATTGTTTATACCCATGATACCGGTCTGGATTATATCACCTATAGCGATTATG
AACTGGATCCGGCAAATCCGCTGGCAGGCGGTGCAGCATGGATTGAAGGTGCATTTGTTCCGCCTAGCG
AAGCACGTATTAGCATTTTTGATCAGGGCTATCTGCATAGTGATGTTACCTATACCGTGTTTCATGTG
TGGAATGGTAATGCATTTCGCCTGGATGATCATATTGAACGTCTGTTTAGCAATGCAGAAAGCATGCG
TATTATTCCGCCTCTGACCCAGGATGAAGTTAAAGAAATTGCACTGGAACTGGTTGCAAAAACCGAAC
TGCGTGAAGCATTTGTTAGCGTTAGCATTACCCGTGGTTATAGCAGCACACCGGGTGAACGTGATATT
ACCAAACATCGTCCGCAGGTTTATATGTATGCAGTTCCGTATCAGTGGATTGTTCCGTTTGATCGTATT
CGTGATGGTGTTCATGCAATGGTTGCACAGAGCGTTCGTCGTACACCGCGTAGCAGCATTGATCCGCAG
GTTAAAAATTTTCAGTGGGGTGATCTGATTCGTGCAGTTCAAGAAACCCATGATCGTGGTTTTGAAGC

ACCGCTGCTGCTGGATGGTGATGGTCTGCTGGCCGAAGGTAGCGGTTTTAATGTTGTTGTGATTAAAG
ATGGTGTGGTTCGTAGTCCGGGTCGTGCAGCACTGCCTGGTATTACCCGTAAAACCGTTCTGGAAATTG
CAGAAAGCCTGGGTCATGAAGCAATTCTGGCAGATATTACCCTGGCAGAACTGCTGGATGCAGATGAA
GTTCTGGGTTGTACCACCGCAGGCGGTGTTTGGCCGTTTGTTAGCGTGGATGGTAATCCGATTTCAGAT
GGTGTTCCGGGTCCGATTACCCAGAGCATTATTCGTCGTTATTGGGAACTGAATGTTGAAAGCAGCAG
CCTGCTGACACCGGTTCAGTATTAATAAgtcgac

9.2.2 (*S*)-selective ω-TA from *Arthrobacter sp.*

caattcATGGGTCTGACCGTGCAGAAAATTAATTGGGAACAGGTGAAAGAATGGGATCGCAAATATCTG
ATGCGTACCTTTAGCACCCAGAATGAATATCAGCCGGTTCCGATTGAAAGCACCGAAGGCGATTATCT
GATTACACCGGGTGGTACACGTCTGCTGGATTTTTTTAATCAGCTGTGCTGTGTTAATCTGGGTCAGA
AAAATCAGAAAGTGAATGCAGCCATTAAAGAAGCACTGGATCGTTACGGTTTTGTGTGGGATACCTAT
GCCACCGATTATAAAGCAAAAGCCGCAAAAATTATTATTGAAGATATTCTGGGAGATGAAGATTGGCC
TGGTAAAGTTCGTTTTGTTAGCACCGGTAGCGAAGCAGTTGAAACCGCACTGAATATTGCACGTCTGT
ATACCAATCGTCCGCTGGTTGTTACCCGTGAACATGATTATCATGGTTGGACCGGTGGTGCAGCAACCG
TTACCCGTCTGCGTAGCTTTCGTAGCGGTCTGGTTGGTGAAAATAGCGAAAGCTTTAGCGCACAGATT
CCGGGTAGCAGCTGTAGCAGCGCAGTTCTGATGGCACCGAGCAGCAATACCTTTCAGGATAGCAATGG
CAATTATCTGAAAGATGAAAACGGTGAACTGCTGTCTGTTAAATATACCCGTCGCATGATTGAAAATT
ATGGTCCGGAACAGGTTGCAGCAGTTATTACCGAAGTTAGCCAGGGTGTTGGTAGCACCATGCCTCCG
TATGAATATGTTCCGCAGATTCGCAAAATGACCAAAGAACTGGGTGTTCTGTGGATTTCTGATGAAGT
TCTGACCGGTTTTGGTCGTACCGGTAAATGGTTTGGCTATCAGCATTATGGTGTTCAGCCGGATATTA
TTACCATGGGTAAAGGTCTGAGCAGCAGCAGCCTGCCTGCAGGTGCAGTTGTTGTGAGCAAAGAAATC
GCAGCCTTTATGGATAAACATCGTTGGGAAAGCGTTAGCACCTATGCAGGTCATCCTGTGGCTATGGC
TGCCGTTTGTGCAAATCTGGAAGTGATGATGGAAGAAAATCTGGTTGAACAGGCCAAAAATAGCGGTG
AATATATCCGTAGCAAACTGGAACTGCTGCAGGAAAAACATAAAAGCATTGGCAATTTTGATGGTTAT
GGCCTGCTGTGGATTGTTGATATTGTGAATGCCAAAACCAAAACCCCGTATGTTAAACTGGATCGCAA
TTTTCGTCATGGCATGAATCCGAATCAGATTCCGACCCAGATCATTATGGAAAAAGCCCTGGAAAAAG
GTGTTCTGATTGGTGGTGCAATGCCGAATACCATGCGTATTGGTGCAAGCCTGAATGTTAGCCGTGGC
GATATTGATAAAGCAATGGATGCACTGGATTATGCCCTGGATTATCTGGAAAGCGGTGAATGGCAGCA
GAGCTAAgtcgac

9.2.3 (*S*)-selective ω-TA from *Chromobacterium violaceum*

caattcATGCAGAAACAGCGTACCACCTCTCAGTGGCGTGAACTGGATGCAGCACATCATCTGCATCCGT
TTACCGATACCGCAAGCCTGAATCAGGCAGGTGCACGTGTTATGACCCGTGGTGAAGGTGTTTATCTG
TGGGATAGCGAAGGCAACAAAATTATTGATGGTATGGCAGGTCTGTGGTGTGTTAATGTTGGTTATGG
TCGCAAAGATTTTGCAGAAGCAGCACGTCGTCAGATGGAAGAACTGCCGTTTTATAATACCTTTTTTA

AAACCACCCATCCGGCAGTTGTTGAACTGAGCAGCCTGCTGGCCGAAGTTACACCGGCAGGTTTTGATC
GTGTGTTTTATACCAATAGCGGTAGCGAAAGCGTTGATACCATGATTCGCATGGTTCGTCGTTATTGG
GATGTTCAGGGCAAACCGGAAAAAAAAACCCTGATCGGTCGTTGGAATGGTTATCATGGTAGCACCAT
TGGTGGTGCCAGCCTGGGTGGTATGAAATATATGCATGAACAGGGTGATCTGCCGATTCCGGGTATGG
CACATATTGAACAGCCGTGGTGGTATAAACATGGCAAAGATATGACACCGGATGAATTTGGTGTTGTT
GCAGCACGTTGGCTGGAAGAAAAAATTCTGGAAATTGGTGCCGATAAAGTTGCAGCATTTGTGGGTGA
ACCGATTCAGGGTGCAGGTGGTGTTATTGTTCCGCCTGCAACCTATTGGCCTGAAATTGAACGTATCTG
CCGCAAATATGATGTTCTGCTGGTTGCCGATGAAGTTATTTGTGGTTTTGGTCGTACCGGTGAATGGT
TTGGTCATCAGCATTTTGGTTTTCAGCCGGACCTGTTTACCGCAGCCAAAGGCTTATCTTCTGGCTATC
TGCCGATTGGTGCAGTTTTTGTTGGTAAACGTGTTGCAGAAGGTCTGATTGCAGGCGGTGATTTTAAT
CATGGCTTTACCTATAGCGGTCATCCGGTTTGTGCAGCAGTTGCACATGCAAATGTTGCAGCACTGCGT
GATGAAGGTATTGTTCAGCGCGTGAAAGATGATATTGGTCCGTATATGCAGAAACGTTGGCGTGAAAC
CTTTAGCCGTTTTGAACATGTTGATGATGTTCGTGGTGTTGGTATGGTTCAGGCATTTACCCTGGTGA
AAAACAAAGCAAAACGCGAACTGTTTCCGGATTTTGGTGAAATTGGCACCCTGTGCCGTGATATTTTT
TTTCGCAATAATCTGATTATGCGTGCCTGTGGTGATCACATTGTTAGCGCACCGCCTCTGGTGATGACC
CGTGCCGAAGTTGATGAAATGCTGGCCGTTGCAGAACGCTGTCTGGAAGAATTTGAACAGACCCTGAA
AGCACGTGGTCTGGCCTAAgtcgac

9.2.4 AlaDH from *Bacillus subtilis*

ATGTTTTTTTATTTTCTTAATCAAACAAAGAATTTTCCAAAATATCAAGCTACACTAAAAATATCACA
TATACAGGAGGAGACAGATATGATCATAGGGGTTCCTAAAGAGATAAAAAACAATGAAAACCGTGTCG
CATTAACACCCGGGGGCGTTTCTCAGCTCATTTCAAACGGCCACCGGGTGCTGGTTGAAACAGGCGCGG
GCCTTGGAAGCGGATTTGAAAATGAAGCCTATGAGTCAGCAGGAGCGGAAATCATTGCTGATCCGAAG
CAGGTCTGGGACGCCGAAATGGTCATGAAAGTAAAAGAACCGCTGCCGGAAGAATATGTTTATTTTCG
CAAAGGACTTGTGCTGTTTACGTACCTTCATTTAGCAGCTGAGCCTGAGCTTGCACAGGCCTTGAAGGA
TAAAGGAGTAACTGCCATCGCATATGAAACGGTCAGTGAAGGCCGGACATTGCCTCTTCTGACGCCAA
TGTCAGAGGTTGCGGGCAGAATGGCAGCGCAAATCGGCGCTCAATTCTTAGAAAAGCCTAAAGGCGGA
AAAGGCATTCTGCTTGCCGGGGTGCCTGGCGTTTCCCGCGGAAAAGTAACAATTATCGGAGGAGGCGT
TGTCGGGACAAACGCGGCGAAAATGGCTGTCGGCCTCGGTGCAGATGTGACGATCATTGACTTAAACG
CAGACCGCTTGCGCCAGCTTGATGACATCTTCGGCCATCAGATTAAAACGTTAATTTCTAATCCGGTCA
ATATTGCTGATGCTGTGGCGGAAGCGGATCTCCTCATTTGCGCGGTATTAATTCCGGGTGCTAAAGCTC
CGACTCTTGTCACTGAGGAAATGGTAAAACAAATGAAACCCGGTTCAGTTATTGTTGATGTAGCGATC
GACCAAGGCGGCATCGTCGAAACTGTCGACCATATCACAACACATGATCAGCCAACATATGAAAAACA
CGGGGTTGTGCATTATGCTGTAGCGAACATGCCAGGCGCAGTCCCTCGTACATCAACAATCGCCCTGAC
TAACGTTACTGTTCCATACGCGCTGCAAATCGCGAACAAAGGGGCAGTAAAAGCGCTCGCAGACAATA

CGGCACTGAGAGCGGGTTTAAACACCGCAAACGGACACGTGACCTATGAAGCTGTAGCAAGAGATCTA
GGCTATGAGTATGTTCCTGCCGAGAAAGCTTTACAGGATGAATCATCTGTGGCGGGTGCTTAA

9.2.5 FDH from *Komagataella pastoris*

attcgagctcaaggagatatagatATGAAAATCGTTCTCGTTTTGTACTCCGCTGGTAAGCACGCCGCCGATGAA
CCAAAGTTGTATGGTTGTATCGAAAATGAATTGGGTATTAGACAATGGCTTGAGAAGGGCGGCCATGA
ATTGGTTACTACATCAGACAAAGAGGGTGAAAACTCTGAGTTAGAAAAGCACATTCCTGACGCTGATG
TGATTATTTCCACTCCATTCCATCCAGCCTACATCACGAAGGAGAGAATCCAAAAAGCCAAGAAGCTG
AAGTTGTTGGTCGTTGCTGGTGTCGGTTCCGACCACATTGACTTGGACTACATTGAACAAAATGGCCT
AGATATTTCGGTCCTAGAGGTTACTGGTTCCAACGTTGTTTCAGTGGCTGAGCATGTCGTTATGACTA
TATTGAACTTGGTGAGAAACTTTGTTCCAGCTCACGAGCAAATTGTTAACCACGGCTGGGACGTTGCT
GCCATCGCCAAGGACGCCTACGATATCGAAGGTAAGACCATCGCAACAATTGGTGCTGGAAGAATTGG
TTACAGAGTCTTAGAGAGACTTGTGGCTTTCAACCCTAAGGAATTGTTGTACTACGACTACCAAGGTC
TTCCAAAAGAGGCCGAGGAAAAAGTTGGTGCCAGAAGAGTCGACACTGTCGAGGAGCTGGTTGCTCAA
GCCGATGTTGTTACCGTCAATGCCCCACTGCACGCAGGTACTAAGGGTTTAGTTAACAAGGAGCTTCTG
TCCAAGTTCAAGAAGGGTGCTTGGTTGGTTAACACAGCCAGAGGTGCCATCTGCAATGCTCAAGATGT
CGCTGATGCCGTTGCATCTGGTCAATTGAGAGGTTACGGTGGTGACGTCTGGTTCCCTCAGCCAGCTCC
AAAGGACCATCCATGGAGAGATATGAGAAACAAGTACGGATACGGAAACGCCATGACTCCTCATTACT
CAGGTACCACTTTGGACGCCCAGGTCAGATATGCCGAAGGTACCAAGAACATCTTGAACTCATTCCTTA
CCAAGAAGTTTGACTACAGACCTCAAGATGTCATTCTTTTGAACGGTAAGTACAAGACCAAGGCTTAT
GGTAATGACAAAAAGGTCGCATAAtctagagtcg

9.2.6 GDH from *Bacillus megaterium*

attcgagctcaaggagatatagatATGTATAAAGATCTGGAAGGCAAAGTGGTGGTGATTACCGGCAGCAGCAC
CGGCCTGGGCAAAAGCATGGCGATTCGTTTTGCGACCGAAAAAGCGAAAGTGGTGGTTAACTATCGCA
GCAAAGAAGATGAAGCGAACAGCGTGCTGGAAGAAATTAAAAAAGTGGGCGGCGAAGCGATTGCGGT
GAAAGGTGATGTGACCGTGGAAAGCGATGTGATTAACCTGGTGCAGAGCGCGATTAAAGAATTTGGCA
AACTGGATGTGATGATTAACAACGCGGGCCTGGAAAATCCGGTGAGCAGCCATGAAATGAGCCTGAGC
GATTGGAACAAAGTGATTGATACCAACCTGACCGGCGCGTTTCTGGGTAGCCGTGAAGCGATTAAATA
CTTCGTGGAAAACGATATTAAAGGCACCGTGATTAACATGAGCAGCGTGCATGAAAAAATTCCGTGGC
CGCTGTTTGTGCATTATGCGGCGAGCAAAGGCGGCATGAAACTGATGACCGAAACCCTGGCCCTGGAA
TATGCGCCGAAAGGCATTCGTGTGAACAACATTGGCCCGGGTGCGATTAACACCCCGATTAACGCGGA
AAAATTTGCGGATCCGGAACAGCGTGCGGATGTGGAAAGCATGATTCCGATGGGCTATATTGGCGAAC
CGGAAGAAATTGCGGCGGTGGCGGCGTGGCTGGCCAGCAGCGAAGCGAGCTATGTGACCGGCATTACC
CTGTTTGCGGATGGCGGCATGACCCAGTATCCGAGCTTTCAGGCGGGTCGTGGCTAATAAtctagagtcg

9.2.7 PtDH from *Pseudomonas stutzeri*

attcgagctcaaggagatatagatATGCTGCCGAAACTGGTCATCACCCATCGTGTGCACGAAGAAATCCTGCAA
CTGCTGGCTCCGCATTGCGAACTGATTACCAATCAGACCGACAGCACTCTGACCCGTGAAGAAATTCTG
CGTCGCTGCCGTGACGCACAGGCAATGATGGCGTTTATGCCTGATCGTGTGGATGCTGATTTCCTGCAG
GCTTGTCCGGAACTGCGCGTAATTGGCTGCGCGCTGAAAGGTTTCGACAATTTCGACGTTGATGCGTGT
ACTGCTCGTGGCGTGTGGCTGACCTTCGTTCCGGACCTGCTGACTGTGCCGACTGCGGAACTGGCTATC
GGCCTGGCGGTTGGTCTGGGCCGTCACCTGCGTGCAGCAGACGCGTTTGTTCGTTCTGGTAAATTTCGC
GGCTGGCAGCCTCGCTTTTACGGTACCGGTCTGGACAACGCGACCGTGGGCTTCCTGGGTATGGGTGCA
ATCGGTCTGGCGATGGCTGATCGTCTGCAGGGTTGGGGCGCTACCCTGCAATACCACGCGCGTAAAGCT
CTGGATACGCAAACGGAACAACGCCTGGGTCTGCGTCAAGTGGCTTGCAGCGAGCTGTTCGCTTCTAGC
GACTTCATTCTGCTGGCTCTGCCGCTGAACGCAGATACCCTGCACCTGGTCAACGCGGAACTGCTGGCG
CTGGTACGTCCGGGCGCACTGCTGGTTAACCCGTGCCGTGGCTCTGTGGTCGACGAAGCCGCAGTTCTG
GCGGCTCTGGAACGCGGTCAACTGGGCGGCTACGCGGCGGACGTGTTTGAGATGGAAGATTGGGCGCG
TGCTGATCGTCCGCAGCAGATTGATCCAGCCCTGCTGGCGCACCCGAACACGCTGTTTACTCCGCACAT
CGGTTCTGCAGTACGTGCGGTTCGTCTGGAGATCGAGCGTTGTGCGGCTCAGAACATTCTGCAGGCGCT
GGCGGGCGAGCGTCCGATCAACGCAGTGAATCGCCTGCCGAAAGCCAACCCTGCGGCTGACTAAtctagag
tcg

CURRICULUM VITAE

PERSONAL DATA

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EDUCATION

Since 10/2011	**Graz University of Technology** **Master of Science in "Biotechnology"** Master Thesis: "Design and Application of a Whole-cell-Catalyst for the Asymmetric Amination of Ketones" Univ.-Prof. Dipl.-Ing. Dr.techn. Wolfgang Kroutil, Department of Organic and Bioorganic chemistry, Karl-Franzens-University of Graz Project lab course: "Promiscuity of Old Yellow Enzymes: Unexpected Isomerization Reaction" O.Univ.-Prof. Dr.phil. Kurt Faber, Deparment of Organic and Bioorganic chemistry, Karl-Franzens-University of Graz
10/2007 – 07/2011	**Karl-Franzens-University of Graz** **Bachelor of Science in "Enviromental System Sciences; Chemistry"** Bachelor Thesis: "Novel blue light excitable luminesence europium (III) complexes for application in optical oxygen sensors" Univ.-Prof. Dipl.-Chem. Dr.rer.nat. Ingo Klimant, Institute of Analytical Chemistry and Food Chemistry, Graz University of Technology
09/1999 – 06/2007	**BRG / BORG Kirchdorf/Krems** Matura passed with distinction (average 1,0)
09/1995 – 06/1999	**Elementary School Magdalenaberg**

PRACTICAL EXPERIENCE

09/2012 – 02/2013	**ACIB - Austrian Centre of Industrial Biotechnology** **Research Assistant** Research work in the field of biocatalysis in cooperation with company SANDOZ
07/2012 – 08/2012	**University of Graz, Department of Organic and Bioorganic chemistry** **Summer student internship** "Optimization of the reaction conditions of norcoclaurine synthase" Prof. Univ.-Prof. Dipl.-Ing. Dr.techn. Wolfgang Kroutil
07/2008 – 08/2008	**Donau Kanol GmbH & Co KG** **Trainee in Quality Management**

PUBLICATIONS

R. C. Simon, J. H. Sattler, **J. E. Farnberger**, C. S. Fuchs, N. Richter, F. Zepeck, W. Kroutil, *Enzymatic Synthesis of the Silodosin Amine Intermediate*. *Tetrahedron: Asymmetry* **2014**, *25*, 284-288

ADDITIONAL QUALIFICATION

Languages	**German** (native) **English** (fluent) **Spanish** (proficient)
Soft Skills	Presentation Techniques Stress and Time Management
EDV	MS Office, ChemBio Office, CLC Main Workbench
Personal Interests	Literature and Arts, Volleyball, Climbing, Running