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Development of Dynamic Kinetic Resolution Processes for Biocatalytic Production of Natural and Nonnatural L-Amino Acids

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Abstract:

Two different dynamic kinetic resolution processes for the production of a number of natural and nonnatural L-amino acids at 100% chemical and optical yield have recently been established at Degussa. The first process is based on the dynamic kinetic resolution of 5-monosubstituted hydantoins using tailor-made whole-cell biocatalysts coexpressing a L-carbamoylase, a hydantoin racemase, and a hydantoinase. The hydantoin-converting pathway was optimized by adjusting expression levels of the respective enzymes as well as by inverting the enantioselectivity of the D-selective hydantoinase. This resulted overall in a 50-fold improved productivity and significant reduction of biocatalyst cost. The second process is based on the dynamic kinetic resolution of *N*-acetyl amino acids using an acylase in combination with a novel racemase from *Amycolatopsis orientalis* subsp. *lurida*. This racemase could overcome the problem of substrate inhibition and requirement of high concentrations of divalent metal ions which limits the use of other *N*-acylamino acid racemases described in the literature.

1. Introduction

The worldwide sales of formulated chiral drugs in single-enantiomer dosage forms is growing at an annual rate of 13% and reached more than $130 billion (U.S.) in 2000 as shown in Figure 1. This growth is expected to continue as more and more drugs are developed which target enzymes, hormones, or receptors. In these cases chirality plays a crucial role in the desired interactions. Pharmaceutical as well as fine chemicals companies focusing on the production of drugs and intermediates are therefore challenged to devise new chiral technologies and improve enantioselective processes.[1]

Today, classical diastereoisomeric crystallization of salts and kinetic resolution are still the most prevalent methods of separating enantiomers on a commercial scale. However, these methods are limited to only 50% yield of the desired enantiomer. If the other enantiomer is not required, which is mostly the case, 50% of the starting material is either wasted or needs to be recycled in an additional racemization step. Waste disposal as well as costly racemization and additional separation steps strongly compromise the economies of these processes and represent excellent targets for the development of superior processes.

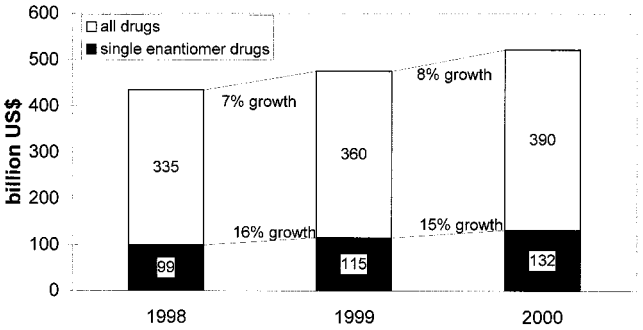

Figure 1. **Global sales of all drugs and single enantiomer drugs between 1998 and 2000. Source: *Chem. Eng. News* 2002, *78*, 45−57; *Chem. Eng. News* 2001, *79* 79−97.**

Approaches that yield optically pure products at up to 100% chemical yield such as the use of the chiral pool,[2] asymmetric syntheses,[3] and dynamic kinetic resolution of racemic mixtures[4,5] are therefore highly competitive and often superior. In this context, the use of biocatalysts has found widespread application and is rapidly moving from an "expensive enabling tool" toward a "lowest-cost" approach.[6] A key factor for the successful development of biocatalytic processes is fast access to inexpensive biocatalysts at large scale with suitable properties such as high activity, high selectivity, and high stability. Unfortunately, industrial enzymes are often not available off-the-shelf for a desired reaction or show properties incompatible with industrial demands. However, the application of recombinant DNA technology allows us to rapidly screen[7] and design biocatalysts[8] for new applications.

Here we provide two recent examples for the integration of biological tools such as design and screening of novel biocatalysts in the development of dynamic kinetic resolution processes for the production of optically pure amino acids which are used in infusion solutions, as feed and food additives, as intermediates for pharmaceuticals, cosmetics, and pesticides, and as chiral synthons in organic synthesis.[9]

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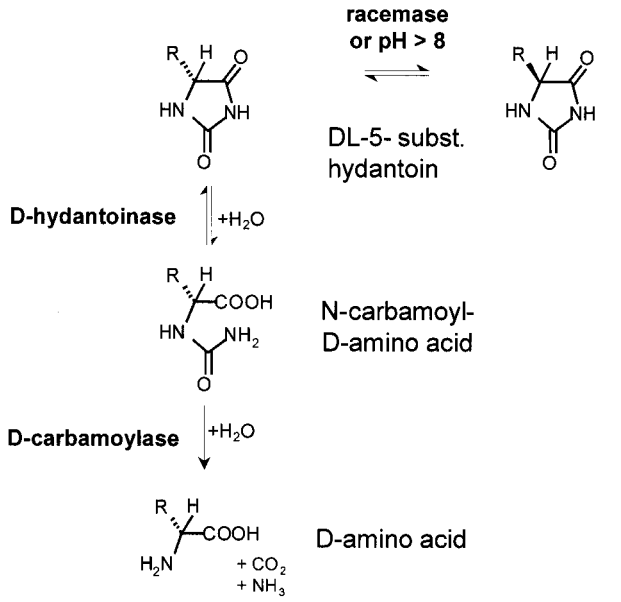

Figure 2. **Reaction scheme of the D-hydantoinase process.**

The first example discusses the design of a whole-cell biocatalyst for the production of L-amino acids based on the hydantoinase process. The second example shows how the currently applied acylase processes can be dramatically improved by integrating a new racemase made available through powerful molecular screening technologies.

2. L-Hydantoinase Process Optimization by Design of a Recombinant Whole-Cell Biocatalyst Using Directed Evolution

The hydantoinase process of which the involved reactions are shown in Figure 2 was first introduced in the 1970s for the production of D-amino acids such as D-phenylglycine and *p*-OH-phenylglycine.[10] Today, it is commercially applied at a scale of >1000 tons per year for the above-mentioned amino acids which are used as side chains for the β-lactam antibiotics ampicillin and amoxicilin.

As 5-monosubstituted hydantoins racemize spontaneously or are enzyme-catalyzed[11,12] under biotransformation conditions, a 100% yield of optically pure D- or L-amino acid can be reached. Both straightforward synthesis of racemic 5-monosubstituted hydantoins from cheap starting materials through the reactions shown in Figure 3 and low byproduct formation add to the attractiveness of the hydantoinase process for the industrial production of optically pure natural and nonnatural amino acids.[13]

Despite a number of reports of strains with L-selective hydantoin-hydrolyzing enzymes[14−16] the commercial application of the hydantoinase process is still limited to the production of D-amino acids. Processes for the production of L-amino acids are limited by low space−time−yields and high biocatalyst costs. Driven by the attractiveness of the hydantoinase process Degussa has started a R&D program to establish a commercially feasible process for the production of natural and nonnatural L-amino acids. Despite initial progress in reducing the biocatalyst production cost, increasing the activity of the biocatalyst and improving the space−time−yield[17,18] process economics were still prohibitive for commercialization of this process especially for low priced amino acids such as L-methionine.

Recently, a new generation of a L-hydantoinase process was developed based on a tailor-made recombinant whole-cell biocatalyst. Further reduction of biocatalyst cost by use of recombinant *Escherichia coli* cells overexpressing a hydantoinase, carbamoylase and hydantoin racemase from *Arthrobacter sp.* DSM 9771 were achieved. To improve the hydantoin-converting pathway expression levels of the different genes were balanced on the basis of differences in the specific activities of the enzymes. This has been accomplished by using different gene doses coding for the respective enzymes similar to the strategy provided by Wilms et al.[19] This highly active recombinant whole-cell biocatalyst could be produced in high-cell density fermentation at m^3-scale at concentrations above 50 g/L dry cell weight which further reduced the biocatalyst production cost compared the original *Arthrobacter* strain.

Despite this progress the D-selectivity of the hydantoinase towards different substrates[20] was still limiting its application for a number of products. For example, for some aliphatic hydantoins such as DL-methylthioethylhydantoin (DL-MTEH) the D-enantiomer is hydrolyzed much faster by the "L"-hydantoinase from *Arthrobacter* than the L-enantiomer. Due to this D-selectivity of the hydantoinase the conversion of DL-MTEH leads to a strong accumulation of *N*-carbamoyl-D-methionine during the course of the reaction carried out with a wild-type whole cell biocatalyst. In principle, the accumulated *N*-carbamoyl-D-methionine slowly converts into L-methionine due to the reversibility of the hydantoinase reaction and the coupled hydantoin racemization and L-specific decarbamolyation, but the space−time−yield of the L-hydantoinase process for the desired 100% conversion of the hydantoin into the L-amino acid is rather low.[21] To further improve the space−time−yield of this process, an L-selective hydantoinase was required. As screening did not provide better hydantoinases we tried to invert the enantioselectivity of the hydantoinase by directed evolution. Indeed, we could demonstrate for the first time that inversion of enantioselectivity can be rapidly achieved by this approach.[22] The mutants shown in Table 1 differing in only two effective amino acid substitutions showed remarkable differences in

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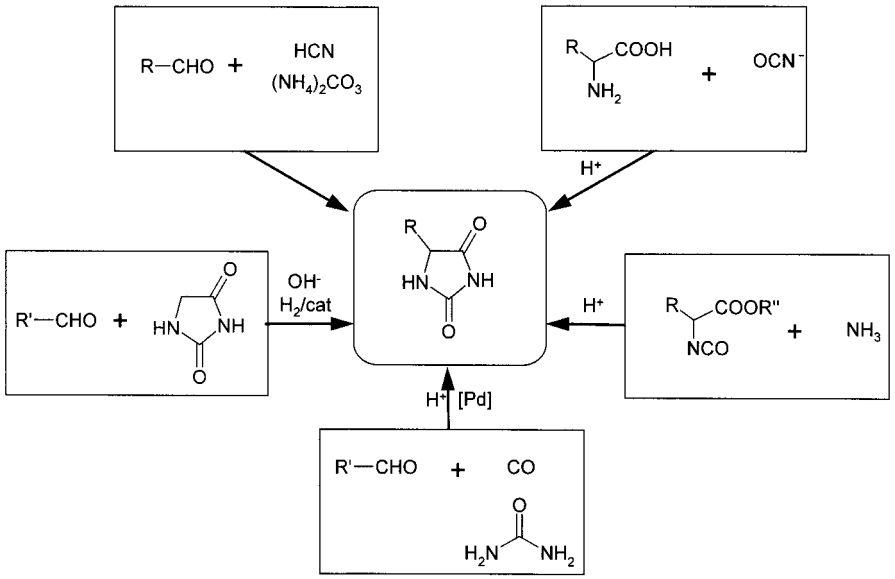

Figure 3. **Different routes for the synthesis of 5-monosubstituted hydantoins.**

Table 1: **Mutants created by directed evolution and their effect on the hydantoinase properties**

clone name	mutation	effect of mutations compared to wild-type
11DH7	L95I, Q251R[a]	reduced D-selectivity
1CF3	V154A	increased D-selectivity
22CG2	L95I, Q251R, V180A	reduced D-selectivity and increased activity
Q2H4	L95F, Q251R, V180A	L-selectivity and increased activity

[a] Mutation Q251R was found to be neutral as its introduction to wild-type did not change the enzyme properties.

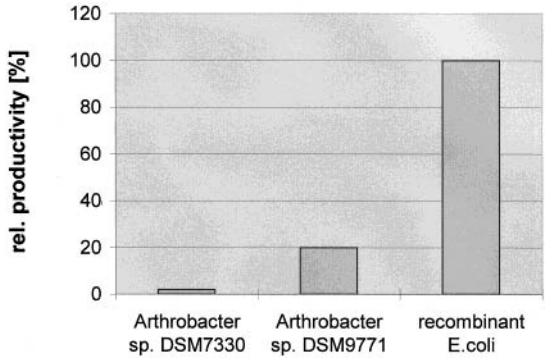

Figure 4. **Comparison of the relative productivities (g L^{-1} h^{-1}) for L-methionine production with the wild-type strains *Arthrobacter* sp. DSM 7330, *Arthrobacter* sp. DSM 9771 after process optimization and the recombinant *E. coli* coexpressing an evolved L-hydantoinase, a L-carbamoylase, and a hydantoin racemase.**

enantioselectivity. One mutant (1CF3) produced the D-enantiomer at an enantiomeric excess of 90%, whereas an L-selective mutant (Q2H4) produced the opposite enantiomer with 20% ee at 40% conversion. Although the L-selectivity of the designed enzyme was not impressive and leaves room for further improvements, the productivity of the process could be dramatically improved as shown in Figure 4 These improvements have been confirmed at m^3 scale using a simple batch reactor concept coupled to a continuous

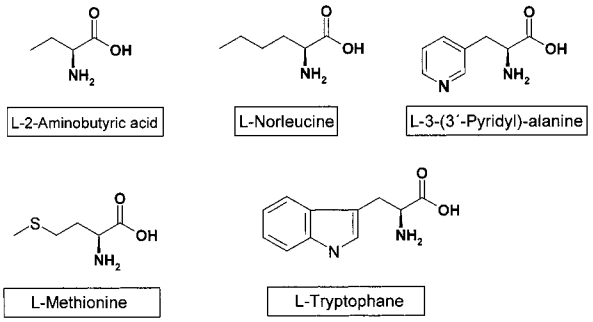

Figure 5. **Examples of natural and nonnatural L-amino acids available from the L-hydantoinase technology.**

centrifuge for cell separation. A new platform technology for the production of a large variety of optically pure natural and nonnatural L-amino acids at a theoretical 100% chemical and optical yield has therefore been established for which some examples of accessible amino acids are given in Figure 5.

3. L-Acylase Process Optimization by Introduction of Novel Racemases Found by Genetic Screening

The acylase process was established at Degussa in the 1970s for the production of L-methionine and other proteinogenic and nonproteinogenic L-amino acids such as L-valine, L-phenylalanine, L-norvaline or L-α-aminobutyric acid. Currently, several hundred tons per year of L-methionine are produced by this enzymatic conversion using an enzyme membrane reactor.[23]

The starting material for the acylase process is a racemic mixture of *N*-acetyl-amino acids which are chemically synthesized, for example, by acetylation of DL-amino acids with acetyl chloride or acetic anhydride in alkali in a Schotten−Baumann reaction.[24] The kinetic resolution reaction of *N*-acetyl-DL-amino acids is achieved by a stereo-

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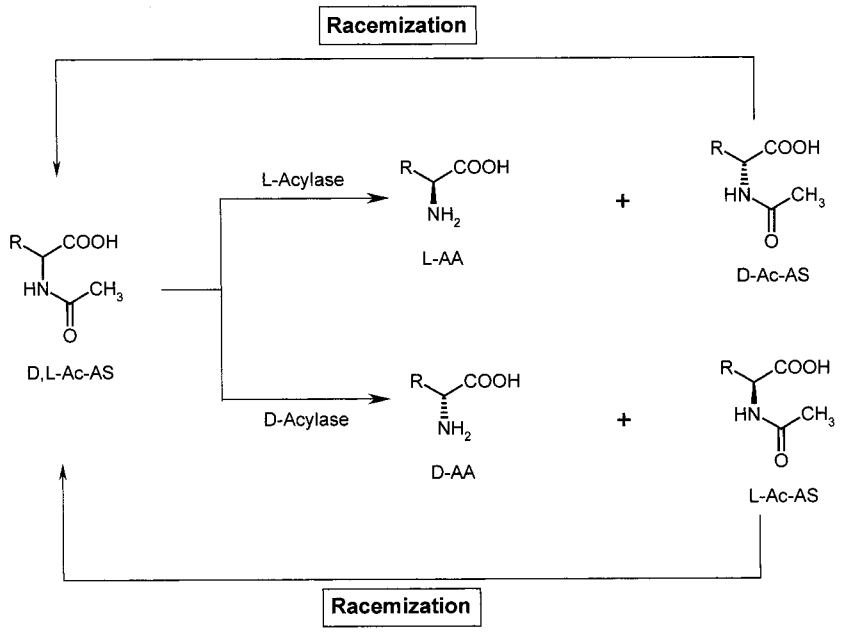

Figure 6. **Reaction scheme of the acylase process.**

specific L-acylase from *Aspergillus oryzae* which hydrolyzes only the L-enantiomer and produces a mixture of the corresponding L-amino acid, acetate, and *N*-acetyl-D-amino acid. After separation of the L-amino acid by a crystallization step, the remaining *N*-acetyl-D-amino acid is recycled by thermal racemization under drastic conditions (Figure 6). D-Amino acids are also accessible by chemical hydrolysis of the *N*-acetyl-D-amino acid or directly by use of D-selective acylases. Selective in situ racemization of *N*-acetyl-amino acid could dramatically improve the acylase process by eliminating costly racemization and separation steps. Therefore a number of companies have screened for *N*-acylamino acid racemases.[25,26]

Such an *N*-acylamino acid racemase (AAR) activity was found by Tokuyama et al. in various actinomycetes strains.[27] The gene for the *N*-acylamino acid racemase from *Amycolatopsis* sp. TS-1-60 was cloned and overexpressed in *E. coli*, and the gene product was characterised.[28,29] The requirement for a high concentration of divalent heavy metal ions (e.g., cobalt ions > 10 mM) for optimal enzyme activity, substrate inhibition at concentrations above 50 mM and inhibition by L-methionine at less than 100 mM severely restrict the use of this enzyme in a commercial process.[30]

To obtain *N*-acylamino acid racemases with superior properties 31 different actinomycetes strains were examined in a genetic screening using PCR and Southern hybridization methods to detect the genes of this enzymes (AAR). A 504-bp DNA fragment from *A. orientalis* subsp. *lurida* was amplified using a PCR approach with degenerated primer pools derived from the highly conserved KXK-motif of the enolase superfamily[31] and from the N terminus of two known *N*-acylamino acid racemases.[28] With this DNA-fragment as probe in hybridization experiments, we could detect and isolate a 2.5-kb *Eco*RI DNA fragment with the *aar* gene from *A. orientalis* subsp. *lurida*. Furthermore, these experiments showed only weak or no signals in 30 other actinomycetes strains.[32]

Cloning and sequencing of a 2.5-kb *Eco*RI DNA fragment from *Amycolatopsis orientalis* subsp. *lurida* revealed the coding gene of a *N*-acylamino acid racemase, which had identities to the racemase gene of *Amycolatopsis* sp. TS-1-

Table 2: **Substrate specificity of *N*-acylamino acid racemase from *A. orientalis* subsp. *lurida*[a]**

substrate	relative activity (%)
N-acetyl-D-alanine[c]	3
N-acetyl-D-aminobutyric acid[c]	11
N-acetyl-D-methionine[b,c]	100
N-acetyl-D-naphthylalanine[c]	0
N-acetyl-D-phenylalanine[b]	76
N-acetyl-D-valine[b]	83
N-acetyl-L-*tert*-leucine[c]	0
N-acetyl-L-methionine[b,c]	130
N-acetyl-L-phenylalanine[b]	30
N-acetyl-L-tyrosine[c]	30
N-acetyl-L-valine[b]	22
N-benzyloxycarbonyl-L-phenylalanine[c]	0
N-chloracetyl-L-phenylalanine[c]	7

[a] The specific activity with *N*-acetyl-D-methionine was taken as 100%. [b] The specific activities were investigated by the standard assay[31] and analyzed by HPLC. [c] Specific activities were analyzed by HPLC or the polarimetric assay or both.

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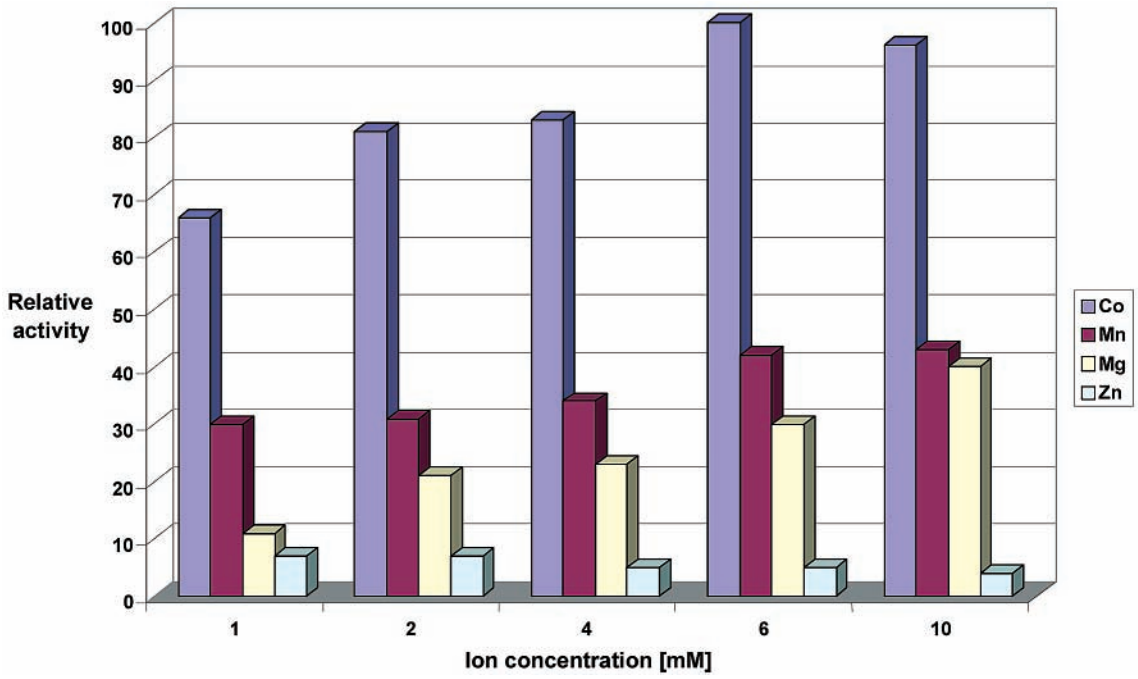

Figure 7. **Effect of various metal ions in different concentrations on the relative enzyme activity of *N*-acylamino acid racemase from *A. orientalis* subsp. *lurida*.**

60[29] of 86% at the level of DNA and 90% at the level of amino acids. The heterologous overexpression in *E. coli* resulted in a specific activity of about 0.2 units/mg (total protein) of this racemase. A two-step purification with heat treatment followed by a anion-exchange chromatography led to nearly homogeneous enzyme.[32]

This *N*-acylamino acid racemase catalyzed the racemization of various industrially important aromatic as well as aliphatic *N*-acylamino acids which are listed in Table 2. The K_m value and the V_{max} calculated from Michaelis−Menten plots were 24 mM and 8 units/mg for *N*-acetyl-D-methionine and 35 mM and 13.7 units/mg for *N*-acetyl-L-methionine. The racemization reaction is subject to substrate inhibition with a K_i of 457 or 398 mM for *N*-acetyl-D- and -L-methionine, respectively. In contrast, L-methionine was no substrate for *N*-acylamino acid racemase (data not shown). The K_m value and the V_{max} were 23 mM and 6.5 units/mg for *N*-acetyl-D-phenylalanine, and 5 mM and 1.5 units/mg for *N*-acetyl-L-phenylalanine, as well as 148 mM and 27.3 units/mg for *N*-acetyl-D-valine, and 42 mM and 2.5 units/mg for *N*-acetyl-L-valine. The racemization of the latter substrates was not subject to substrate inhibition in the tested range of substrate concentrations up to 250 mM.[32]

Further physicochemical characteristics of the purified enzyme of *A. orientalis* subsp. *lurida* were similar to those from *Amycolatopsis* sp. TS-1-60 and *S. atratus*,[28] such as a pI of 4.4, a pH optimum about 8, and stability at 50 °C for 30 min. The M_r of about 300 for the native enzyme and the M_r of 40 for the subunit suggests that the enzyme is a homooctamer.[32]

Figure 7 summarizes the effect of various metal ions in different concentrations on the activity of racemase. *N*-acylamino acid racemase activity was highest with Co^{2+}, followed by Mn^{2+} and Mg^{2+}, whereas Zn^{2+} resulted only in a low level of activity.[32] Interesting for the use of the AAR in a commercial production process is that the enzyme is fairly active at cobalt concentrations below 1 mM.

Another important point for the industrial application of the enzyme is that the AAR from *A. orientalis* subsp. *lurida* exhibits a much lower substrate inhibition (K_i = 400 mM) compared to the enzyme from *Amycolatopsis* sp. TS-1-60 which already shows inhibition above 50 mM.[30] The combination of this new enzyme together with L- or D-specific acylases could expand the, thus far, limited scope of the acylase process and presents a valuable platform for the production of chiral synthons at 100% optical and chemical yield.

4. Conclusions

Modern biological methods such as novel screening technologies, protein engineering by directed evolution, and recombinant expression of enzymes and whole enzymatic pathways are highly promising tools for the development of new biocatalytic processes. Fast access to improved enzymes or enzymes catalyzing novel reactions are expected from them. In addition, these tools enable us to design whole-cell biocatalysts comprising new pathways by combining different enzymes from different sources which can be used to perform chemically often impossible multistep syntheses in a single reactor.

The design presented above of a recombinant whole-cell biocatalyst by directed evolution as well as the introduction of a new *N*-acylamino acid racemase by DNA-based screening are excellent examples that demonstrate the power of these approaches. As a result of this work we could establish two new dynamic kinetic resolution processes for the production of L-amino acids. Obviously, both approaches have a much higher potential than currently used processes

based on classic kinetic resolution such as the acylase or amidase process. Whether the L-hydantoinase process or the combination of an L-acylase with and *N*-acylamino acid racemase is preferred is strongly dependent on the specific case and will be influenced by the biocatalyst properties such as substrate specificity as well as the access to the cheapest substrate and the easiest product isolation. These two "100% optical and chemical yield approaches" provide us with a high degree of flexibility for the production of a large number of different L- or D-amino acids, which is especially important for fast changing targets and short development periods typical for the fine chemicals industry serving the needs of pharmaceutical industry.

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