Development of a biocatalytic production process for (S)-α-hydroxy ketones

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“And remember, the only difference between science and fooling around is to write everything down!”

The Mythbusters

For my family. The whole of it.
Abstract

Enantiomerically pure α-hydroxy ketones are versatile building blocks for the pharmaceutical industry. Since the highly chemo- and stereoselective synthesis of these compounds is very challenging with traditional chemical synthesis, the use of biocatalysis for the α-hydroxy ketone production is an interesting alternative. One of the most promising alternatives is the use of thiamine diphosphate (ThDP)-dependent enzymes, which are able to produce these hydroxy ketones by carboligation of two inexpensive aldehydes. The enzyme toolbox available in the Biocatalysis & Biosensors group at IBG-1, Forschungszentrum Jülich GmbH, includes numerous ThDP-dependent enzymes, which are able to synthesise a wide palette of hydroxy ketones with high selectivity. However, most of the wild-type enzymes are (R)-selective for the carboligation if at least one of the aldehydes used for the carboligation is aromatic, making the synthesis of aromatic and mixed aliphatic/aromatic (S)-hydroxy ketones more challenging. In this work, with the Acetobacter pasteurianus pyruvate decarboxylase variant E469G (ApPDCE469G), one of the few designed (S)-selective variants, a lab scale production process for (S)-phenyl propionyl carbinol (PPC) has been developed. In order to do this, several tasks have been performed:

- Reaction characterization and optimization: By combining appropriate choice of substrates and solvent as well as reaction engineering the specific space-time-yield could be increased up to 61 fold, proving the immense impact of suitable reaction condition on both, catalyst productivity and selectivity. Further, the enantiomeric excess of the product could be improved from 89 % up to 98 % (S)-PPC. Especially avoidance of organic co-solvents, used as substrate solubility enhancers, as well as the exchange of the donor aldehyde by its corresponding α-keto acid, which is decarboxylated to the aldehyde prior to carboligation by the same enzyme, were essential for the enhancement.

- Use of whole cell: alternatively to purified enzyme, the use of whole cells for the production of (S)-PPC has also been evaluated and optimized. Recyclability of the cells was determined and the reaction was scaled up in a 500 mL stirred tank reactor with a substrate fed, achieving product concentrations over 10 g/L/d. The drawbacks of the whole cell catalysis were a slightly lower enantiomeric excess of the product compared with pure enzyme and the occurrence of a new by-product. This procedure was also successfully tested for the production of (S)-phenylacetylcarbinol from pyruvate and benzaldehyde in order to verify that the process extrapolation to other (S)-hydroxy ketones is possible.

- Development of an enantiomeric excess enhancement strategy: In order to make use of the easy applicable whole cell catalysis and to overcome the drawback of only moderate stereoselectivity, a new strategy to increase stereoselectivity, called 'chiral polishing' was invented. By using a second, strictly (R)-selective ThDP-dependent enzyme, which is able to cleave (R)-α-hydroxy ketones into aldehydes but does not except the (S)-enantiomer the undesired (R)-enantiomer can be removed and the
aldehydes formed are again available for the carboligation step by the ApPDCE469G yielding a highly (S)-selective product.
Kurzfassung.


- Entwicklung einer Strategie zur Steigerung der Enantiomerereinheit: Um das leicht anwendbare Ganzzell-Katalyse Verfahren anwendbar zu machen und um den
Nachteil der nur moderate Stereoselektivität zu überwinden, wurde eine neue Strategie zur Steigerung der Enantiomerenreinheit erfunden. Durch die Verwendung eines zweiten, rein (R)-selektiven ThDP-abhängigen Enzyms, das in der Lage ist, (R)-α-Hydroxyketone in Aldehyde zu spalten, wird das entsprechende (S)-konfigurierte Enantiomer angereichert. Die gebildeten Aldehyde stehen darüber hinaus wieder für die von der ApPDCE469G katalysierten Carboligation zur Verfügung, was sowohl die Ausbeute wie auch die Enantiomerenreinheit des (S)-konfigurierten Produkts weiter erhöht.
List of publications

- Álvaro Gómez Baraibar, Eric von Lieres, Wolfgang Wiechert, Martina Pohl, and Dörte Rother: Effective production of (S)-α-hydroxy ketones: an reaction engineering approach. *In press*

- Dörte Rother, Martina Pohl, Torsten Sehl and Álvaro Gómez Baraibar: 2-Schritt Synthese zur Herstellung von (1S,2S)-Norpseudoephedrin (Cathine) durch Kopplung einer (S)-selektiven Lyase und einer (S)-selektiven Transaminase. Patent application.
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Abbreviations:

AI: Auto induction media

ApPDC: *Acetobacter pasteurianus* pyruvate decarboxylase

BA: Benzaldehyde

BSA: Bovine serum albumin

BZ: Benzoin

DOE: Design of Experiments

DNA: Deoxyribonucleic acid

EcSucA: subunit of α-ketoglutarate dehydrogenase from *Escherichia coli*

ee: enantiomeric excess

IPTG: Isopropyl β-D-1-thiogalactopyranoside

KBA: 2-ketobutyric acid

HBP: 2-Hydroxy butyrophenone

HK: alpha-Hydroxy ketone

HPP: Hydroxy propiophenone

KP: Potassium phosphate buffer

LB: Luria Bertani Broth

NMR: Nuclear magnetic resonance

PAC: Phenyl acetyl carbinol

PfBAL: Benzaldehyde lyase from *Pseudomonas fluorescens*

PFR: Plug flow reactor

PPC: Phenyl propionyl carbinol

PpBFD: Benzoylformate decarboxylase from *Pseudomonas putida*

PROP: Propanal

PYR: Pyruvate

SFC/MS: Super critical fluid chromatography/mass spectrometry
SSTY: Specific space time yield

STR: Stirred tank reactor

STY: Space time yield

ThDP: Thiamine diphosphate

ZmPDC: pyruvate decarboxylase from *Zymomonas mobilis*
1. Introduction

1.1. History of biocatalysis

Even though human kind has used and modified living beings since prehistoric times, like the creation of crops during the agricultural revolution in Mesopotamia, the use of enzymes as a catalyst (biocatalysis) and specially its understanding is relatively new.

The word enzyme, from the Greek “within yeast”, was first used by the German scientist Kühne in 1876 [1], giving a name for all non-living extracts catalyzing a chemical reaction. Some years later, in 1894, Fischer [2] realized that these enzymes were responsible for the optical selectivity presented by living microorganisms which had been previously described by Louis Pasteur in 1848 [3]. Even though the mechanism behind this selectivity was not fully understood, the enzymatic synthesis started being used empirically for biocatalytic production processes, like the synthesis of (R)-mandelonitrile using plant extract [4]. The explanation of the enzyme nature was a matter of strong debate, until the crystallization of urease by Summer in 1926 [5] proved that these enzymes were in fact of proteins. This observation granted him the Nobel Prize in chemistry in 1946. During the 20th century, the use of biocatalysis continued growing with more applications mainly for the production of natural compounds, such as steroids [6], until the use of protein engineering enabled the possibilities to improve catalysts [7] and modifying them to accept non-natural substrates. This revolution, enabled by the discovery of the DNA structure by Watson and Crick in 1953 [8], is considered the 2nd wave of biocatalysis [9] and expanded the use of biotechnology for the production of pharmaceutical intermediates and fine chemicals.

During the first and second waves of biocatalysis, biocatalytical production processes were designed taking into account the limitations of the enzyme [10], but once the combination of protein engineering via site-directed mutagenesis, directed evolution, gen synthesis and bioinformatics allowed more reliable protein modification, the enzymes could be modified and adopted to practically any production process [11-14]. This state of the art is considered the current third wave of biocatalysis [9].

Apart from the mentioned use for production of natural compounds [15], one of the main applications of biocatalysis is the synthesis of pharmaceuticals [16-18]. About 50 % of the drugs used nowadays are chiral compounds and over 90 % of them are used as racemic mixtures [19]. Due to the different pharmacological activity of the enantiomers, the use of these racemic mixtures can have a negative influence on the pharmacological effect of the drug as well as be the cause of undesired side effects [20]. In order to produce enantiomerically pure drugs by traditional chemical synthesis, multi-step syntheses including an appropriate protection group strategy are usually required [21, 22]. Since this option is inefficient, expensive and often environmentally harmful [23, 24], there has been a trend to develop more sustainable production processes (i.e. green chemistry) [25], with high atom efficiency and low environmental impact. Due to the high selectivity displayed by the
enzymes and their renewable nature, the use of biocatalysis for single enantiomer drug production is one of the most promising options to achieve this ambitious goals [9, 26-28].

In the future of this field is aiming for the use of multi-step syntheses [29], combining engineered enzymes and green chemistry approaches in order to produce new synthetic pathways converting inexpensive raw materials into highly complex molecules with applications in diverse fields, like specialty chemicals [11, 30], bio-plastics [31, 32] or biofuels [33]. Even though nowadays the use of biocatalysis is still small compared with traditional petrochemical production processes, the field is growing steadily and the perspectives for the next decades are highly positive [34, 35].

1.2. Enzyme sources

Enzymes are classified according to the Enzyme Commission (EC) enzymes according to the type of reaction catalyzed (Table 1).

Table 1: Enzyme types classification

<table>
<thead>
<tr>
<th>EC number</th>
<th>Name</th>
<th>Function</th>
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</thead>
<tbody>
<tr>
<td>EC 1</td>
<td>Oxidoreductases</td>
<td>Redox reactions</td>
</tr>
<tr>
<td>EC 2</td>
<td>Transferases</td>
<td>Transfer of a functional group</td>
</tr>
<tr>
<td>EC 3</td>
<td>Hydrolases</td>
<td>Bond hydrolysis</td>
</tr>
<tr>
<td>EC 4</td>
<td>Lyases</td>
<td>Bond cleavage without hydrolysis or redox reaction</td>
</tr>
<tr>
<td>EC 5</td>
<td>Isomerases</td>
<td>Isomerization of single molecules</td>
</tr>
<tr>
<td>EC 6</td>
<td>Ligases</td>
<td>Covalent bond creation using energy (ATP-dependent)</td>
</tr>
</tbody>
</table>

In order to obtain a specific enzyme for the production of a desired product there are different possibilities available:

- Database mining: by using databases such as BLAST [36, 37] it is possible to find candidates which might fit to the process needs. This can be done by searching proteins with relevant similarities to known useful catalysts.
- Exploiting enzyme promiscuity: the use of well-known enzymes for catalysis of other reactions than the physiological ones is another possibility [38].
- Creation of metagenomic libraries: only a small percentage of the organism present in nature would grow in an axenic culture [39]. By using a metagenomic approach, the whole proteome diversity of a sample can be revealed increasing the number of candidates for your optimal catalyst, provided that the respective genes can be expressed in the chosen host organism [40, 41]. Also, sequencing of microorganisms from extremophiles, is gaining importance for the discovery of active catalyst at conditions such as high temperatures, high salt concentrations or extreme pH-values [42].
- Directed evolution: the introduction of random changes into the coding gene and the subsequent screening of respective variants in order to find improved variants has been possible since the 70’s when whole organisms were treated with different chemicals or UV light in order to induce mutations [43]. After the invention of the PCR, directed evolution of enzymes became possible in the 90’s [44-47]. Even though this technique has produced several successful results [48], the use of random mutagenesis is not very efficient since a high percentage of the mutations produce inactive proteins or show no effect [49]. Modern techniques like site-saturation mutagenesis [50] or combinatorial active site saturation [51] allow a more efficient evolution, but usually require some knowledge about the protein.

- Rational or semi-rational design: when the knowledge about a catalyst is advanced enough (usually by knowing the sequence, structure and the function) chances to predict the effects of mutagenesis increase, especially if the mechanism of an enzyme is well understood [52]. This technique has been proven useful to enhance promiscuous activity in enzymes [53], to modify the enantioselectivity [54-56], and to increase its stability and activity [57]. The development of new *in silico* simulation techniques is becoming a powerful tool to enhance this strategy [58-60].

1.3. Advantages of biocatalysis over traditional chemical production

The use of biocatalysis in industrial syntheses has several advantages over traditional chemical approaches:

- High selectivity and effectiveness: the turnover numbers of enzymes are usually orders of magnitudes higher than for organometallic catalyst [61]. Further, enzymes usually present excellent selectivity, being able to catalyse the synthesis of specific isomers or enantiomers in high purities. Also the big variety of enzymes and the possibility of modifying them in order to increase the available reactions makes possible to have an enzyme performing virtually every possible reaction that can be done chemically.

- Mild reaction conditions: even though the new protein engineering techniques allows the modification of enzymes to adopt to non-physiological reaction conditions, the classic enzyme catalysis is conducted in aqueous buffer at neutral pH, with mild temperature and without organic solvents or heavy metals. Due to this characteristic, strong acidic or basic conditions or toxic compounds are reduced or completely eliminated, making biocatalysis an environmentally friendly alternative to organic chemical synthesis [62]. Also the lower temperature required compared to traditional chemical approaches decreases the amount of energy needed, making the process energetically cheaper and reducing CO₂ production [63]. This feature converts enzymes in one of the most promising actors in the green chemistry field [23, 64].
- Renewable and biodegradable: the production of enzymes is done by cultivation. Even though this process requires energy [65], no petrol derivate chemicals are usually employed, making the catalyst production potentially entirely renewable. Also the enzymes can be easily biodegraded, reducing costs of waste treatment and its environmental impact.

Despite the mentioned advantages, biocatalysis has still some drawbacks which are hindering its application in industrial processes:

- Low stability of the catalyst under process conditions: Chemical processes often run under conditions far from physiological values which can have a negative impact on the enzyme stability [13]. Since the cost of the catalyst is a big contributor to the overall process costs (especially when is used in pure form) [66] each gram of catalyst must be used as effectively and as long as possible. Due to this fact, often biocatalytic processes are performed under non-optimal conditions in order to avoid catalyst inactivation. Even though there are techniques to increase the stability of enzymes, like immobilization or protein engineering [7], they usually do not achieve the required values for being cost effective.

- Absence of appropriate catalysts and long development time: even though it is theoretically possible to catalyze practically any organic reaction by an enzyme, the catalyst discovery and development can require iterative engineering rounds which may significantly increase the time that the process needs to be market ready [16]. Due to the short life of patents in pharmaceutical industry, this can be a major hurdle for the application of biocatalysis in industrial scale [16]. One of the options to overcome this challenge is the creation of enzyme toolboxes consisting of collections of well characterized enzymes. Screening of such toolboxes for appropriate enzymes may significantly reduce the number of steps needed to identify the right catalyst.

- High water requirement: the production of protein via cultivation requires high amounts of water due to the inability of the bacteria for growing over certain cell densities. The amount of energy and the resources (glucose) used for fermentation makes this step the main contributor to the environmental impact of the biocatalyst production [65]. Also since the process is usually done with genetically modified organisms, the treatment of the waste water can prevent its reutilization [21].

- Competition with industrial chemistry: even if the biocatalytic alternative is cost effective and environmentally friendly, companies have to invest large amounts of money in order to reengineer their production processes. Due to this reason, biocatalysis has been limited to processes where no chemical alternative was feasible or where the chemical reaction was much more expensive e.g. due to the number of steps. Even though the adaptation of enzymes by protein- and reaction engineering to process requirements can overcome this problem in the future. Further, if new production sites are built, biocatalytic alternatives can be taken into account.
1.4. Biocatalytic process development

Once the enzyme for product synthesis has been found or engineered, a production process has to be developed. The main aim of this procedure is to design a robust production method which maximizes the product relative to the amount of catalyst needed. Every biocatalytic process consists of three steps (Figure 1), which can be radically different depending on the type of reaction and the catalyst limitations:

- Upstream processing: production of the catalyst.
- Reaction: substrates are converted into products by the action of the enzyme.
- Downstream: product recovery and purification and catalyst recovery (if possible).

![Diagram of biocatalytic process](http://en.wikipedia.org/wiki/File:Continuous_bach_reactor_CSTR.svg)

Figure 1: Blueprint of a generic biocatalytic production process. The complexity of the upstream part depends on the format of the catalyst: 1) Whole cell, 2) Crude extract, 3) Purified protein or 4) Optional: Immobilized catalyst. In the reaction, the catalyst converts the substrate(s) into product(s). The downstream part consists in the product recovery and its further purification via different techniques. Tank picture taken from Wikimedia Commons.

For each of the mentioned steps, several considerations have to be made. The optimization of each of the process parts plays a critical role in the overall production output, stressing the importance of process development.

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1 http://en.wikipedia.org/wiki/File:Continuous_bach_reactor_CSTR.svg
In some cases, steps can be integrated. These options can have a positive impact on the process complexity as well as solve some problems that are associated with certain reactions:

- **Fermentation**: the biotransformation is carried out in a stirred tank reactor by the growing cells, integrating upstream and reaction in one pot. This option eliminates the time and costs required for catalyst purification and its re-buffering. It can be used when the metabolism of the growing cell does not interfere with the reaction by substrate or product degradation or by-product formation and also when the substrate and the product are not inhibiting the cell growth. Even though the production by fermentation can be useful for process simplification, separation of upstream and reaction step enables to modify and control the catalyst and substrates concentrations more accurately [16]. Also, the reaction has to be compatible with the whole cell catalyst, as will be explained in the upstream section.

- **In situ** product removal: The product is removed from the reaction while it is still running [67]. This can be done by different methods, like adsorption on resins [68], pervaporization or extraction into a second organic phase. It is a useful alternative in those cases where the catalyst is inhibited by high concentrations of products or the equilibrium is not favored [67].

1.4.1. **Upstream**

The upstream part of the process involves the preparation of the catalyst, its recovery and modifications if required. In order to do this, the encoding gene has to be cloned, and an appropriate recombinant host has to express the protein. Expression optimization is a critical step in process development since it determines the catalyst availability. In order to increase the amount of active protein produced by the host, several parameters have to be optimized, like the concentration of the inducing agent (usually IPTG or lactose), the temperature or the cultivation media. Once this is done the catalyst can be used in three different formats:

- **Whole cell**: The whole cell is used as catalyst without any further treatment. This is considered to be the cheapest catalyst format [66, 69] since its recovery can be done by inexpensive methods such as centrifugation or filtration. But whole cell biocatalysis can have some drawbacks like side reactions by the cell proteins, interaction with the metabolism or mass transfer problems due to the cell membrane. The cells can be used as catalyst during fermentation integrating upstream and biotransformation steps (see above) or as “resting” cells. For the latter mode, cells are separated from the cultivation vessel and resuspended in an appropriate reaction medium for the reaction step, where the lack of nutrients leaves the cells metabolically inactive. This procedure has the advantage of avoiding interactions between the cell metabolism and the biocatalytic reaction. Another
advantage of whole cell catalysis is that no extra cofactors or the addition of extra enzymes for their regeneration is usually needed, making this catalyst format extremely useful for reactions where expensive cofactors are needed, (i.e. redox reactions or oxidations) [29, 70, 71]. Also, using metabolic engineering, multi-step synthesis can be carried out in whole cell, reducing dramatically the catalyst production costs [10].

- Crude extract: cells are disrupted before the reaction step. The destruction of the membrane eliminates possible mass transfer problems between the inside of the cell and the reaction media. Another possible advantage is that this method allows a better control of the enzyme environment since this is no longer operating inside the cell [72].

- Pure enzyme: the use of pure enzyme eliminates problems associated with the other proteins present in the cell as well as any mass transfer problems related to the membrane. The catalyst can be produced in an expression host or in a cell-free system [72, 73] and then purified by different methods. However, the use of pure enzyme is usually avoided in industrial applications due to the high costs for the purification step.

Another interesting option to be considered during upstream development is the immobilization of the catalyst [74]. This procedure is used to increases the stability and the recyclability of the catalyst and can be very useful for enzymes with short half-life in non-immobilized form [75]. The main drawbacks of this process are reduction of activity and the increment of catalyst costs [66].

1.4.2 Reaction

The reaction is the core of the biocatalytic process. In this step the substrates are converted into products by the biocatalyst. The reaction is usually carried in aqueous buffer but there are other options in non-conventional media [76]:

- Organic solvents: the reaction can be performed either in pure organic solvent, a biphasic system of buffer and solvent or in a monophasic mixture of some water soluble organic solvent and buffer. This option can be used in order to increase substrate or product solubility, reduce side reactions, in situ feed or in situ product removal or biocatalyst stabilization. Nevertheless, the use of organic solvents may alter the enzyme stability and its performance as well as has a negative impact on the environmental friendliness of the process, if the organic solvent cannot be fully recycled.

- Ionic liquids: ionic liquids are considered to be a green alternative to organic solvents because they have no vapor pressure. The advantages and drawbacks are the same as with organic solvents [77].
- Neat substrate systems: in some cases the reaction can be carried out in pure substrate. This option can be used when the substrate is liquid, is not toxic for the catalyst, and there is no substrate inhibition. The advantage of this reaction media is the highest product concentrations that can be achieved [78, 79].

- Supercritical fluids: the use of supercritical fluids such as CO₂ is considered to be a green alternative to organic solvents. It increases the solubility and the diffusion of substrates and products and it can be converted into a non-toxic gas just by recovering atmospheric pressure and temperature [80].

- Gas phase: when the substrates are highly volatile, substrate saturated gas can be used as reaction media by flowing through the immobilized catalyst [81].

The performance of a biocatalytic reaction is influenced by several different factors which should be taken into account when a production process is being designed.

- Substrate concentration: This is one of the most critical parameters. If the production of high product concentrations is intended, the substrate present in a reaction is going to be the limiting factor. Therefore, addition of high substrate concentrations is necessary but there are some cases where this can be a problem: substrate inhibition and low substrate solubility. Usually, the enzymatic reaction speed increases with increased substrate concentration until the maximal velocity \( V_{\text{max}} \) is reached, but some enzymes are inhibited by certain substrates. Also, the substrate can produce an emulsion when concentrations over its solubility limit are reached. This would generate a second phase which deactivates many enzymes due to interphase toxicity.

- Temperature: the reaction speed is usually enhanced with increasing temperature, until thermal denaturation overcomes activation. Due to these contradictory trends, the optimal temperature should be chosen in order to maximize the productivity and to minimize denaturation taking into account the catalyst activity during the applied reaction time.

- pH: Another crucial parameter is the pH-value. Every enzymatic reaction has an optimal pH value where it runs at maximal speed. Also the pH can affect the biocatalyst stability and in some cases it is involved in background reactions. Further, the pH-value can also be important in low-water reaction systems. Due to the pH-memory effect an enzyme “remembers” the last pH it “saw”, e.g. in the last purification step before lyophilization. Using this effect, the activity of enzymes may be tuned in non-conventional media.

- Buffer salt: In order to keep a constant pH, an appropriate buffer salt has to be chosen. Some of these compounds can have a negative effect on the catalyst activity or its stability, so a buffer screening is usually one of the first steps in catalyst characterization. Also the temperature at which the reaction is going to take place should be considered since changes in the temperature can produce variations in the pH depending on the used buffer salt. In some cases, buffers can also be involved in background reactions [82]. Another aspect which has to be taken into account is the
price of the salt, especially in industrial processes since it can be a big contributor to the final product price.

- Ionic strength: the salt concentration (mainly buffer) may have a strong effect on the catalyst solubility and has to be in a range which allows an optimal catalyst load.

- Enzyme concentration: the importance of this parameter is more economical than scientific, since the catalyst cost is one of the main contributors to the final product costs [66]. Therefore, the enzyme concentration should be kept as low as possible meanwhile keeping the productivity over certain limits.

The biocatalytic reaction takes place in a reaction vessel and can be run in different modes of operation depending on the reaction specifications. There are three types of reaction modes (Figure 2):

- Batch reactor: the substrates are placed in the tank together with the catalyst. The reaction is run until it stops either due to full conversion, reached equilibrium or catalyst inactivation. This is the simplest operation mode.

- Fed batch reactor: once the reaction starts, substrate is constantly fed over time. Under ideal conditions, the substrate concentration should be kept constant while the product concentration continuously rises. This operation mode can be used in order to keep the reaction equilibrium favorable, to increase the final product concentration, to overcome low substrate solubility or to avoid substrate inhibition [21, 83].

- Continuous reactor: during the reaction substrate is continuously fed and the product is removed without stopping the reaction [84, 85]. Under ideal conditions, the concentrations of substrate and product should be constant over time. This mode has the same advantages as the fed batch but also eliminates the possible product inhibition or equilibrium problems due to its accumulation [86].

Figure 2: Reactor operation modes and their respective reaction profile. Tank picture taken from Wikimedia Commons.
Depending on the reaction type, the reaction media, the biocatalyst format and the chosen mode of operation, the reaction can be carried out in different types of reactors. Even though there are many different reactor types, they are usually variation of only two principles, which will be discussed on the examples of the stirred tank reactor and the plug flow reactor (Figure 3):

- **Stirred tank reactor (STR):** this reactor consists of a vessel of variable size equipped with a stirrer to provide homogeneous mixing in the tank. The catalysts stability may be reduced due to shear stress as well as gas-liquid or liquid-liquid interphases in case of a biphasic system. The STR can be operated in different modes depending on the reaction as it has been explained in the previous section.

- **Plug flow reactor:** the catalyst is immobilized in a reaction chamber and the substrate is pumped through. Due to this, the reaction is progressing along the length of the reactor with spatially constant substrate and product concentrations. This reactor type can be used when there is a strong substrate or product inhibition or when the catalyst is highly sensitive to shear stress.

![Figure 3: Reaction profiles of the two main reactor types: the stirred tank reactor (STR) operated in batch mode and the plug flow reactor (PFR). Tank picture taken from Wikimedia Commons.](image)

### 1.4.3. Downstream processing

Once the reaction step is completed, the product has to be recovered. This step of the process is usually not so problematic since several scalable technologies from traditional chemical syntheses can be used [69]:

- **Filtration:** filtration through a membrane it is an easy method for separation of particles of different size which can be used for product recovery. Depending on the
pore size of the membrane, it can be used either for separating solid product (which has precipitated or crystallized) or for separation of the catalyst from the reaction media. This technique is also an interesting option for in situ product removal in a continuously operated tank. If the product is extracted by a concentration gradient without applying extra pressure on the membrane, the process is called dialysis. This method can be enhanced by the use of an electric field (electrodialysis), which is a useful method for separation of charged molecules like lactic acid [87].

- Extraction: the most widespread method for product recovery in biocatalysis is the liquid-liquid extraction of the aqueous reaction system with an organic solvent that is not water miscible. Even though this is a cost-efficient and effective method, it may decreases the environmental friendliness of the production process, if the organic solvent cannot be fully recycled and if waste water is contaminated. Subsequently, the products must further be purified either using chromatography (see below) or by distillation or crystallization.

- Adsorption: the product can be absorbed onto a resin or activated carbon. Different types of resins can have affinity for different types of product, like ions or hydrophobic compounds.

- Chromatography: chromatography includes an extend variety of separation methods which can be used for separation of practically all types of molecules. It can be used for product recovery or for product purification after an extraction step.

1.4.4. Process feasibility analysis:

As it was stated previously, an efficient biocatalytic process is generally more environmental friendly than the competing chemical production but the production costs are usually higher. In order to evaluate the economic viability of a process, several critical parameters which can have a strong impact on the final product costs have to be carefully analyzed:

- Catalyst availability: catalyst costs are one of the main contributors to the final product costs in a biocatalytic process. Therefore, the production of active catalyst has to be as efficient as possible, taking into account the amount of biocatalyst obtained per gram of cell as well as the specific activity. In order to do this, characteristics like host, expression vector, growth, and induction method have to be optimized. Another critical aspect is the recyclability of the catalyst and its stability. In order to improve these parameters, immobilization can be an interesting option. In any case, this method increases the catalyst costs, so it has to be planned carefully [66].

- Gram of product per gram of catalyst: due to the high costs of the catalyst, the amount of product obtained per gram of enzyme has to be maximized in order to keep the process cost low. This value varies by orders of magnitude depending on the catalyst format, due to the difference in upstream costs. Apart of achieving high
yields, in order to increase the product obtained per gram of catalyst, the recyclability of the catalyst is critical [88].

- Specific space time yield (SSTY): defined as the amount of product that can be obtained per mg catalyst in a period of time. The SSTY is one of the most critical parameters of the process. In order to increase it, the reaction conditions have to be optimized.

- Final product concentration: in order to keep the downstream process cost effective, the final concentration of the product is critical. Low product concentration can be due to unfavorable reaction equilibrium, product inhibition or low substrate solubility. The different strategies to overcome these problems are listed in Table 2.

- Conversion: in order to keep high atom economy, this parameter should be as high as possible. Conversion can also have a high impact in the downstream processing cost, since low conversion means that the product has to be separated from the substrate.

- Stereoselectivity: as it was stated in Chapter 1.3, one of the main advantages of biocatalysis is the possibility to produce chiral compounds. This is of capital importance in pharmaceutical industry since the FDA and other regulatory organizations require any impurities, such as an unwanted enantiomer, to be below 1%. This means that without any other contaminant present, the concentration of the desired stereoisomer of a drug should be over 98%.

Table 2: Possible solutions for different causes of low product concentration

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low substrate solubility</td>
<td>Use of a cosolvent</td>
</tr>
<tr>
<td></td>
<td>Emulsion of substrate</td>
</tr>
<tr>
<td></td>
<td>Fed-batch</td>
</tr>
<tr>
<td>Substrate inhibition</td>
<td>Enzyme engineering</td>
</tr>
<tr>
<td></td>
<td>Fed-batch, CSTR</td>
</tr>
<tr>
<td>Product inhibition</td>
<td>Enzyme engineering</td>
</tr>
<tr>
<td></td>
<td><em>In situ</em> product removal</td>
</tr>
<tr>
<td>Unfavorable equilibrium</td>
<td>Increase substrate concentration</td>
</tr>
<tr>
<td></td>
<td>Product/by-product <em>In situ</em> removal</td>
</tr>
<tr>
<td></td>
<td>Temperature variation</td>
</tr>
</tbody>
</table>

All the process parameters are depending on one factor: the market price of the product. Low prized bulk chemicals would require outstanding catalyst recyclability, enormous SSTY, and specially high product concentrations. Due to these facts industrial biocatalysis has been focusing on pharmaceuticals, fine chemicals or products which are unavailable by traditional chemical synthesis or where production was not economically feasible by chemical means. Nowadays, with the improvement of protein engineering making enzymes more active, more selective and especially more resistant towards reaction conditions, the production costs are decreasing and biocatalysis is becoming each day a more competitive technology.

1.5. α-Hydroxy ketones
α-Hydroxy ketones, like acyloins or benzoins, are a family of molecules which present an alcohol group adjacent to a ketone (Figure 4).

![Figure 4: General formula of an α-hydroxy ketone.](image)

This type of compounds can be found in several pharmaceuticals like urease inhibitors [89], Alzheimer’s medication [90] or antifungals [91] and they are also precursors of other important building blocks such as amino alcohols [92] or diols [93] (Figure 5).

![Figure 5: Examples of compounds derived from α-hydroxy ketones or derivates. From Hoyos et al. 2010 [94].](image)
1.5.1. Chemical production

Due to the chiral nature of the OH group, the production of enantiomerically pure α-hydroxy ketones can be challenging but there are still several ways of producing these compounds by traditional chemical synthesis:

- **α-Hydroxylation**: by using a chiral oxidant, a ketone can be hydroxylated producing an α-hydroxy ketone [95].
- **Ketohydroxylation**: an olefin is oxidized by a strong oxidant yielding an α-hydroxy ketone [96].
- **Asymmetric oxidation/reduction**: enol ethers [97] or enolates, diols [98] or diketones can be used as substrates for asymmetric oxidation/reduction using a chiral oxidant.
- **Oxidative kinetic resolution**: racemic α-hydroxy ketones can be resolved by oxidation using a pro-chiral catalyst and molecular oxygen [99, 100].
- **Asymmetric condensation**: the acyloin condensation of two aldehydes has been known for more than a century [101]. Nowadays, this reaction is conducted in a biomimetic way by using thiazolium salts as catalysts [102, 103].

Even though there are some examples of successful chemical synthesis of α-hydroxy ketones, usually multiple reaction steps, low yields and especially low chemo- and enantioselectivity are very common drawbacks of these production methods [104]. The use of a biocatalytic approach can overcome these hurdles.

1.5.2. Biocatalytic production

Apart from the described chemical synthetic routes, a biocatalytic approach is possible [94]. There are mainly three enzymatic methods available:

- **Redox reactions**: enantiomerically pure α-hydroxy ketone can be produced either by oxidation of α-diols or by reduction of α-diketones. Since redox reactions require the regeneration of expensive cofactors, this processes can be best performed using either whole cell catalysis or multi-enzymatic systems with the addition of extra ketones, alcohols or sugars in order to regenerate the cofactors.
- **Dynamic kinetic resolution using hydrolases**: another option is the use of kinetic resolution of racemic α-hydroxy ketones. This step can be easily catalyzed by a lipase, but it has the disadvantage of having a maximum yield of 50 % of the starting compound. In order to overcome this limitation, racemization of the remaining substrate can be performed either enzymatically or chemically.
- **Carboligation of aldehydes or α-ketoacids using thiamine diphosphate (ThDP) dependent enzymes**: as it will be further discussed in the following section, ThDP-dependent enzymes are able to produce enantiomerically pure α-hydroxy ketones by carboligation of two inexpensive aldehydes or the corresponding α-ketoacids. This option combines cost-effective substrates with the theoretical yield of 100 % [105]
1.6. ThDP dependent enzymes

ThDP-dependent enzymes are a very well-known family of enzymes which use the ThDP (the biologically active form of vitamin B₃) (Figure 6) as cofactor in order to catalyze a broad range of C-C, C-N, C-S, C-O ligase and cleavage reactions [106]. Due to their ligase activity, this family of enzymes has a strong potential for industrial application, therefore there has been an intense effort in understanding the 3D structure of several ThDP-dependent enzymes. The sequences of these proteins are compiled in the ThDP-dependent Enzyme Engineering Database (TEED) [107] and a numbering system for a clear identification of structurally equivalent amino acid residues in the enzymes has been proposed [108].

![ThDP structure](image)

**Figure 6: Ylide form of the ThDP.**

ThDP-dependent enzymes are able to catalyze several C-C-ligase reactions using aldehydes or ketones [106] as substrate like the Stetter like 1,4 addition of aldehydes [109], but one of the best described reactions is the C-C formation by a benzoin condensation-like reaction of two aldehydes yielding an α-hydroxy ketone [109-111]. The reaction mechanism is one of the best understood examples of umpolung chemistry (Figure 7). The charged ylide form of ThDP may nucleophilically attack an aldehyde forming a carbanion-enamine, which may subsequently reaction with a second aldehyde yielding the α-hydroxy ketone. Without any enzyme present, ThDP itself is able to catalyze these reactions but at extremely slow speed [112].
Due to the specificity of these enzymes and the diversity of substrate acceptance [105, 113-115], during the last decade the group of Prof. Martina Pohl has compiled an enzymatic "toolbox" of ThDP-dependent enzymes, which can be used for production of many different \( \alpha \)-hydroxy ketones with high chemo- and stereoselectivity [106, 116, 117]. With the addition of new wildtype enzymes and variants the palette of products which are possible to access is expanding. An interesting example is the carboligation of acetaldehyde and benzaldehyde (Figure 8). This reaction can in principle yield four different \( \alpha \)-hydroxy ketones, each of them as enantiomers, but within the "toolbox" there are different enzymes which will yield only one enantiomer specifically.
Table:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R)-Acetoin</td>
<td>EcSucA [118]</td>
</tr>
<tr>
<td>(S)-Acetoin</td>
<td>ApPDCE469G [55]</td>
</tr>
<tr>
<td>(R)-PAC</td>
<td>EcAHAS [119]</td>
</tr>
<tr>
<td>(S)-PAC</td>
<td>ApPDCE469G [55]</td>
</tr>
<tr>
<td>(R)-HPP</td>
<td>PbBAL [120]</td>
</tr>
<tr>
<td>(S)-HPP</td>
<td>PbBFD [121, 122], PbBFDL461A [123]</td>
</tr>
<tr>
<td>(R)-Benzoin</td>
<td>PbBAL [113, 124]</td>
</tr>
<tr>
<td>(S)-Benzoin</td>
<td>Under development</td>
</tr>
</tbody>
</table>

Figure 8: Possible carboligation products of benzaldehyde and acetaldehyde. The different enzymes in the toolbox can be used for the production of a specific enantiomer. Extracted from Hailes et al. 2009 [125]. EcSucA: subunit of α-ketoglutarate dehydrogenase from *Escherichia coli*, ZmPDC pyruvate decarboxylase from *Zymomonas mobilis*; PbBAL: benzaldehyde lyase from *Pseudomonas fluorescens*, ApPDC pyruvate decarboxylase from *Acetobacter pasteurianus*, PbBFD: benzoyl formiate decarboxylase from *Pseudomonas putida*.

1.6.1. Chemoselectivity

As it has been stated above, ThDP-dependent enzymes show a pronounced chemoselectivity. This characteristic is controlled by the binding order of substrates. The first substrate binding to the ThDP will donate the ketone part to the product, while the hydroxy group is introduced by the second substrate. The binding order is determined by the topography of the active site. As was explained before, the carboligation of benzaldehyde and acetaldehyde can yield four different products but when both substrates are used in
presence of ApPDC, the products would be only phenylacetylcarbinol (PAC) and acetoin since the active site of the this enzyme is too small for fitting the benzaldehyde in the donor position. On the other hand, BAL presents a larger donor binding site and when this enzyme is used, benzaldehyde is the preferred donor, yielding 2-hydroxypropiophenone (HPP) and benzoin (Figure 9).

Figure 9: Explanation of the chemoselectivity in ThDP-dependent enzymes. The binding order of the substrates to the ThDP will determine the final product. In this example, the carboligation of acetaldehyde (square) and benzaldehyde (hexagon) can yield two different products: HPP or PAC.

1.6.2. Stereoselectivity

Also the stereoselectivity of ThDP-dependent enzymes relies on a very well understood mechanism: when both substrates are reacting in a parallel orientation, the product will be (R)-configured. In case that the substrates are aligned in an antiparallel orientation, the product will have (S)-configuration (Figure 10). Due to the topology of the active site of enzymes in this family, the main part of wildtype ThDP-dependent decarboxylases are (R)-selective. The only known exception so far is the benzoylformate decarboxylase (BFD) from Pseudomonas putida which catalyzes the formation of (S)-HPP with an ee of 92 % [123]. This particular enzyme presents a morphological unit called “S-pocket” which allows the stabilization of the aromatic substrate in an antiparallel orientation relative to the aliphatic one. This “S-pocket” morphology is also present in other enzymes of this family, but is mostly blocked by some amino acid residues. Nevertheless, by mutating these amino acids it is possible to open this “S-pocket” inverting the stereoselectivity of the enzyme with different degrees of success. A positive example of this possibility is the rational design of the ApPDCE469G.
Figure 10: Explanation of the mechanism behind stereoselectivity determination of the ThDP-dependent enzyme ApPDC. When both substrates react in a parallel orientation, the product is \((R)\)-configured. This reaction occurs in wildtype ApPDC (A). In ApPDCE469G (B), the mutation opens the so-called “S-pocket” allowing benzaldehyde to react in an antiparallel orientation, yielding an \((S)\)-configured product. Adapted from Rother et al. [55].

1.7. ApPDCE469G

The PDC is a member of the decarboxylases family, the largest of the five ThDP-dependent enzyme families [126]. As many ThDP-dependent enzymes, PDC is a tetrameric protein, with four active sites which are located in the binding area between two monomers (Figure 11).
In *Acetobacter pasteurianus* PDC plays a central role for the oxidative metabolism. Its physiological function is the decarboxylation of pyruvate to acetaldehyde in the aerobic metabolism of lactate (Figure 12)[127], whereas in other organisms the PDC is usually involved in ethanol synthesis.

![3D structure of ApPDC. From RCSB database. PDB code: 2BVI.](image)

Figure 11: 3D structure of ApPDC. From RCSB database. PDB code: 2BVI.

![Proposed role of PDC in the oxidative metabolism of Acetobacter sp.](image)

Figure 12: Proposed role of PDC in the oxidative metabolism of *Acetobacter sp.* L/D –lactate is oxidized by L- and D- lactate dehydrogenases (LDH) to pyruvate which is further decarboxylated by the ApPDC (red box) into acetaldehyde. This acetaldehyde is further oxidized to acetate by different aldehyde dehydrogenases (ALD). Refined from Raj et al. 2001 [127].
The decarboxylation mechanism of this enzyme is analogous to the one published by Tittman et al. [128] for PDC of Zymomonas mobilis: the pyruvate is bound to the ThDP forming lactyl-ThDP (L-ThDP) which is transformed to the carbanion/enamine 2-hydroxyethyl-ThDP (HEThDP-) by decarboxylation. A protonation step generates the conjugated acid HEThDP and finally the acetaldehyde is released regenerating ThDP (Figure 13). Not physiologically, this enzyme is known to catalyze the carboligation of acetaldehyde and benzaldehyde yielding (R)-PAC with an ee of 93 % [55] following the reaction schemes presented in Figures 7 and 13.

![Reaction mechanism of ApPDC](image)

Figure 13: Reaction mechanism of ApPDC. The enzyme is able to catalyze two kinds of reactions: Decarboxylation of an \( \alpha \)-ketoacid (i.e. pyruvate) (left) and the carboligation of two aldehydes (i.e. acetaldehyde and benzaldehyde) (right). The protonation step of the carbanion enamine into the conjugated acid prior to acetaldehyde release is conducted by glutamate 469. Adapted from Meyer et al. [129]

Interestingly, this enzyme presents a large “S-pocket”, which is blocked by a glutamate residue located in position 469. By exchanging this amino acid by glycine, the “S-pocket” can be opened, allowing the benzaldehyde molecule to approach in an antiparallel orientation with respect to ThDP-bound acetaldehyde, producing (S)-configurated PAC with an ee of 89 % [55]. Additionally, glutamate 469 plays an important role for the decarboxylation reaction. In the wildtype enzyme this residue catalyzes the protonation of the carbanion-enamine intermediate prior to the release of acetaldehyde [130]. Since this amino acid is not present in the variant, this step is not so favorable, decreasing considerably the decarboxylase activity of the variant relative to the wildtype. However, the carbanion-enamine is able to react with another aldehyde such as benzaldehyde, which produces the carboligation step yielding the desired (S)-PAC product.
1.8. Aim of the project

Even though the palette of α-hydroxy ketones, which is possible to produce, is increasing due to the enlargement of the ThDP-dependent enzymes in the toolbox, there are certain products which are still challenging to produce. One of the best examples is the production of the (S)-enantiomers of the already mentioned PAC and its derivates which are not accessible by chemical approaches. Also the biocatalytic production presents challenges: When one of the substrates for the carboligation has an aromatic ring, most of the described wildtype enzymes from the structural family of decarboxylases are (R)-selective, making the production of aromatic (S)-configured α-hydroxy ketones more difficult. The ApPDCE469G variant described before is the only designed enzyme so far being able to catalyze the formation of (S)-PAC derivates by carboligation of benzaldehyde and an aliphatic aldehyde (Figure 14). The production of these specific compounds is of special interest since (R)-PAC is currently used as precursor for several pharmaceuticals such as ephedrine, pseudoephedrine or norephedrine [131]. The access to the (S)-configured PAC would lead to the production of the complementary enantiomers which can have distinct physiological effects as the ones currently used.

![Conversion: 2 % ee: 61 % (S)](conversion2.png)

![Conversion: 33 % ee: 89 % (S)](conversion33.png)

![Conversion: 20 % ee: 85 % (S)](conversion20.png)

Figure 14: Initial results for the carboligation of different aliphatic aldehydes with benzaldehyde catalyzed by the ApPDCE469G under standard conditions [55].

By design of the ApPDCE469G variant, a first step in the direction of (S)-selective carboligation of an aromatic and an aliphatic aldehyde yielding PAC derivates was made, but yield and enantiomeric excess of this reaction were far from optimal for technical application. Therefore, the aim of this project was to develop a feasible production process for (S)-PAC derivates using the ApPDC variant in order to produce as much product as possible with the highest isomeric and enantiomeric purity. The model reaction used for the process development was decided to be phenyl propionyl carbinol (PPC), which can be produced by the carboligation of benzaldehyde and propanal. This compound was selected since the initial screening revealed best conversion and ee were the highest in the series tested so far (Figure 14).
The described ApPDCE469G variant is considered to be the best catalyst currently available for this reaction. Due to this, further protein modification was considered to be out of the scope of this work which will be focused on the production process development. Instead, a biocatalytic production process of (S)-PPC was developed and optimized.

Even though the use of pure enzymes is the most widespread in academia, industry tends more to the use of whole cell catalysis due to easier production and lower costs. Therefore, both approaches, biotransformation with purified enzyme as well as whole cell catalysis should be analyzed and advantages and drawbacks of them compared.

In order to obtain maximal specific space time yields with the highest degree of purity, the reaction conditions should be optimal. Optimization should include all factors which affect the outcome of the reaction: the concentrations of catalyst, substrate(s), product(s), as well as the reaction medium including all reaction conditions. Therefore the following aspects of reaction optimization as well as process designs had to be in focus to reach the aim of gaining a technically useful process with high productivity and excellent stereoselectivity:

- ApPDCE469G is able to catalyze the carboligation reaction by using either two aldehydes or an aldehyde and an α-ketoacid which is decarboxylated prior to C-C bond formation. Due to this characteristic of the enzyme, propanal or the corresponding α-ketoacid, α-ketobutyric acid (KBA) can be used as donor for the carboligation with benzaldehyde. The use of both donors should be compared in terms of reaction kinetics, product yield, enantiomeric excess and by-product production.
- The use of organic cosolvents is a very common to increase the solubility of hardly water-soluble substrates. One of the most common solvents used to increase benzaldehyde solubility is DMSO [132], but Gerhards et al. determined that the addition of this compound can decrease both productivity and ee of the product when the ApPDCE469G is catalyzing a carboligation reaction between acetaldehyde and benzaldehyde. Since the reaction studied in this recent work was different, the use of DMSO had also to be assessed.
- Several parameters involved in reaction performance (temperature, pH, substrate concentration, and enzyme concentration) should be optimized in order to achieve the highest possible productivity and enantiomeric purity. Here classical reaction optimization processes should be compared to iterative processes.
- Once the reaction is optimized the possibility of an scale-up should be assessed. In order to do this, several parameters like the reactor type and operation mode have to be carefully evaluated. Concerning the downstream step, in order to recover and purify the product, an easy laboratory scale method should be developed.
Once the process is outlined a feasibility analysis should be performed in order to assess its potential industrial production process. Additionally, this analysis could also help to identify possible bottlenecks were future research efforts should focus.

Finally, the production of different (S)-hydroxy ketones should be evaluated in order to assess how transferable the process is and which key experiments should be done in order to adapt it to the production of different compounds.

2. Materials and methods

2.1. Chemicals

All chemicals, except propioin, were purchased from Sigma Aldrich in the maximum grade of purity available. Propioin was purchased from TCI Europe.

2.2. Cost calculation

In order to calculate the economic costs of certain parts of the process, the prices used as reference were the ones given by Sigma Aldrich in the highest purity available. In order to have a closer value to the bulk product price, the price for the largest trading unit provided was chosen. The prices of all the compounds were annotated at the same day (16th of August 2013) in order to avoid price fluctuations.

Table 3: Prices of the different compounds from the Sigma Aldrich catalogue in August 2013. All prices relate to the largest trade unit provided by this company in the highest degree of purity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
<th>Price (€)</th>
<th>Product code</th>
</tr>
</thead>
<tbody>
<tr>
<td>ThDP</td>
<td>100 g</td>
<td>540</td>
<td>C8754-100G</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>1 kg</td>
<td>140</td>
<td>63138-1KG</td>
</tr>
<tr>
<td>KBA</td>
<td>25 g</td>
<td>82.7</td>
<td>K401-25G</td>
</tr>
<tr>
<td>Propanal</td>
<td>20 kg</td>
<td>274</td>
<td>W292303-20KG-K</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>500 mL</td>
<td>131</td>
<td>418099-500ML</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>2.5 kg</td>
<td>324</td>
<td>P3786-2.5KG</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1 kg</td>
<td>112</td>
<td>P5655-1KG</td>
</tr>
<tr>
<td>DMSO</td>
<td>1 L</td>
<td>350</td>
<td>D8418-1L</td>
</tr>
<tr>
<td>Na PYR</td>
<td>500 g</td>
<td>375</td>
<td>P2256-500G</td>
</tr>
</tbody>
</table>

The price of the catalyst was assumed to be 100 €/kg in case of whole cell and 1000 €/kg for purified enzyme based on the values described by Tufvesson et al. [66].
2.3. Catalyst preparation

2.3.1. Protein production

The ApPDCE469G gen was cloned into a pET22b (Novagen) (EMD Milipore, USA) vector and transformed into *Escherichia coli* BL21 (DE3) (Novagen) (EMD Milipore, USA) as described elsewhere [55]. Bacteria were cultivated in auto induction (AI) medium (described in Table 4) over 48 hours. Cells were harvested by centrifugation and the supernatant was removed. The resulting pellet was frozen at -20°C. These frozen cells were used as catalyst in whole cell experiments.

Table 4: Components of the auto induction method used for cell production.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium Phosphate (PK) buffer at pH 7</td>
<td>100 mM</td>
</tr>
<tr>
<td>Peptone</td>
<td>12 g/L</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>24 g/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.5 g/L</td>
</tr>
<tr>
<td>Lactose</td>
<td>2 g/L</td>
</tr>
<tr>
<td>Glycerin</td>
<td>5 ml/L</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>1 ml/L</td>
</tr>
</tbody>
</table>

2.3.2. Protein purification and storage

In order to produce the enzyme, 30 grams of frozen cells produced as explained in Chapter 2.3.1, were resuspended in 100 mL of 50 mM potassium phosphate (PK) buffer with 2.5 mM ThDP and 2.5 mM MgSO₄. Afterwards, 1 mg/mL of lysozyme was added and the suspension was incubated for 30 min in an ice bath with slow magnetic stirring. Cells were then disrupted using 4 rounds of ultrasonification of 5 min each with an amplitude of 70 % and a cycle of 0.5 seconds. The crude extract was centrifuged at 20000 rpm for 45 min at 4°C and filtered using Whatman™ filters (GE Healthcare, UK) of 0.45 µm pore size and injected on a 18 mL Ni-NTA superflo 100 mL column (Quiagen, Germany) in an Åkta purifier (GE healthcare, USA). After a washing step using 50 mM PK buffer with 2.5 mM ThDP and 2.5 mM MgSO₄ buffer containing 50 mM imidazole, the protein was eluted with 50 mM PK buffer with 2.5 mM ThDP and 2.5 mM MgSO₄ buffer with 250 mM imidazole. The protein fraction was desalted using an Amicon® cell (EMD Milipore, USA) with a 120 KDa membrane and the resulting solution was lyophilized. The lyophilized powder was stored at -20°C.

Protein purity was determined by SDS-PAGE using a NuPAGE® Tris-Acetate Mini Gels (Life Technologies, USA) with the protocol established by the provider.
2.4. Analytic setup

2.4.1. HPLC

Product concentration was determined by HPLC analysis using a Hewlett Packard 1100 series (Hewlett Packard, USA) device with an ID column provided by Chiral Technologies Europe (France). The measurements were done by taking 50 µL of reaction media which were diluted 1:10 in acetonitrile in order to stop the reaction. This step was producing also enzyme precipitation and the samples were centrifuged at 14000 rpm in an Eppendorf centrifugue during 3 minutes. Then the upper 100 µL of the mixture was taken into an HPLC vial with a micro insert and analyzed by HPLC. The method consisted of an isocratic elution with 35% acetonitrile and 65% deionized water with a flow of 1 mL/min for 20 minutes. The method was calibrated for an injection volume of 20 µL.

Since this column enables the determination of product concentration and enantiomeric excess, it was possible to substitute the previous analytic method consisting of a measurement with a Hibar 250-4 Lichrospher 100 rp-8 (5 µm) (Merk Milipore, USA) column in reverse phase for concentration determination and a second measurement using a chiral OD column provided by Chiral Technologies Europe (France) for determination of enantiomeric excess. Also, the Hibar column was not able to produce complete peak separation when whole cell catalysis was used, as it is described in the Results chapter.

The use of 4-hydroxy acetophenone as injection standard was initially assessed but due to the accuracy of the HPLC injection system which become obvious after performing several experiments, it was considered as unnecessary.

Table 5: Retention times of the different compounds involved in the reaction using the described HPLC method.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S)-PPC</td>
<td>7.4</td>
</tr>
<tr>
<td>(R)-PPC</td>
<td>9.6</td>
</tr>
<tr>
<td>(S)-PAC</td>
<td>5.5</td>
</tr>
<tr>
<td>(R)-PAC</td>
<td>6.7</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>8.0</td>
</tr>
</tbody>
</table>

2.4.2. GC

Detection of the aliphatic by-product propioin was done by a 6890 N Network GC system (Agilent technologies, USA). The product was extracted with ethyl acetate (200 µL solvent per 100 µL sample) and injected in a Lipodex E column. The method started at 80°C for 5 min, raised up to 180°C in one minute and kept that temperature for 3 min. Retention time for propioin: (S)-propioin 2.9 min, (R)-propioin 3.2 min.
2.4.3. Protein concentration determination

The protein concentration was determined by the Bradford test [133] using BSA as standard in the main part of the experiments. In the test related to chiral polishing, the protein concentration was determined with a Direct detect device (Merck Milipore, USA).

2.5. Production of reference compounds

Some of the α-hydroxy ketones studied in this work are not commercially available and they were synthetized in the laboratory.

\((S)\)-PAC was produced enzymatically using \(ApPDCE469G\) by carboligation of benzaldehyde and pyruvate. 50 mM benzaldehyde and 400 mM pyruvate were added in a 50 mL shake flask with variable amounts of enzyme (usually 0.1 mg/mL) in 50 mM PK buffer at pH 7 with 0.1 mM of ThDP and 2.5 mM MgSO\(_4\). The reaction was monitored by HPLC and it was run until the conversion exceeded 80 %. Then extra benzaldehyde was added up to a 50 mM concentration. Once the reaction stopped, the product was extracted three times with ethyl acetate, dried using anhydrous NaSO\(_4\) and concentrated using a rotatory evaporator. Afterwards, the product was purified by flash chromatography using silica gel 60 (Roth, Germany) as stationary phase in variable volume depending on the amount of product to purify. The mobile phase consisted of 80 vol% petrol ether and 20 vol% ethyl acetate. After this step, the product was concentrated one more time with the rotatory evaporator. The pure compound was stored under argon at 4 °C. The purity determination was done by \(^1\)H-NMR [55].

\((S)\)-PPC was also enzymatically produced using the same procedure as described for \((S)\)-PAC but with α-ketobutyrate (KBA) instead of pyruvate. The product was extracted 3 times with ethyl acetate, dried using anhydrous NaSO\(_4\) and concentrated using a rotatory evaporator. Afterwards, the product was purified by flash chromatography with a mobile phase consisting in 85 vol% petrol ether and 15 vol% ethyl acetate and concentrated one more time. The pure compound was stored under argon at 4 °C.

2.6. Protein expression optimization

In order to maximize the protein amount obtained by cell cultivation, different induction strategies were tested. The cells were produced using autoinduction medium or Luria-Bertani broth (LB) with different amounts of IPTG (0.5 or 1 mM) which was added once the culture was over 0.6 OD, temperatures (20 or 30 °C) and cultivation times (24 or 48 h). After harvesting, the different cultures were diluted to an OD of 1 in a volume of 40 mL in order to obtain the same amount of cells in each sample. The cells were centrifuged and the supernatant was removed.
Finally, the amount of protein was measured by a carboligation test: the cells were re-buffered in 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO₄, with 15 mM of benzaldehyde and 150 mM of KBA. After 6 hours of incubation at 30°C the amount of PPC was measured by HPLC.

2.7. Standard experimental reaction conditions

Unless otherwise specified, all tests were performed in 1.5 mL glass vials with a reaction volume of 1 mL containing 50 mM PK buffer, pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO₄ and 0.1 mg/mL of enzyme. The incubation conditions were 30°C and 1000 rpm in a Thermomixer comfort shaker (Eppendorf, Germany).

2.7.1. Substrate characterization

The reaction was run at standard conditions with benzaldehyde concentrations between 10 and 20 mM. Each vial contained either propanal or KBA in a concentration between 40 and 100 mM. The reaction was incubated during 24 hours and measurements with HPLC and GC were done at times 0, 8 and 24 h. All tests were done in duplicate.

2.7.2. Reaction kinetics

In order to determine the kinetic parameters of the carboligation using KBA and benzaldehyde as substrates, two different experiments were done: First, the reaction was run at a fixed concentration of benzaldehyde (30 mM) and varying KBA concentrations between 10 and 300 mM. Second, the reaction was run at a fixed concentration of KBA (300 mM) and varying benzaldehyde concentrations between 4.5 and 45 mM. In both cases, the reaction was run in 50 mM PK buffer, pH 7, 0.1 mM ThDP, 2.5 mM of MgSO₄ and 0.05 mg/mL of enzyme over 30 min at 30°C and 500 rpm. Measurements were performed at times 0, 5, 10, 15, 20 and 30 min and the samples were analyzed by HPLC with the method explained in Chapter 2.4.1. From these data, the initial reaction rate was calculated, plotted and kinetic parameters were calculated using Origin. The experiments were done in triplicate.

2.7.3. Effect of DMSO

The reaction was run with different concentrations of DMSO from 0 % (buffer control) to 30 % v/v. The reaction took place in 50 mM PK buffer, pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO₄, 150 mM of KBA, 30 mM of benzaldehyde and 0.1 mg/mL of enzyme at 30°C and 500 rpm. Measurements were done at times 0 and 17 hours and samples were analyzed by HPLC and GC with the methods explained in Chapter 2.4.1. and 2.4.2. in order to determine
product and byproduct concentrations and their respective ee. Experiments were done in duplicate.

2.7.4. Reaction optimization

The reaction was run using a different temperatures, buffer salts, pH-values, enzyme- and substrate concentrations (Table 6). The reaction was run for 8 h at 500 rpm. Measurements were done at times 0, 4 and 8 hours and the samples were analyzed by HPLC and GC with the methods explained in Chapters 2.4.1. and 2.4.2. End point measurements were used for comparison between the different tested conditions. This reaction time was chosen in order to study the effects of the reaction conditions on the enzyme performance for a longer reaction interval, and not only under initial rate conditions.

Table 6: Variations of studied parameters for reaction optimisation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tested range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>30°C-42°C</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>6 to 42 mM</td>
</tr>
<tr>
<td>α-ketobutyric acid</td>
<td>10 to 300 mM</td>
</tr>
<tr>
<td>pH</td>
<td>4-8</td>
</tr>
<tr>
<td>Buffer</td>
<td>Potassium phosphate buffer (pH adjusted with KOH and H3PO4)</td>
</tr>
<tr>
<td></td>
<td>MES-buffer (pH adjusted with HCL and NaOH)</td>
</tr>
<tr>
<td>enzyme concentration</td>
<td>0.08 to 0.165 mg/mL</td>
</tr>
</tbody>
</table>

For the first round of experiments, the parameter values from literature [55] were used as a starting point and some variations were included in order to identify which parameter had the strongest impact on the specific space-time-yield (SSTY) and on the ee. The full description of the experimental condition can be found in the Annex. After running these experiments, results were analyzed in order to identify most promising conditions and a new set of experiments was designed. This iterative approach was repeated until no further improvement of SSTY or ee was achieved.

2.7.5. Whole cell biotransformations

Unless otherwise is specified, the reaction with whole cell were performed in 1.5 mL glass vials with a reaction volume of 1 mL. Reaction media: 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO4. The samples were incubated at 30 °C and 500 rpm in an Eppendorf shaker Thermomixer comfort shaker (Eppendorf, Germany).
2.7.5.1. Optimal cell load

The reaction was run in 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO\textsubscript{4} at 30°C and 500 rpm. Different amounts of wet cells were added as catalyst from 0 (negative control) to 100 mg/mL. The substrate concentration was 30 mM benzaldehyde and 150 mM KBA. The reaction was studied during 4 hours and samples were taken at times 0, 1, 2, 3 and 4 hours. Samples were analyzed by HPLC with the method explained in Chapter 2.4.1.

An experiment comparing wet cells and crude extract was done by running the reaction 50 mM PK buffer at pH 7 with 5 mM of MgSO\textsubscript{4}, 150 mM of KBA and 30 mM of benzaldehyde at 30°C and 500 rpm. 20 mg/mL of wet cell were used as catalyst but for one of the reaction vials the cells were previously disrupted by using 4 rounds of ultrasounds of 5 min each with amplitude of 70 % and a cycle of 0.5 seconds. This process lysates the cell producing crude extract which was used as catalyst (without further processing) in the second set of reactions. The reaction was studied in duplicate for 24 hours and measurements were taken at time 0, 0.5, 1, 1.5, 2 and 24 hours. Samples were analyzed by HPLC with the method explained in Chapter 2.4.1.

Comparison between wet cell and lyophilized cell was done by running the reaction in 50 mM PK buffer at pH 7 with 5 mM of MgSO\textsubscript{4}, 150 mM of KBA and 30 mM of benzaldehyde at 30°C and 500 rpm. Some frozen cells were lyophilized, which results in a reduction of weight of 75 %. Due to this, in order to have similar catalyst load, the reaction with wet cell was run with 10 mg/mL of catalyst and the reaction with lyophilized cells with a catalyst concentration of 2.5 mg/mL. The reaction was studied in duplicate during 18 hours and measurements were taken at time 0, 0.45, 1.5, 2.25 and 18 hours. Samples were analyzed by HPLC with the method explained in Chapter 2.4.1.

2.7.5.2. Influence of ThDP

The reaction was run with different amounts of ThDP between 0 mM (negative control) and 1 mM in 50 mM PK buffer at pH 7 with 5 mM of MgSO\textsubscript{4}, 150 mM of KBA and 30 mM of benzaldehyde at 30°C and 500 rpm with a cell concentration of 10 mg/mL of wet cells. The reaction was studied for 3 hours with measurements at time 0, 1, 2, and 3 hours. Data were analyzed by HPLC with the method explained in Chapter 2.4.1.

2.7.5.3. Optimization of the KBA concentration

The reaction was run at different KBA concentrations between 10 mM and 600 mM in 50 mM PK buffer at pH 7, with 2.5 mM of MgSO\textsubscript{4}, 0.1 mM ThDP, and 30 mM of benzaldehyde, 30°C and 500 rpm with a wet cell concentration of 10 mg/mL. The reaction was studied
during 20 hours with measurements at time 0, 1, 2, 3 and 20 hours. The data was analyzed by HPLC.

2.7.5.4. Optimization of the benzaldehyde concentration

The reaction was run at different benzaldehyde concentrations between 5 mM and 200 mM in 50 mM PK buffer at pH 7, with 2.5 mM of MgSO₄, 0.1 mM ThDP, and 300 mM of KBA, 30°C and 500 rpm with a cell concentration of 10 mg/mL of wet cell. The reaction was studied for 18 hours with measurements at time 0, 1, 2, 3 and 18 hours. Data were analyzed by HPLC.

2.7.5.5. Optimal pH

The reaction was run at different pH values between 6 and 8 in 50 mM PK buffer with 2.5 mM of MgSO₄, 0.1 mM ThDP, 150 mM of KBA and 30 mM of benzaldehyde at 30°C and 500 rpm with a cell concentration of 10 mg/mL of wet cell. The reaction was studied for 3 hours with measurements at time 0, 1, 2, and 3 hours. Data were analyzed by HPLC.

2.7.5.6. Optimal temperature

The reaction was run at different temperatures between 20°C to 40°C in 50 mM PK buffer at pH 7, with 2.5 mM of MgSO₄, 0.1 mM ThDP, 150 mM of KBA and 30 mM of benzaldehyde and 500 rpm with a cell concentration of 10 mg/mL of wet cell. The reaction was studied for 20 hours with measurements at time 0, 1, 2, 3 and 20 hours. Data were analyzed by HPLC.

2.7.6. Whole cell recyclability

The reaction was run with different substrate concentrations as specified in Table 7 in 50 mM PK buffer at pH 7, with 2.5 mM of MgSO₄, 0.1 mM ThDP, and 500 rpm with a cell concentration of 15 mg/mL of wet cell. The reaction was run during 24 hours and the cells were recovered by centrifugation (2 min 14000 rpm). The supernatant was analyzed by HPLC, the cells were washed in buffer 2 times and the reaction was restarted by adding fresh substrate. The cycle was repeated 5 times.
Table 7: Reaction conditions for the recyclability test.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Benzaldehyde (mM)</th>
<th>KBA (mM)</th>
<th>Cell (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>150</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>150</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>150</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>300</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>450</td>
<td>15</td>
</tr>
</tbody>
</table>

A second experiment was performed in the same way but incubating some cells for a variable number of days in 50 mM PK buffer with standard cofactors concentrations but without substrates in order to measure if the compounds involved in the carboligation reaction were producing some damage in the cells. After this buffer incubation, the reaction was started in the same way described before and the results were analyzed.

2.7.7. Scale-up

The reaction was run in a Dasgip® (Dasgip, Eppendorf, Germany) 500 mL continuously stirred tank reactor (CSTR) at 30°C, 150 rpm and pH 7 controlled with KOH and H₃PO₄ in a 120 mL vol. The buffer used was 50 mM PK at pH 7 with 2.5 mM of MgSO₄, 0.1 mM ThDP. The substrate concentrations were 333 mM of KBA and 17 mM benzaldehyde and 12.5 mg/mL of wet cell as catalyst. The reaction was studied for 20 h with measurements at time 0, 1, 2 and 20 hours. Data were analyzed by HPLC with the method explained in Chapter 2.4.1.

2.7.8. Fed-batch mode

The reaction was run in two Dasgip® 500 mL reactor at 30°C, 150 rpm and pH 7 controlled with KOH and H₃PO₄ in a 170 mL vol. The buffer used was 50 mM PK, pH 7, with 2.5 mM of MgSO₄, 0.1 mM ThDP. The substrate concentrations were 471 mM of KBA and 18 mg/mL of wet cell as catalyst. Both tanks had different starting benzaldehyde concentration: the first one was loaded with 23.5 mM and the second one with 47 mM. Distilled benzaldehyde was fed at a rate of 100 µl/h. The reaction was studied for 21 h with measurements at time 0, 1, 2, 3, 4, 5 and 21 hours. Data were analyzed by HPLC with the method explained in Chapter 2.4.1.

Another experiment was performed using pyruvate instead of KBA. The initial substrate concentrations were 500 mM PYR and 47.1 mM of benzaldehyde. The reaction was studied for 24 h with measurements at time 0, 1, 2, 3, 4, 5, 20 and 24 hours. Data were analyzed by HPLC with the method explained in Chapter 2.4.1.
2.7.9. Chiral polishing

The chiral polishing reaction was studied using 10 or 20 mM of (S)-PAC with an ee of 68 %. BAL and ApPDCE469G were added in concentrations of 2.2 and 2.1 mg/mL respectively (concentrations determined by the Direct detect device). Also pyruvate was added in a concentration 20 times higher than PAC (200 mM and 400 mM, respectively). The reaction media was 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO₄ at 30°C and 500 rpm. The reaction was studied for 24 h with measurements at time 0, 3.3, 17.3 and 24 hours. Data were analyzed by HPLC with the method explained in Chapter 2.4.1.

To find optimize the concentrations of BAL and ApPDCE469G for the chiral polishing, a second test was done. The reaction was performed in 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO₄ at 30°C and 500 rpm. The substrate concentration was 17 mM of (S)-PAC with a 68 % ee and 100 mM pyruvate. Enzymes were used in different concentrations as explained in Table 8. The reaction was studied for 24 h with measurements at time 0, 3, 5, 20 and 24 hours. Data were analyzed by HPLC. The experiment was performed in duplicate.

Table 8: Protein concentrations in the different experiments for the chiral polishing experiment

<table>
<thead>
<tr>
<th>Tube</th>
<th>BAL (mg/mL)</th>
<th>ApPDCE469G (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.80</td>
<td>0.40</td>
</tr>
<tr>
<td>2</td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td>3</td>
<td>0.40</td>
<td>0.80</td>
</tr>
</tbody>
</table>

3. Results and discussion

3.1. Protein expression optimization and catalyst production

In order to obtain as much overexpressed active protein as possible from the minimum amount of cell, an induction optimization experiment was performed. Two different media were used: auto induction media (AI) and Luria Broth (LB) media containing different amounts of isopropyl β-D-1-thiogalactopyranoside (IPTG). The cultivation took place at different temperatures for 24 or 48 h. The conditions of the experiment are described in Table 9.
Table 9: Experimental conditions in the induction optimization experiment.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Harvest time after induction(h)</th>
<th>Medium /IPTG conc.</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>AI</td>
<td>20°C</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>AI</td>
<td>30°C</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>LB/IPTG 0.5 mM</td>
<td>20°C</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>LB/IPTG 1 mM</td>
<td>20°C</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>LB/IPTG 1 mM</td>
<td>30°C</td>
</tr>
<tr>
<td>6</td>
<td>48</td>
<td>AI</td>
<td>20°C</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>AI</td>
<td>30°C</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>LB/IPTG 0.5 mM</td>
<td>20°C</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>LB/IPTG 1 mM</td>
<td>20°C</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>LB/IPTG 1 mM</td>
<td>30°C</td>
</tr>
</tbody>
</table>

In order to test the amount of active enzyme inside the cell, a carboligation test was performed. This method was preferred over the traditional SDS-PAGE since its results are quantitative and focus more in the active protein than in its absolute amount. The samples were diluted to the same optical density. Since the amounts of cells were considered to be similar, the differences in the PPC production should be related to the active protein concentration inside the bacteria. The results are shown in Figure 15:

Figure 15: PPC produced after 6 h of reaction by whole cell biocatalysis from different cultivation conditions. For details see Tab. 9. The carboligation test was performed in 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO₄, with 15 mM of benzaldehyde and 150 mM of KBA. Incubation: 30 °C and 500 rpm. Experiment performed in duplicate.

With an initial substrate concentration of 15 mM of benzaldehyde (and 150 mM KBA), the conversion in the main part of the reaction vials is over 100 %. Additionally, the error bars are quite significant. This can be due to the fact that a non-optimal HPLC analytic method
was used. Also the capacity of the whole cell was underestimated, producing much higher yields than expected. Since the reaction run until benzaldehyde was depleted, it is difficult to compare which of the conditions yielded higher amount of active protein. Despite these problems, some trends can be seen:

- The induction with 1 mM IPTG at 30 °C produces less active protein than any other set of conditions.
- When AI media is used at 20 °C, the activity after 48 hours of cultivation after induction was higher, compared to 30 °C, and the activity after 48 hours of cultivation after induction was lower than after 24 hours. This can be due to lactose depletion due to higher metabolism related to the higher temperature or a less accurate folding process.
- Whole cell catalysis can be used for PPC production.

It was concluded that the use of AI media at 20 °C with 48 h cultivation was the optimal induction method and it was used afterwards for large scale cultivation (5 L). These cells were used for all further studies in this project either as whole cell catalysts or for production of purified enzyme using immobilized metal-affinity chromatography as described in the Materials and Methods section. A typical chromatogram is presented in Figure 16.
Figure 16: Chromatogram of the affinity chromatography performed to purify ApPDCE469G. Protein elution was monitored at 280 nm (blue line). The conductivity is shown as green line and can be used for tracking the imidazole concentration as explained in Chapter 2.3.2.

After the purification, the elution peak was collected and desalted using an Amicon cell yielding highly pure protein as it can be seen in the SDS gel presented in Figure 17. The purified protein was then lyophilized and the resulting powder was analyzed by Bradford test, showing a protein content of 23 % w/w.

Figure 17: SDS gel of different steps of the ApPDCE469G purification. FT: flow through peak. WP: wash peak. FT/2: flow through peak diluted 50 %. Cell: elution peak loaded in the Amicon® cell for desalting. ApPDCE469G labeled with an arrow.
3.2. Reaction optimization for (S)-PPC production using pure enzyme

In order to increase the enzyme specific space-time-yield (SSTY) as much as possible using a minimum amount of enzyme, optimal reaction conditions should be determined. In order to perform this task, an optimization of all the factors which have an impact on the reaction performance such as the donor substrate and its concentration, the addition of a cosolvent to increase benzaldehyde solubility, as well as temperature and pH.

3.2.1. Choice of donor substrate

As explained in the introduction (chapter 1.7), the ApPDC is able to use two different types of donor substrates for the carboligation: aldehydes and the α-ketoacids, which are decarboxylated prior to the carboligation reaction. Therefore, in order to produce PPC, there are two possible substrates to use together with benzaldehyde as the acceptor: propanal or α-ketobutyric acid (KBA) (Figure 18).

![Figure 18: (S)-PPC synthesis by the ApPDCE469G. Apart of benzaldehyde, the second substrate can be either propanal (top) or KBA (bottom) in which case a previous decarboxylation step releasing CO₂ is required](image)

Since it is known that the substrate ratio can produce high variations in the enzyme performance [84, 120, 132], the reaction was studied using different concentrations of propanal, KBA and benzaldehyde. After 24 h of reaction, the (S)-PPC yield (Figure 19) and its ee (Figure 20) were determined. Also, the concentration of propioin was measured (Figure 22). This compound is produced by the self-carboligation of propanal and is the main by-product of the reaction. Its production should be reduced as much as possible in order to decrease the downstream cost.
Figure 19: PPC concentration after 24 h of reaction under different substrate concentrations using either propanal (Prop, red columns) or α-ketobutyric acid (KBA, blue series) and benzaldehyde. Reaction performed in 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO$_4$, and 0.1 mg/mL of enzyme. Incubation at 30 °C and 1000 rpm. Experiments performed in duplicate.

Figure 19 shows that when KBA is used, the production of PPC is between 1.4 and 2-fold higher compared to the use of propanal as a donor. According to the kinetics reported by Rother et al. [55] for the carboligation of propanal and benzaldehyde, a concentration of 60 mM of propanal would yield maximal reaction velocity. This was confirmed in the present study since the final PPC concentration is practically the same when 100 mM or 60 mM of propanal is used. This trend can be observed when the benzaldehyde concentration is either 10 or 20 mM. However, the studies with KBA gave significant better results compared to propanal up to 100 mM of KBA. Therefore, a more detailed kinetic study was performed as it will be discussed later in this chapter.

The reason behind the different performance of the enzyme with the two different donor substrates can be easily explained using the mechanistic model proposed by Meyer et al. [129] for the PDC of Zymomonas mobilis, which was presented in the Introduction (Chapter 1.7). Due to the mutation, the $\text{ApPDCE469G}$ has a decreased decarboxylase activity [55] since the carbanion-enamine cannot be protonated to the conjugated acid by the E469 (Figure 13). This step prevents the release of propanal after KBA decarboxylation, making the carboligation step more favorable. On the other hand, when propanal is bound to the ThDP forming hydroxypropyl-ThDP, the deprotonation required to reach the carbanion-enamine state is also slower, making the carboligation with propanal much slower than in the $\text{ApPDCwt}$.

Concerning the enantiomeric purity of the product ($S$)-PPC the results shown in Figure 20 suggest that higher ee values are possible when KBA is used instead of propanal. These
results are most pronounced when propanal or BA are used in high excess relative to benzaldehyde. When KBA is used, the ee seems to be independent from the substrate concentrations, reaching a value about 96.5 % under all the conditions tested. On the other hand, when propanal is used instead of KBA, the ratio between propanal and benzaldehyde seem to decisive for the final product ee as can be seen in Figure 21.

Figure 20: (S)-PPC ee after 24 h of reaction under different substrates concentrations using either propanal (Prop, red columns) or α-ketobutyric acid (KBA, blue columns) and benzaldehyde. Reaction performed in 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO₄, and 0.1 mg/mL of enzyme. Incubation at 30 °C and 1000 rpm. Experiment was performed in duplicate.

Figure 21: (S)-PPC ee after 24 h of reaction produced by different substrate ratios of propanal and BA. Reaction performed in 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5
mM of MgSO$_4$ and 0.1 mg/mL of enzyme. Incubation at 30 °C and 1000 rpm. Experiments performed in duplicate.

The mechanistic model of Meyer et al. described in Chapter 1.7, does not provide an explanation for the decrease in selectivity when propanal is used. Gerhards et al. described [134] that the use of organic solvents decreases the S-selectivity of the ApPDCE469G. The proposed explanation behind this fact is that the solvent can compete with the substrates at the entrance to the S-pocket, preventing the aromatic ring of the benzaldehyde to be arranged in an anti-parallel manner and therefore making the S-configuration less favorable. Even though propanal is not considered as a solvent, the effect can be the same, lowering the ee of the S-product. This could explain the linear decrease of ee related to the substrate ratio: when propanal is in relative abundance to benzaldehyde, its arrangement in the S-pocket is statistically more probable. On the other hand, KBA is the larger and more hydrophilic molecule. These two factors prevent its stabilization in the S-pocket making the entrance of benzaldehyde more probable. This theory can explain the fact that higher ee is obtained when KBA is used instead of propanal.

Finally, despite the fact that standard deviations are considerable, the use of KBA seem to reduce the amount of propioin produced an average of 93.5 %. This can be seen in Figure 22. The propioin concentration produced seems to be related to the excess of propanal or KBA over benzaldehyde. The large error bars can be due to the combination of two factors: propanal volatility and analytic method. Due to the high volatility of the propanal, pipetting a precise amount of this product is challenging, so the substrate concentration can have severe variations. This effect is not so notable in the PPC production since the propanal concentrations are high enough to assure maximal velocity. On the other hand, the kinetic parameters of the selfigation of propanal are not known and it is possible that small differences in substrate concentrations in the tested range have a big impact in the reaction velocity. Also the measurement of propioin concentration by GC requires an extraction step with ethyl acetate. This step can compromise the reproducibility of the measurement.
Figure 22: Propioin produced after 24 h of reaction depending on different substrate concentrations using either propanal (Prop, red columns) or 2-ketobutyric acid (KBA, blue columns) and benzaldehyde. Reaction performed in 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO₄, and 0.1 mg/mL of enzyme. Incubation at 30 °C and 1000 rpm. Experiments performed in duplicate.

The mechanistic model of the enzyme can also explain this third advantage of the KBA over propanal. Once the carbanion-enamine is formed, carboligation with an aldehyde in solution is the next step of the reaction. When propanal is present in the reaction, two aldehydes (propanal and benzaldehyde) compete as acceptors for C-C coupling. If a second molecule of propanal is bound to the carbanion-enamine, propioin would be released. On the other hand, when KBA is used as a substrate, the only aldehyde available for carboligation would be benzaldehyde, and no propioin would be formed. The fact that some traces of propioin are produced when KBA is present it is probably due to the fact that some KBA is decarboxylated and propanal is released. It has been reported that the pyruvate decarboxylase activity of the variant is only 2 % of the wild type [55] and it is possible to assume also a strong reduction when KBA is used, but it still can produce some small amounts of propanal.

Summarizing, when KBA is used, higher yields of product with higher ee and smaller amounts of by-product are formed. Nevertheless, the price of the α-ketoacid is usually higher than the price of aldehydes. In this specific case, the cost of 1 g KBA is 424-times higher compared to 1 g of propanal. In order to assess if higher yields obtained with KBA compensate the higher price, a cost analysis was performed. To do this, the costs of the substrates in the tested concentrations were calculated for a reaction volume of 1 L. Prices of the buffer salts and the cofactors were added and price per gram of PPC was calculated. Results can be found in Figure 23.
Figure 23: Cost per gram of product under different reaction conditions. Costs were calculated using Sigma Aldrich prices for the highest purity and the biggest scale available. The calculation includes the buffer components, cofactors and substrates. Since the enzyme is not commercially available, its price was not taken into account.

Despite the 400-times higher price for KBA, the higher productivity with this donor substrate reduces the impact on the final product costs. Depending on the conditions, the costs for the final product are only 5-14 times higher with KBA relative to propanal. However, costs for downstream processing are not included, which would be favorable for KBA due to the higher ee, higher final product concentration and lower by-product formation. As aldehydes can reduce the half-life of an enzyme, KBA is also advantageous with respect to the stability of the biocatalyst.

Conclusively, depending on the scale and demands on product purity the use of KBA over propanal can be a cost efficient alternative to propanal despite is higher price.

Another advantage of KBA is that when used in high concentrations (100 mM), the solubility of benzaldehyde is increased (data not shown). This can be due to a mild amphiphatic nature of KBA. This feature allowed performing kinetic measurements using benzaldehyde concentrations up to 45 mM without the formation of a second phase which were not studied in previous work due to solubility limitations [55]. In order to determine the kinetic parameters, the reaction was run with a fixed amount of benzaldehyde and different amounts of KBA and vice versa. The results are plotted in Figure 24.
Figure 24: Michaelis-Menten plots of the reaction: benzaldehyde fixed at 30 mM (left) and KBA fixed at 400 mM (right). Experimental points are labeled in blue. The red line shows the calculated value for the Michaelis-Menten equation with the parameters given below the chart. The calculations were done with initial rate activity at the conditions reported in the Materials and Methods section. Experiments were performed in triplicate.

As it can be clearly seen, both substrates show typical hyperbolic Michaelis-Menten kinetics. The $V_{\text{max}}$ values are reached $>150$ mM KBA and $>30$ mM benzaldehyde. Therefore, these concentrations were used for next experiments.

### 3.2.2. Solvent choice

One of the main challenges for this reaction is the low solubility of benzaldehyde in a buffered system. In order to overcome this challenge, a frequent option is to add an organic solvent. This option has been explored extensively by Gerhards et al. [134] showing that the addition of different organic solvents to the ApPDCE469G reduces its yield and the $ee$ of the $S$-product when acetaldehyde and benzaldehyde are used as substrate. Since the reaction with KBA is different, the effect of DMSO on the reaction performance was studied. This specific solvent was used due to the fact that it is one of the most commonly used cosolvents in biotransformations with ThDP-dependent enzymes [132]. The reaction was studied with different volumes. Results are displayed in Figure 25.
Figure 25: Relative PPC concentration after 17 hours of reaction with different concentrations of DMSO from 0 vol% (Ctrl) to 30 vol%. Reaction performed in 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO₄, 150 mM KBA, 30 mM benzaldehyde and 0.1 mg/mL of enzyme. Incubation at 30 °C and 1000 rpm. Experiment performed in duplicate.

The results of this test show clear coincidence with data presented by Gerhards et al. [134]: the final yield is reduced with increasing DMSO concentration, reaching a maximum reduction of 50% in presence of 30 vol% of the cosolvent.

Also the ee of the product is decreased up to 8.4% when 30 vol% DMSO is used (figure 26). The theory behind this fact is the same as explained before (Chapter 3.2.2) for the decrease of ee when propanal is used instead of KBA: the blockage of the S-pocket by the solvent molecule prevents the antiparallel orientation of benzaldehyde.

Figure 26: (S)-PPC ee after 17 hours of reaction with different concentrations of DMSO from 0 vol% (Ctrl) to 30 vol%. Reaction performed in 50 mM PK buffer at pH 7 with 0.1 mM ThDP,
2.5 mM of MgSO$_4$, 150 mM KBA, 30 mM benzaldehyde and 0.1 mg/mL of enzyme. Incubation at 30 °C and 1000 rpm. Experiments were performed in duplicate.

Due to these two negative effects on the reaction performance, the use of DMSO is not advisable. Also, the correlation between these results and those of Gerhards et al. [134] lead to the assumption that the enzyme would interact in the same way with other solvents of appropriate size to enter the S-pocket. Therefore, other alternatives like methyl tert-butyl ether (MTBE) or 2-methyl tetrahydrofuran (2-MTHF) [135] were not tested. It has been reported that the use of DMSO is able to enhance the half-life of the enzyme [132] but due to the negative impact on yield and ee, this effect was not studied in this work.

From the economical point of view, the use of DMSO is also not advisable since it increases the costs for downstream processing, because removal of DMSO from the final product is difficult [132]. Additionally, it was found that by adding this solvent, the costs of the reaction media is increased, since DMSO is more expensive than phosphate buffer. Combining the higher costs with the lower yields, the price per gram of product increases notably, as can be seen in Figure 27:

![Figure 27: Economic evaluation of the use of DMSO. The plot shows the price per liter of reaction medium (blue) including the buffer salt, substrates, cofactors and the different DMSO concentrations from 0 vol% (ctrl) to 30 vol% and the production costs per gram of product (red) under these conditions. The costs were calculated using prices from Sigma Aldrich for the highest purity and the biggest scale available. The cost includes the buffer components, solvent, cofactors and substrates. Since the enzyme is not commercially available, its price was not taken into account. As can be seen, costs of the reaction media increase linearly with the DMSO concentration. Nevertheless, due to lower yields, the cost per gram of the product increases much faster: the price of reaction media when 30 vol% DMSO is used is around 3-times higher than the reaction in buffer, but the price per gram of PPC is 6-times higher. Combining this fact with](attachment:figure27.png)
the higher downstream cost for solvent removal when DMSO is added, one can conclude that the use of DMSO is not an economically feasible option.

3.2.3. Optimization of the reaction conditions

The reaction conditions are a critical aspect in a production process. In order to obtain maximal specific space-time-yields (SSTY), all parameters have to be chosen carefully. The usual approach to achieve this goal is the study of the optimal pH and temperature as well as the kinetics of the enzyme. However, in this case there are some extra challenges that make this basic characterization insufficient: the ee of the product is affected by some reaction conditions in an opposite way than the SSTY. Further, there are complex relations between the different parameters, producing different effects due to combination of factors. Therefore, a compromise had to be found concerning the optimal reaction conditions.

In order to overcome these challenges, an iterative optimization using a computer-based design of experiment (DOE) strategy was used. The reaction was studied using different reaction conditions. Afterwards, the results were analyzed trying to determine how each parameter affects the ee and the final conversion. This was done in the following manner: the reaction was run for 8 hours and the end point measurement was used for calculating the SSTY. End point measurement and final conversion were used instead of initial rate activity in order to take the stability of the enzyme into account. For example, a reaction at higher temperature may have a higher initial rate than at lower temperature, but if the catalyst is sensitive to this parameter, the final conversion can be reduced, because the enzyme is more rapidly deactivated. By using a longer reaction time this contradictory effects can be taken into account.

By using the reaction conditions reported in literature [55] as a starting point, a first set of experiments was designed. Considering previous results, the use of DMSO was avoided and propanal was replaced by KBA as a donor. The design of each new round of experiments was suggested using the Kriging algorism [136]. The Kriging method allows a mathematical approximation and visualization of ee and SSTY over the entire parameter space. This information is used to plan the next round of experiments with parameters that are potentially Pareto optimal. In addition, the Kriging algorism provides an estimate of how accurately the true ee and SSTY are mathematically approximated from the given experimental data. This information is used to plan additional experiments in sparsely sampled regions of the parameter space and in regions with conflicting experimental data. In total 8 different rounds of experiment were performed until no further improvement on SSTY or in the ee was achieved, which are more detailed explained below. Results of the iterative round are described in Table 10. More detailed information is described in the Annex. To visualize the optimization strategy a Pareto diagram (Figure 28) was used that combines individual experimental values with a general trend.
Classical experimental design strategies, separately performed to optimize $ee$ and SSTY, would only yield the extreme cases along the coordinate axes of a Pareto diagram (Figure 28). In contrast, the experimental design strategy followed in this study generates the entry Pareto diagram. After each experimental round the parameter values of the Pareto optimal points were compared to the entire database, including previous rounds, in order to determine trends, gaps and inconsistencies. The next round of experiments was carefully planned with a variable number of experiments, around 10, in order to follow these trends, close gaps, and check for inconsistencies. Notably, this procedure resulted in a constant improvement of Pareto optimal points from round to round.

Table 10: Results of iterative reaction optimization. For each round of experiments, the experimental parameters, aims of the respective experimental round, results, and the maximum values of SSTY and $ee$ are listed.

<table>
<thead>
<tr>
<th>Round</th>
<th>parameters</th>
<th>aims of study</th>
<th>result</th>
<th>max values</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>pH 6-8, Temperature 30-37 °C, Substrates 15-30 mM, Enzyme 0.1 mg/mL</td>
<td>First rough estimate of effect of different parameters on SSTY and $ee$</td>
<td>SSTY increased with substrate concentration and temperature</td>
<td>SSTY: 10.3 mM/h*mg, $ee$: 96.9%</td>
</tr>
<tr>
<td>II</td>
<td>pH 7, Temperature 30 °C, BA 30 mM, KBA 60-360 mM, Enzyme 0.165 mg/mL</td>
<td>Study effect of KBA concentration on SSTY, Determine effect of KBA concentration on $ee$</td>
<td>SSTY increased with KBA concentration, Higher $ee$ achieved, KBA/BA ratio seems to affect the $ee$</td>
<td>SSTY: 10.8 mM/h*mg, $ee$: 97.9%</td>
</tr>
<tr>
<td>III</td>
<td>pH 7, Temperature 30°C, BA 6-42 mM, KBA 400 mM, Enzyme 0.165 mg/mL</td>
<td>Study effect of BA concentration on SSTY, Determine effect of substrate concentration on $ee$</td>
<td>SSTY increased with BA concentration, Lower $ee$ than in round II, KBA/BA ratio not optimal</td>
<td>SSTY: 14.5 mM/h*mg, $ee$: 95.4%</td>
</tr>
<tr>
<td>IV</td>
<td>pH 4-5, Temperature 30°C, BA 10-20 mM, KBA 40-100 mM, Enzyme 0.08 mg/mL</td>
<td>Study effect of lower substrate concentration on SSTY, determine effect of lower pH on $ee$</td>
<td>SSTY reduced severely, Low pH seems to be less effective, High $ee$ achieved</td>
<td>SSTY: 4.3 mM/h*mg, $ee$: 98%</td>
</tr>
<tr>
<td>V</td>
<td>pH 6-7, Temperature 30-37°C, BA 30-42 mM, KBA 300-400 mM, Enzyme 0.12 mg/mL</td>
<td>Optimized SSTY (Vmax), Higher $ee$</td>
<td>SSTY increased $ee$ decreases with temperature</td>
<td>SSTY: 15.9 mM/h*mg, $ee$: 97.4%</td>
</tr>
<tr>
<td>VI</td>
<td>pH 6.5-7, Temperature 30-37°C, BA 30-42 mM, KBA 240-400 mM, Enzyme 0.12-0.165</td>
<td>Optimized SSTY, Determine the effect of enzyme concentration</td>
<td>Slight increase in SSTY, Slight increase in $ee$, Enzyme concentration has</td>
<td>SSTY: 17 mM/h*mg, $ee$: 97.4%</td>
</tr>
</tbody>
</table>
| VII | pH 7  
Temperature 30°C  
BA 10-30 mM  
KBA 10-100 mM  
Enzyme 0.165 mg/mL | Lower SSTY due to lower substrate concentration  
Higher ee by optimal KBA/BA ratio | No increase in SSTY  
High ee but far below the Pareto line | SSTY: 3.7 mM/h*mg  
ee: 97.9% |
| VIII | pH 7  
Temperature 42°C  
BA 25-35 mM  
KBA 120-420 mM  
Enzyme 0.165 g/mL | High temperature should increase SSTY  
Lower ee | Reduced SSTY  
High temperature decrease enzymes stability  
Ee was low, but not as low as expected | SSTY: 10.6 mM/h*mg  
ee: 96.4% |

Due to the amount of data and the competing trend between SSTY and ee, it was not possible to obtain an absolute maximum for both SSTY and ee. In order to visualize this fact, the results were plotted in a Pareto chart [137] (Figure 28):
Figure 28: Pareto chart of the reaction optimization experiment. Each data group corresponds to a different set of experiments as described in Table 10. Each experiment was performed in duplicate; the error bars are not shown for the sake of data clarity. The full results are shown in the Annex. The reaction conditions on the labeled points are described in the table below the figure and correspond to: 1) Result published by Rother et al. [55] 2) highest ee, and 3) highest SSTY obtained within the Pareto line.

As it can be seen in Figure 28 that the reaction performance has a strong dependence on the reaction conditions. The initial point was calculated from literature data, it has the lowest SSTY and ee of all the conditions tested. This is probably due to the fact that the reaction time used was 72 hours instead of 8 h (this study), which can produce big variations in the SSTY. Also, DMSO was used as a cosolvent and propanal was used as a donor instead of KBA and both options have been proved in this work to have a negative impact on the catalyst performance.

<table>
<thead>
<tr>
<th>Point</th>
<th>BA (mM)</th>
<th>KBA/Propanal (mM)</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Enzyme (mg/mL)</th>
<th>ee (%)</th>
<th>SSTY (mM/h*mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>18</td>
<td>7</td>
<td>30</td>
<td>0.3</td>
<td>89.0</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>360</td>
<td>7</td>
<td>30</td>
<td>0.165</td>
<td>97.9</td>
<td>10.8</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>300</td>
<td>6.5</td>
<td>37</td>
<td>0.12</td>
<td>96.6</td>
<td>17.0</td>
</tr>
</tbody>
</table>
Overall, a maximal ee of 98 % could be achieved. Increasing this value further would probably require a modification of the catalyst. The point number 2 (Figure 28) gives the highest SSTY for this ee. Every set of parameters with higher SSTY shows a decrease in ee.

Looking at the data more closely, the interplay between the parameters makes it difficult to analyze their isolated effect on the reaction but, nevertheless, it is possible to determine some trends:

- Substrate concentrations have a strong influence on the SSTY. As can be inferred from the kinetic data, maximal SSTY were obtained under high substrate loads of both substrates (over 200 mM KBA and over 30 mM BA). On the other hand, when the reaction is not running at saturated KBA concentrations, low benzaldehyde concentrations (under 30 mM) limit the SSTY and seem to reduce also the ee of the product.
- pH values outside the range from 6 to 7.5 are not optimal since either the SSTY or the ee are reduced.
- Temperature has a positive impact on SSTY until 40 °C. Above this value the effect is reversed, probably due to enzyme inactivation. Concerning the ee, lower temperatures (30 °C) where observed to be optimal.
- The enzyme concentration has no impact on the ee. Even though higher enzyme concentrations speed up the reaction and thus increase the final product concentration, the SSTY is not affected in the tested concentration values [138].

After the 8 rounds of optimization, the enzyme performance was highly improved compared to the starting point. The SSTY was increased 61.3 fold according to the literature data [55] up to a value of 17 mM/(h*mg enzyme). Also the ee was enhanced up to a 98 %, which is 9 % higher than for the earlier reported conditions. This is of special importance since the increment of ee at values over 95 % requires higher free Gibbs energy than at lower ee values due to the logarithmic relation between these two factors [134] (Figure 29).
As it was explained before that the highest ee and SSTY were achieved using different reaction conditions, so the optimal conditions should be decided depending on the process needs: if high ee is required, substrate saturating conditions, pH 7 and 30 °C can be used. On the other hand, if the aim is to obtain high amounts of product, the temperature can be increased to 37 °C, but this will decreases the ee to 96.5 %.

3.3. Whole cell biocatalysis for (S)-PPC and (S)-PAC production

As was explained before, the use of whole cell can be a cost-effective alternative to purified enzyme in a biocatalytic reaction due to the fact that cell disruption and protein purification are not required. The industrial production of PAC is currently done by fermentation using yeast [131], in a process where benzaldehyde is fed to the growing cells which catalyze the carboligation with acetaldehyde produced by the glucose metabolism. Due to this, fermentation cannot be used for PPC synthesis, since a mixture of PAC and PPC would be produced. On the other hand, using metabolically inactive resting cells can be a feasible option. As was explained before (Chapter 3.1), the use of cells resuspended in buffer was tested in order to determine optimal growth conditions for protein production with positive results. Therefore, the possibility of whole-cell biocatalysis was further evaluated.

Figure 29: \( \Delta G \) values of different enantiomeric excesses. From Gerhards et al. ([134])
3.3.1. Whole cell reaction setup

The first step was to find the optimal cell load. In order to determine this, the reaction was run using different cell concentrations with a fixed amount of substrates over a 4 hours taking samples every hour. The results are displayed in Figure 30.

Figure 30: PPC production over time with different cell concentrations. Reaction performed in 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO₄, 150 mM KBA and 30 mM benzaldehyde. Incubation at 30 °C and 500 rpm. Experiments were done in duplicate.

Figure 30 clearly shows that full conversion was achieved in 4 hours with the minimal catalyst load used (25 mg/mL). With higher cell loads the reaction was already completed within one hour. Afterwards product degradation can be detected. This fact can be explained by the degradation of PPC produced by the cell metabolic background.

Figure 31 shows that the effect of the cell concentration on the product yield after one hour is not linear. Additionally, the diagram shows some more than 100 % of conversion in some points. This was due to the low performance of the HPLC method when whole cell were used since the PPC peaks overlap with a by-product which was never detected in reactions were pure enzyme was present. This by-product is believed to be HBP (Figure 8), an isomer of PPC since the retention time using the HPLC method described in the Materials and Methods section is similar to one of the enantiomers of HBP. Nevertheless, further attempts of determination of the identity of this by-product by ¹H-NMR and SFC/MS have not been successful.

In order to improve the analytics, a new method using a different column was developed for whole cell analytics. Therefore and also in order to avoid by-product accumulation, it was decided to use a lower cell load (10 or 15 mg/mL) in the following experiments and to limit the reaction time to 3 hours. By doing this, the by-product concentration could be kept below the detection limit.
Figure 31: Product concentration after one hour of reaction obtained with different cell loads. Reaction performed in 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO$_4$, 150 mM KBA and 30 mM benzaldehyde. Incubation at 30 °C and 500 rpm. Experiments were performed in duplicate.

The whole cell catalysis was also compared with crude cell extract in order to check if the diffusion of substrates or products through the membrane can produce some limitations but the results were similar. Also the use of lyophilized cells was assessed, showing no improvement over the wet cell apart of the easier handling. Since nor crude extract nor lyophilized cell presented any advantage, wet cell were used for all further experiments.

3.3.2. Reaction optimization

Since the whole cell approach showed a great potential in order to achieve high product concentrations, more research was done in this aspect. Since the reaction conditions were found to have such a high impact on the performance of the carboligation when purified enzyme was used as catalyst, another optimization process was done with whole cells in order to assess how product yield and ee could further be enhanced. In this case, the iterative process used for the isolated enzymes (Chapter 3.2.3.) was replaced by a traditional optimization approach, were the effect of each parameter on the reaction was studied independently of the others.

3.3.2.1. ThDP dependence

One of the most frequently reported advantages of working with whole cell biocatalysis is that there is no need of adding extra cofactors, since these are produced inside the cell by its
metabolism. Dominguez de Maria et al. [120] proved this statement true for the ThDP-dependent enzymes BAL and BFD. In contrast, in this work the reaction was studied with different cofactor concentrations showing a different trend. The reaction was run for 3 hours with 0-1.1 mM ThDP and a fixed concentration of MgSO$_4$ of 5 mM. This excess of MgSO$_4$ was used in order to assure the binding of all present ThDP to the enzyme. The results are plotted in Figure 32.

![Figure 32: (S)-PPC produced after 3 hours of reaction with different amounts of ThDP and 5 mM MgSO$_4$ in whole cell biocatalysis. The reaction was performed in 50 mM PK buffer at pH 7 with 150 mM of KBA and 30 mM of benzaldehyde at 30°C and 500 rpm with a cell concentration of 10 mg/mL of wet cells. Experiments were performed in duplicate. As can be clearly seen, without the addition of extra ThDP, no product formation is observed. Nevertheless, when 0.1 mM ThDP is present (standard conditions) the reaction runs at maximum velocity. The ThDP concentrations did not show any impact on the ee which was between 96.8 % and 97.0 % in all samples. Due to high concentrations of recombinant enzyme inside the cell, it is possible that the cells cannot provide sufficient ThDP by metabolic, making external addition essential to achieve full activity. Even though this is a clear disadvantage as the addition of 0.1 mM of ThDP would increase the costs of the reaction media by 0.27 €/liter, this is an extremely low increment compared with the costs for KBA (Chapter 3.2.1.). The use of a different cultivation conditions may overcome this limitation.

3.3.2.2. Substrate optimization

The effect of different substrate concentrations was also studied in the whole cell system. The reaction was run with different KBA concentrations and a fixed concentration of
benzaldehyde in order to check optimal conditions. An end point measurement was used for comparison. Measuring the initial reaction rate for kinetic determination was not considered as useful since it is difficult to assess the amount of enzyme inside the cell and therefore, the \(K_m\) and \(V_{\text{max}}\) values would not have been accurate. The results are shown in Figures 33 and 34.

Figure 33: Whole cell biocatalysis of PPC. The PPC concentration was determined after 20 hour for various starting concentrations of KBA, keeping the benzaldehyde concentration at 30 mM. Reaction performed in 50 mM PK buffer at pH 7, with 2.5 mM of MgSO4, 0.1 mM ThDP, 30°C and 500 rpm with a wet cell concentration of 10 mg/mL. Experiments were performed in duplicate.
Figure 34: ee of the (S)-PPC produced after 20 hours of reaction under different KBA concentrations. Reaction performed in 50 mM PK buffer at pH 7, with 2.5 mM of MgSO4, 0.1 mM ThDP, 30 mM benzaldehyde, 30°C and 500 rpm with a wet cell concentration of 10 mg/mL. The low (S)-PPC concentration on the two lower KBA concentrations difficults the accurate assessment of the ee, resulting in large error bars. Experiment performed in duplicate.

As can be seen in the previous figure, an increment in the KBA concentration has a positive impact on the reaction performance up to 600 mM without any substrate inhibition being observed. This is consistent with the kinetics of the purified enzyme (Figure 24, chapter 3.2.1.), but the data suggest that the whole cells require two times more KBA in order to reach maximal speed compared to the isolated enzyme. This can be due to several reasons, like differences between inner to outer cell conditions or diffusion limitations due to the cell membrane. A significant effect on the ee of the product was not observed.

The subsequent optimization of the benzaldehyde concentrations for the whole cell biocatalysis is shown in Figure 35. Due to its low solubility in aqueous media, the concentration of benzaldehyde is the limiting factor for the achievement of high product concentrations. One of the options to meet this challenge is the use of an emulsion. In order to test this possibility, the concentration of benzaldehyde was studied up to 200 mM, which is much higher than its solubility limit (ca. 35 mM). Therefore, some of the reaction vials contained an aqueous-benzaldehyde two phase system. The use of benzaldehyde emulsions for PAC production using a partially purified fungal PDC has been reported to produce high product concentrations of >400 mM without damaging the enzyme [139]. However, ApPDCE469G seems to be more sensitive, because the product concentration decreased > 60 mM benzaldehyde (Figure 35).
Figure 35: PPC concentration after 3 hours of reaction in the presence of different benzaldehyde concentrations. The reaction took place in 50 mM PK buffer at pH 7, with 2.5 mM of MgSO$_4$, 0.1 mM ThDP, 300 mM of KBA, 30°C and 500 rpm with a cell concentration of 10 mg/mL of wet cell. Experiments were performed in duplicate.

Figure 36: ee of the (S)-PPC produced after 3 hours of reaction under different BA concentrations. The reaction took place in 50 mM PK buffer at pH 7, with 2.5 mM of MgSO$_4$, 0.1 mM ThDP, 300 mM of KBA, 30°C and 500 rpm with a cell concentration of 10 mg/mL of wet cell. Experiment performed in duplicate. Due to the small standard deviation (below 0.1 %), the error bars are difficult to appreciate.

Once the emulsion is produced, the performance of the reaction is reduced. This can be due to catalyst deactivation due to interphase contact or to a negative effect produced by the benzaldehyde. The inhibitory effect on the catalysts produced by aldehydes has been reported previously on purified enzyme [122, 140] and it seems to apply also to whole cell catalysis. The most interesting effect observed with high concentrations of benzaldehyde is the significant increase in product ee, which is shown in Figure 37. As it was previously
mentioned, lower benzaldehyde concentrations would statistically decrease the chances of benzaldehyde to enter the S-pocket, reducing therefore the ee of the S-product. Even though this effect can only be seen in the reaction with pure enzyme when the KBA is not used in saturating concentrations (Chapter 3.2.3.), it is much clearer when whole cells are used. This could be due to a difference in concentration between the inner cell and the media caused by diffusion but when whole cell was compared with crude extract, no clear difference was found (data not shown).

3.3.2.3. Optimal pH

Also an optimal pH was determined. The effect of this parameter on the reaction performance can be different when whole cells are used as catalyst, since the value can vary between the outer cell medium and the inner cell conditions. The reaction was studied between pH 6 to 8 and an end point measurement of the final PPC concentration and ee was compared in Figure 37:

Figure 37: Effect of pH in the (S)-PPC concentration (left) and its ee (right) after 3 hours of reaction with whole cell catalysis. The reaction was performed in in 50 mM PK buffer with 2.5 mM of MgSO₄, 0.1 mM ThDP, 150 mM of KBA and 30 mM of benzaldehyde at 30°C and 500 rpm with a cell concentration of 10 mg/mL of wet cell. Experiments were done in duplicate.

As it can be clearly seen, the optimal pH-values for productivity and ee differ: while the maximum conversion was achieved at pH 7, ee was slightly higher at lower pH. This effect on the ee was also observed with pure enzyme (data not shown).

The pH optimal of the whole cell reaction shows similarities with the described for the purified enzyme (Chapter 3.2.3.), but in the case of whole cells the pH-optimum of other enzymes being active on the products must be taken into account. Some of these enzymes may be able to degrade or isomerize the (S)-PPC affecting the ee or the yield.
3.3.2.4. Temperature effect

Finally, the temperature effect on the production of (S)-PPC by whole cell biotransformation was determined. Therefore, the reaction was run at different temperatures and product concentration was compared after 3 hours. The results are displayed in Figure 38:

![Graph showing temperature effect on (S)-PPC production](image)

Figure 38: Temperature effect on the whole cell biocatalysis yielding (S)-PPC. The left chart shows the product concentration after 3 hours of reaction under different temperatures and the its ee can be found in the right chart. The reaction was performed in 50 mM PK buffer at pH 7, with 2.5 mM of MgSO₄, 0.1 mM ThDP, 150 mM of KBA and 30 mM of benzaldehyde and 500 rpm with a cell concentration of 10 mg/mL of wet cell. Experiment performed in duplicate.

As can be seen, the final concentration of PPC almost doubled, when the reaction temperature was increased from 20°C to 40°C. In the other hand, the ee shows the opposite trend: the higher the temperature, the lower the ee. The trend is similar to results observed with isolated enzyme (chapter 3.2.3.), but more pronounced with whole cells. This is probably related to the metabolic background of the cell. Even though only three different temperatures were tested, the trend was considered to be clear and no further optimization of this parameter was done. Since there are many options in order to increase the product concentration, but not so many to increase the ee, it was decided to use a maximum of 30 °C in whole cell reactions.

3.3.3. Catalyst recyclability

Another advantage of the use of whole cells can be recyclability of the catalyst. Industrially, this can be done by centrifugation or filtration and the recovered catalyst can be reused in a second cycle. Since the price of the catalyst is considered to be one of the main contributors to the final product price in a biocatalytic production process [66], reuse of the cells is highly desirable.

In this work, the recyclability of the cells was studied as follows: the reaction was run for 24 hours at different substrate concentrations and the cells were recovered by centrifugation, the product concentration was measured in the supernatant and the pellet was washed in
buffer. Then, fresh substrate was added and the reaction was restarted. This cycle was repeated five times. Due to the different tested in substrate concentrations, the yield was expressed as a percentage of the yield achieved after the first cycle. Results are displayed in Figure 39:

![Figure 39: Recycling studies of cells for the production of (S)-PPC using different substrate concentrations (see right table). Each color represents a different experiment. The reaction was performed in 50 mM PK buffer at pH 7, with 2.5 mM of MgSO₄, 0.1 mM ThDP, and 500 rpm with a cell concentration of 15 mg/mL of wet cell. Experiments were performed in duplicate. As shown in Figure 39 the recyclability of the wet cells by centrifugation was found to be very poor. After just one cycle, the catalyst performance was reduced by an average of 65 %. After 4 cycles, the yield was reduced to about 11 %. The different substrate concentrations resulted in very small variations but some trends can be seen: in vials 4 + 5 with higher concentration of KBA (350 mM and 400 mM), the stability of the cells seemed to be slightly higher. This can be due to two reasons: KBA has some stabilizing effect or removal of benzaldehyde from the media by a faster carboligation reduces the negative influence of the benzaldehyde on the biocatalyst [132, 141].

In order to clarify this point, a second experiment was made. In this case two reactions were recycled during 5 days following the same procedure as before but using different benzaldehyde concentrations. The experimental conditions are described in Table 11. As a control cells in three reaction vials were incubated in buffer without substrates. This was done in order to determine if the activity decay was due to mechanical stress produced by the recycling process or due to compounds involved in the reaction. After this incubation the reaction was started by substrate addition. The experimental setup is described in Table 11 and the results are plotted in Figure 40.
Table 11: Experimental design of the recycling test. The substrate concentrations of each vial are described in the left table. In the right one, the experimental plan is explained: when the reaction is started, when the reaction is analyzed or when the cells are incubated in buffer without substrates.

<table>
<thead>
<tr>
<th>Vial</th>
<th>BA (mM)</th>
<th>KBA (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>150</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
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<td>20</td>
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<tr>
<td>5</td>
<td>20</td>
<td>150</td>
</tr>
</tbody>
</table>

<table>
<thead>
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<th>Cycle</th>
<th>Vial</th>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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</thead>
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<td>Analysis</td>
<td>Analysis</td>
<td>Analysis</td>
<td>Analysis</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Start</td>
<td>Analysis</td>
<td>Analysis</td>
<td>Analysis</td>
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<tr>
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<td>Buffer</td>
<td>Buffer</td>
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<td>Start</td>
<td>Analysis</td>
</tr>
</tbody>
</table>

Figure 40: Decay of the relative yield of PPC after different reaction cycles. The missing points corresponds to the cycles were the cells were incubated in buffer without substrates as is explained in Table 11. The reaction was performed in 50 mM PK buffer at pH 7, with 2.5 mM of MgSO$_4$, 0.1 mM ThDP, and 500 rpm with a cell concentration of 15 mg/mL of wet cell. Experiments were performed in duplicate.

As it can be clearly seen that the addition of extra benzaldehyde has just some minor effect on the catalyst recyclability. Further the data show that incubation of the cells in buffer without substrates is actually more harmful than to run the reaction. This indicates that the mechanic stress of the centrifugation and the washing step is highly harmful for the cell.

Due to the strong decay in biocatalyst performance, recycling is not an advised option. It is possible that other methods of recovery, such as microfiltration, can be less aggressive for the catalyst but that option has not been explored in this work. Another option could be the use of an alternative *E. coli* strain or a different and more resistant host. In any case, the low
recyclability in aqueous media can be a critical step for industrial applicability so more effort in catalyst stabilization should be considered.

3.3.4. Scale up

Once the reaction was characterized and optimized, the scale of the reaction was increased from 1 mL of reaction in 1.5 mL vials to 170 mL in a 500 mL STR in order to test the scalability of the reaction. In order to avoid purification of large amounts of protein for these up-scale experiments, the reaction was run using whole cells for 24 hours and the results are displayed in the next Figure:

![Course of the carboligation reaction for (S)-PPC production using whole cell biocatalysis in a 500 mL STR reactor operated in batch mode.](image)

Figure 41: Course of the carboligation reaction for (S)-PPC production using whole cell biocatalysis in a 500 mL STR reactor operated in batch mode. The reaction media was 50 mM PK buffer at pH 7 with 2.5 mM MgSO_4_, 0.1 mM ThDP, 333 mM KBA, 17 mM benzaldehyde and 12.5 mg/mL of wet cell to a final volume of 120 mL. The temperature was set up to 30 °C, the pH was controlled with KOH and H_3PO_4 and the stirring speed was set to 150 rpm.

The results of this experiment shown that the use of whole cell in an STR is a feasible option. During the first two hours of the reaction, the product concentration grows almost linearly with a SSTY of 0.43 mM/h*mg of cell (71 mg/l*h*mg cell). The reaction continues until the benzaldehyde is totally consumed, achieving a final PPC concentration of 13.5 mM (81 % conversion) with a final ee of 90 %. This comparatively low ee can be explained by the quick depletion of benzaldehyde, since it was observed (Chapter 3.3.2.2.) that in the presence of low concentrations of this substrate the ee is reduced.

Another problem was the accumulation of the by-product HBP over time, which competes for the available benzaldehyde, thereby limiting the conversion.
Figure 42: Accumulation of the by-product during the course of the reaction in the STR. For details see Figure 41. Due to the unconfirmed identity of the by-product, the peak area measured by the HPLC is given.

Even though this problem can be overcome in mL scale by decreasing the amount of catalyst and reaction time, in large scale those options would limit the final product concentration making the process less economically efficient. Given the initial benzaldehyde concentration of 17 mM and a conversion of 81% into PPC and assuming that no benzaldehyde is lost, it is possible to assume that the by-product maximum concentration is around 3 mM. This by-product accumulation can increase the complexity of the downstream process.

3.3.5. Fed batch

Since the batch reaction in the STR run until the benzaldehyde was fully converted, it was considered that with higher benzaldehyde concentrations higher product concentrations could be achieved. Due to the low solubility of benzaldehyde it is not possible to increase its concentration over 50-60 mM without generating an emulsion. As was described in Chapter 3.3.2.2., an emulsion of benzaldehyde decreases the catalyst performance so this approach should be avoided. There are two ways to overcome this challenge: addition of an organic solvent or the use of a fed strategy. As was explained before, the addition of a water-soluble organic solvent (DMSO in this work) has been proven to be inefficient. Thus, the use of a fed-batch strategy was the only possible option.
Figure 43: Course of the carboligation reaction for \((S)\)-PPC production using whole-cell biocatalysis in a 500 mL STR operated in fed-batch mode. The reaction media consisted in 170 mL of 50 mM PK buffer at pH 7, with 2.5 mM MgSO$_4$, 0.1 mM ThDP, 471 mM KBA, 47 mM benzaldehyde and 18 mg/mL of wet cell as catalyst. Pure benzaldehyde was pumped with a rate of 100 µl/h (0.1 g/h). The tank was set up at 30°C, 150 rpm and the pH 7 controlled with KOH and H$_3$PO$_4$.

The results in Figure 43 show that the product concentration rises almost linearly during the first five hours of the reaction with a SSTY of 0.6 mM/(h*mg cells) (99 mg/(h*l*mg cells)), which is 39 % increase of the SSTY achieved in the batch process. Additionally, the final concentration of PPC is 63 mM (10.3 g/l). Also the final product ee was 93.6 % which is 3.6 % better than obtained in the batch process. This can be due to the fact that the benzaldehyde concentration is kept high, avoiding ee decrease by substrate depletion as it was explained in Chapter 3.3.2.2. Nevertheless, the batch process presented a better conversion (81 %) meanwhile the fed-batch only reached a 33 % of conversion due to the high amount of benzaldehyde which was added (around 185 mM).

A closer look to the data in Figure 43 demonstrates that the speed of the fed is not optimal: in the first hours, the reaction is faster than the feed, decreasing the benzaldehyde concentration in the media. After some time, the reaction slows down and the substrate accumulates. This decrease in reaction speed can be due to inactivation of the catalyst either by contact with benzaldehyde or by the stirring of the tank, which would be consistent with the results presented in Chapter 3.3.3. about the mechanical sensitivity to the cells. Another option would be a depletion of KBA. Even though the cell can probably metabolize KBA, the concentration of this compound is in high excess (471 mM), so this possibility is not as probable. A third option could be product inhibition. This extreme has not been observed so far, but the concentrations achieved in this experiment are higher than those obtained in any small-scale experiment before.
Another advantage encountered in this operation mode was that the accumulation of the by-product was smaller than expected: even though the by-product area increased 1.7 times respect the one in the batch process (Figure 44), the product concentrations was 4.5 fold higher, meaning that the relative concentration of byproduct was smaller in the fed-batch process than in the batch one.

Figure 44: Accumulation of by-product in the STR when PPC production is performed with whole cells in batch mode (blue) or in fed-batch mode (red). For further details see legends of Figures 41 and 43.

Since a fed-batch strategy results in high product concentrations, the production of (S)-PAC was also tested in order to evaluate if the production process can be extrapolated to other (S)-hydroxy ketones. The reaction conditions were the same as the ones used for (S)-PPC production but the substrate used was pyruvate instead of KBA. Results are displayed in Figure 45.
Figure 45: Course of the carboligation reaction for (S)-PAC production in a 500 mL STR operated in fed-batch mode with a benzaldehyde feeding rate of 0.1 g/hour in a 170 mL volume. The reaction conditions were the same as explained in figure 43 with the difference that 500 mM pyruvate was used instead of KBA.

The final PAC concentration was 156.4 mM (23.4 g/l), which is 2.5 fold the amount of PPC produced under equivalent reaction conditions. Also the conversion is 84 % which is even higher than the 81 % achieved in the batch production of (S)-PPC reported previously in chapter 3.3.4. Since the reaction is not linear after the second measurement due to the rapid benzaldehyde depletion, the calculation of the SSTY is not very reliable. Even though such a high product concentration was achieved, the ee of (S)-PAC was only 43 % which is much too low to be technically useful. Rother et al. [55] described that the ee of (S)-PAC is lower than the (S)-PPC when purified ApPDCE469G is used, (Figure 14 in Chapter 1.8.) and the difference seems to be even higher when whole cells are used. Another reason for such a low ee can be the depletion benzaldehyde in the first hours of the reaction, overcoming the capacity of the feed. The use of higher feed rates could overcome this problem and help to achieve even higher product concentrations. Also in this case, the by-product formation was observed. In this case, the by-product considered to be hydroxy propiophenone (HPP).

This experiment proved that by using a feed strategy it is possible to overcome the problems produced by the low benzaldehyde solubility, achieving product concentrations above the industrial minimum. Nevertheless, the production of by-product is an important challenge. This can be overcome by using another E. coli strain with different background metabolism or by using pure enzyme. Also in the case of (S)-PAC formation, the ee of the product was lower than expected. In case that optimizing feeding rate to avoid substrate depletion does not solve the problem, this process would not be useful for the production of this compound. In order to solve this issue, further protein engineering should be done or another way has to be found to increase ee. One possibility will be described in the next chapter.

3.4. Chiral polishing

Chiral polishing is a process developed during this work that can be used to improve the ee of (S)-PAC derivatives. As it was explained in the introduction, when the carboligation involves one or more aromatic substrates, the main part of the ThDP-dependent enzymes are (R)-selective. One of these, BAL, is able to produce (R)-HPP and (R)-benzoin with high ee by carboligation of acetaldehyde and benzaldehyde but it is also able to catalyze the backwards reaction, cleaving an (R)-hydroxy ketone in two aldehydes. Interestingly, BAL does not accept (S)-hydroxy ketones as substrates and thanks to this characteristic, this enzyme can be used for production of (S)-hydroxy ketones, like (S)-benzoin [142], via racemic resolution. Nevertheless, since the benzaldehyde is favored as carboligation
substrate, the reaction equilibrium has to be shifted by addition of large amounts of acetaldehyde.

In this work, the combination of BAL with ApPDCE469G was used in order to enhance the ee of the (S)-hydroxy ketones produced by fed-batch: PPC and PAC. Since BAL is not able to accept α-ketoacids as substrates [143], reaction equilibrium could be shifted by adding pyruvate or KBA. In that way, BAL would cleave (R)-PAC or (R)-PPC into benzaldehyde and an aliphatic aldehyde (acetaldehyde in case of PAC and propanal in case of PPC) and ApPDCE469G would use that benzaldehyde as a substrate together with the additional α-ketoacid (Figure 46). This procedure differs from racemic resolution in the starting material: the resolution substrate is a racemic mixture and the yield is limited to a 50 %. In this method the starting material is already asymmetric and the ee is “polished” without loss of any product to a theoretical yield of 100 %.

![Chemical reaction diagram](image)

Figure 46: Principle of chiral polishing used in this work: ApPDCE469G uses benzaldehyde and KBA in order to catalyze the synthesis of (S)-PPC in excess. (R)-PPC is then cleaved by BAL yielding benzaldehyde and propanal which can be reused by ApPDCE469G.

The proof of concept was done by using (S)-PAC as starting material, which was synthetized as described in the Materials and Methods section. This compound was chosen instead of (S)-PPC due to the higher economical interest and the fact that the ee of (S)-PAC after carboligation is much lower than of (S)-PPC, so any changes would be more clearly visible. Enzymatically produced (S)-PAC with an ee of 68 % was added in two different concentrations to a reaction vial containing BAL and ApPDCE469G as well as pyruvate in a 20-fold excess relative to (S)-PAC in order to enhance the carboligation speed (Figure 47).
Figure 47: Development of the ee over time of different concentrations of (S)-PAC using the chiral polishing technique. The reaction media was 50 mM PK buffer at pH 7 with 0.1 mM ThDP, 2.5 mM of MgSO₄ at 30°C and 500 rpm. PfBAL and ApPDCE469G were added in concentrations of 2.2 and 2.1 mg/mL respectively. Pyruvate concentration was 20 times higher than the (S)-PAC concentration: 200 mM for the 10 mM (S)-PAC sample and 400 mM for the 20 mM one.

As can be clearly seen, the ee of (S)-PAC increased steadily over time up to a 98.1 % in less than 24 h. The reaction with a lower starting concentration of (S)-PAC was faster but both of them reached approximately the same level. The use of this technique proved to be useful for production of (S)-PAC and (S)-PPC (data not shown) and it can probably be used for the production of other (S)-hydroxy ketones which are not available via asymmetric synthesis or which show too low ees.

3.5. Process metrics and cost calculations

In order to assess the industrial applicability of a biocatalytic production process, several aspects of the process performance are taken into account. Apart of the product concentration of > 1 g/L barrier [27] another important parameter is the amount of product obtained per gram of catalyst. As a rule of thumb, this number has to be over 1000 g/g of pure enzyme or over 15 g/g of whole cell [16] due to the cheaper production of this catalyst format. Also the space time yield (STY) should be over 0.1g/l*h ([27]). This ensures that a minimal product concentration is achieved in a reasonable time. Additionally, for pharmaceutical products, the maximum amount of impurities allowed by the FDA is 1 %. Since the non-desired enantiomer is considered a by-product, in absence of another undesired compounds the final ee of a pharmaceutical product is required to be over 98 %. In any case, if the product is not a pharmaceutical, the enantiomeric purity is not so critical.
In order to calculate this, the data from the fed-batch experiment were used since running the reaction in a 0.5 L STR is the closest experiment to a pilot plant that has been performed in this work. Due to the low recyclability shown by the cells, it was considered that only one batch was run with one cell batch, and then the catalyst was discarded. The results were calculated for (S)-PAC and (S)-PPC and the results are compiled in Table 12.

Table 12: Comparison of productivity data from 0.5 L STR data for the production of (S)-PAC and (S)-PPC with minimal metric values for production of fine chemicals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Minimal value for industrial feasibility</th>
<th>(S)-PAC</th>
<th>(S)-PPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product concentration</td>
<td>1 g/L [27]</td>
<td>23.4 g/l</td>
<td>10.3 g/l</td>
</tr>
<tr>
<td>Product per catalyst</td>
<td>15 g/g (whole cell) [16]</td>
<td>1.3 g/g</td>
<td>0.6 g/g</td>
</tr>
<tr>
<td>ee</td>
<td>98 %</td>
<td>43 %</td>
<td>93.6 %</td>
</tr>
<tr>
<td>STY</td>
<td>0.1 g/l*h [27]</td>
<td>1 g/l*h</td>
<td>0.5 g/l*h</td>
</tr>
</tbody>
</table>

Using the presented method, the final product concentration and STY achieved are several times higher than the minimum required for industrial production of fine chemicals. Nevertheless, there are still two points where the prerequisites are not met:

- **Product per catalyst:** in both cases (PAC and PPC), the amount of product obtained from a gram of catalyst is one or two orders of magnitude below the minimum required. In order to overcome this critical problem, the feeding speed of benzaldehyde can be optimized in order to produce higher yields and the catalyst should be recycled. Due to the strong inactivation of the whole cells (chapter 3.3.2.2.), which was mainly caused by the treatment of the cells during recovery, a less inactivating catalyst recovery should be used. Therefore, whole cell coating and/or immobilization should be evaluated to increase the catalyst half-life.

- **ee:** As was explained in the Introduction (Chapter 1.4.4.), the minimal ee required for a pharmaceutical product is 98 %. Since none of the produced compounds is known to be used as drug, this limitation should not be applied. If the product is used as a building block (as the PAC is used for pseudoephedrine production), the ee of the final product will depend on how efficient the subsequent steps of the drug synthesis are. In any case, as it was mentioned before, the ee of the (S)-PAC can probably be enhanced by optimizing the feeding speed, but is probably never going to reach values over 98 % due to the limitations of the catalyst. An efficient downstream method, like the chiral polishing method (chapter 3.4.), can overcome this problem but a more cost efficient solution would be to modify the catalyst by enzyme engineering or the development of new variants of different ThDP dependent enzymes such as *Escherichia coli* 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase (MenD) [144].
The data from the pure enzyme was not taken into account for the metrics calculations due to several reasons: the reaction always took place in batch mode, the longest reaction time measured at optimal conditions was 8 hours, and due to the lack of immobilization, enzyme recovery would have been challenging. Nevertheless, the ee of the (S)-PPC produced by pure enzyme was clearly higher and no by-product was detected, which are two advantages which may contribute to compensate for the higher production cost for a pure enzyme.

Despite the huge improvement in (S)-PAC derivatives production achieved in this work, there are still two factors which should be further improved for full industrial feasibility. Due to the unavailability of the produced compounds, this method seems to be an interesting option for lab scale production of (S)-PAC derivatives. Further enzyme modification, the development of an efficient catalyst recyclability method and optimization of the feeding rate for benzaldehyde could overcome the mentioned hurdles and bring this production process closer to market.

Using the data obtained from the fed batch experiments, the product price per gram was calculated in 13.7 €/g for the PPC and 1.7 €/g for the PAC. This calculation only takes into account the costs for the buffer salts, the cofactors, and the substrates. If the price of the catalyst is assumed to be a maximum of 100 € for kg of whole cell [66], the catalyst price would increase the product price by 0.17 € for PPC and 0.08 € for PAC, making the α-ketoacid the main contributor to the product cost (95 % of the PPC and 81 % of the PAC final costs).

The product costs in industrial scale could be significantly lower than described in this work, since all costs were calculated based on Sigma Aldrich prices and bulk chemicals can be remarkably cheaper. Even though this calculations do not take into account equipment costs, the energy or the labor used, the market prices for these specific α-hydroxy ketones are in the several hundred euros per gram range (i.e. (S)-benzoin provided by Sigma Aldrich 1920 €/g), giving plenty of space for cost effective production.

4. Conclusions and future perspectives

In this work, all the different parts of a biocatalytic production process for (S)-2-hydroxy ketones have been assessed on the example of (S)-selective PPC synthesis.

Upstream: the catalyst availability is a critical point in the process applicability. Therefore, protein expression was optimized, finding the optimal cultivation conditions to be 20 °C in autoinduction medium for 48 hours. Compared with the previously published cultivation procedure [55], the use of these optimized conditions increased the enzyme yield 27-fold.
Even though the catalyst production was clearly enhanced, the use of other induction conditions could be useful to increase also the production of intracellular ThDP. This would solve the problem of cofactor scarcity and decrease the production costs by making external cofactor addition unnecessary.

Carboligation reaction: the use of an $\alpha$-ketoacid instead of an aldehyde for the production of $(S)$-hydroxy ketones has been proven to be an interesting option since it results in higher product yields, higher $ee$, and decreases the formation of aliphatic $\alpha$-hydroxy ketones (acetoin, propioin) as by-products. Using $\alpha$-ketobutyric acid, the reaction conditions for the production of $(S)$-PPC were optimized using an iterative approach. The result of this procedure was an increment in 10 % of the $ee$ up to a 98 % and an increase in the SSTY over 60-fold compared to published data [55].

Also the use of whole cells was assessed, proving to be an extremely productive option. The reaction with this format of catalyst was optimized and scaled up into a 500 mL STR. It was proven that the use of a fed-batch strategy with continuous feed of benzaldehyde overcomes the problem of its low solubility, increasing the product yield over the 10 g/L barrier in less than 24 h, thereby keeping the $ee$ of the product $(S)$-PPC over 90 %. This technique was used also for the production of $(S)$-PAC producing even higher yields (over 20 g/L). Nevertheless, the $ee$ of this product was with 43 % far from optimal.

Even though a lot of work has been done for reaction optimization, there are still some options which can be highly positive for the overall production. The use of faster benzaldehyde feed rates can probably enhance both yield and $ee$ of the products. Additionally, by running the reaction at lower temperature, the $ee$ of the product could be increased. This option can be especially interesting for the PAC production, helping to enhance low enantiomeric purity.

Downstream: a combination of product extraction by using an organic phase and a subsequent flash chromatography for purification, have been proven to be a useful recovery method for the laboratory scale. Nevertheless, other techniques can be more suitable for larger production scales.

A new approach to increase the $ee$ of $(S)$-hydroxy ketones was developed, using a combination of two ThDP-dependent enzymes. This method is similar to dynamic kinetic resolution, but the substrate has a low $ee$ and is not a racemic mixture. One of the enzymes (BAL) cleaves the unwanted $(R)$-enantiomer into the original substrates (benzaldehyde and acetaldehyde/propanal), and the second enzyme (ApPDCE469G) uses them for production of the desired $(S)$-enantiomer. This technique has been applied successfully to $(S)$-PAC, increasing the $ee$ from a 68 % up to a 98 % in less than 24 h. A further optimization of this procedure as well as the use of whole cell catalysts could be extremely interesting. Also the application for the production of different $(S)$-$\alpha$-hydroxy ketones can broaden the product platform accessible by the ThDP dependent enzymes.
Further, recycling of the whole cell biocatalyst was tested, but the stability of the cells was proved to be quite low, with a decrease of a 65% of activity in just one reaction cycle. Here further investigations including the coating and/or immobilization of the cells are necessary. This particular point is of special interest since the recyclability of the catalyst is a key point for the development of a cost-effective biocatalytic production process.

Econometric evaluation of the process has shown that even though major improvements on productivity had been achieved, like product concentrations over 10 g/L, some parameters still need to be improved in order to produce an industrially ready production process. Probably the most critical point is the amount of product obtained per gram of catalyst, which due to the low recyclability of the cells, is below the industrial requirements (see Table 12). As was mentioned before, in order to overcome this hurdle, a better recycling system and catalyst stabilization should be explored. Nevertheless, due to difficulties of the synthesis of these (S)-α-hydroxy ketones, this process is a good starting point for lab scale production.

In conclusion, the synthesis of (S)-α-hydroxy ketones with the developed production process was shown to be feasible in laboratory scale. There are several potent options to increase the process output without further protein modification. As a general conclusion, the use of an α-ketoacid instead of the corresponding aldehyde as donor and high concentrations of benzaldehyde as the acceptor in a fed-batch reactor using whole cells is probably the most efficient option for the production of this family of compounds.

5. References:


Annex
Conditions tested for the multiparametric reaction optimization

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<td>0,1</td>
<td>96,36 ± 0,04</td>
<td>9,78 ± 0,77</td>
</tr>
<tr>
<td>VIII</td>
<td>30</td>
<td>420</td>
<td>7</td>
<td>42</td>
<td>0,1</td>
<td>96,37 ± 0,02</td>
<td>9,21 ± 0,88</td>
</tr>
<tr>
<td>VIII</td>
<td>35</td>
<td>120</td>
<td>7</td>
<td>42</td>
<td>0,1</td>
<td>96,21 ± 0,01</td>
<td>9,16 ± 0,29</td>
</tr>
<tr>
<td>VIII</td>
<td>35</td>
<td>360</td>
<td>7</td>
<td>42</td>
<td>0,1</td>
<td>96,33 ± 0,05</td>
<td>9,41 ± 0,25</td>
</tr>
</tbody>
</table>

Jülich, den 13. Oktober 2013