

AMBIOCAS Description of Main Results/Foregrounds

The project results were developed in each work package (WP) and at each partner in a highly collaborative manner that allowed the supra-disciplinary approach to deliver a number of scientific advances with a symbiotic interaction that enhanced value.

In WP 1: Development of Libraries of Transaminases and HTS Method. Use in cascade reactions A comprehensive set of high throughput screens were developed that enable improved mutants to be identified for any desired target transamination reaction. These then allowed libraries of transaminases to be constructed that were screened to identify the enzymes for the synthesis of new chemical targets. These were then applied in synthetic cascades in WP2. **WP 2: Multi-Enzyme Co-Factor Recycling and Redox Cascades** led to the demonstration of several synthetic multi-enzyme cascades with transaminases as one of the key steps for the synthesis of wide range of targets. An important advance was that co-factor balanced alcohol substrate oxidation-transaminase amination cascade with cofactor recycle was demonstrated. Use of transaminases in organic solvents was also demonstrated for first time. These results were combined for the synthesis of diamines suitable for consumer polymers allowing them to be synthesized from cheap diols. These results were transferred to **WP 3 Application: Cyclohexanol Cascade and Amine Synthesis** where a six step enzymatic cascade to convert the readily available commodity chemical cyclohexanol into 6-aminocaproic acid was demonstrated as feasible. Some other amine synthetic targets were also studied. In **WP 4 Enzyme Design: Enzyme Crystallization and Bioinformatics** transaminase crystal were prepared and structures were obtained to high enough resolutions so that this can be used as models to design improved enzymes.

WP 5 Process Engineering and Scale-Up delivered results by developing scaled-down engineered test rigs that delivered insights into reaction bottlenecks, thermodynamic and kinetic analysis of transaminase reactions that was used to identify bottlenecks. These were applied to the analysis of problem commercial targets and yielded comprehension of the fundamentals of reactions. **WP 6 Enzyme Support Engineering and Application** made supported enzyme formulations with cofactor recycling needed for applications available for industry screening. This was the first time co-factor recycling systems were developed to interact efficiently with supported transaminases. Finally in **WP 7 Economic Modelling, Environmental Impact and LCA** the collaboration developed economic models that can be used identify full process economics and complemented with Life Cycle Analysis that can identify greenhouse gas advantages. These all demonstrated clearly the economic and environmental advantages of the technology developed in AMBIOCAS.

More detailed descriptions of these results are now described.

WORKPACKAGE 1. The development of libraries of ω -transaminases and HTS methods. Use in cascade reactions.

1. New ω -Transaminase Enzyme Genes Identified, Cloned and Libraries Created by Directed Evolution.

It was an early success of the AMBIOCAS project that a growth screen was used to identify 25 micro-organisms with potential transaminase activity and then used to obtain new transaminases. Efforts to identify one of these which was putatively (*R*)-selective, were unsuccessful. Following on from those site saturation mutagenesis libraries were created in several transaminase genes to give libraries of novel genes. These were then screened using the solid phase High Throughput Screens (HTS) developed in the project to identify new novel activities. One of these variants has been investigated for its expanded substrate spectrum in the small binding pocket and offers potential novel activity. Hence platforms of new enzymes were available as a foundation for the AMBIOCAS project.

2. Novel Liquid Phase High Throughput Screens Invented

One problem with identifying improved transaminase enzymes was the lack of suitable methods to efficiently screen large numbers of enzymes.

High throughput screening method was developed for using enzyme cascade to identify specific activities needed to convert target substrates into desired products. This is based on detecting the amino acid product with a coupled amino acid oxidase-horse radish peroxidase colour screen.

A liquid phase micro-titre plate based assay has been developed employing an enzymatic cascade to identify specific activities needed to convert target substrates into desired products. This is based on detecting the amino acid product from substrate deamination with a coupled amino acid oxidase-horse radish peroxidase colour screen. Performed in a spectrophotometer, this screen is ideally suited to assaying isolated transaminase enzymes for activity against a panel of amines and is able to generate quantitative data. By using either an L- or D-AAO this screen is able to cope with either S- or R-selective transaminases (**Figure 1**).

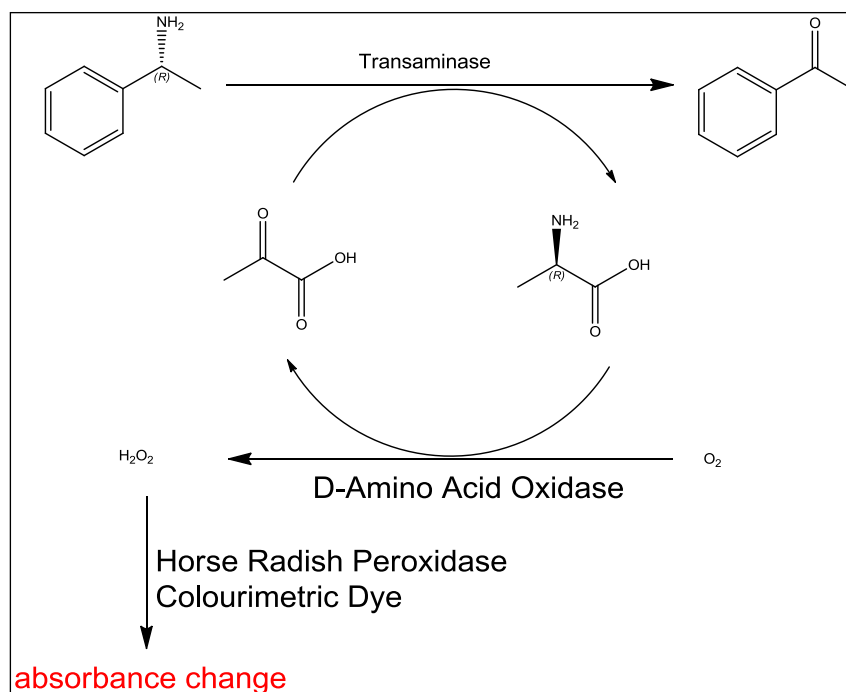


Figure 1: Liquid Phase HTS representation.

3. Solid Phase Screen for (R)-Selective Transaminase

High throughput screening method capable of use for in directed evolution experiments were also developed which were based on the further development of the liquid phase assay. Both the TA and D-AAO are co-transformed together into *E. coli* and this prevents diffusion of intermediates and localise colour change to individual active colonies with high contrast (solid arrow, active colony; hollow arrow, inactive colony in **Figure 2**).

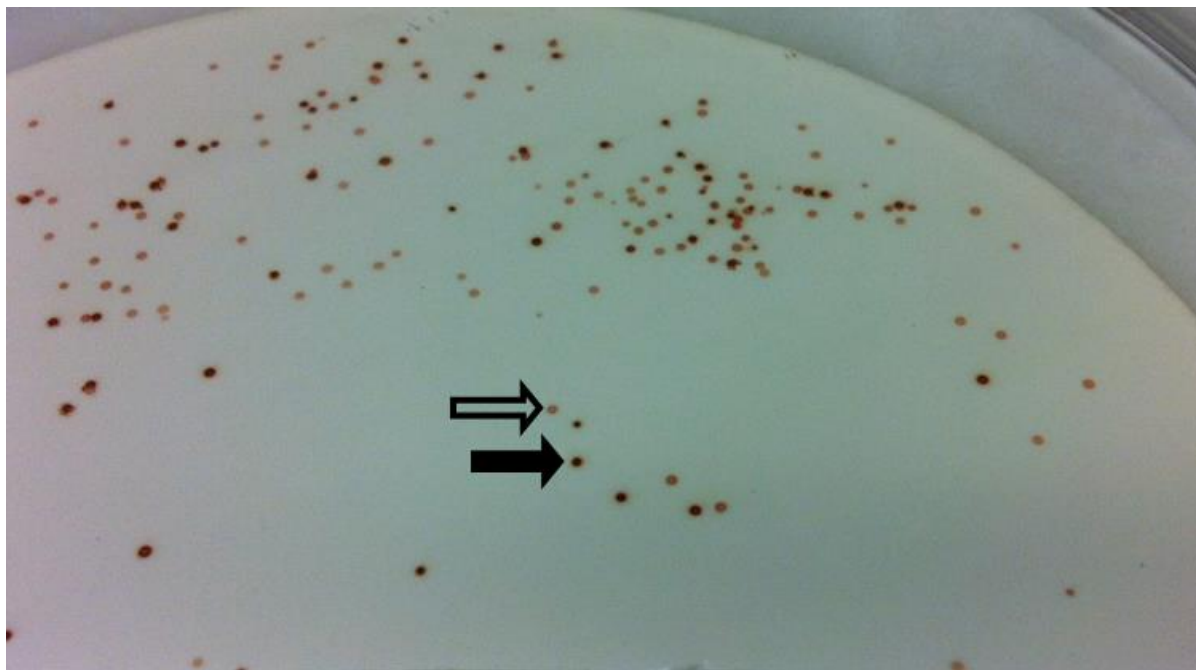


Figure 2: Solid Phase HTS method image.

This was a particularly important breakthrough because solid phase HTS are capable of speedier identification of new enzymes because of its higher capacity than liquid phase methods and this method is capable of identifying the (*R*)-selective enzymes which were very rare at this stage in **AMBIOCAS**.

4. Solid Phase Screen for (S)-Selective Transaminase

A complementary S-selective solid phase high throughput screen has also been developed. To circumvent toxicity associated with an L-AAO, this screen employs an alanine racemase to convert the L-alanine generated by the TA into D-alanine which may be detected with the same D-AAO/HRP cascade as before. It is required to express the alanine racemase on a separate membrane as a continuous 'lawn' of cells to avoid toxicity associated with the co-expression of AlaR and D-AAO. These two solid phase screens are able to screen a large number of protein variants allowing directed evolution of transaminases to be carried out efficiently without the requirement for specialised equipment (**Figure 3**).

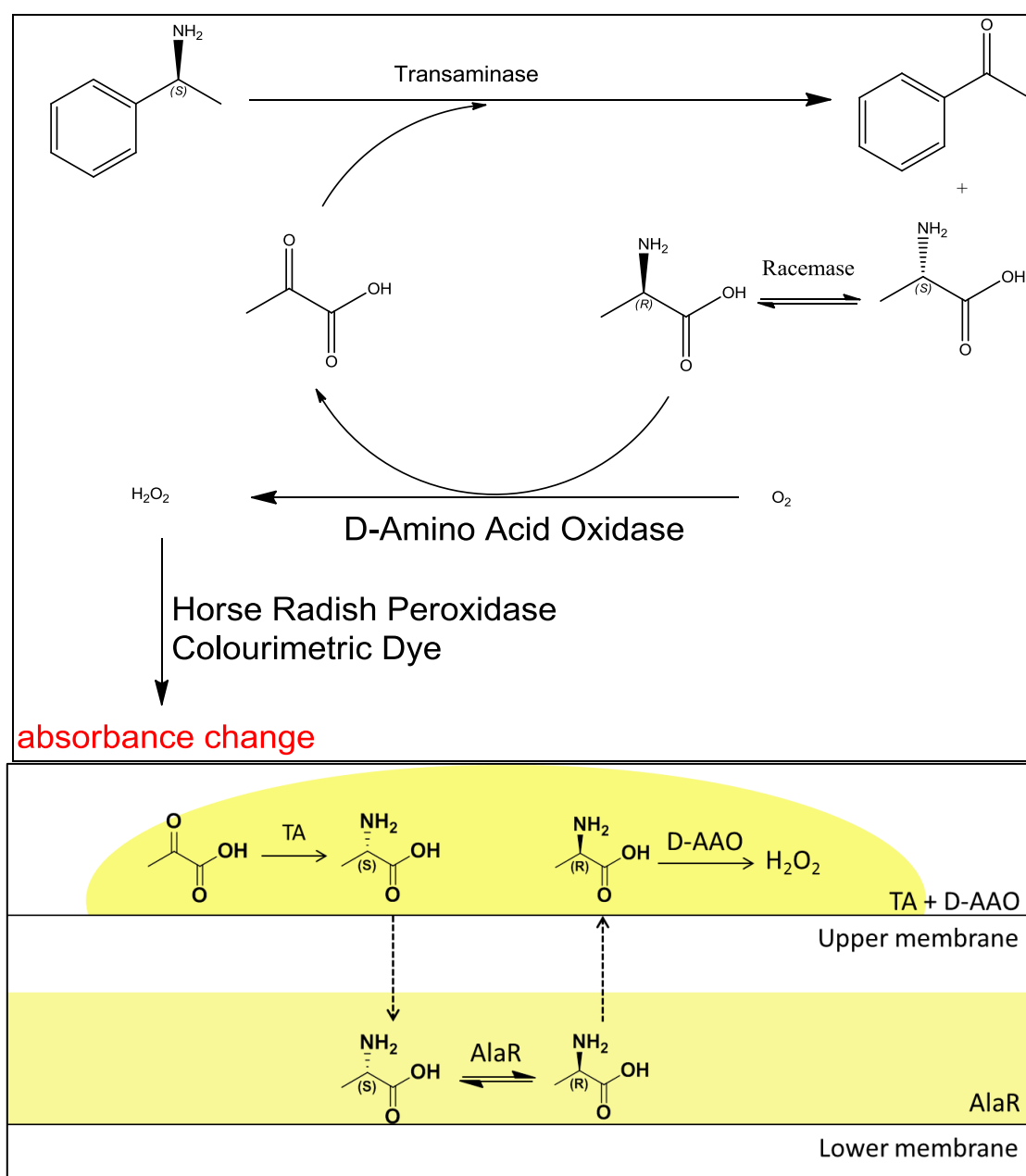


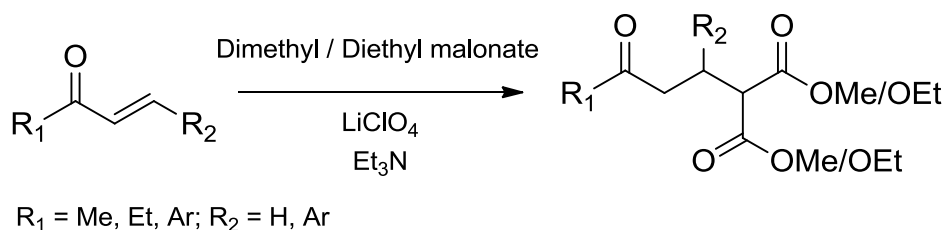
Figure 3: Solid Phase Screen for S-selective Transaminase

5. Key Substrates Synthesized

To be able to investigate the proposed enzymatic cascades to synthesise heterocyclic compounds that were of interest as building blocks for pharmaceutical syntheses it was first necessary to synthesise the substrates identified as necessary starting materials.

These are of the type shown below in **Scheme 1**.

These have been synthesised to be used as starting materials for the enzyme catalysed cascade synthesis of lactams. The chemistry was based on that published by Saidi et al. and features a selective Michael addition of active methylene compounds under solvent-free conditions



Scheme 1: Synthetic Method used to make Key Materials

These substrates were used in cascade reactions later in the project. This was a demonstration of one of the key disciplines of synthetic organic chemistry on a scale not normally used in academic biocatalysis studies.

6. Cascades by Spontaneous Product Cyclisation

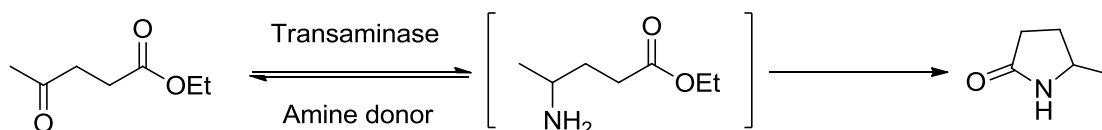
One problem that had been frequently identified as a limitation of transaminase chemistry was that the equilibrium is unfavourable and only relatively small amounts of products are formed,

Spontaneous cyclisation of the product amine has previously been shown to shift the equilibrium of the transamination in favour of the products.

An investigation of the effect of buffer, co-solvent and pH on the biotransformation with the aim of optimising the reaction and subsequently broadening the substrate scope with two alternative amine donor systems was implemented.

1. LDH/GDH system using alanine as the amine donor
2. IPA system – using excess isopropylamine (IPA) used as amine donor

Ethyl 4-oxopentanoate as was chosen as the test substrate, post transamination this substrate would undergo spontaneous cyclisation into 5-methyl-2-pyrrolidinone (**Scheme 2**).



Scheme 2: Transamination of ethyl 4-oxopentanoate to give 5-methyl-2-pyrrolidinone.

Initially the alanine LDH/GDH system was used with transaminase ATA117, at pH 7.5. The combinations of three buffers and two solvent systems were tested and 20% DMSO in HEPES buffer was the best combination.

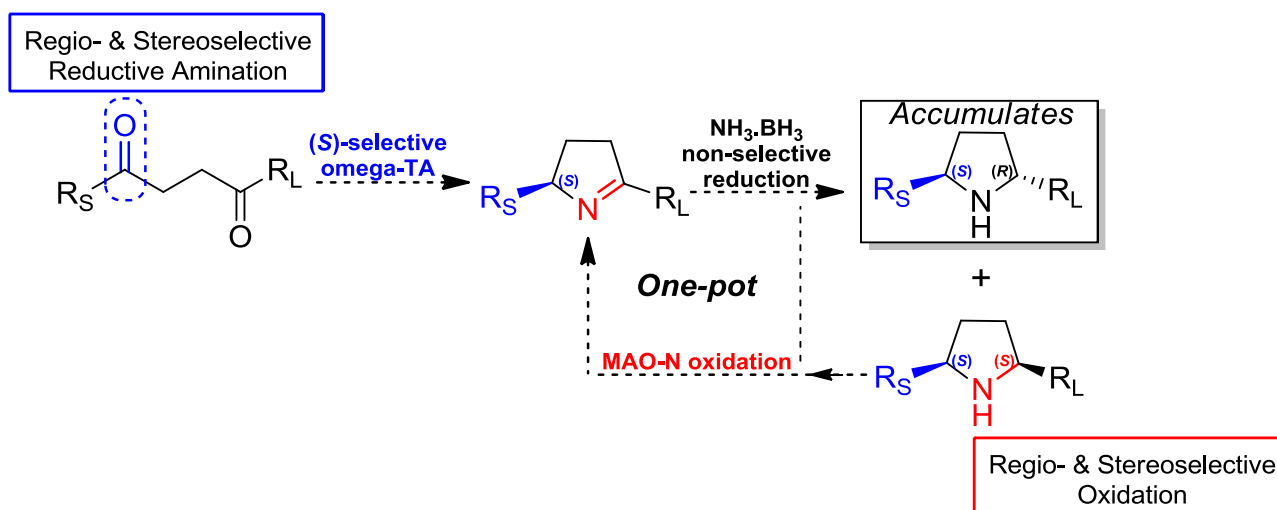
The biotransformations were completely enantioselective, with only one cyclised enantiomer seen when analysed by GC (when compared to the racemic standard). Due to the known selectivity of ATA-117 it is presumed that the *R*- enantiomer is formed.

LDH/GDH is an enzymatic cascade based on Lactate Dehydrogenase/Glucose Dehydrogenase cascade to remove pyruvate formed from alanine donor in the reaction.

7. Dual regio- and stereoselective ω -transaminase/MAO-N cascade process for the synthesis of 2,5-disubstituted pyrrolidines

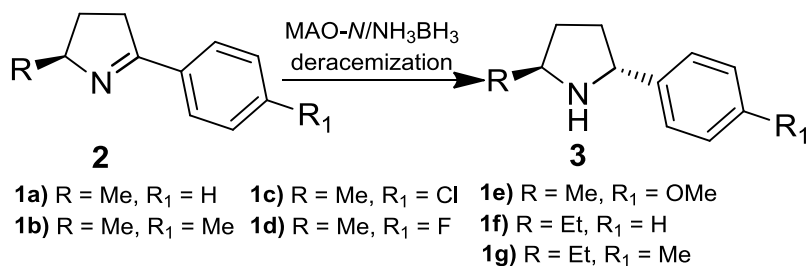
The conversion of a series of achiral 1,4-diketones to the corresponding 2,5-disubstituted pyrrolidines *via* an enzymatic cascade process involving an ω -transaminase (ω -TA) and monoamine oxidase (MAO-N) process was developed in the **AMBIOCAS** project.

This cascade involves an initial asymmetric transamination reaction to yield a chiral amine formed by the ω -TA. This is then unaffected by this second chemo-enzymatic stage using a dynamic kinetic resolution based on a monoamine oxidase (MAO-N) enzymatic oxidation and a chemical reduction (Scheme 3). In these examples the borane-ammonia complex $\text{BH}_3\text{-NH}_3$ was successfully used as the chemical reductant.



Scheme 3: Representation of enzymatic cascade to synthesize chiral cyclic amines.

This method was demonstrated using the examples in Scheme 4 which proved the exquisite regio- as well as stereoselectivity displayed by the engineered MAO-N variants



Scheme 4. MAO-N/ NH_3BH_3 mediated deracemization.

Overall this provides a highly efficient, protecting group free strategy for the synthesis of 2,5-disubstituted pyrrolidines and more generally demonstrates the potential of multistage regio-, and stereoselective enzymatic cascade processes in the synthesis of important target structures.

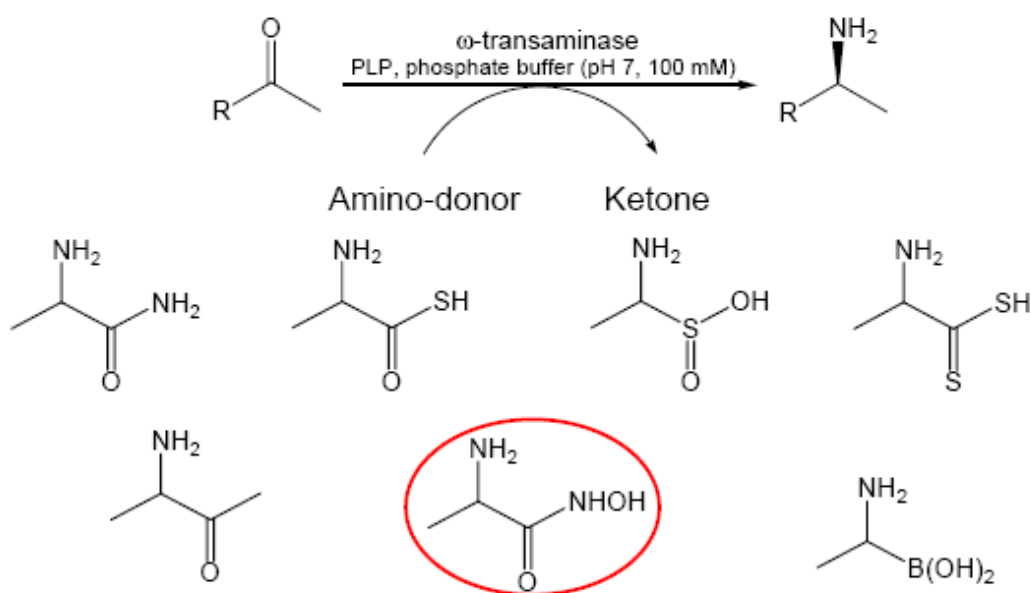
WP2: Multi-Enzyme Co-Factor Recycling and Synthetic Cascades

8. Alternative Amine Donors Found

One approach investigated early in the **AMBIOCAS** project was the potential to apply alternative amine donors to improve the reaction conversions which were frequently known to be low.

Using the theoretical free energy for various donors (see Scheme 5 below) were calculated. Experimental testing and these calculations indicated that alanine hydroxamic acid was a possible more reactive alternative amine donor in a cascade where the keto side product gets removed or recycled. However, for applications it might be required, that the enzymes transforming alanine hydroxamic acid and its co-product pyruvate hydroxamic acid, have to be adapted to be fully compatible with these compounds to achieve faster reactions. Enzymes would need to be adapted are transaminases, amino acid dehydrogenase, and lactate dehydrogenase.

Alternative amine donors



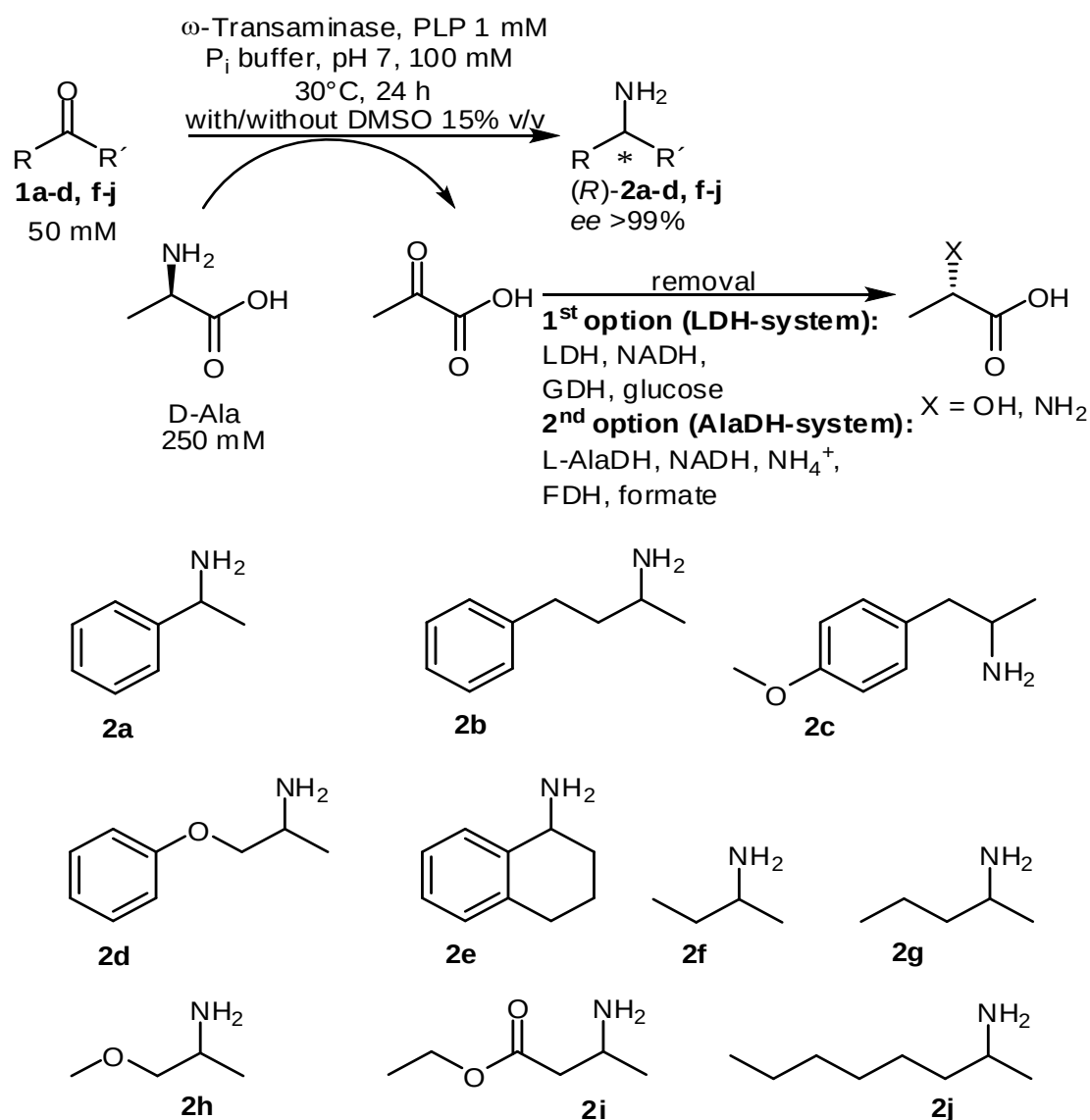
Scheme 5: Alternative Amine Donors Investigated

Although this approach gave results that indicated these were actually superior in terms of conversion the costs of these new donors and the new enzyme development requirements meant this approach was not taken further,

9. (R)-Selective Chiral Amine Synthesis

One of the limitations of transaminase chemistry at the start of the **AMBIOCAS** project was that (*R*)-enantiomer selective enzymes were not readily available.

To expand the toolbox of transaminases testing with various substrates with a focus access to the (*R*)-enantiomeric amines was done. Four (*R*)-selective ω -transaminases were employed for the asymmetric reductive amination of a set of prochiral ketones for the synthesis of bioactive amines with potential application in the synthesis of pharmaceuticals. All substrates examined were converted into the corresponding (*R*)-amines in optically pure form using the (*R*)- ω -transaminases from *Arthrobacter* sp. (ArR- ω TA), *Aspergillus terreus* (AT- ω TA) and *Hyphomona neptunium* (HN- ω TA) and a variant of a transaminase from *Arthrobacter* sp. (ArRmut11- ω TA). Only ArRmut11- ω TA transformed α -tetralone with perfect stereoselectivity. On the other hand, ArRmut11- ω TA transformed most of the other ketones tested with low stereoselectivity, while all other three (*R*)-selective transaminases displayed perfect stereoselectivity (*ee* >99%) for the accepted substrates. All transaminases tested tolerated DMSO as co-solvent, which led to improved conversion compared to the reaction without DMSO addition. (Scheme 6)



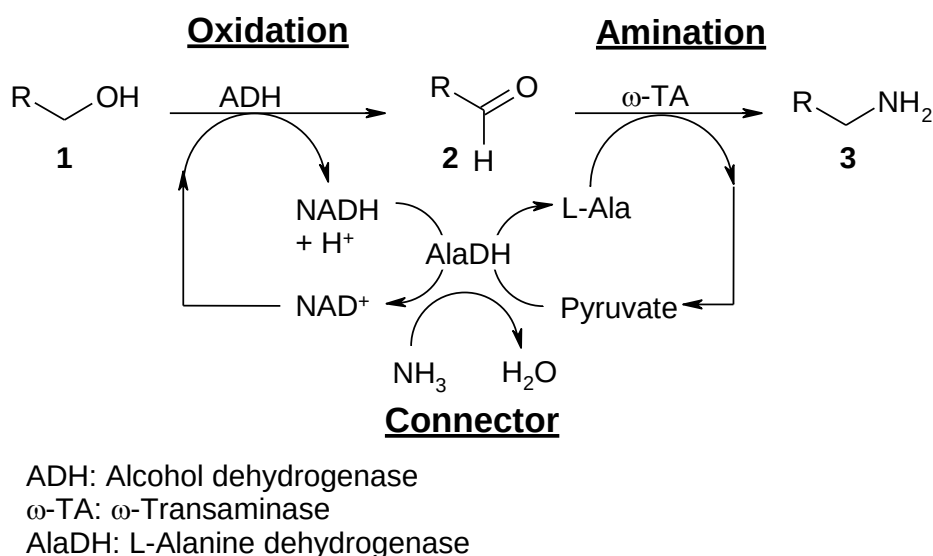
Scheme 6: (*R*)-Enantiomer Amine Synthesized in AMBIOCAS project.

10. The Artificial Bio-Amine Pathway

The artificial bio-amine pathway was a new concept that formed part of the novelty of **AMBIOCAS** project where the design of the desired cascade was to use an artificial pathway to transform alcohols into amines, ideally in a redox-neutral fashion and thus no external oxidation or reduction equivalents should be consumed.

This is similar to the well-known “hydrogen borrowing” metal catalysed amination sequence of alcohols that has been widely studied using precious metal homogeneous catalysis. This is of particular interest to the pharmaceutical industry as it removes alkylating agents used in conventional amine synthesis which have been identified as potential genotoxic intermediates (PGI) due to their ability to alkylate DNA and as such are strictly controlled by the regulatory authorities (FDA);

The first step in this method is an oxidation catalysed by an alcohol dehydrogenase (ADH) consuming NAD^+ leading to the formation of the aldehyde and NADH . In the second sequential step the ω -transaminase (ω -TA) responsible for the amination of the intermediate aldehyde requires an amine donor and L-Alanine as an amine donor (at the start of the project this was the only known amine donor for ω -transaminases which could be recycled *in situ*). In the transamination step alanine is transformed into pyruvate as a side product. and then for the regeneration of L-alanine from pyruvate an L-alanine dehydrogenase (AlaDH) was used which consumes ammonium and NADH . The required NADH can be taken from the oxidation step where the NADH was liberated. Thus, the AlaDH connects the oxidation step with the amination step, regenerating the required NAD^+ of the oxidation by transferring the hydride from the NADH – the side product of the oxidation - to the amination step by regenerating the amine donor alanine from pyruvate. Consequently only an ADH can be used in the oxidation step and not an alcohol oxidase, because only the ADH enables the hydride transfer via NADH ; an oxidase consume molecular O_2 leading to hydrogen peroxide as a side product, which cannot be used in the desired subsequent reduction step (Scheme 7).



Scheme 7: Artificial pathway for the bio-amination of primary alcohols.

This concept was successfully implemented in the project.

11. Amination of *sec*-Alcohols using the Artificial Bio-Amine Pathway

The artificial bio-amine pathway was demonstrated as a cascade for the transformation of *sec*-alcohols into the corresponding amine led to conversions of up to 60% (Table 1).

Conversions:

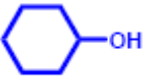
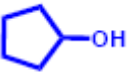
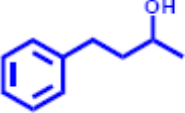
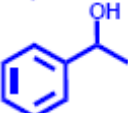
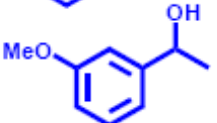
→		up to 43 % Amine*
→		up to 64 % Amine*
→		up to 66 % Amine*
→		up to 25 % Amine*
→		up to 7 % Amine*

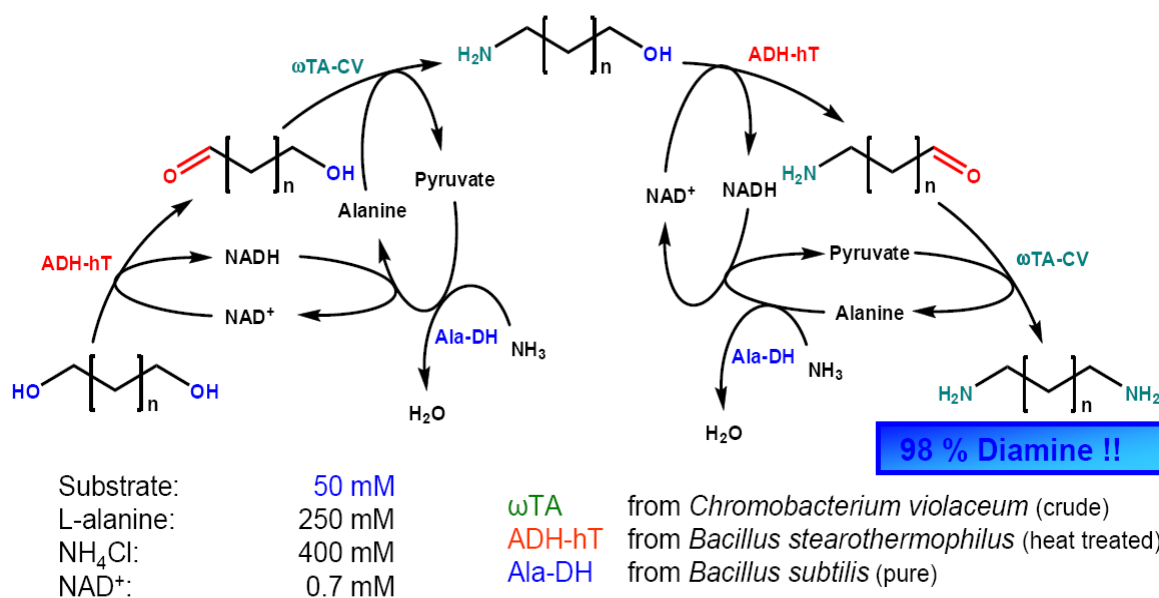
Table 1: Results of conversion of *sec*-alcohols into amines.

However various results indicated that the 66% transformation to amine represents a thermodynamic limitation, probably due to thermodynamic equilibrium (all compounds are in equilibrium and the cascade above has no further external driving force).

12. Amination of Primary Alcohols and Diols using the Artificial Bio-Amine Pathway

The redox-neutral artificial bio-amine pathway cascade concept for primary alcohols gave similar results to secondary alcohols with modest conversions obtained. However in case of diols, especially 1,8-octandiol, 1,10-decandiol, 1,12-dodecandiol surprise results were obtained when using co-solvents to improve the solubility of the substrates. For instance 1,10-decandiol, 1,12-dodecandiol were transformed to the corresponding diamines with up to 98% conversion in a 4-step linear sequence in a redox-neutral fashion after reaction optimization. These diamines have potential to serve as building blocks for polymers where linear diamines have many applications (Scheme 8)

■ Amination of primary Alcohols



n = 6, 8

Scheme 8: Transformation of diols to diamines via a 4-step sequence with internal cofactor recycling.

13. ω -Transaminases in Organic Solvents

One very interesting invention and development arising from the **AMBIOCAS** project was the advance of using transaminases in reactions performed in organic solvents.

Although other enzymes have been applied in organic solvents this technique had not been demonstrated for this particular family of enzymes.

As a result of this work in **AMBIOCAS** ω -Transaminases have been demonstrated to give good results when applied in organic solvents.

They have been demonstrated to accept 2-propylamine as the amine donor (which is not the case when they are used in aqueous solution) to give higher reaction rates (up to 10-fold) and a simplified work-up procedure.

This breakthrough result has been patented.

14. The Nylon-6 Cascade Proof of Principle

One of the major cascade reactions identified as a key component of the **AMBIOCAS** reaction was the conversion of cyclohexanol into 6-aminohexanoic acid. In this development all the enzymes needed for a bio-Nylon cascade had been identified. These enzymatic cascades can offer the possibility to perform various catalytic reactions simultaneously. Consequently multistep reaction could be performed transforming cheap substrates to valuable products via reaction cascades. This is represented in Figure 4.

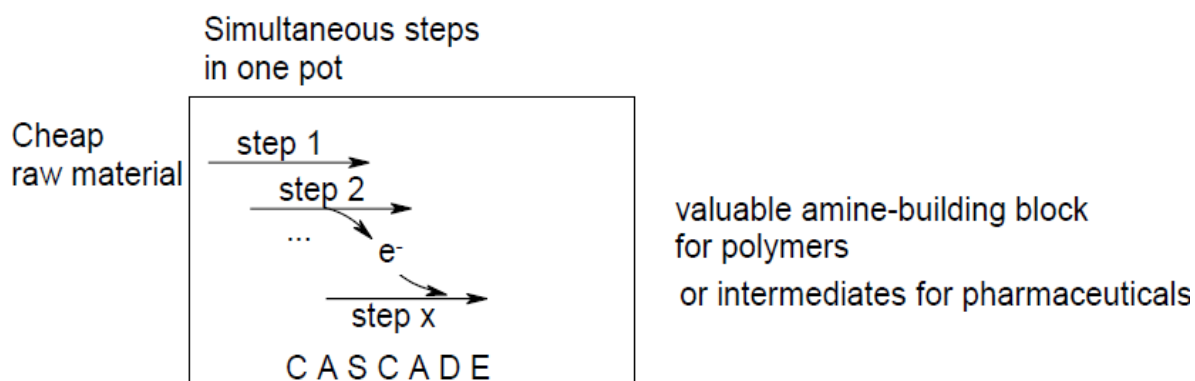


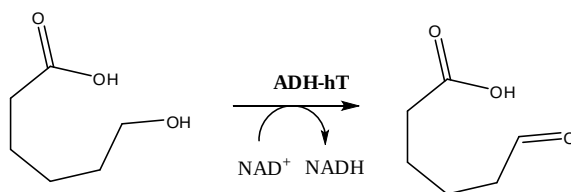
Figure 4: Concept of Bio-Nylon in AMBIOCAS

This is especially appealing if redox balancing is implemented whereby a reduction step can be combined with an oxidation step and the formal abstracted electrons in the oxidation step are reused in the reduction step. This redox-neutral concept avoids additional oxidation and reducing agents and can result in co-factor balancing. For cascades from alcohols to amines a testing system was successfully tried. These enzymatic cascades can offer the possibility to perform various catalytic reactions simultaneously. Consequently multistep reaction could be performed transforming cheap substrates to valuable products via reaction cascade.

A detailed study of the kinetics of transaminase enzymes have allowed a full model for the reaction to be developed which has given insight into the factors that define the limits of the transaminase reactions and allow the an operating window to be understood for reaction development.

15. Enzymatic oxidation of 6-hydroxyhexanoic acid to 6-oxohexanoic acid

To test the concept of the full bio-nylon cascade the individual reactions were developed separately. For this key oxidation the reaction was monitored to check whether the activity measured by the UV-assay would also be of practical interest. The oxidation was tested in a coupled substrate approach using chloroacetone as the oxidant. More than 80 % conversion into 6-oxo hexanoic acid was obtained. Addition of MgCl_2 led to identical results (Scheme 9).

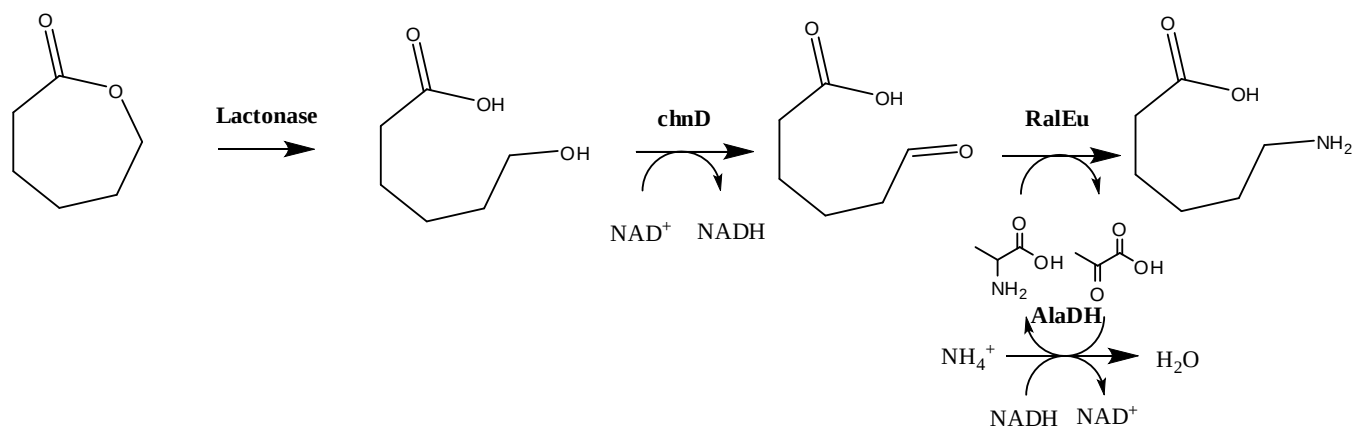


Scheme 9: Key oxidation step in Bio-Nylon cascade

16. Cascade reaction from ϵ -caprolactone to 6-aminohexanoic acid

This key cascade was established in practice in two different ways.

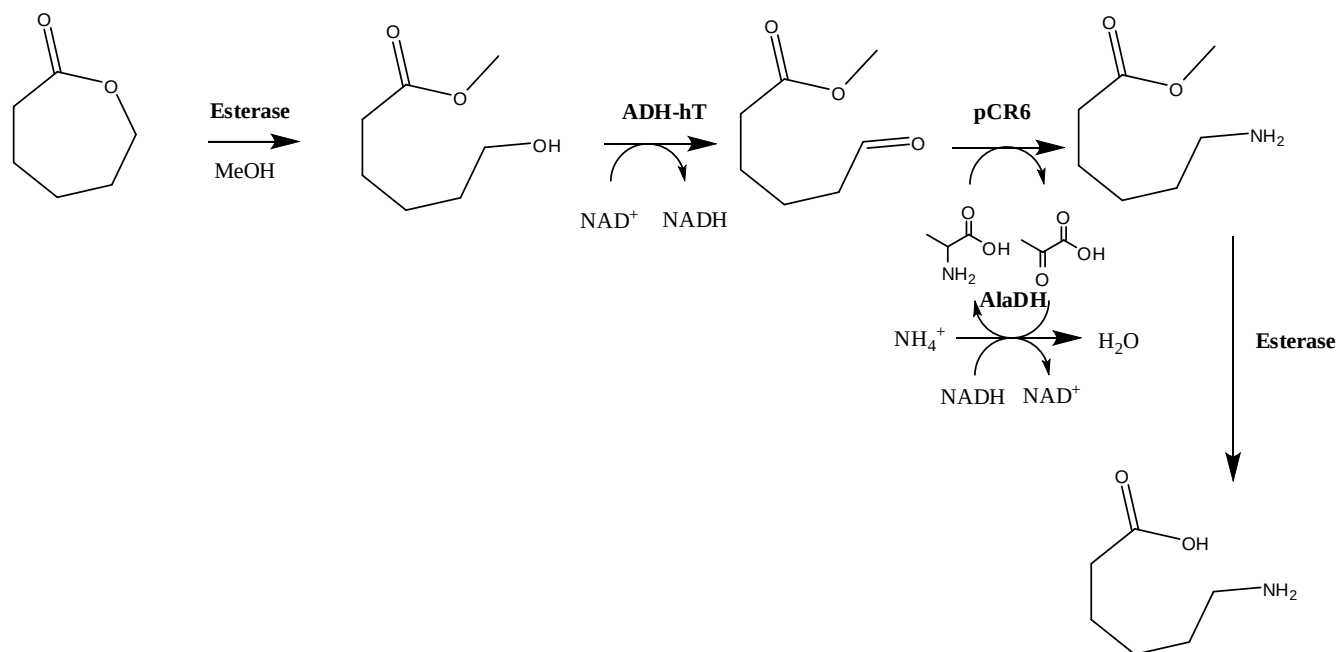
In the first a lactonase was applied to open the 7-membered ϵ -caprolactone ring, and the ω -hydroxyacid obtained was oxidized by a highly specific ADH (6-hydroxyhexanoic acid dehydrogenase *chnD* from *Arthrobacter* sp.) and the resulting 6-oxohexanoic acid was aminated by a β -amino acid transaminase from *Ralstonia eutropha*. In order to overcome severe inhibition resulting from the five times excess of alanine the concentration of the donor was reduced to an equimolar concentration (50 mM). (Scheme 10)



Scheme 10: System 1 for caprolactone cascade.

About 30 % of the product was obtained in this method.

The second system uses an esterase from horse liver (obtained from Sigma Aldrich) in a buffer containing methanol (10 % v/v) to open the lactone ring by formation of the 6-hydroxyhexanoic acid methyl ester. The ester formed is oxidized by an ADH from *Bacillus stearothermophilus* (ADH-hT)^[1], and the resulting aldehyde is aminated by an ω -transaminase from *Paracoccus denitrificans*^[2]. Only 6-aminohexanoic acid was detected up to date, which could be explained by a deprotection of the ester by the esterase.



Scheme 11: System 1 for caprolactone cascade.

Full conversion to the desired 6-aminocaproic acid product was observed.

WP4: Enzyme Crystallization and Bioinformatics.

17. High Purity Enzymes Prepared

The enzymology and modelling work in **AMBIOCAS** required production of high purity enzymes.

Three ω -transaminase pET21a(+) constructs were obtained from project partner **UNIGRAZ**. Two were (S)-selective transaminases being from *Arthrobacter* species (Ars- ω TA) and the other is from *Bacillus megaterium* (BM- ω TA). Both have a molecular weight of 53 kDa, bind pyridoxal phosphate (PLP) as a cofactor and show 95% sequence identity to each other. In addition, one (R)-selective transaminase from an *Arthrobacter* species (ω TAR) was obtained. All constructs contained a His6-tag behind a stop codon, allowing the introduction of a His6-tag into the protein by site directed mutagenesis.

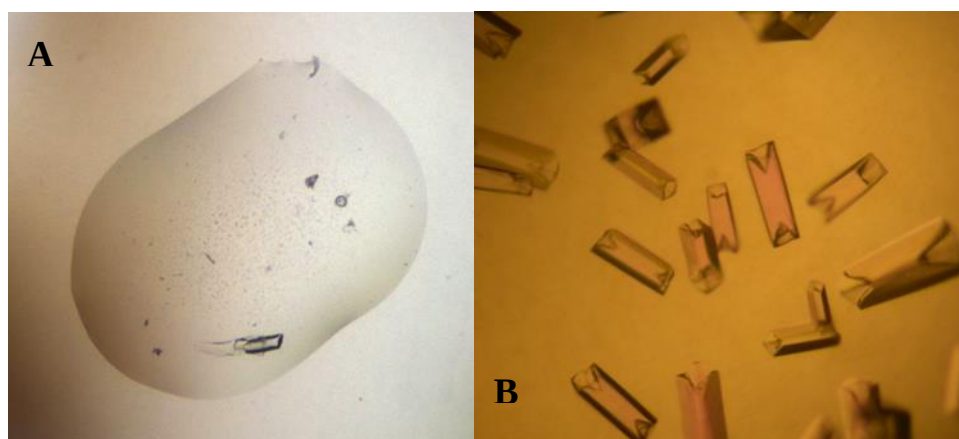
Highly purified protein preparations were needed for crystallization experiments and therefore expression and purification of the transaminases was done by successful introduction of the His₆-tag into the constructs by removing the stop-codon by site-directed mutagenesis and performed expression testing for both the Ars- ω TA(His₆) and BM- ω TA(His₆) constructs.

After the successful expression, a similar purification protocol using Ni²⁺-NTA affinity chromatography and anion exchange chromatography was also used for purifying ω TAR. PLP was added to all purification buffers. The purity of the protein after these two chromatographic steps was high enough for use in crystallization experiments and no further purification was needed. At the end of the purification procedure, a yield of 10-15 mg/L culture was observed and subsequently the protein was concentrated 30 mg/mL.

18. Enzyme Crystallization and Crystallography

The high purity enzymes obtained were then crystallized for x-ray crystallography.

Firstly optimization screens (pH, salts and precipitant concentration) were set up to improve the quality of the crystals obtained from the purified proteins and improvement of the cryo-conditions, used for freezing the crystals yielded good quality crystals (Figure 5). X-ray diffraction images of the crystals were then obtained and analyzed and the information used in modeling and further enzyme design.



BM- ω TA crystals used for X-

Figure 5: Photographs of transaminase enzyme crystals.

19. Scale Down Cascade Test Model Developed

Effective estimation of the kinetic parameters in biocatalytic reactions is vital when building process models for evaluating different process technology options. A new methodology to estimate parameters for biocatalytic reactions is proposed taking into account the positive aspects of different approaches.

A Process Model to provide a rapid assessment of the performance of different routes and to identify bottlenecks and solution strategies for implementation was developed (Figure 6).

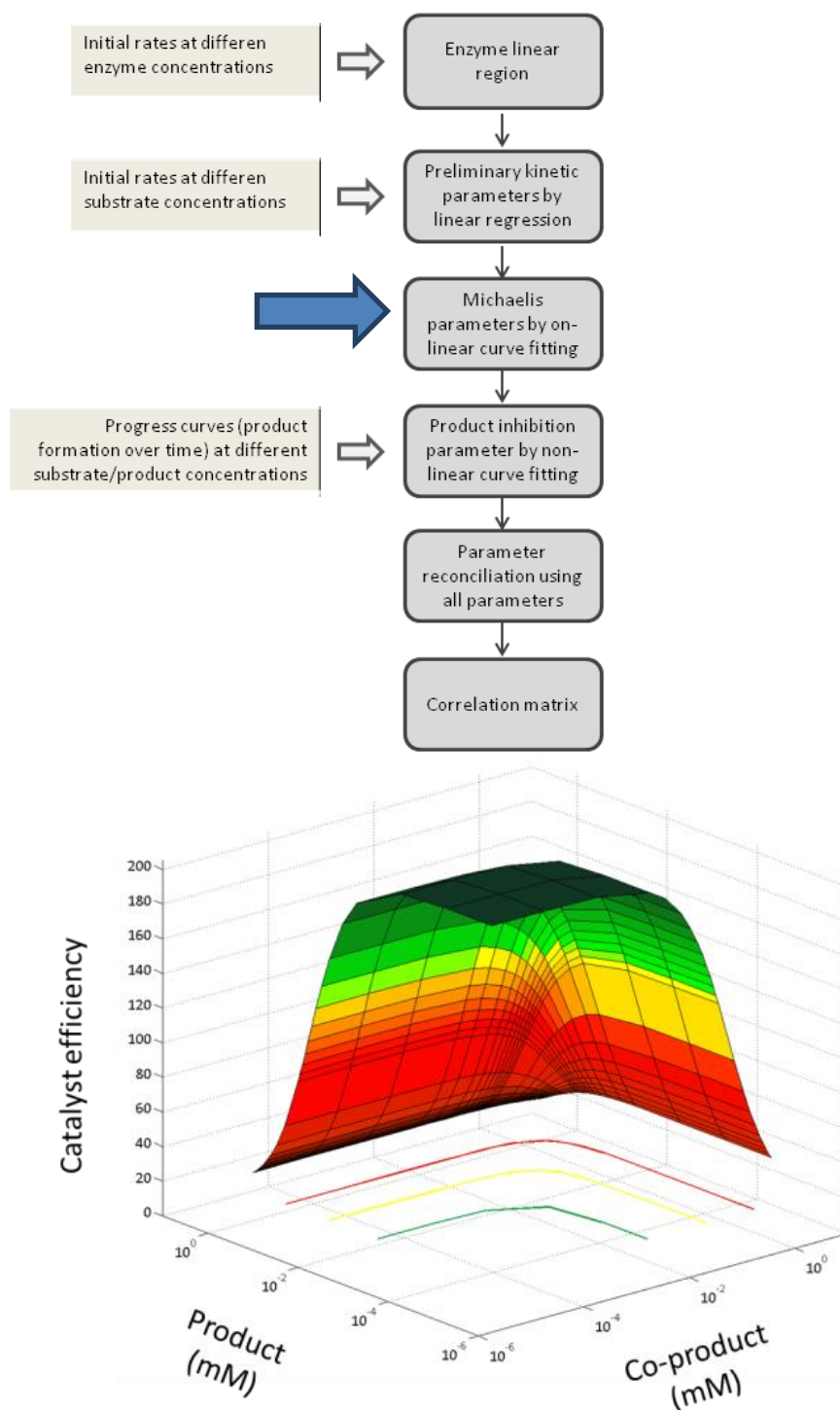


Figure 6: New methodology for parameter estimation and building transaminase kinetic models

20. Identification of reaction thermodynamics as key process parameter

The reaction thermodynamics (ΔG 's free energy) was identified as having a key impact on process performance and feasibility of those involving transaminase enzymes. Furthermore it was seen that the choice of amine donor has a major impact on the ΔG of reaction. A new method for determining the ΔG was developed and ΔG for the most common amine donors were determined. This is represented in Figure 7 and some representative data shown in Table 2.

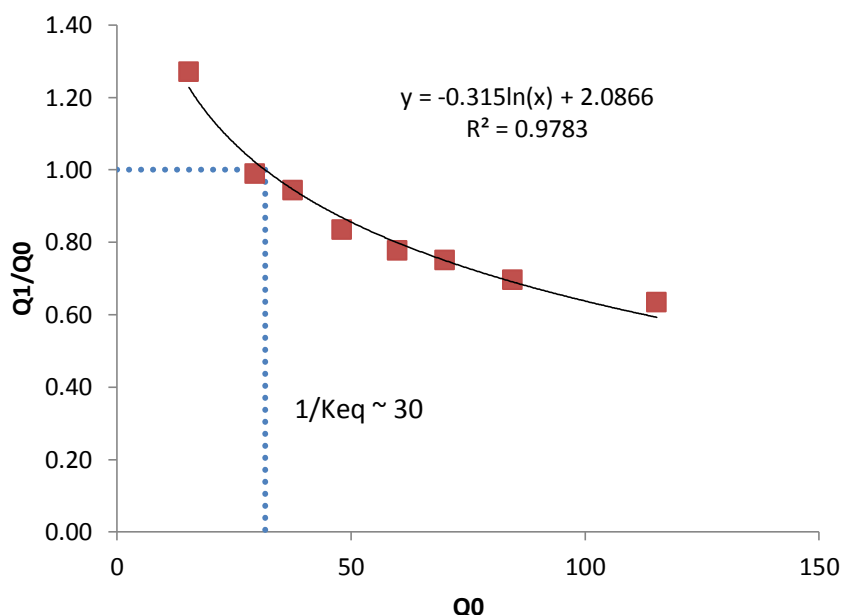


Figure 7: New methodology for determining K equilibrium of reaction. This example shows the reaction between acetophenone and 2-propylamine to yield 2-phenylethylamine and acetone.

Reaction system	AD:	Alanine	2-propyl amine
		$3 \cdot 10^{-5}$	$3 \cdot 10^{-2}$
		$6 \cdot 10^{-4}$	$6 \cdot 10^{-1}$
		$1 \cdot 10^{-3}$	(1)
		$1.7 \cdot 10^{-2}$	$1.7 \cdot 10^1$
		(1)	$1 \cdot 10^3$

Table 2: Thermodynamic data for amination reactions obtained by this method

21. Bottlenecks in reactions identified using a bioprocess engineering approach

Suitable scale-down reactors to test co-factor recycling methods have been designed as described earlier and have led to the development of process models to evaluate alternative modes of operation based on identification of bottlenecks. This has been used to assess the ω -transaminase reaction in terms of kinetics, equilibrium, amine donors, enzyme stability and thermodynamics and has been developed to screen a range of reaction conditions including novel amine donors to improve the approach to transaminase applications. These factors can be presented graphically to identify an operating window where the main operating factors are quantified and targets for performance defined (Figure 8).

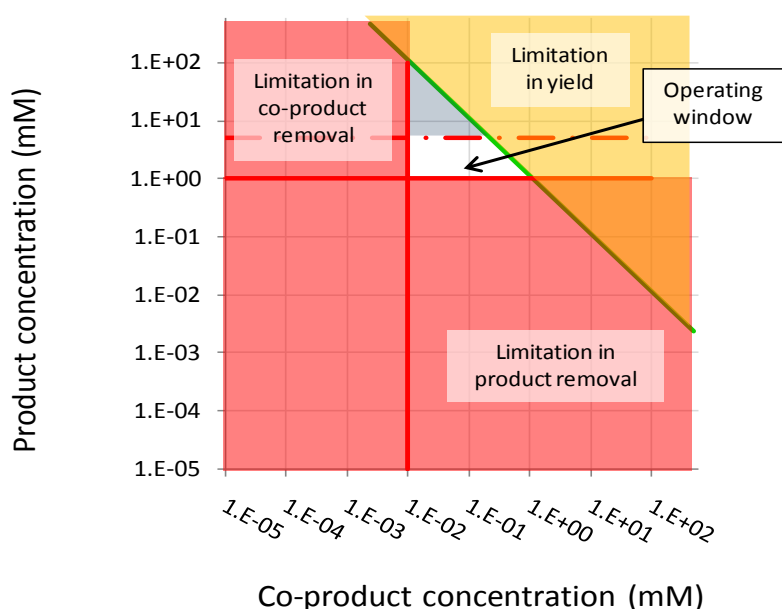


Figure 8: Operating window model for transaminases developed in AMBIOCAS

A detailed study of the kinetics of transaminase enzymes have allowed a full model for the reaction to be developed which has given insight into the factors that define the limits of the transaminase reactions and allow that an operating window to be understood for reaction development.

22. A methodology for cascade selection

A detailed study for selecting the most suitable cascade system was carried out for different cascade systems previously described. Thermodynamic, kinetic, stability and process aspects were considered in a methodological manner in order to discard non-viable options and to identify the most suitable ones by the application of setting threshold values for key parameters (Figure 9).

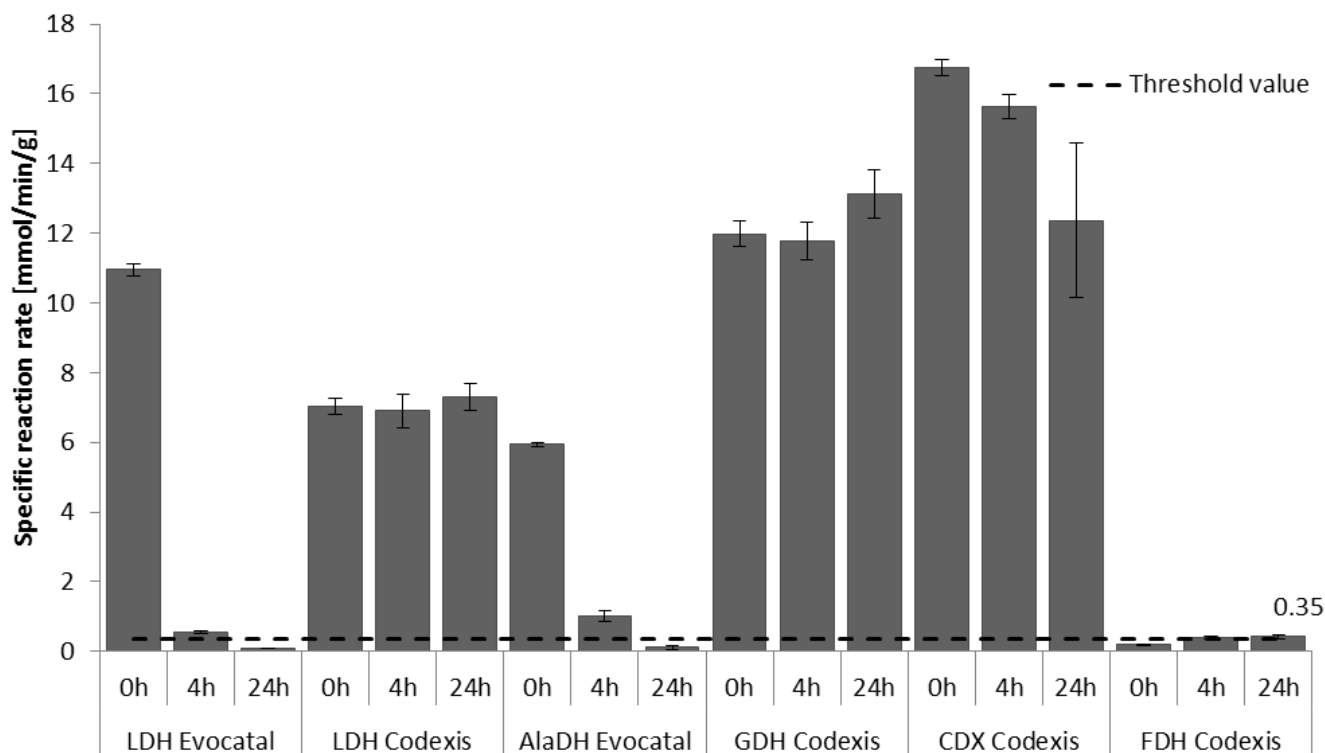
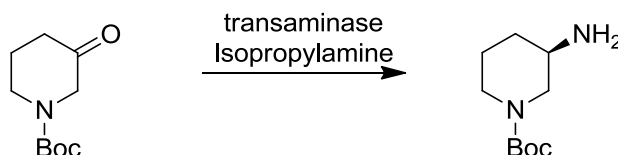


Figure 9: Activity and stability assessment of alternative cascade options.

23. Selection of process strategy and scale-up of synthesis of (S)-(+)-3-Amino-1-Boc-piperidine

(S)-(+)-3-Amino-1-Boc-piperidine was identified by industrial partner Dr Reddy's as a target molecule for synthesis by transaminases. By using the developed scale down systems and methodologies a suitable route was identified and scaled to 40 g/L substrate (very near the identified threshold of 50 g/L). High conversion (<95%) and enantiomeric excess (>99%) was found with 20 g/L substrate in 25 ml reactors with over-head stirring (Scheme 12).



Scheme 12: Synthesis of key intermediate of industrial interest

24. Stabilization of Transaminases by Enzymatic Supports

A comparison of various immobilisation strategies for optimal use of ω -transaminases has been implemented which has identified suitable methods for supports for use in cascades with cofactor recycling. These immobilised catalysts showed increased storage stability over the free enzyme and an enhanced performance under process conditions with respect to recycling and stability (Figure 10). Optimized CLEA[®] formulations were particularly robust.

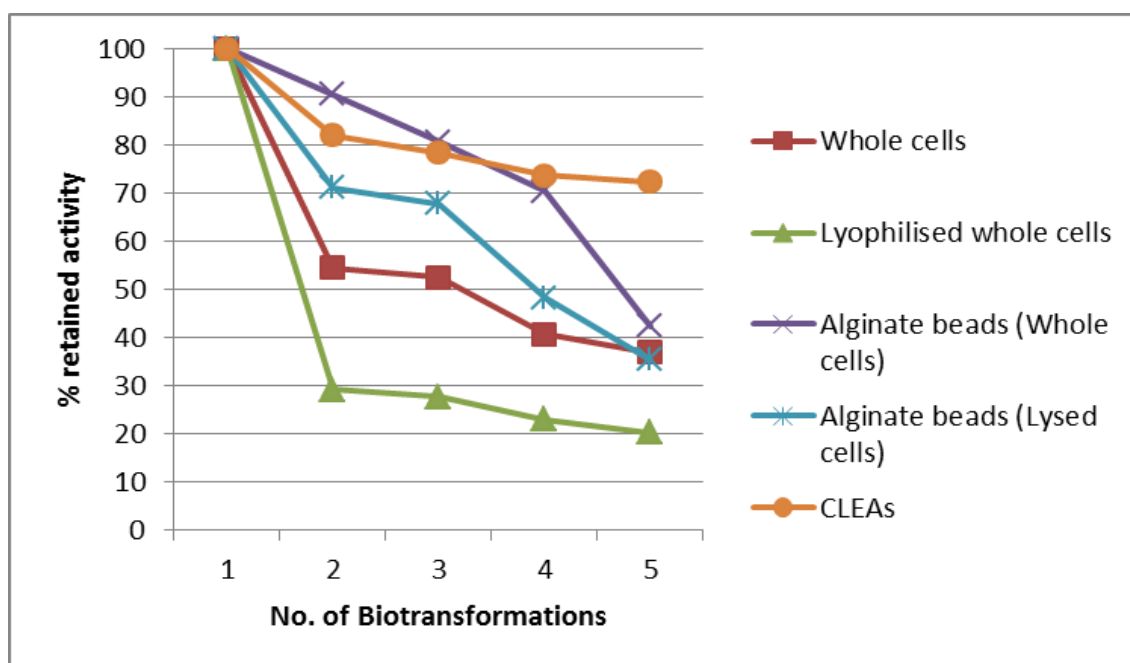


Figure 10: Improved Transaminase Enzyme Stability on Supports

25. Successful ω -transaminase CLEA[®] formulations have been prepared using commercial enzymes that show impressive activity.

Transaminase CLEAs have successfully been prepared from seven transaminase enzymes; three from **UNIMAN** and four from commercial sources. These CLEAs have been characterized against the free enzymes, on storage and in test reactions and showed a highly promising performance compared to the free enzymes. The formulation for each CLEA has been optimised for temperature, pH, PLP concentration, co-solvent concentration, aggregant, cross-linking agent and capping. The CLEAs have been tested in the full cascade reaction using benzyllacetone and alanine to produce chiral amine products. Proof has been delivered for the recycling of the CLEAs without the loss of activity in between reaction cycles and under process conditions. The transaminase CLEAs are in the process of being commercialised with an industrial partner and will be shortly included in our catalogue.

26. Transaminase combi-CLEAs for Application

The full cascade system requires a number of enzymes namely transaminase, alanine dehydrogenase or lactate dehydrogenase and either formate dehydrogenase or glucose dehydrogenase. In addition to single CLEAs that were made of all the enzymes, there was also many combi-CLEAs where two or more enzymes were incorporated into the same biocatalyst. Examples of these combi-CLEAs include: transaminase/alanine dehydrogenase and lactate dehydrogenase/glucose dehydrogenase. A triple combi-CLEA was also generated by combining a transaminase with lactate dehydrogenase and glucose dehydrogenase so that all of the co-factor recycling can be achieved with one biocatalyst. These triple combi-CLEAs have shown to be effective in the synthesis of chiral amines which means that each enzyme is performing its task in the cascade. The CLEAs and combi-CLEAs were also trialled under process conditions and some showed impressive activity when compared to the free enzyme (Figure 11).

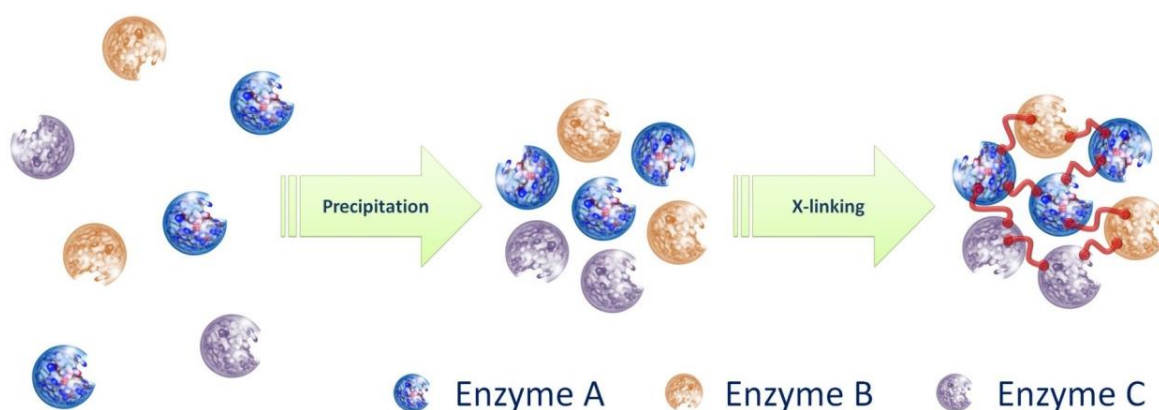


Figure 11: Triple combi-CLEA formation

27. Development of a metrics based method for assessing bioprocess feasibility

Process cost estimation is extremely useful both in production as well as in R&D to guide activities directed at developing, implementing and improving processes. Much useful information can be obtained about the drivers and bottlenecks preventing the immediate implementation of an effective and economic process, even at an early stage of development (where the uncertainties are considerable). In **AMBIOCAS** decisive steps were taken towards a metrics based method for rapidly assessing bioprocess feasibility in early stages and with minimal data at hand.

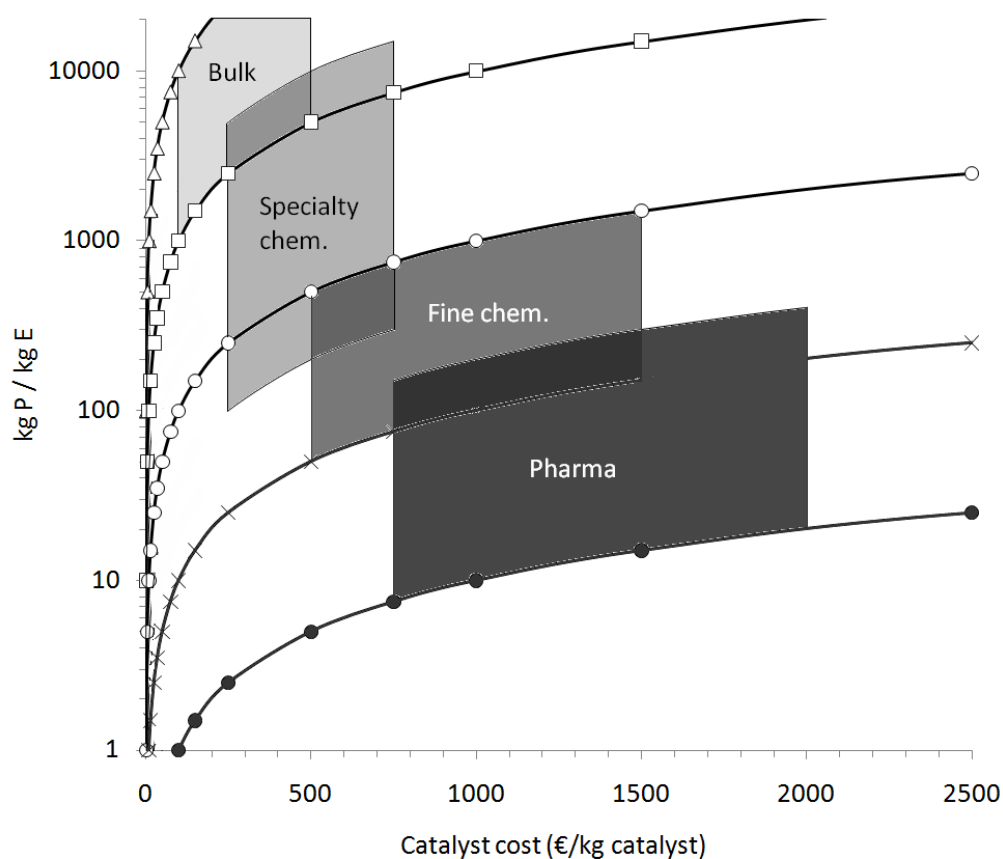


Figure 12: Effect of biocatalyst cost and allowable cost contribution on the requirements for biocatalyst productivity in terms of kg product per kg biocatalyst used for production of bulk, fuel or specialty chemicals using immobilized enzymes. Allowable cost contribution of 0.01€/kg Δ , 0.1€/kg $\square$, 1 €/kg $\circ$, 10€/kg $\times$, 100€/kg $\bullet$.

28. Economic assessment of the transaminase route to Nylon-6 Intermediate

An evaluation of the process economy showed that in order to compete economically with the current industrial process (current selling price is 2.70 €/kg) the co-factor generation and alanine recycling systems need to be improved (Figure 11).

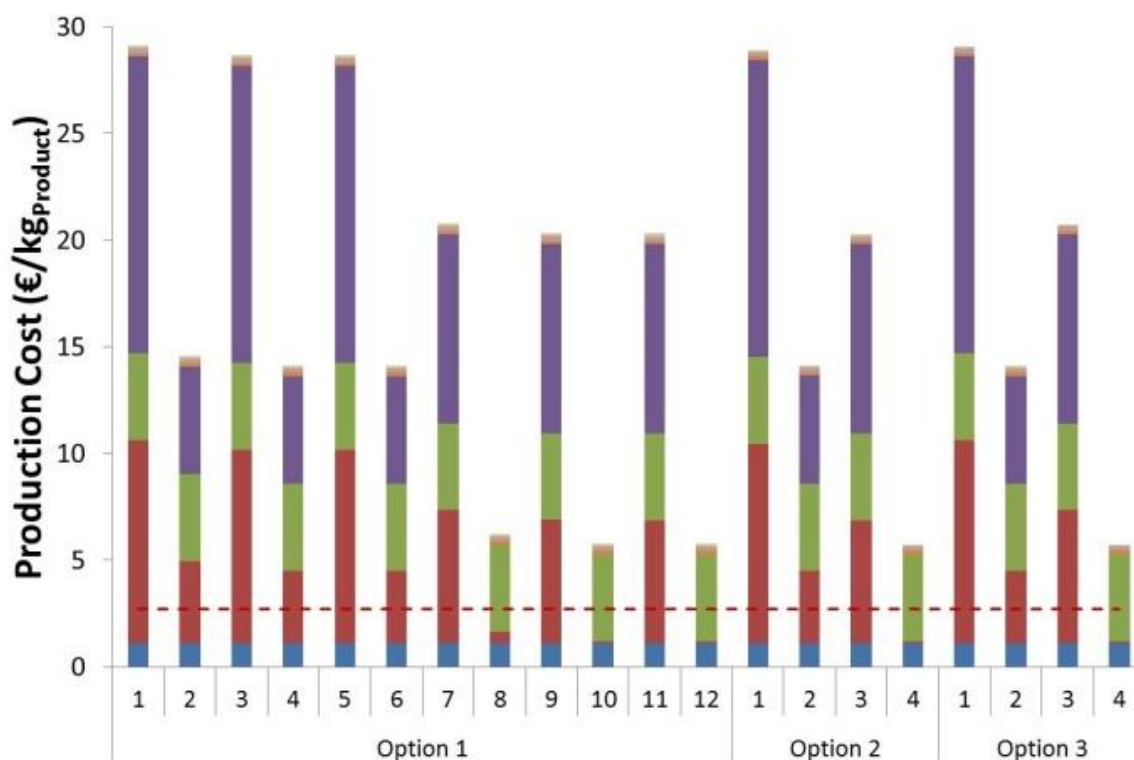


Figure 11: Production cost for the different process design option for production of 6AHA;
Legend: ■ cyclohexanol, ■ biocatalyst, ■ alanine, ■ cofactors, ■ glucose, ■ NH₄Cl, ■ utilities, ■ labour, ■ CAPEX and --- current ε-caprolactam selling price

29. Sustainability assessment of the transaminase route to Nylon-6

An LCA study of different process alternatives identified that the starting material cyclohexanol and the alanine contributed more than 80% of the total impact (Global Warming Potential).

The biocatalytic route shows some savings in the primary energy demand and the greenhouse gas emissions, however (as is common for biobased products) for the rest of the evaluated environmental impacts (photochemical ozone creation, eutrophication and acidification potential) the performance of the biocatalytic synthetic route is not better than the environmental performance of the conventional chemical route.