

Faculté des bioingénieurs

Biocatalytic chiral amine synthesis coupled with in situ chemocatalytic substrate production

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Abstract

Transaminases are promising biocatalysts that transfer an amine group to a ketone, enabling the synthesis of chiral amines; key components in over 40% of pharmaceuticals and other fine chemicals. Their high enantioselectivity and mild operating conditions help reduce the need for costly and environmentally intensive purification processes. However, the practical application and industrial-scale adoption of transaminases are hampered by substrate-induced enzyme inactivation.

This master thesis addresses this issue through the development of a cascade reaction in which another non-inhibiting compound is used as a starting substrate and converted in situ to the substrate of the amine, enabling the progressive and controlled synthesis of the inhibitory ketone. The model reaction involves the one-pot conversion of 1-phenylethanol into 1-phenylethylamine, via oxidation to acetophenone followed by the transamination step. The proposed approach couples transaminase activity with a selective oxidation step catalyzed by gold nanoparticles supported on silica (Au/SiO₂). This inorganic catalyst exhibits effectiveness under mild conditions compatible with enzymatic reactions.

A compatibility window between the enzyme and inorganic catalyst was identified, considering interference with the enzyme, the cofactor and the amine donor. To mitigate these interferences, the enzyme was immobilized at room temperature on a commercial resin to reduce catalyst inhibition. Additionally, the concentration of the amine donor was reduced from 500 mM to 60 mM. Immobilization was further improved by applying a step of incubation with the cofactor (PLP), contributing to improved enzyme stability and alleviating the need for high PLP concentration in the reaction (which help preserve the activity of the gold catalyst).

Although the implementation of the chemoenzymatic cascade introduced additional challenges for the enzyme, leading to a lower catalytic activity compared to transamination alone, it nevertheless effectively addressed the issue of substrate inhibition. This was confirmed by the comparable enzymatic activity observed when using 10 mM or 40 mM of 1-phenylethanol as a starting material, whereas similar concentrations of acetophenone would have resulted in a 75% loss of activity.

Keywords

Biocatalysis, transaminase, enzyme, hybrid chemo-enzymatic catalysis, chiral amine, gold nanoparticles, transamination, oxidation.

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Table of Abbreviations

AASG	Aerosol-assisted sol-gel
Ac	Acetophenone
ADH	Alcohol dehydrogenase
APIs	Active pharmaceutical ingredients
APTES	(3-aminopropyl)triethoxysilane
ATA	Amine transaminase
DPPA	3,3-diphenylpropionic acid
ee	Enantiomeric excess
EP	Epoxy resin
GC	Gas chromatography
GLYMO	(3-glycidyloxypropyl)trimethoxysilane
HFA	Hydrophilic functional amino
HPLC	High-performance liquid chromatography
IPA	Isopropylamine
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NP	Nanoparticles
PhEtOH	1-phenylethanol
PLP	Pyridoxal-5'-phosphate
PMP	Pyridoxamine 5'-phosphate
SEM	Scanning electron microscopy
TA	Transaminase
TEMPO	2,2,6,6-tetramethyl-1-piperidinyloxy
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction
α -TA	α -transaminase
ω -TA	ω -transaminase

State of the Art

I. Molecular asymmetry

Chirality plays a central role in both chemistry and biology, particularly in the structure and function of molecules. It is defined as the property of a molecule being non-superimposable on its mirror image¹. In 1848, Louis Pasteur first discovered chirality through optical activity experiments, observing that sodium ammonium tartrate tetrahydrate crystals could rotate plane-polarized light in different directions². As illustrated in **Figure 1**, he identified two enantiomers of tartaric acid, L-(+)- and D-(-)-, which respectively rotate polarized light clockwise (positive rotation) or counterclockwise (negative rotation)³, and with two asymmetric centers.

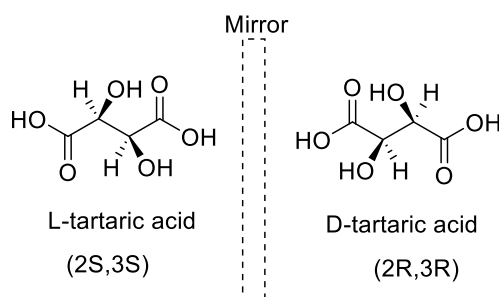


Figure 1. D- and L- tartaric acid enantiomers.

Today, enantiomers are primarily distinguished using two systems:

- The R/S system, determined by the Cahn-Ingold-Prelog priority rules, assigns an absolute configuration based on the spatial arrangement of substituents around the chiral center⁴.
- The (+)/(-) system classifies enantiomers according to the direction in which they rotate plane-polarized light: "+" for rightward (dextrorotatory) and "-" for leftward (levorotatory) rotation, determined experimentally using a polarimeter⁵.

In a chiral environment, like living systems, two enantiomers may exhibit distinctly different behaviors⁶. Although these stereoisomers appear identical in terms of molecular formula, composition and connectivity⁷, they can nonetheless display very different properties such as odor perception, biological activity, toxicity, taste and enzyme interactions^{5,8,9}. Inversion of a chiral center can shift the perception of R-carvone (spearmint-like odor) and S-carvone (caraway-like odor)¹⁰. Likewise, there is a single variation in orientation between an illicit drug of abuse and medication under strict prescription or a less exciting over-the-counter product in the case of S- or R-methamphetamine¹¹.

II. Importance of chiral separation

Pharmaceuticals, agrochemicals and cosmetics are technological sectors with a significant interest in chiral molecules due to their unique properties¹². These industries, which rely on large-scale production, would benefit from obtaining enantiomerically pure compounds rather than racemic mixtures, an equimolar mixture of the two enantiomers⁴.

Notably, chiral drugs account for over 72% of chiral production, and this proportion continues to grow¹. Until recently, many chiral drugs were marketed as racemates due to enantioseparation challenges¹². However, it is now clear that typically only one enantiomer provides the desired therapeutic effect¹³. Consequently, single-enantiomer drugs have become the standard^{5,12,14}: between 1990 and 2006, the proportion of new drugs marketed as pure enantiomers increased from approximately 20% to nearly 90%, clearly reflecting the direction of pharmaceutical R&D¹⁵. Since 2016, the EMA has not approved a single racemic drug, and between 2013 and 2022, the FDA has approved only one racemate per year on average¹⁶.

As the production of enantiopure compounds is costly, relying either on complex synthetic routes or expensive purification steps, enantiopure molecules possess a significantly higher added value compared to a 50:50 racemic mixture of the two enantiomers, with prices often ranging from 5 to 100 times higher depending on the molecule and the purification process. 1-phenylethanol, for example, is sold as a racemate at \$161 per kilogram¹⁷, whereas the pure S-enantiomer costs \$251 for just five grams¹⁸, equivalent to \$50,200 per kilogram, corresponding to about a 312-fold higher cost for the enantiomerically pure form.

This considerable economic gap highlights the growing need for more efficient, selective, and sustainable methods to produce enantiopure compounds. Traditional techniques for obtaining enantiopure compounds can be optimized or replaced. Mounting pressure on the chemical industry to improve process life cycle assessments has driven the search for alternative techniques¹⁹. Life cycle analyses of API production have shown that the manufacturing of material inputs accounts for more than 80% of the overall life cycle environmental impact. While the environmental impact of existing methods is a concern, it is above all their relatively low yields that remain the main limitation²⁰.

Due to their identical physico-chemical properties in an achiral environment, enantiomers cannot be separated by conventional analytical methods. It is therefore necessary to introduce a chiral environment to enable discrimination between them¹³. Two principal strategies are commonly employed: the historical “racemic” approach, where a non-selective synthesis is followed by racemate purification, and the “chiral” approach, which relies on asymmetric synthesis to selectively produce a single enantiomer¹⁴.

II.1. Enantioseparation

Although various approaches exist for separating enantiomers, such as enantioselective liquid-liquid extraction membrane-based methods, selective crystallization and chromatographic methods are widely considered as the most flexible and productive strategies for racemate purification¹, therefore the most widespread industrially used method to obtain enantiomerically pure compounds. However, the achievable yields are limited to 50%, because racemates contain only 50% of the desired enantiomer²¹. These methods often rely on the formation of diastereoisomers, which are also stereoisomers, molecules with the same atomic connectivity, but non-superimposable. Yet, unlike enantiomers, they are *not* mirror images of each other (**Figure 2**). Compared to enantiomers, they are easier to separate because, beyond having different chemical properties, they often exhibit distinct physical properties (e.g. melting point, solubility, vapor pressure...) ^{1,13}.

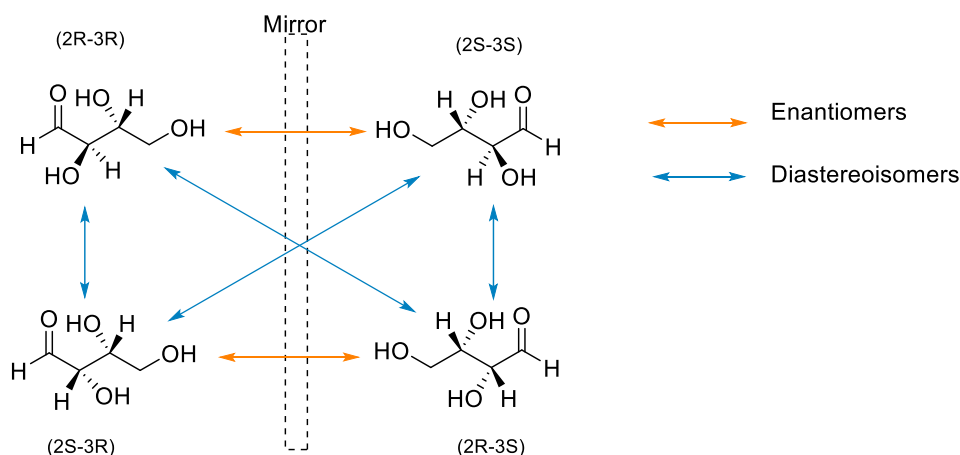


Figure 2. Erythrose diastereoisomers and enantiomers.

However, crystallization often requires additional steps to improve overall yield, typically involving repeated racemization to recycle the undesired enantiomer, thereby extending the process before achieving the desired purity²². In the case of high-performance liquid chromatography, precolumn derivatization is frequently time-consuming, solvent- and reagent-intensive, and thus costly, which has motivated the search for alternative strategies²³.

II.2 Asymmetric synthesis

Remarkable progress has been made in the field of asymmetric synthesis, also called enantioselective or stereoselective synthesis¹⁴, lately as it permits to selectively produce one enantiomer, limiting the production of by-products and improving the atom economy²⁴, making it a greener alternative²⁵. These methods rely on the introduction of a chiral element, such as a chiral catalyst, reagent, or auxiliary, into the reaction system in order to induce the selective formation of one stereoisomer over the other²⁶.

Recognized as a key methodology for generating chiral molecules from achiral precursors²², asymmetric catalysis, or enantioselective catalysis, was honoured with the Nobel Prize in Chemistry in 2021⁷. This approach relies on the use of a chiral catalyst to selectively promote the formation of one enantiomer over the other during a chemical reaction starting from a prochiral substrate²⁷. Upon interaction with the chiral environment of the catalyst, the substrate can lead to two distinct diastereomeric transition states. Ideally, one of these pathways exhibits a significantly higher activation energy²⁸, favouring the formation of a single enantiomer through kinetic discrimination. This selectivity arises from the chiral environment of the catalyst's active site, whose conformation stabilizes one transition state over the other²⁹.

III. Chiral amines

Among chiral molecules of pharmaceutical relevance, chiral amines are particularly important due to their key role as building blocks in the synthesis of high-value active pharmaceutical ingredients (APIs)³⁰. Moreover, 40–45% of pharmaceutical molecules, agrochemicals, and other industrially relevant fine chemicals incorporate a chiral amine³¹. Examples are represented on **Figure 3**.

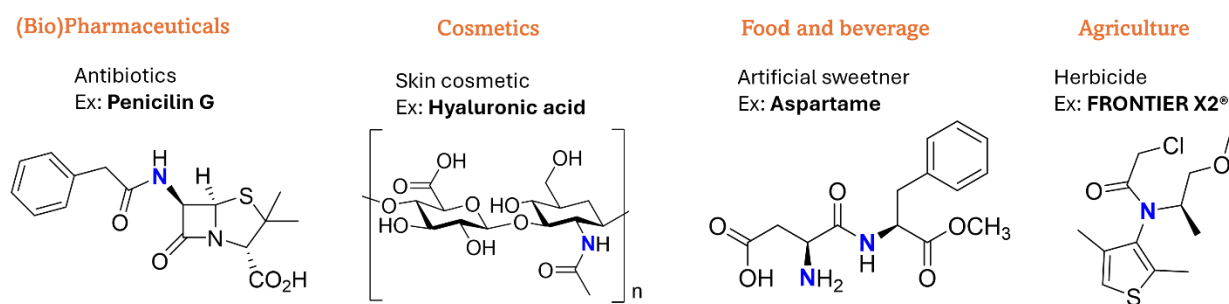


Figure 3. Examples of molecules containing chiral amines, and their field of application.

Beyond their function in APIs, chiral amines serve as chiral auxiliaries or resolving agents, further broadening their applicability³¹. Nevertheless, their synthesis often relies on non-green processes catalysed by costly organometallic complexes containing toxic heavy metals (e.g., Ru, Rh, Pd)³² which typically demand harsh operating conditions, exhibit imperfect enantioselectivity, and have limited reusability³³. Another strategy involves the asymmetric hydrogenation of the corresponding imine or enamide, catalyzed by a transition metal complex³⁴.

As the demand for greener and more sustainable processes intensifies, alternative approaches are urgently needed. In this regard, biocatalysis has emerged as a particularly attractive strategy, enabling the asymmetric synthesis of chiral amines with reduced ecological impact.

IV. Biocatalysis

IV.1 Concept and applications

Nature has likely developed the most optimized catalytic pathways, enabling the highly selective production of molecules under mild conditions such as low temperature, close to neutral pH, and aqueous environments³⁴.

Growing demand for the environmentally friendly, highly selective, and efficient organic synthesis of complex molecules has driven the adoption of enzymes in chemical synthesis¹⁹. Enzymes are proteins that act as natural catalysts. They can function within biological systems as well as *in vitro* to accelerate not only the formation of essential biological building blocks, such as amino acids, nucleotides, and sugars, but also the biosynthesis of highly complex natural and non-natural products. Beyond their role in metabolism and cellular regulation, enzymes are increasingly used in chemical synthesis to catalyze challenging transformations with outstanding enantioselectivity, enabling access to complex molecules that are difficult or inefficient to produce by traditional synthetic routes¹⁹. This exceptional selectivity arises from the precise three-dimensional geometry of their active site, which creates a chiral environment that strongly favours the formation of one enantiomer over the other^{35,36}.

Biocatalysis is now widely recognized as a viable alternative to other asymmetric catalysts in the development of synthetic pathways¹⁹, with established applications in the pharmaceutical, chemical, cosmetic, agricultural, and food industries. The large-scale viability of biocatalysis is demonstrated by the production of polyacrylamide (30,000 tons/year) using nitrile hydratases³⁷ and the synthesis of agrochemicals³⁸. To reduce their environmental impact, major corporations are increasingly investing in biocatalysis as a means to develop new materials and more sustainable industrial processes³⁹. Compared to traditional methods, biocatalysis enables operations under milder temperature and pressure conditions^{19,40}.

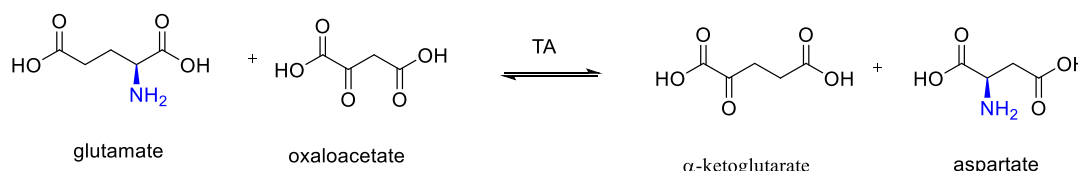
Enzymes are specific to a particular type of reaction and are classified according to the transformation they catalyze⁴¹. Notably, certain enzymes are dedicated to the synthesis of chiral amines, making them attractive alternatives to traditional catalytic methods.

IV.2 Enzymes for the synthesis of chiral amines

Exploration of protein-catalysed reactions in bioprocesses has led to the development of biosynthetic enzymes. Two primary classes of enzymes, transaminases (TA) and reductive aminases, have been identified to facilitate the conversion of prochiral ketones or aldehydes into chiral amines^{33,39}. In this thesis, we will focus on the promising transaminases.

IV.2.1. Transaminase

Transaminases, also known as aminotransferases, catalyse hundreds of metabolic reactions in the human body involving amino compounds, including amino acids, amino sugars, and polyamines⁴². These enzymes redistribute nitrogen among amino acids and corresponding oxoacids (**Scheme 1**), playing a fundamental role in both protein metabolism and gluconeogenesis, they are crucial for normal physiological function in multiple organs⁴³.



Scheme 1. Example of a natural TA-catalyzed equilibrated reaction in living organisms for amino acids metabolism.

Depending on the position of the amino group relative to the carboxyl group, TAs are classified as α -transaminases (α -TAs) or ω -transaminases (ω -TAs). The latter exhibit broader substrate specificity⁴⁴ accepting a wider variety of amine and ketone structures, which makes them particularly valuable in the pharmaceutical field. As a result, ω -TAs are more commonly used in biocatalysis and have a broader range of synthetic applications⁴⁵. They also benefit from more favourable equilibrium constants compared to α -TAs⁴⁴.

Amine transaminases (ATAs) transfer an amino group from an amino donor to an amino acceptor in the presence of pyridoxal-5' phosphate (PLP, **Figure 4**), its cofactor³⁴, exhibiting high enantioselectivity in the direct synthesis of primary and secondary chiral amines from prochiral ketones or aldehydes⁴⁴. They can also utilize readily available and inexpensive chiral donors, making them particularly attractive for industrial applications^{33,42}.

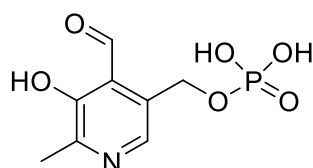


Figure 4. Pyridoxal 5'-phosphate structure, cofactor of PLP-dependent enzymes.

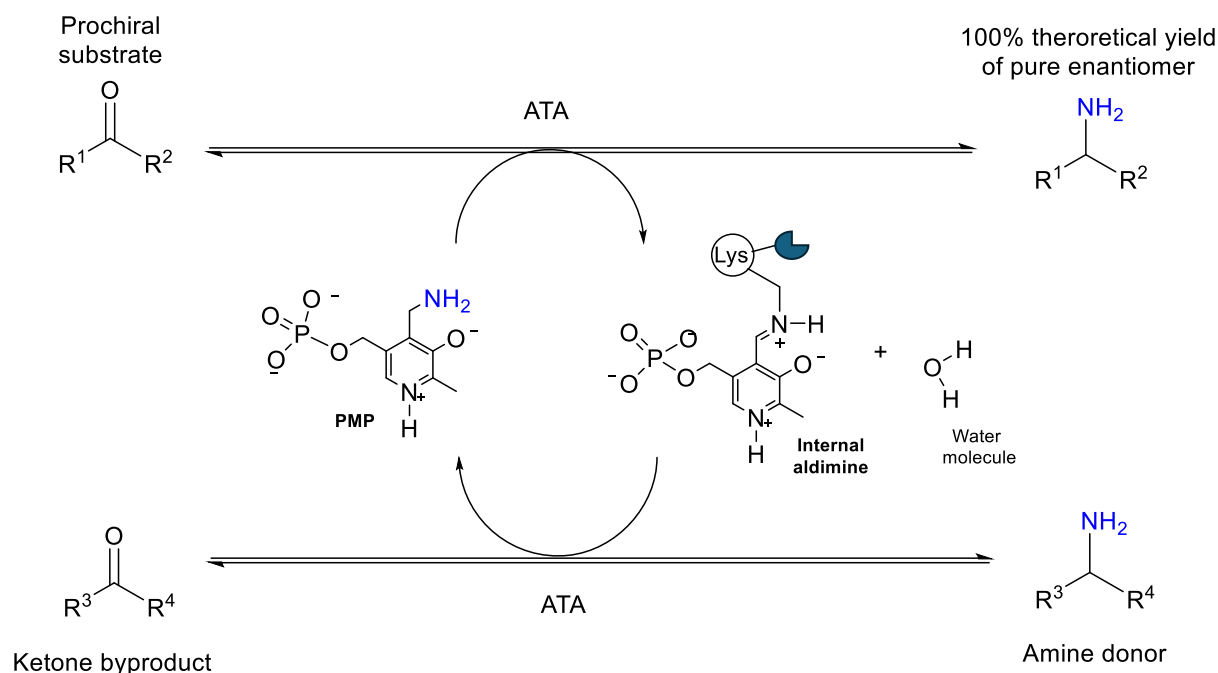
Codexis has developed ATA_117 (**Figure 5**), a mutant of a particular amine transaminase originating from the soil microorganism *Arthrobacter sp.* KNK 168^{46,47}. This enzyme is commercially available, extensively characterized, and well-suited for laboratory-scale experiments⁴².



Figure 5. ATA_117 three-dimensional structure from AlphaFold3.

ATAs function as homodimers. Each monomer houses half of an active site, and both monomers must assemble into a dimer to bring the active sites together and enable catalytic activity. The substrate binding sites consist of both a large and a small pocket, which must align appropriately for efficient catalysis. The active site is made of three pockets, one to bind the cofactor, and the two others to bind small and large substrate⁴².

PLP is the active form of B6 vitamin⁴⁸. In transamination mechanism, it acts as the transient keeper of the amino group. Transamination reaction can be considered as two half-reactions. In the first half-reaction, the amino group from the amine donor displaces the lysine-bound Schiff base in the internal aldimine of the enzyme, resulting in the formation of an external aldimine intermediate. This step is followed by a transaldimination and subsequent release of the corresponding α -keto acid or ketone, accompanied by the conversion of the pyridoxal 5'-phosphate cofactor into its aminated form, pyridoxamine 5'-phosphate (PMP). In the second half-reaction, the amino group of PMP is transferred to an acceptor substrate, leading to the formation of a new imine and the subsequent release of the corresponding amine product. The catalytic cycle is then completed by the reconstitution of the internal aldimine through Schiff base formation with the catalytic lysine residue⁴⁷ (**Scheme 2**).



Scheme 2. Asymmetric synthesis of a chiral amine starting from a prochiral ketone catalyzed by an ATA and PLP mechanism. Adapted from⁴⁷.

PLP-dependent enzymes are highly valued for their broad catalytic versatility, which renders them particularly effective and adaptable biocatalysts in a wide range of synthetic applications^{45,47}.

IV.2.2. Industrial application

Thanks to the extensive knowledge already available on these enzymes, transaminases have been successfully applied in various processes, in some cases even outperforming previously used chemo-catalytic systems in terms of selectivity, efficiency, and sustainability^{40,49–51}.

Sitagliptin synthesis by transaminases is a striking example of industrial-scale organic synthesis⁵². Although improvements have been introduced for the chemo-catalytic route, biocatalysis still delivers more attractive results.

To further broaden their industrial applicability, a key approach, common to all catalysts, is to render ATAs heterogeneous through immobilization.

IV.3. Enzyme immobilization

Interest is getting bigger towards the immobilization of ATAs onto solid supports, getting the best of recyclability, reusability and making them more robust to certain conditions for more catalyst stability⁵³.

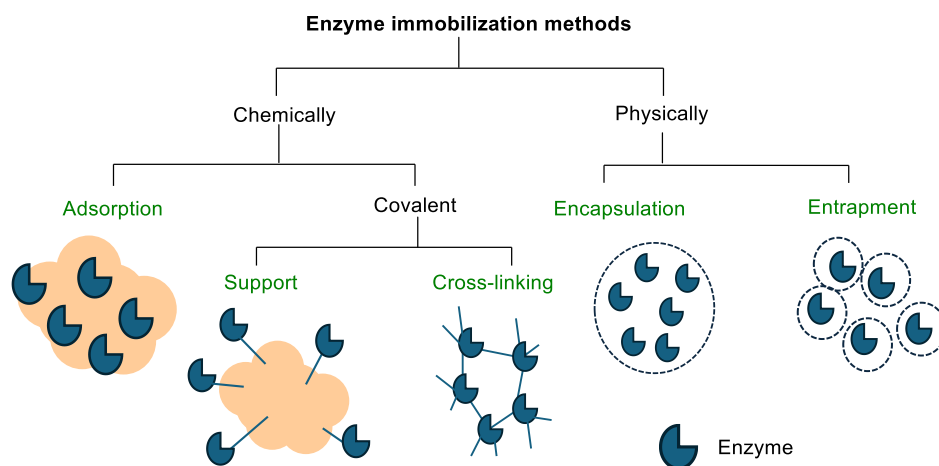
IV.3.1. Methods

Beyond facilitating separation, immobilization frequently enhances stability towards storage and reaction conditions⁵⁴. The increased robustness widens the process window

(higher operational temperatures, pH, improved resistance to organic solvents and ionic force) making them more suitable for chemical reactions⁵⁴.

Two main challenges must be addressed: first, ensuring a robust attachment chemistry to prevent enzyme leaching; and second, preserving enzymatic activity, as immobilization can also lead to partial deactivation⁴².

A range of techniques exist for enzyme immobilization (**Scheme 3**). Enzymes are either reversely adsorbed or covalently bonded on a support or among enzymes by cross-linking. Physical confinement offers additional options, such as encapsulating the enzyme within a semipermeable polymeric or inorganic shell, or entrapping it inside porous matrices⁵⁴. Regardless of the method chosen, each technique converts a soluble enzyme into an insoluble catalyst, with the intent to make it reusable and process-tolerant. Immobilization thus markedly expands the practical utility of biocatalysts in industrial organic synthesis.



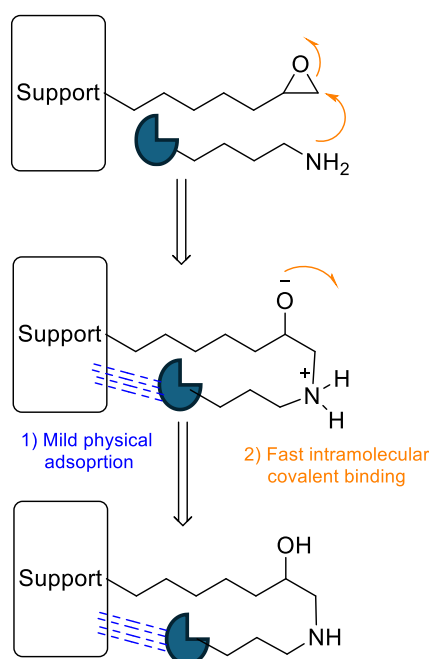
Scheme 3. Schematic classification of enzyme immobilization strategies. Adapted from ⁵⁴.

The most robust immobilization is achieved through covalent grafting, in which the enzyme forms a stable covalent bond with a functionalized or non-functionalized surface⁵⁴. A particular attention should be given to the link between the support and the enzyme since it could influence the properties or activity of the enzyme. Organic support materials frequently present inherent functional groups on their surfaces that serve as natural anchoring sites for enzymes. Moreover, these supports can be obtained in a wide range of chemical compositions⁴².

IV.3.2. Epoxy-functionalized resins

Epoxide-functionalized supports are commonly used due to their ability to react under mild conditions with various nucleophilic groups present on protein surfaces, such as amino, thiol, or phenolic groups⁵⁵. For a binding with an amino group, these reactions typically result in the formation of amino-alcohol linkages, for example, between epoxide groups and enzymes surface-exposed lysines⁵⁴. The immobilization generally follows a

two-step mechanism: a rapid, mild physical adsorption of the enzyme onto the support surface is followed by a covalent coupling reaction between the adsorbed enzyme and the epoxide functionalities (**Scheme 4**). Sepabeads®-type epoxy resins are particularly well-suited for industrial use due to their high mechanical stability, chemical robustness, and high surface density of epoxy groups, which facilitates strong and dense enzyme-support interactions. These functionalized materials also exhibit excellent stability in aqueous suspensions and during storage⁵⁵.



Scheme 4. Covalent enzyme immobilization on epoxide-functionalized support. Adapted from⁵⁴.

ReliZyme EP403 and ReliZyme HFA403 are two widely used poly(methyl methacrylate) resins commonly employed for the immobilization of enzymes, and they have already been used to fix α -TA³⁰. They are constituted of porous and hydrophilic polymethylacrylate, activated with a functional group: respectively epoxy and epoxy-amino (**Figure 6**), for covalent immobilization.

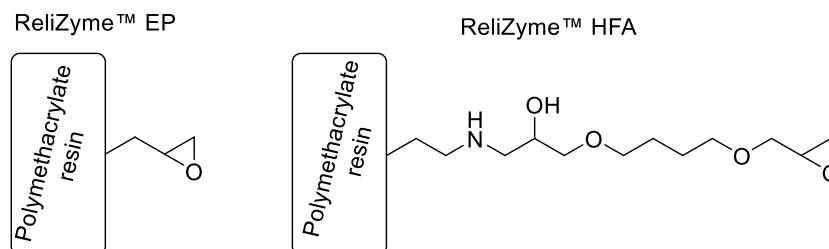


Figure 6. Structures of polymethacrylate resins functionalized with epoxy (EP) and amino-epoxy (HFA) groups.

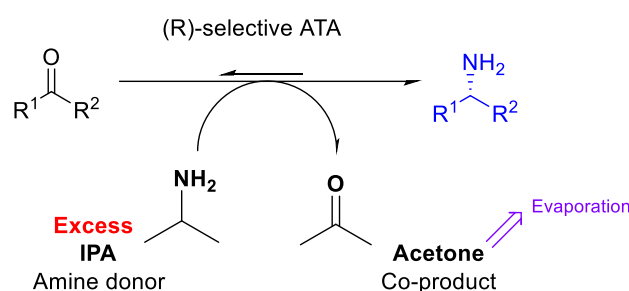
In particular, the longer and less polar spacer arm of HFA contributes to greater flexibility of the immobilized enzyme. Its lower polarity also makes it more suitable for enzymes acting on hydrophobic substrates, such as aromatic compounds⁵⁶.

With the enhanced properties of immobilized enzymes, it opens the way for a wider range of applicability for biocatalysts. Nevertheless, the use of enzymes in synthetic chemistry is not without challenges, as they are subject to several intrinsic constraints.

IV.4. Challenges related to transaminases

Transamination reactions frequently encounter unfavourable thermodynamics^{40,45,57,58}, particularly when the synthesized chiral amine is not an amino acid⁴². Consequently, research efforts have focused on developing equilibrium-shifting strategies^{33,45}.

Easy solutions include the possibility of using large excess of amine donor to push the equilibrium in the right direction^{45,59}. Using isopropylamine (IPA, **Scheme 5**) as a readily available and cost-effective amine donor for industrial applications involving transaminase is a possible strategy⁶⁰. One key advantage of this choice is that it releases acetone as the co-product, which can be easily evaporated, thus pulling the reaction equilibrium toward the formation of the desired chiral amine by removing the co-product during the reaction^{19,45,57,61}.



Scheme 5. Model transamination using isopropylamine (IPA) as the amine donor forming acetone as the co-product. The absolute configuration shown corresponds to R^1 having higher CIP priority than R^2 .

On the other hand, transaminase systems are challenging to scale up due to their sensitivity to inhibition by ketones, which act both as substrates and as co-products^{33,46,59}. Enzyme engineering is being explored as a strategy to enhance the robustness of these biocatalysts, improving their tolerance to substrate⁴⁵. Another alluring strategy is to use cascade methodologies to mitigate enzyme deactivation by gradually introducing the problematic substrate, thereby limiting its inhibitory effect^{19,59}.

V. Catalytic cascades

V.1. Cascade reactions for streamlined synthesis

To solve inhibition issues or to target economic efficiency, a possibility is to couple the enzymatic reaction with another upfront or downstream chemical transformation: starting from a cheaper or non-inhibitory substrate, or rapidly converting unstable or inhibitory products to another useful target⁶².

Such streamlined approach can significantly shorten multi-step syntheses, making it suitable for industrial purposes. Furthermore, employing cascade methodologies provides the added advantage of driving reaction equilibria toward product formation, as each intermediate is immediately consumed in the subsequent step¹⁹.

Such cascade reactions are gaining traction in the context of greener synthesis of high value chemicals. Recent developments in both academia and industry have broadened the scope of cascade approaches to produce structurally challenging organic compounds¹⁹. The high selectivity of enzymes eliminates the need for protective groups and lengthy intermediate isolation and purification^{19,34,52}.

V.2. Categorization

Cascades reactions can be categorized into two operational modes: sequential and concurrent (simultaneous). In the concurrent mode, all catalysts are introduced at the beginning of the reaction and work under uniform conditions, without parameter adjustments. Conversely, in the sequential mode, the second catalyst is introduced only after the first step has reached completion. At this stage, additional components such as cofactors or solvents may be introduced, and reaction parameters, including temperature, pressure, pH, or substrate concentration, can be modified to optimize the subsequent transformation³⁴. A wide range of biocatalytic cascades has been developed, encompassing linear, cyclic, and parallel designs, from single- to multi-step, performed either in vitro or in vivo¹⁹.

V.3. Biocatalytic cascades in the context of transamination

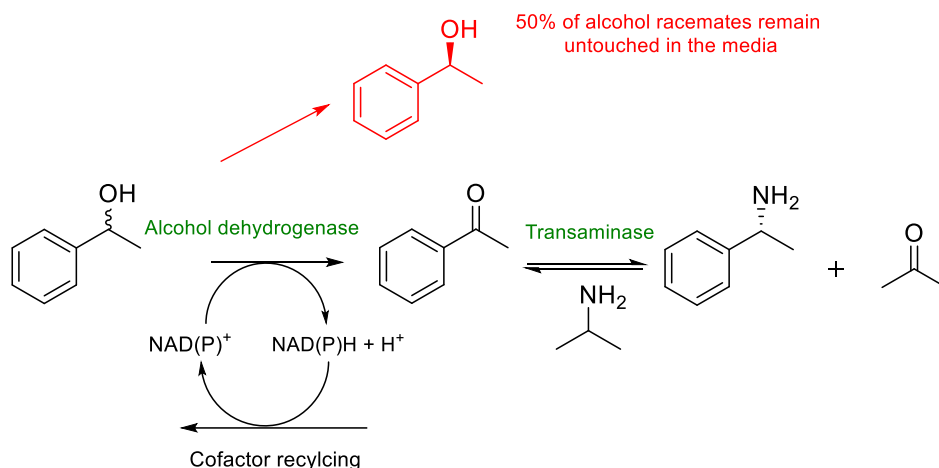
These enzymatic-cascades typically enhance molecular complexity by introducing chirality or new functionalities, often transforming inexpensive achiral materials into high-value chiral products^{19,22}. Applying enzyme cascades could help overcoming possible thermodynamic problems and avoid substrate inhibition issues⁶³, two issues often encountered when using transaminases.

V.3.1. *Two-enzymes biocatalytic cascade starting from alcohol oxidation*

Alcohols are particularly used substrates for organic synthesis, and oxidative conversion provides a convenient pathway to activate them for downstream transformations⁶⁴. Notably, certain enzymes can selectively oxidize alcohols into their corresponding ketones. But unless the enzyme has been extensively engineered to become ambidextrous⁶³, it generally cannot convert both alcohol enantiomers⁶⁵. This is due to the high enantioselectivity that characterizes most enzymes, a feature that commonly makes them particularly attractive for organic synthesis. As a result, the reaction yield is limited to a maximum of 50%, with the non-converted enantiomer remaining in the reaction medium throughout the process (**Scheme 6**).

Such processes using ambidextrous alcohol dehydrogenases have recently been proposed by Tian and Li⁶⁵. However, achieving this level of enzyme flexibility requires the development of specific enzyme mutants and solid expertise in protein engineering. In their strategy, the engineered dehydrogenase is combined with a transaminase to form an effective cascade system.

Beyond the challenge of enantioselectivity, a key consideration in enzymatic processes is the requirement for cofactors and their regeneration. Oxidation-capable enzymes such as alcohol dehydrogenases are often nicotinamide-dependent, relying on cofactors like NAD⁺ or NADP⁺ (**Scheme 6**). Their use presents notable drawbacks such as the high cost of these cofactors and the necessity for efficient recycling systems⁶⁵. This often involves introducing an additional enzyme, thereby increasing the overall complexity of the process.



Scheme 6. Biocatalytic cascade using an alcohol dehydrogenase and a transaminase to avoid substrate inhibition.

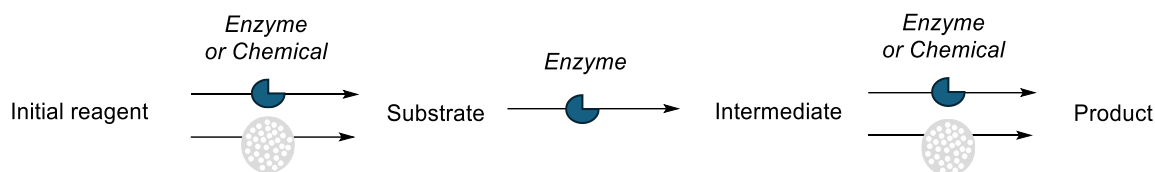
Since cofactors are often used in stoichiometric amounts, failing to recycle them can compromise sustainability, especially at an industrial scale. Addressing this challenge has been a major milestone in advancing biocatalysis for industrial organic synthesis¹⁹. An appealing and streamlined strategy consists in coupling a transaminase with a complementary catalyst that enables the transformation of both alcohol enantiomers.

V.3.2. Chemo-enzymatic cascades

To overcome the challenges associated with introducing a second enzyme, the use of a heterogeneous chemo-catalyst can be considered as an alternative for the alcohol oxidation.

In chemo-enzymatic systems, at least one step remains enzymatic, but additional transformations are carried out using chemo-catalysts (**Scheme 7**). When both biocatalysts and chemo-catalysts are combined within a single, uninterrupted sequence, the process is referred to as hybrid catalysis, chemo-enzymatic cascade or hybrid bio-organocatalytic cascade¹⁹. Respective catalysts can be either soluble or immobilized or solid. In some

cases, both the chemo- and the enzymatic functions are placed on the same bi- or multi-functional catalyst, which is referred to as a “hybrid chemo-enzymatic heterogeneous catalyst”⁶⁶. These one-pot cascades hold significant potential for organic synthesis.



Scheme 7. Biocatalytic linear cascades.

In addition to linear hybrid cascades, orthogonal and cyclic cascades have also been developed. Parallel cascades exist as well, although in this case, the term "cascade" often refers not to shared substrates, but rather to a shared cofactor, a strategy commonly employed for cofactor recycling⁶⁷.

However, the combination of different catalytic classes (biocatalysts and chemo-catalysts) within a single batch reactor presents several challenges, particularly concerning the compatibility of optimal operating conditions. While enzymes typically function efficiently under mild operating conditions, many chemo-catalysts require harsher conditions: elevated temperatures, organic solvents, non-neutral pH, leading to operational constraints^{34,68}. Furthermore, stability issues and mutual deactivation are frequently encountered when two catalysts of distinct natures are employed simultaneously in a one-pot system. Establishing a system where catalysts function concurrently remains a significant challenge. To achieve this, it is essential to identify a compatibility window in which different catalysts can coexist while maintaining sufficient activity to ensure an acceptable overall yield. Consequently, multi-pot, multi-step cascades are traditionally preferred, as they offer greater flexibility in optimizing individual reaction steps³⁴. Few examples of fully concurrent catalytic cascades are reported in the literature compared to sequential systems^{34,69}. In the last 30 years, the utilisation of chemo-enzymatic processes is mainly limited to dynamic kinetics resolutions and enzymatic processes with concomitant regeneration of cofactors³⁴.

Chemoenzymatic cascades, however, have already been explored in academic, notably for the synthesis of chiral amines. These approaches typically begin with a chemo-catalytic step such as oxidation, hydration, or isomerization applied to inexpensive starting materials (racemic alcohols, alkenes, allyl alcohols, or alkynes), leading to the formation of a prochiral ketone intermediate. This intermediate can then undergo transamination catalyzed by an enzyme to afford the desired chiral amine in a single enantiomeric form³⁴. In the context of this thesis, we will focus on the in situ generation of the prochiral ketone via oxidation of an alcohol.

V.4. Metal-catalyzed oxidation

Several strategies exist for the oxidation of alcohols, including the use of stoichiometric chemical oxidants and chemo- or bio-catalytic approaches^{70,71}. Commonly used strong chemical oxidants include chromic acid derivatives, TEMPO-based systems (2,2,6,6-tetramethylpiperidin-1-oxyl), and other metal-free oxidants^{71–73}. However, these methods often suffer from significant drawbacks, such as the generation of toxic waste and the need for harsh reaction conditions⁷³, notably involving the addition of stoichiometric quantities of toxic inorganic reagents to the reaction medium⁷⁴ and thus requiring protection and deprotection steps⁷¹, conditions that are generally incompatible with biocatalysis, and in any case not in line with the principles of green chemistry. Thus, catalytic routes are preferred.

V.4.1 *Platinum-group metals*

Among heterogeneous catalysts, those based on platinum-group metals (platinum, palladium⁷⁵, ruthenium, and rhodium) are the most commonly employed for oxidation reactions in organic synthesis⁷⁶. Processes based on these active metals are already well established, achieving high conversions to ketones, aldehydes, or carboxylic acids while operating at moderate temperatures⁷⁵. However, in addition to their high cost^{77,78}, several limitations have been reported, including lack of selectivity due to overoxidation⁷⁸, product or substrate poisoning⁷⁷, low thermal stability⁷⁸, particle leaching^{79,80}, and catalyst agglomeration^{80,81}.

With a delay of 20 to 30 years compared to the previously mentioned metals, gold made a spectacular and disruptive entry into the field of homogeneous organic synthesis, perhaps because it was long considered “too noble to be used in chemistry”⁸². While also known to suffer from some of the above-mentioned issues, gold-based catalysts are an important focal point when destined to be combined with biocatalysis, due to their outstanding performance under mild conditions, including very low temperatures⁸³.

V.4.2 *Gold-based oxidation catalysts*

Although gold was long considered catalytically inert, studies have demonstrated that supported gold nanoparticles (Au NPs) exhibit high catalytic activity, particularly in liquid-phase oxidations⁸⁴. Au NPs enable the selective oxidation of alcohols and alkanes^{84,85}, reactions typically associated with low conversion rates and poor selectivity when performed with other metals⁸⁵.

Gold is now considered a catalyst of choice for oxidation reactions, thanks to its ability to operate under mild conditions and its high selectivity⁸⁵, with minimal loss of activity or metal leaching. These features offer clear advantages over palladium or platinum catalysts⁷⁵. Unlike these two metals, gold is not susceptible to oxygen poisoning⁸⁵ or overoxidation and can efficiently activate mild oxidants⁷⁵, making it particularly well-

suited for environmentally friendly and selective oxidation processes⁸⁵. Molecular oxygen (O_2), often used as the oxidant, is efficiently activated by gold thanks to its resistance to oxygen deactivation^{84,86}.

Gold can be supported on a variety of oxide materials to achieve effective dispersion, including CuO, NiO, SiO_2 , TiO_2 , and ZrO_2 . Among these, silicon dioxide (SiO_2) is particularly attractive for catalytic applications due to its low cost, its thermal stability, and its chemical inertness⁸⁷. Furthermore, just as polymeric resins, SiO_2 offers excellent tunability for surface functionalization⁸⁸. This adaptability enhances its utility in catalysis, as it is compatible with derivatization strategies, such as the grafting of organic modifiers like (3-aminopropyl)triethoxysilane (APTES) or (3-glycidyloxypropyl)trimethoxysilane (GLYMO), required for biomolecule immobilization⁸⁹. However, the combination of a silica support with gold remains challenging because of the inherently low affinity between the two materials. As a result, conventional methods such as impregnation or deposition-precipitation often led to poorly controlled syntheses, typically yielding gold nanoparticles up to four times larger than desired⁸⁸.

To enable efficient gold-catalyzed oxidation, it is essential to develop synthesis methods that yield well-defined Au NPs, as the catalytic performance of gold is highly structure-dependent^{75,85,87}. Various strategies have been reported in the literature to produce gold nanoparticles with sizes below 5 nm, well-dispersed on different supports. Among them, sol-immobilization and impregnation are widely used. Some approaches require post-synthesis calcination or the use of stabilizing agents to improve nanoparticle size control and dispersion, thereby enhancing catalytic performance and overall conversion⁸⁵.

In this context, our laboratory has developed a dedicated synthesis route to highly dispersed Au/ SiO_2 catalysts. Using a one-pot aerosol-assisted sol-gel (AASG) method, mesoporous silica microspheres are produced, featuring well-dispersed gold nanoparticles⁸⁸. This technique couples aerosol processing with sol-gel chemistry, which traditionally involves hydrolysis and polycondensation of molecular precursors to form inorganic networks⁹⁰. In the AASG approach, a precursor solution is atomized into fine airborne droplets that undergo rapid solvent evaporation upon exposure to a heated zone⁹¹. During this drying phase, the sol-gel reactions proceed in situ, leading to the direct formation of solid microspheres without generating liquid by-products⁸⁸. The combination of rapid aerosol drying and controlled inorganic condensation ensures excellent homogeneity, high textural control, and efficient dispersion of metal nanoparticles on the support (**Figure 7**). This makes AASG particularly well suited for the synthesis of supported metal catalysts⁹¹⁻⁹³.

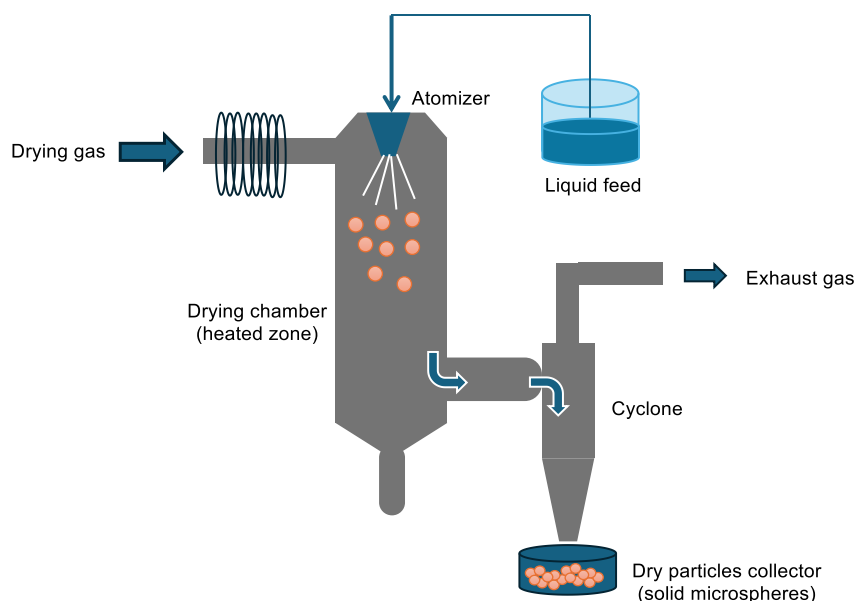


Figure 7. Spray-dryer for formation of well-dispersed gold nanoparticles catalyst.

For the synthesis of Au/SiO₂ catalyst, precursor solution typically contains a silica source, a thiol-based gold-stabilizing agent, a structure-directing surfactant, and a gold precursor⁸⁸. The resulting material is subsequently calcined in static air to remove organic components and develop the desired porosity. The use of the thiol stabilizer effectively delays gold nanoparticle formation, promoting high dispersion and enhanced thermal stability within the silica matrix⁸⁸.

This catalyst showed good performances in the selective oxidation of glycerol to dihydroxyacetone⁹⁴. In the context of the present thesis, it appears as a suitable candidate to catalyze the oxidation of the alcohol that would be used as the starting point of the process, thereby enabling a subsequent transamination step with reduced risk of substrate inhibition.

VI. Objectives

The objective developed is to unlock the enantioselective synthesis of chiral amines by transamination by coupling the enzymatic reaction with an upstream chemo-catalytic synthesis of the prochiral ketone, starting from non-inhibitory alcohols. To do so, the idea is to develop a chemo-enzymatic catalytic system that would achieve the two parts of the reaction in a one-pot one-step cascade fashion.

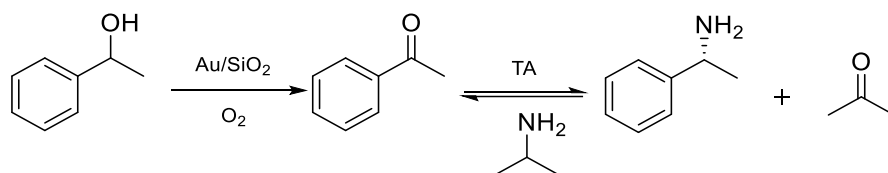
Working on a specific model reaction (see Strategy), we step ourselves the following sub-objectives:

1. Find suitable operation conditions for both catalysts,
2. Prove the feasibility of the one-pot one-step reaction,
3. Demonstrate the beneficial effects of the coupling (as compared to the reaction that starts from the prochiral ketone).

VII. Strategy

The model cascade converts 1-phenylethanol into 1-phenylethylamine (**Scheme 8**) by:

- 1) In situ oxidation to acetophenone using Au/SiO₂,
- 2) Coupling with transamination catalyzed by ATA_117 enzyme.



Scheme 8. Model transamination starting from an alcohol to a chiral amine.

The development of the hybrid chemo-enzymatic cascade follows four streamlined stages. First, each catalyst is independently optimized: enzyme immobilization is tuned for stability and activity, while the chemical catalyst is characterized and tested under mild conditions to define its best operating parameters. Second, compatibility between both catalysts is established by identifying conditions that allow their simultaneous operation without mutual deactivation; reaction parameters are adjusted as needed to create a suitable operational window. Third, the fully integrated cascade is executed by combining all optimized conditions into a single process. Finally, experimental data are rigorously analyzed to elucidate reaction mechanisms and inform the design of future hybrid systems.

Experimental

I. Materials

Acetophenone (AcPh ; >98.0%), *3,3-diphenylpropionic acid* (DPPA ; >97%), *isopropylamine* (IPA ; >99%), *(3-Mercaptopropyl) trimethoxysilane* (MPTMS ; >96%), *(±)-1-phenylethanol* (>98%), *2-phenylethanol* (>98%), *1-phenylethylamine* (>98%), *pyridoxal-5'-phosphate* (PLP ; >98%), *tetraethyl orthosilicate* (TEOS ; >98%) were purchased from TCI.

Bradford Reagent, *hypotaurine* (>98%), *(R)-(+)-1-phenylethylamine* (>99%), pluronic F-127, *sodium hydroxide* (NaOH ; >97%) and *trifluoroacetic anhydride* (>99%) were purchased from Sigma-Aldrich.

Ethanol absolute, *ethyl acetate* (for HPLC) and *hydrochloric acid* (HCl ; 37%) were purchased from VWR BDH Chemicals.

Gold precursor *hydrogen tetrachloroaurate (III) trihydrate* (HAuCl₄·3H₂O ; 99.99%, Au min 49%) was purchased from Alfa Aesar, *di-potassium hydrogen phosphate* (K₂HPO₄ ; >99%) from Carl ROTH and *potassium phosphate monobasic* (KH₂PO₄ ; 99%) from Acros Organics.

The amine resin (Relizyme™, HFA112/S) was purchased in M-size from Resindion S.r.l. The commercial enzyme ATA_117 (Codexis) was kindly provided by Ludivine van den Biggelaar.

Most experiments were conducted in the Heterogeneous (Bio-) Catalysis Laboratory at the University of Louvain (Louvain-la-Neuve, Belgium). The production, purification, and characterization of His-tagged transaminase were performed in the Laboratory of Biophysics, Biochemistry and Microorganism Genetics (team of Prof. Patrice Soumillon) at the same university.

II. Methods

II.1. Chemo-catalyst synthesis

Au/SiO₂ catalyst was originally developed in the laboratory, during the PhD thesis of Margot Van Der Verren⁹⁴, and an adapted protocol was followed in the context of this master's thesis.

Solution A was prepared by mixing 34.23 g of TEOS (Tetraethyl orthosilicate) and 1.75 g of MPTMS (3-Mercaptopropyl-trimethoxysilane) with 60.05 g of acidified water (0.1 M HCl). In a separate vessel, 11.70 g of Pluronic F127 was dissolved in 24.02 g of absolute ethanol and 135.08 g of deionized water. Both solutions were stirred at room temperature overnight. Chloroauric acid trihydrate (HAuCl₄·3H₂O) was then added to solution A to achieve a nominal gold loading of 1 wt. % in the final material. After 10 minutes of stirring, solution A was poured into solution B and the combined mixture was stirred for a further

30 minutes. This premix was atomized using a Buchi 290 Spray Dryer at 5 psi, and the resulting droplets were carried through a tubular quartz reactor maintained at 175°C. The dry powder was collected on a ceramic crucible covered with aluminum foil, then calcined in static air at 550 °C using a heating ramp of 1 °C/min for 5 hours.

II.2. Characterization methods

II.2.1. Infrared spectroscopy FTIR-ATR

Fourier Transform Infrared Spectroscopy in Attenuated Total Reflectance mode (FTIR-ATR) was used to measure the decrease in radiation intensity as a function of wavenumber, corresponding to the absorption of infrared light by molecular vibrations in the sample. This technique involves directing an infrared beam onto a diamond crystal with a high refractive index and infrared transparency. When the beam reaches the interface between the crystal and the solid sample, it undergoes total internal reflection. At each reflection point, an evanescent wave penetrates a few microns into the sample. This interaction causes partial absorption of the wave by the sample, leading to attenuation of the reflected signal, which is then recorded.

Spectra were collected using a Bruker Equinox IFS55 spectrometer equipped with a DTGS (Deuterated Triglycine Sulfate) detector. Measurements were performed at room temperature, over the range of 4000–300 cm^{-1} , using 100 accumulated scans at a resolution of 4 cm^{-1} , with air used as background. The data were processed using the OPUS software.

II.2.2. Nitrogen physisorption

Specific area surface, pore volume and average pore size are determined via N_2 physisorption. The catalyst sample is weighted and degassed under primary vacuum at 150°C overnight with a Micromeritics VacPrep 061 and then weighted again. Micromeritics TriStar 3000 measures nitrogen physisorption isotherms for adsorption and desorption in liquid nitrogen at 77K.

Specific surface area was determined by the Brunauer-Emmet-Teller Method (BET), in the pressure range between 0.05 and 0.30 (relative pressure). The micropore volume was estimated using the t-plot method based on the Harkins and Jura thickness equation. The average pore diameter was obtained from the adsorption branch of the isotherm using the Barrett-Joyner-Halenda (BJH) method. The adsorption branch was used to avoid overestimation caused by an artefactual peak in the desorption branch, typically associated with the tensile strength effect.

II.2.3. Scanning electron microscopy (SEM)

SEM imaging was carried out on a JEOL 7600F at an accelerating voltage of 5 kV. Samples were mounted on aluminium stubs using carbon tape, and then sputter-coated with an 8 nm gold film under vacuum (Cressington Sputter Coater 208 HR).

II.2.4. X-ray diffractometry (XRD)

X-ray diffraction (XRD) was used to characterize the catalyst structure: both the atomic arrangement within the unit cell and the average crystallite size of the metal nanoparticles. Patterns were collected at room temperature on a Bruker D8 Advance diffractometer in Bragg–Brentano geometry, using Cu K $\alpha_{1,2}$ radiation ($\lambda = 1.5418 \text{ \AA}$) at 40 kV and 30 mA. Scans spanned $2\theta = 5\text{--}100^\circ$ with 0.05° steps and 1.5 s counting time per step. Powdered samples were loaded into 25 mm PMMA holders. Diffraction peaks were integrated in WinPLOTR, the FWHM of each reflection was extracted, and the Scherrer equation was applied to estimate mean crystallite sizes:

$$D = \frac{K \times \lambda}{\beta \times \cos\theta}$$

where D is the crystallite size (in nm),

K is the shape factor (typically 0.9),

λ is the x-ray wavelength (in nm),

β is the full width at half maximum of the peak (in radians),

θ is the Bragg angle (half of the measured 2θ value, in radians).

Bragg's Law was used to relate the diffraction angle to the interplanar spacing d :

$$n\lambda = 2d \sin\theta$$

where n is order of diffraction ($n=1,2,\dots$),

d is the path difference.

II.2.5. X-ray photoelectron spectroscopy (XPS)

XPS analyses were carried out with a SSX 100/206 photoelectron spectrometer from Surface Science Instruments (USA) equipped with a monochromatized micro focused Al X-ray source (powered at 20 mA and 10 kV). The powdery samples were fixed with double-sided tape onto small brass troughs of 6 mm diameter that were placed on a ceramic carousel. The pressure in the analysis chamber was around 10^{-6} Pa. The angle between the surface normal and the axis of the analyser lens was 55° . The analysed area was approximately 1.4 mm^2 and the pass energy was set at 150 eV. In these conditions, the full width measured at half maximum (FWHM) of the Au 4f $_{7/2}$ peak for a clean gold standard sample was about 1.6 eV. A flood gun set at 8 eV and a Ni grid placed 3 mm above the

sample surface were used for charge stabilisation. The following sequence of spectra was recorded: survey spectrum, C 1s, O 1s, Si 2p, Au 4f and C 1s again to check the stability of charge compensation with time. The C_{1s}-(C,H) component of the C1s peak of carbon has been fixed to 284.8 eV to set the binding energy scale. Data treatment was performed with the CasaXPS program (Casa Software Ltd, UK), some spectra were decomposed with the least squares fitting routine provided by the software with a Gaussian/Lorentzian (85/15) product function and after subtraction of a non-linear baseline. Molar fractions were calculated using peak areas normalised based on acquisition parameters and sensitivity factors provided by the manufacturer.

II.3. Quantification methods

II.3.1. Gas chromatography analysis (GC)

Quantification of the molecules obtained during catalytic reactions was carried out by GC equipped with a flame ionization detector (FID) and a Restek Rtx-5 column (30 m x 0.25 mm x 0.25 μ m). The vector gas was helium, the pressure was 10 psi, injector and detector temperatures were set at 250°C. The temperature program of the oven follows this scheme (**Figure 8**):

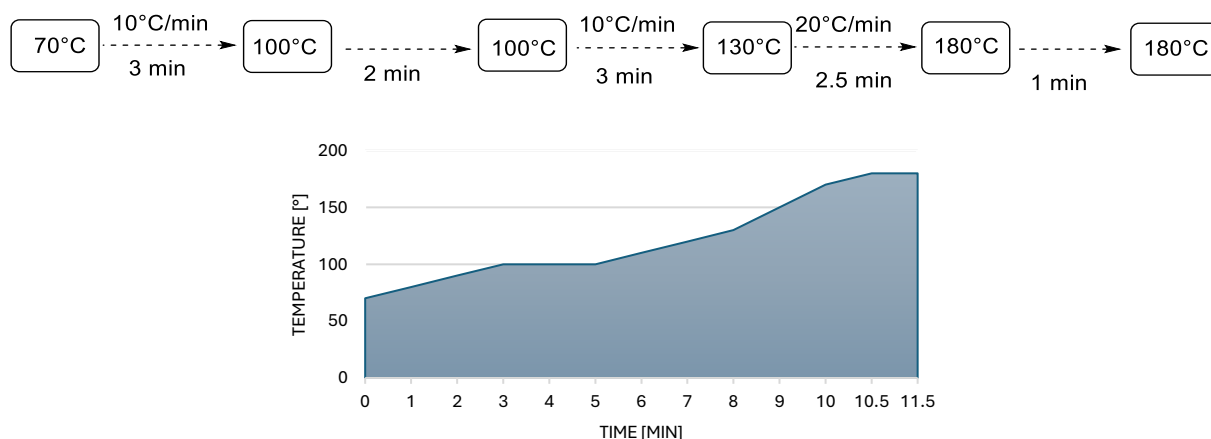


Figure 8. Temperature program for gas chromatography.

Prior to analysis, sample preparation involved collecting 250 μ L of the reaction mixture, followed by deprotonation with 100 μ L of NaOH 2M and liquid–liquid extraction using 500 μ L of ethyl acetate. The mixture was vortexed for 10 seconds and centrifuged for 20 seconds at 6000 rpm to allow phase separation. Then, 400 μ L of the organic (upper) phase was collected and transferred into a glass vial for GC analysis.

2-phenylethanol was used as an internal standard to correct for sample-to-sample variability and injection-to-injection fluctuations during analysis. Under these conditions, baseline separation of the peaks was achieved, and concentrations were calculated from the peak areas after calibration.

II.3.2. Chiral GC

a) Derivatization

To 500 μL of the reaction mixture, 100 μL of NaOH 2M and 250 μL of ethyl acetate were added. The mixture was vortexed for 10 seconds and centrifuged for 20 seconds at 6000 rpm to allow phase separation. The ethyl acetate layer was transferred to 3 mL glass tubes and 50 μL of trifluoroacetic anhydride were added and heated for 30 minutes at 70°C. Samples were evaporated to dryness under a stream of nitrogen and reconstituted with 200 μL of ethyl acetate.

b) Chromatography

Detection of the derivatized 1-phenylethylamine was carried out by GC equipped with a flame ionization detector (FID) and a Agilent CP-Chirasil Val (25 m x 0.25 mm x 0.12 μm). The vector gas is helium, the pressure at the start is 7 psi, injector and detector temperatures are at 250°C. The temperature program of the oven follows this scheme (Figure 9):

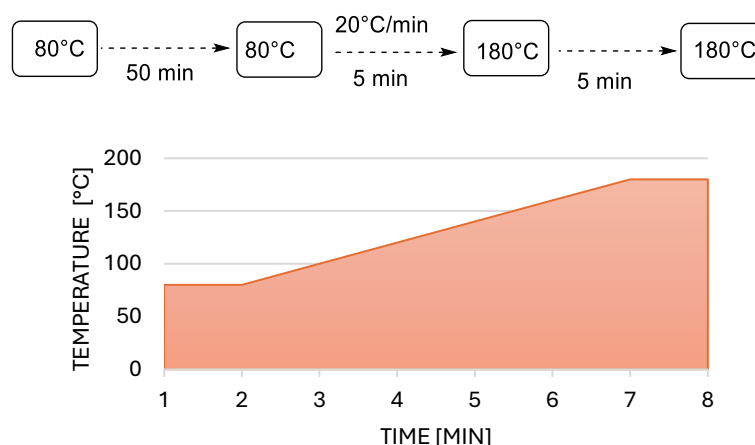


Figure 9. Temperature program for chiral gas chromatography.

“Enantiomeric excess” (ee) is one of the metrics used to quantify the enantiomeric purity of chiral substances. It is calculated with the formula:

$$ee [\%] = \frac{|R - S|}{R + S} * 100$$

where R and S are the molar fractions of the R and S enantiomers. By definition, an ee value of zero corresponds to a racemic mixture. The goal of enantioselective synthesis is to achieve the highest possible ee, ideally approaching 100%. ee was determined by chiral gas chromatography. Separation of the R- and S-phenylethylamine enantiomers was achieved on a chiral stationary phase under normal-phase conditions, using helium as the carrier gas. Under these conditions, baseline separation of the R- and S-

phenylethylamine peaks was routinely achieved, and ee values were calculated from the peak areas.

II.3.3. Bradford colorimetry

This quantification method is used to quantify the protein concentration in solution, primarily to assess immobilization rate on the resin. To perform it, 50 μ L of sample is added to 950 μ L of Bradford reagent and incubated for 10 minutes at room temperature. Measurements were performed in disposable polystyrene cuvettes (1 cm path length), compatible with visible-range spectrophotometry. Absorbance is then measured at 595 nm using a Thermo Scientific Genesys 10S-Vis spectrophotometer. The blank was always measured and contained water or PLP and PBS in the same proportions as in the reaction medium. Protein content is estimated with a calibration curve established using bovine serum albumin (BSA) as the standard. The resulting linear equation was: $A = 1.0611 \times [\text{Protein}]$, with $R^2 = 0.9979$.

II.4. Enzyme production and immobilization

Two forms of the transaminase ATA_117 were employed in this study. One batch consisted of the pure, lyophilized enzyme obtained from surplus material produced by Ludivine Van den Biggelaar's during her thesis. Second batch was a cell-free enzyme preparation (skipping the purification step) produced in the biophysics, biochemistry and microorganisms genetics laboratory at the University of Louvain according to the following protocol. The first batch was used for the majority of experiments because it lacked the undesired compounds found in the cell-free extract.

II.4.1. His-Tagged ATA_117 transaminase production

DNA is inserted into competent bacteria that are transformed (i.e. DNA of interest is incorporated in their own DNA). (ii) Enzyme of interest is overexpressed into bacteria.

a) Bacterial transformation

50 μ L aliquot of BL21 E.coli strains is mixed with 0.16 μ g plasmid, and electroporated using Bio-Rad GenePulser Xcell (2.5 kV, cuvette diameter 2 mm). Then, 1 mL LB medium is added, and the mix is incubated for 1 hour at 37 $^{\circ}$ C, and spread over petri plates, containing LB(Luria-Bertani)-Agar and kanamycin, using glass beads. Petri plate is incubated for 37 $^{\circ}$ C overnight. 15 mL LB medium are mixed with 15 μ L kanamycin (25 g/L) in a 50 mL culture tube (closed but oxygen permeable). This tube is inoculated with a single colony from the plate, and then incubated at 37 $^{\circ}$ C at 180 rpm overnight. Aliquots of this suspension are then mixed with glycerol (final concentration is 20 %vol glycerol), flash-frozen and stored at -80 $^{\circ}$ C. Those aliquots are the transformed cells.

b) Enzyme expression

15 mL LB medium is mixed with 15 μ L kanamycin in a 50 mL culture tube, inoculated with an aliquot of transformed cells, and incubated at 37 °C, at 180 rpm, overnight to obtain a “pre-culture”. 1L LB medium is inoculated with 15 mL of pre-culture suspension in a 2L Erlenmeyer with baffles, and then incubated at 37 °C, at 180 rpm, during 2 to 4 h (in order to be in the exponential phase of the bacterial growth). This phase is reached when optical density (OD) at 600 nm reaches 0.4 or 0.5 (using Nanodrop ND-1000, Isogen Life Science). 500 μ L IPTG (1 M) is added for enzyme expression induction, and then the erlenmeyer is incubated at 37°C overnight at 180 rpm. Culture suspension is centrifuged again (10 minutes, 4000 rpm at 4 °C). Cell pellet is collected and then flash-frozen and stored at -80 °C. Those tubes are the expressed proteins in whole cells.

c) Enzyme recovery

Cell lysis

Expressed proteins in whole cells tube is thawed at room temperature in water bath. Two tablets of protease inhibitor (EDTA- free) are crushed and added to the protein tube during the thawing phase. Then 1 mL lysozyme solution (20 g/L) is added, and the tube is incubated on ice for 90 minutes. Then, sonication is performed (using Ultrasonic Processor 75022 VibraCell™, Sonics®) with amplitude of 70 during 3 minutes on ice. Suspension is then centrifuged at 12000 rpm for 20 minutes at 4 °C. Supernatant is collected and ultra- filtered in 50 mL tubes (Centrifugal Filter Units, Amicon® Ultra-15 10K, Merck Millipore Ltd) by centrifugation at 4 °C at 4000 rpm. Enzyme is aliquoted in 1.5 mL tubes and flash-frozen and stored at – 80°C.

Enzyme concentration

Enzyme concentration is measured using NanoDrop ND-1000 (Isogen Life Science) at 280 nm (using phosphate buffer as a blank).

II.4.2. Enzyme immobilization

Immobilization was typically carried out at room temperature (~22 °C) in a mixture containing 1 mM PLP, 125 mg/mL HFA resin and 1 mg/mL pure enzyme; the volume was then brought to 2 mL with 100 mM PBS (pH 8). For cascade reactions, the total volume was similarly adjusted to 5 mL in conical-bottom tubes. These tubes were placed on a vertical tube rotator at moderate speed and incubated overnight. After immobilization, the supernatant was discarded, and the resin was washed three times with 10 mL deionized water.

When PLP maturation was applied, this step followed the immobilization phase and involved again a 2-hour vertical rotation after adding PLP at a ratio of 1 mL per mg of enzyme. To ensure complete removal of excess PLP, the resin was washed three additional times with 10 mL water.

In both cases, the resulting resin-bound enzyme was recovered as a moist solid and used directly in the reaction medium.

II.5. Catalytic tests

II.5.1. Catalytic performance

To evaluate catalytic performance, the results are expressed in terms of conversion, yield, and selectivity, defined as follows:

Conversion (%) is defined as the proportion of substrate that has reacted relative to its initial molar concentration ($C_{\text{substrate},0}$). It provides an indication of how much of the starting material has been consumed during the reaction:

$$\text{Conversion (\%)} = \frac{C_{\text{substrate},0} - C}{n_{\text{substrate},0}} \times 100$$

However, conversion alone does not necessarily reflect product formation. For instance, losses due to substrate evaporation, adsorption onto the magnetic stir bar or the vessel walls, or side reactions may lead to a lower product yield. Therefore, yield (%) is defined as the amount of product formed relative to the initial concentration of substrate:

$$\text{Yield (\%)} = \frac{C}{C_{\text{substate},0}} \times 100$$

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Finally, selectivity (%) refers to the proportion of the converted substrate that is effectively transformed into the desired product.

II.5.2. Reaction kinetics

Reaction kinetics were monitored by regularly sampling the reaction medium over time. Samples were collected at the initial time point and then every 2 hours, as well as after 24 hours, in order to construct kinetic profiles. For chemo-catalytic experiments, an additional sampling point was included at 1 hour, as the gold catalyst exhibits activity from early stages. In cascade systems, sampling was extended up to one week to capture longer-term dynamics. All samples were subsequently deprotonated, extracted, and analyzed by gas chromatography (GC). In all cases, the time zero sample was collected prior to the addition of the first catalyst: either the enzyme (for enzymatic assays) or the gold catalyst (for chemo-catalytic and cascade reactions). All reactions were conducted under 35 °C.

II.5.3. Enzymatic transamination of acetophenone

A model transamination was used to assess enzymatic activity, converting achiral acetophenone into enantiopure (R)-1-phenylethylamine, using isopropylamine (IPA) as the amine donor. Reactions (2 mL total volume) were run in Eppendorf tubes at 35 °C under gentle stirring. Standard conditions were 500 mM IPA, 1 mM PLP, 20 mM

acetophenone and either free enzyme (1 mg/mL) or its immobilized form, with the remaining volume made up to 2 mL using 100 mM phosphate buffer (pH 8). IPA, PLP and phosphate buffer solutions contain 10 mM 2-phenylethanol as the internal standard.

II.5.4. Chemo-catalytic oxidation of 1-phenylethanol

Chemo-catalytic tests were carried out in 10 mL round-bottom glass flasks under moderate magnetic stirring (2500 rpm). These larger volumes were selected to ensure sufficient interaction between the reaction medium and molecular oxygen, which served as the oxidant for the alcohol oxidation. Standard reaction conditions were 35 °C, 20 mg/mL gold catalyst and 20 mM racemic 1-phenylethanol in 100 mM phosphate buffer (pH 8), with the buffer volume adjusted to bring the total reaction volume to 5 mL, and 10 mM 2-phenylethanol as the internal standard.

II.5.5. Cascade systems

Cascade reactions were carried out under the same conditions as the chemo-catalytic oxidation reactions, with the addition of the immobilized enzyme prior to the first sampling. Isopropylamine was also introduced at either 500 mM or 60 mM, and each reaction included a pre-reaction PLP maturation step.

Results and Discussion

I. Enzymatic catalyst

ATA_117 is notably characterized by two key features: substrate inhibition occurring at relatively low concentrations, and its high enantioselectivity in producing chiral amines. These aspects will be addressed in the following sections to evaluate their applicability for this reaction.

I.1. Acetophenone and 1-phenylethanol inhibition

Literature reports a 50% loss of activity upon exposure to 25 mM acetophenone⁹⁵. To determine the concentration threshold beyond which acetophenone (1-phenylethanol) inhibits the enzyme, various concentrations (0, 5, 10, 20, 30, 40, and 50 mM) were tested. The yield of 1-phenylethylamine was measured after 6 hours of reaction.

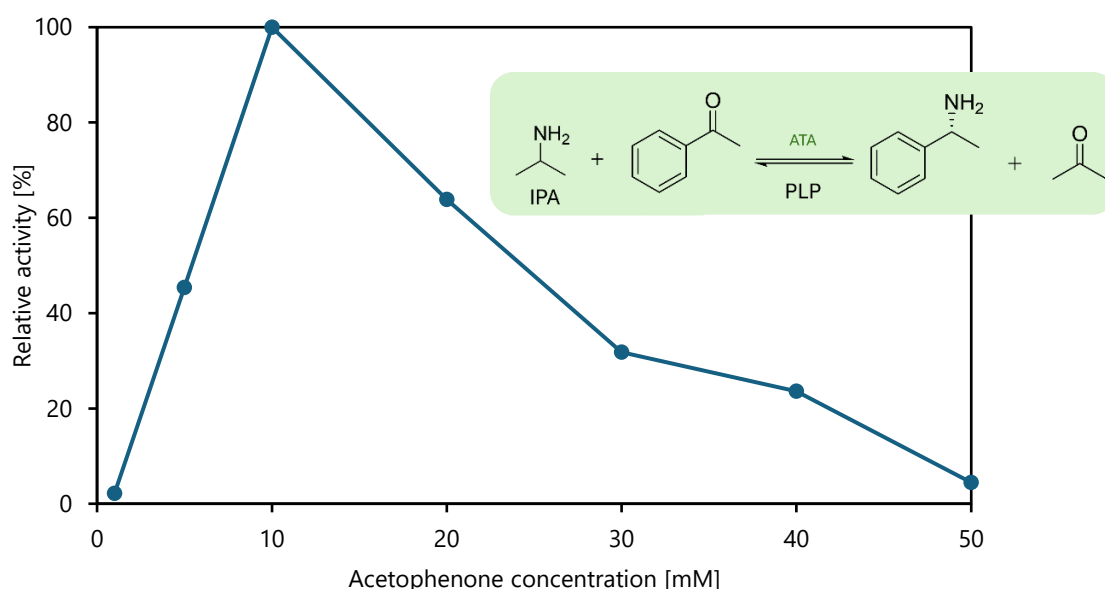


Figure 10. Relative activity of free ATA_117 at varying acetophenone concentrations

Enzymatic activity normalized to the maximum value at 10 mM acetophenone ($0.53 \text{ mmol} \cdot \text{h}^{-1} \cdot (\text{g}_{\text{enzyme}})^{-1}$), across a substrate range of 0–50 mM. Reaction conditions: 1 mM PLP, 500 mM IPA, 100 mM buffer added to reach final volume, and 1 mg/mL free ATA_117.

Substrate effectively causes a concentration dependent enzyme deactivation. On **Figure 10**, relative activity substantially reduces after 10 mM: it loses 70% of its activity at 30 mM. In living organisms, substrate inhibition functions as a form of metabolic regulation. Although its exact mechanism remains unclear, an excess of substrate can bind to secondary sites on the enzyme, leading to protein destabilization⁵⁰.

Our idea to initiate the reaction from 1-phenylethanol to create a progressive “fed-batch effect” and prevent substrate inhibition involves exposing the transaminase to 1-phenylethanol. The potential impact of this exposure on the biocatalyst must therefore

be assessed as well, to determine whether this strategy is indeed appropriate. Thus, enzymatic transamination tests were performed under standard enzymatic conditions and starting from 10 mM of acetophenone, in the presence of various concentrations of 1-phenylethanol ranging from 0 to 50 mM (Figure 11).

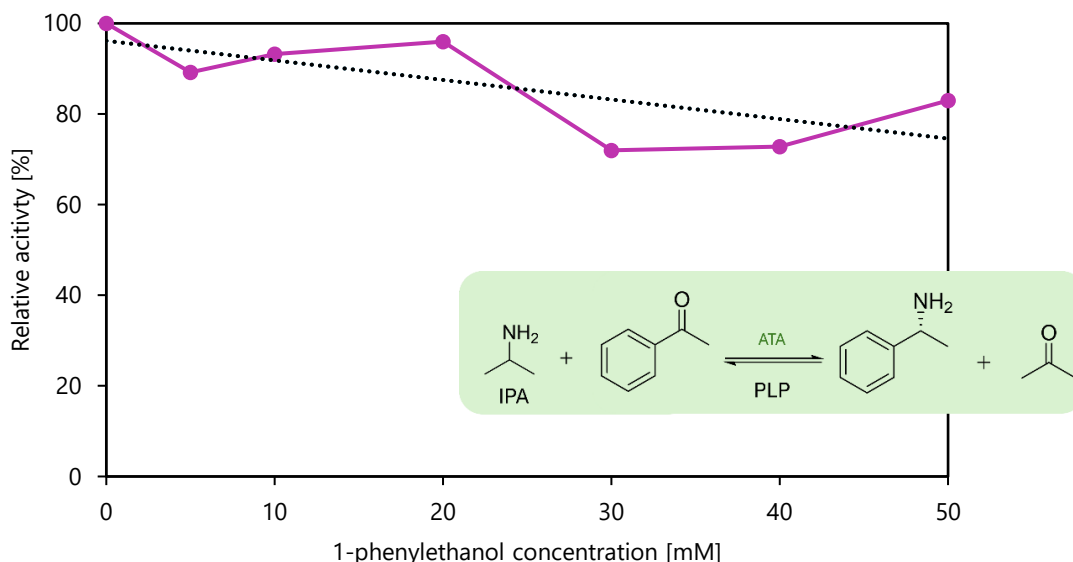


Figure 11. Relative activity of free ATA_117 by 1-phenylethanol at fixed acetophenone concentration

Relative activity measured with 10 mM acetophenone and varying 1-phenylethanol concentrations (0–50 mM), normalized to the maximum activity without 1-phenylethanol ($0.53 \text{ mmol} \cdot \text{h}^{-1} \cdot (\text{g}_{\text{enzyme}})^{-1}$). Reaction conditions: 10 mM acetophenone, 1 mM PLP, 500 mM IPA, 100 mM buffer added to reach final volume and 1 mg/mL free ATA_117.

A slight decreasing trend in 1-phenylethylamine production was observed as the concentration of 1-phenylethanol increased. Compared to acetophenone inhibition, relative enzyme activity remained high (83%) at 50 mM 1-phenylethanol, whereas it drastically decreased to 4% with 50 mM acetophenone. This clearly justifies initiating the reaction from the alcohol and highlights the importance of minimizing acetophenone concentration in the reaction medium.

I.2. Enzyme enantioselectivity

Chiral gas chromatography was used to determine the enantiomeric excess. This chromatogram (Figure 12) displays the two peaks expected when a racemic mixture of phenylethylamine (e.e. = 0%) is injected from a known standard solution. The first peak, eluting at 35 minutes, corresponds to the R-enantiomer, as confirmed by comparison with the standard, while the second peak at 37.5 minutes corresponds to the S-enantiomer.

When the reaction mixture is analyzed, only one peak is observed within this retention time window. Based on the elution time, the product is identified as the R-enantiomer, which is consistent with the use of an R-selective ATA. Since no trace of the S-enantiomer

is detected, the enantiomeric excess is determined to be >99%. This result confirms the enzyme's enantioselectivity.

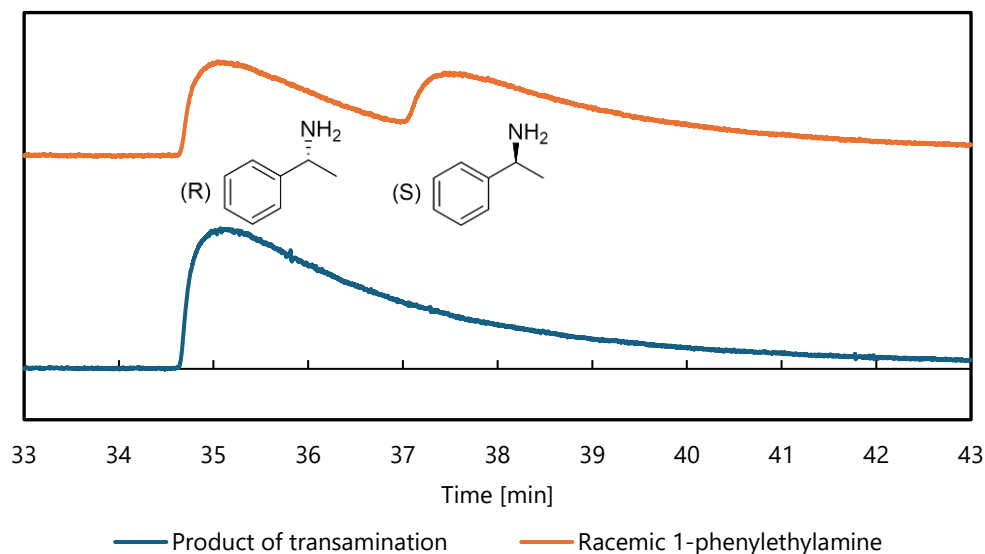


Figure 12. GC chromatogram of racemic 1-phenylethylamine and transamination product
Comparison between a standard racemic 1-phenylethylamine and the product obtained after 24 h of transamination catalyzed by free ATA. Reaction conditions: 10 mM acetophenone, 1 mM PLP, 500 mM IPA, 100 mM buffer added to reach final volume, and 1 mg/mL free ATA₁₁₇.

The catalytic efficiency of ATA₁₁₇ was first evaluated independently from the chemo-catalyst. To assess the stabilizing effect of immobilization, both the free and immobilized forms of the enzyme were tested. These results serve as a reference point for subsequent comparisons upon modification of the reaction conditions.

1.3. Catalytic test

1.3.1. Experimental error

To assess the experimental error, one of the tests was repeated in its entirety, including both the reaction and analytical steps. This repetition accounted for cumulative sources of variability such as weighing inaccuracies, sampling, and instrumental limitations (e.g., micropipette and balance precision).

The repeated experiment involved a transamination reaction under standard conditions using the free enzyme (1 mM PLP, 500 mM IPA, 100 mM buffer, 1 mg/mL enzyme), with the addition of a gold catalyst. The results of this repetition are detailed in Appendix A.1. Determination of experimental error

Based on the results of this repetition, a relative error of 7% will be considered for all activity measurements presented in this thesis.

1.3.2. Immobilization

Immobilizing an enzyme enhances its resistance to reaction conditions and generally improves its stability⁵⁴. In the context of applying this enzyme in a hybrid cascade, achieving optimal stability is a key objective. However, it is also well established that being immobilized typically reduces catalytic activity^{96,97}.

To assess this balance between stability and activity, the catalytic performance of the immobilized enzyme was compared to that of the free enzyme. The evolution of product yield over time for both systems, under identical conditions, is presented in **Figure 13**.

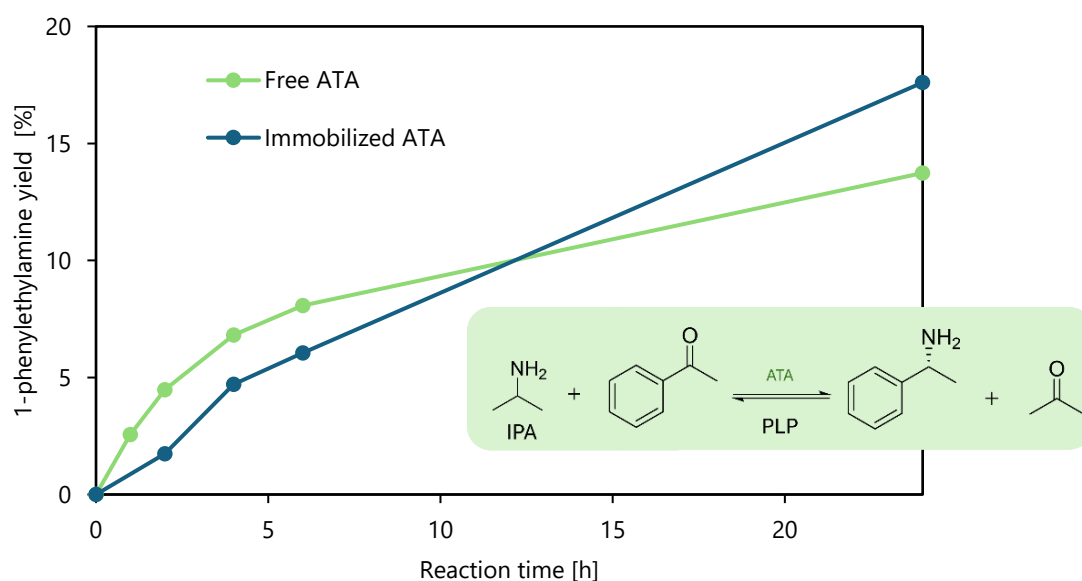


Figure 13. 1-phenylethylamine yield with free and immobilized ATA_117

Comparison of 24 h product formation kinetics for transamination reactions catalyzed by free or immobilized ATA_117 (89% immobilization rate). Classical immobilization: 1 mM PLP, 100 mM buffer to reach final volume, 1 mg/mL enzyme, 125 mg/mL resin. Reaction conditions: 20 mM acetophenone, 1 mM PLP, 500 mM IPA, 100 mM buffer to reach final volume, and 1 mg/mL ATA_117.

During the first 6 hours, both reaction kinetics followed a similar overall trend, although the immobilized enzyme exhibited a noticeable initial delay, achieving approximately half the yield of the free enzyme within the first 2 hours. This initial difference can be explained by partial enzyme loss during the immobilization procedure, as washing steps inevitably remove some enzyme. Bradford assay confirmed this, revealing an immobilization efficiency of 89%, meaning that 11% of the initial enzyme load was lost compared to the free enzyme system, thus contributing to the initially lower conversion.

Consequently, assessing enzymatic activity rather than overall yield provides a more accurate comparison. Initial enzymatic activities were estimated at $0.32 \text{ mmol}\cdot\text{h}^{-1}\cdot(\text{g}_{\text{enzyme}})^{-1}$ for the free enzyme and $0.17 \text{ mmol}\cdot\text{h}^{-1}\cdot(\text{g}_{\text{enzyme}})^{-1}$, for the immobilized enzyme, confirming the observation of an approximately twofold higher initial activity for the free enzyme. This loss is generally attributed to reduced conformational flexibility and/or sub-optimal orientation on the carrier⁹⁶.

Despite this initial setback, the immobilized enzyme rapidly catches up. From 4 h to 24 h it maintains an constant reaction rate, while the free enzyme shows a progressive decline. The sustained slope observed for the immobilized system underscores its superior operational stability, as further discussed in the next section.

I.4. Immobilization conditions optimization

Immobilization conditions were optimized to enhance subsequent catalytic performance. Conducting the immobilization under conditions matching those of the target reaction environment may help preserve, or even improve, the enzyme's activity. Each parameter was therefore systematically evaluated to identify the conditions that best support both effective immobilization and catalytic efficiency.

I.4.1. Immobilization temperature

The temperature selected for catalytic tests was 35°C, as it is suitable for optimal enzymatic activity. However, three different temperatures were explored during the immobilization step (**Figure 14**): 35°C, room temperature¹, and 4°C. Immobilization at room temperature (~22 °C) proved to be significantly more effective, showing a 17% improvement after 24 hours of reaction, compared to 35°C. This condition was therefore chosen for subsequent immobilizations, as it also offered the practical advantage of eliminating the need for additional heating or cooling equipment.

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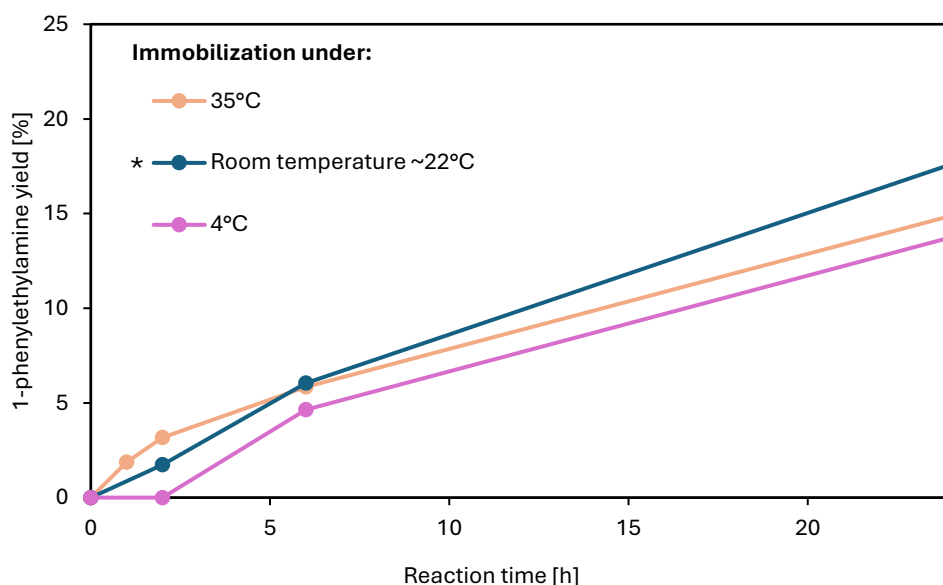


Figure 14. 1-phenylethylamine yield over time with varying immobilization temperatures

Comparison of 24 h product formation kinetics using ATA_117 immobilized at 35 °C, room temperature (~22 °C), or 4 °C. All reactions conducted at 35 °C. Reaction conditions: 20 mM acetophenone, 1 mM PLP, 500 mM IPA, 100 mM buffer to reach final volume, and 1 mg/mL ATA_117.

¹ Classical conditions are systematically indicated by an asterisk (*).

1.4.2. Buffer concentration

Different phosphate buffer concentrations were tested, as ionic strength can affect enzyme activity (Figure 15).

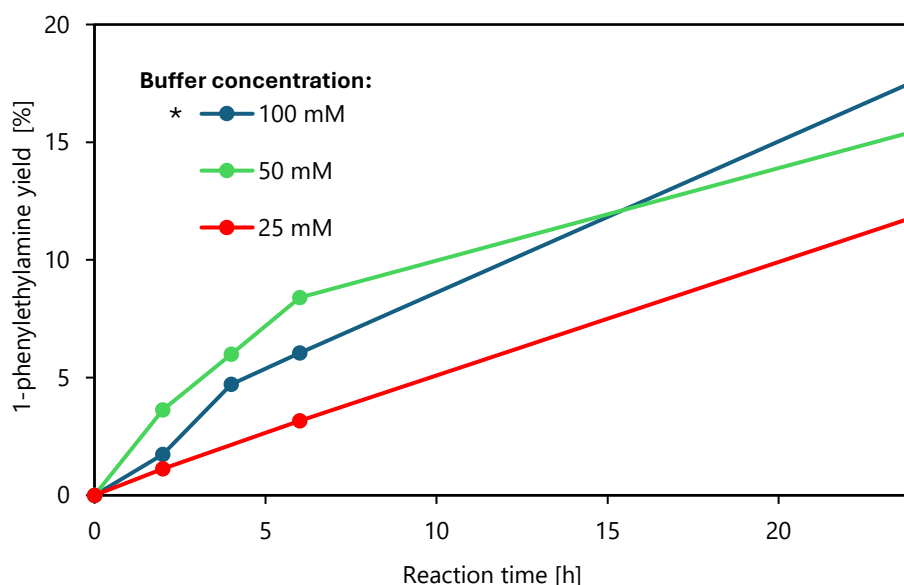


Figure 15. 1-phenylethylamine yield over time with varying buffer concentrations during immobilization

Comparison of 24 h product formation kinetics using ATA_117 immobilized with buffer concentrations of 25, 50 or 100 mM. Reaction buffer concentration maintained at 100 mM. Reaction conditions: 20 mM acetophenone, 1 mM PLP, 500 mM IPA, 100 mM buffer to reach final volume, and 1 mg/mL ATA_117.

A low ionic strength environment facilitates initial enzyme adsorption through residual interactions, a crucial step given the highly hydrophobic nature of epoxy beads⁹⁸. Thus, any approach reducing repulsion between beads and protein should be beneficial. Such adsorption effectively positions the enzyme in proximity to epoxy groups, enabling efficient covalent immobilization. This is evidenced by the observed immobilization rate of 96% achieved when using a 50 mM buffer concentration during immobilization, an improvement compared to the conventional rate of 91% obtained at 100 mM. When subsequently tested under identical reaction conditions, the enzyme immobilized in 50 mM buffer outperformed the conventional immobilization at 100 mM during the initial 4 hours; however, it exhibited lower long-term stability afterward.

With 100 mM buffer, the initial enzyme activity was $0.15 \text{ mmol} \cdot (\text{g}_{\text{enzyme}})^{-1} \cdot \text{h}^{-1}$, whereas it increased to $0.20 \text{ mmol} \cdot (\text{g}_{\text{enzyme}})^{-1} \cdot \text{h}^{-1}$ at 50 mM, illustrating the better conversion at 50 mM buffer in the beginning. In contrast, using a 25 mM buffer resulted in lower activity ($0.13 \text{ mmol} \cdot (\text{g}_{\text{enzyme}})^{-1} \cdot \text{h}^{-1}$) and consistently reduced yields. A lower phosphate buffer concentration can also have an inhibitory effect by inducing a higher cofactor dissociation⁹⁹.

Overall, we decided to keep the 100 mM buffer for immobilization, as it appeared to provide greater enzyme stability and improved conversion after 24 hours. This observation

is supported by Chen *et al.*, who demonstrated that higher salt concentrations reduce the enzyme unfolding rate and exert a stabilizing effect on ATA conformation¹⁰⁰.

1.4.3. Substrate-assisted immobilization

The addition of various concentrations of acetophenone during the immobilization step was also explored (**Figure 16**). As often observed in biocatalysis, incubating an enzyme in the presence of its substrate can help stabilize the enzyme in its active conformation, potentially by improving specific interaction with its substrate^{101,102}. In the case of enzyme immobilization, this has been reported to lead to improved activity⁹⁷. After immobilization in the presence of 0, 5, 10, or 20 mM of acetophenone, the immobilized enzyme was washed and catalytic tests were initiated by adding acetophenone at a concentration of 20 mM in all cases.

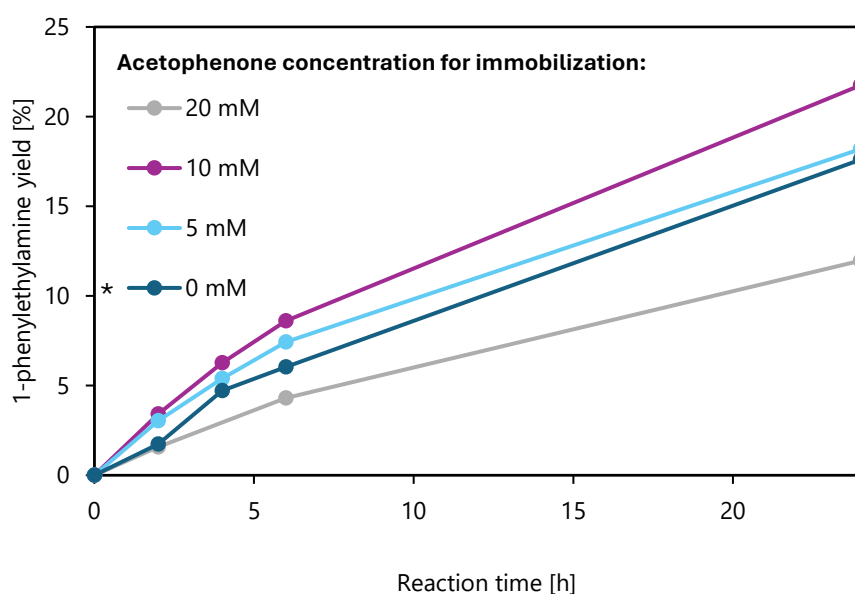


Figure 16. 1-phenylethylamine yield over time with substrate addition during immobilization
Comparison of 24 h product formation kinetics using ATA_117 immobilized in the presence of 0–20 mM acetophenone. Reaction is always initiated with 20 mM acetophenone. Reaction conditions: 20 mM acetophenone, 1 mM PLP, 500 mM IPA, 100 mM buffer to reach final volume, and 1 mg/mL ATA_117.

The addition of 5 mM acetophenone resulted in only a slight improvement, insufficient to justify its use during the immobilization process. In contrast, 10 mM acetophenone proved more effective, with a 24% improvement compared to the yield obtained under standard conditions. However, increasing the concentration to 20 mM caused a significant decrease in yield, as expected, since such a level is known to promote early enzyme deactivation.

1.5. Improved capacities due to immobilization

Immobilization offers key advantages in terms of thermal stability, solvent tolerance, and long-term preservation. Immobilization generally helps the enzyme resist irreversible structural changes during catalysis or storage⁵³.

1.5.1. Resistance to long-term storage

Since free ATA in solution remains stable for only 48 hours, two storage methods were evaluated over a five-months period to assess the long-term storage potential of the immobilized enzyme (**Figure 17**). Storage was assessed as dry resin (without solvent) and as wet resin (suspended in 2 mL of buffer). Samples were stored in a refrigerator at 5°C during the entire storage period.

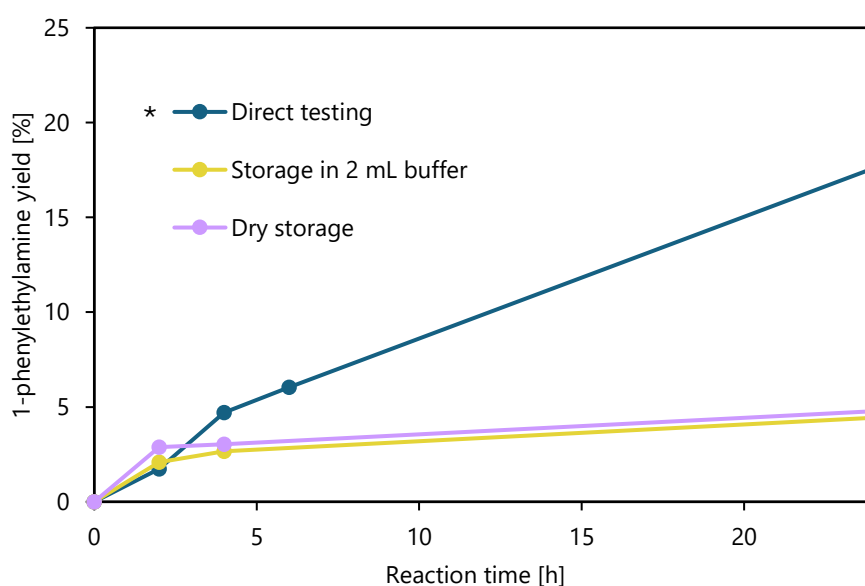


Figure 17. 1-phenylethylamine yield over time after different storage conditions of immobilized ATA_117

Comparison of 24 h product formation kinetics using ATA_117 immobilized under classical conditions and tested immediately, after dry storage, or after wet storage (in 2 mL buffer). Reaction conditions: 10 mM acetophenone, 1 mM PLP, 500 mM IPA, 100 mM buffer to reach final volume, and 1 mg/mL ATA_117.

Immobilization did not prevent activity loss during storage. After four months at 5°C, the immobilized enzyme lost about two-thirds of its activity, regardless of whether it was stored in buffer or without solvent. This indicates that the loss is mainly due to storage duration rather than storage conditions, likely caused by gradual denaturation. Shorter storage periods may result in limited activity loss, which could be relevant for short-term applications.

The concentration of enzyme in the supernatant was measured after storage to evaluate whether any enzyme had detached from the support over time. After 5 months, the immobilization yield was found to be 83%, compared to 91% for freshly immobilized enzyme. This slight decrease suggests a limited loss of bound enzyme during storage,

indicating that the enzyme remained largely attached to the support even after prolonged storage.

In fact, the storage medium plays a critical role, as it can significantly affect protein structure⁵⁰. A likely explanation for the observed activity loss is the instability of ATAs, and proteins in general, in aqueous environments, possibly due to the dissociation of the PLP cofactor from the enzyme, resulting in the formation of the less stable apoenzyme⁵⁰. To mitigate this, it is recommended to remove residual moisture prior to storage, either by drying the resin under mild conditions under vacuum¹⁰³ or by lyophilization, which involves rapid freezing followed by water removal¹⁰⁰. These approaches have been shown to improve enzyme stability over time by preventing degradation in aqueous environments during storage⁵⁰.

1.5.2. Resistance to operating conditions

Aurélien Doutry applied destabilizing conditions such as a high IPA concentration, a higher temperature (50°C), or a partial concentration of organic solvent (10% methanol) to ATA_117 for his thesis, and it allowed to show the effect of immobilization on the resistance, by comparing product yield after 24 hours (**Figure 18**).

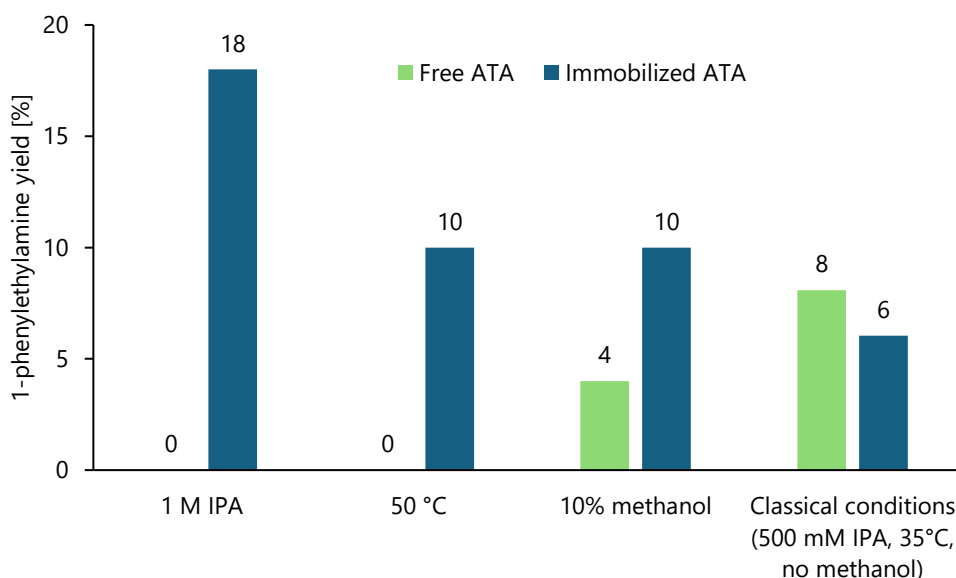


Figure 18. 1-phenylethylamine yield after 24 h under harsh conditions with free and immobilized ATA_117
Comparison of product formation after 24 h under harsher conditions (1 mM IPA, 50 °C, or 10% methanol, Aurélien Doutry's results) and classical conditions (20 mM acetophenone, 1 mM PLP, 500 mM IPA, 100 mM buffer to reach final volume, 1 mg/mL free ATA_117, 35 °C), highlighting the effect of immobilization on enzyme performance.

The immobilized enzyme demonstrated significantly higher catalytic activity than its free counterpart when exposed to challenging conditions such as 1 M isopropylamine (IPA), 50 °C, or 10% methanol, as demonstrated by the higher yields. Although partial activity loss was observed under these stresses, particularly at elevated temperature and in the

presence of organic solvents, the immobilized enzyme retained sufficient functionality to remain catalytically effective.

In contrast, the free enzyme was completely inactivated under 1 M IPA or at 50 °C, highlighting the remarkable stability conferred by immobilization. This enhanced resistance is particularly advantageous when working under non-ideal conditions, such as those required for integrating catalysts of different natures within a hybrid system. The increased tolerance of the immobilized enzyme broadens its applicability across a wider range of operational settings.

While 10% methanol did not lead to full deactivation of the free enzyme, a substantial reduction in activity was still observed. In the case of 1 M IPA, the increased concentration helps shift the reaction equilibrium toward amine formation, which is especially beneficial under thermodynamically unfavourable conditions. However, excessive IPA can promote enzyme inactivation by stabilizing the less robust aminated enzyme-cofactor complex. The dissociation of this complex into PMP and the apoenzyme is known to increase the risk of protein unfolding¹⁰⁴. The complete inhibition observed with the free enzyme under these conditions further underscores its vulnerability compared to the immobilized form.

1.5.3. Leaching test

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Immobilizing the enzyme presents several advantages, but its ability to remain bound to the resin without leaching must be verified. One of the key benefits of immobilization in industrial applications is the heterogeneity of the catalyst, enabling its recovery and potential reuse. To ensure that the enzyme does not leach from the support, a hot filtration test was conducted (**Figure 19**): after six hours of reaction, the resin was removed by filtration, and the supernatant (reaction medium) was monitored over time.

Following catalyst removal, no further product formation was observed up to 43 hours. This confirms that the enzyme did not leach into the reaction medium and that it was fully recovered upon filtration, thereby validating the heterogeneous nature of the catalyst.

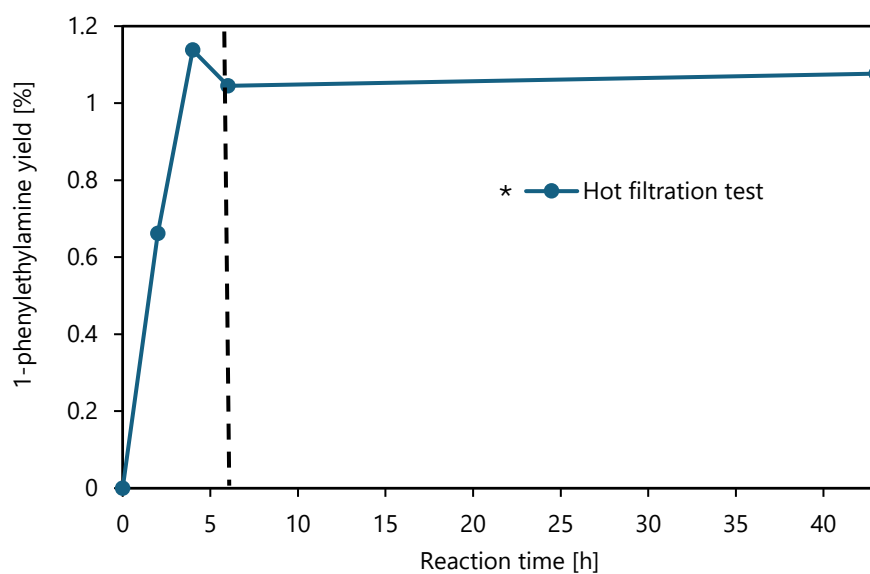


Figure 19. Hot filtration test to assess leaching of immobilized ATA_117

Comparison of 1-phenylethylamine formation over 24 h with resin removal after 6 h (dashed line), to evaluate catalytic activity in the absence of the immobilized enzyme. Reaction conditions: 20 mM acetophenone, 1 mM PLP, 500 mM IPA, 100 mM buffer to reach final volume, and 0.2 mg/mL ATA_117.

II. Inorganic catalyst

The Au/SiO₂ catalyst was characterized and evaluated for its activity in the model reaction involving the oxidation of 1-phenylethanol to the corresponding ketone (acetophenone), in reaction conditions that are compatible with the envisaged combination with an enzyme.

II.1. Selectivity and kinetics of Au/SiO₂ catalyst

The reactions were carried out in a 10 mL round-bottom flask to provide sufficient molecular oxygen for the process, thanks to the larger headspace volume, allowing better gas-liquid contact with the reaction medium (**Figure 20**).

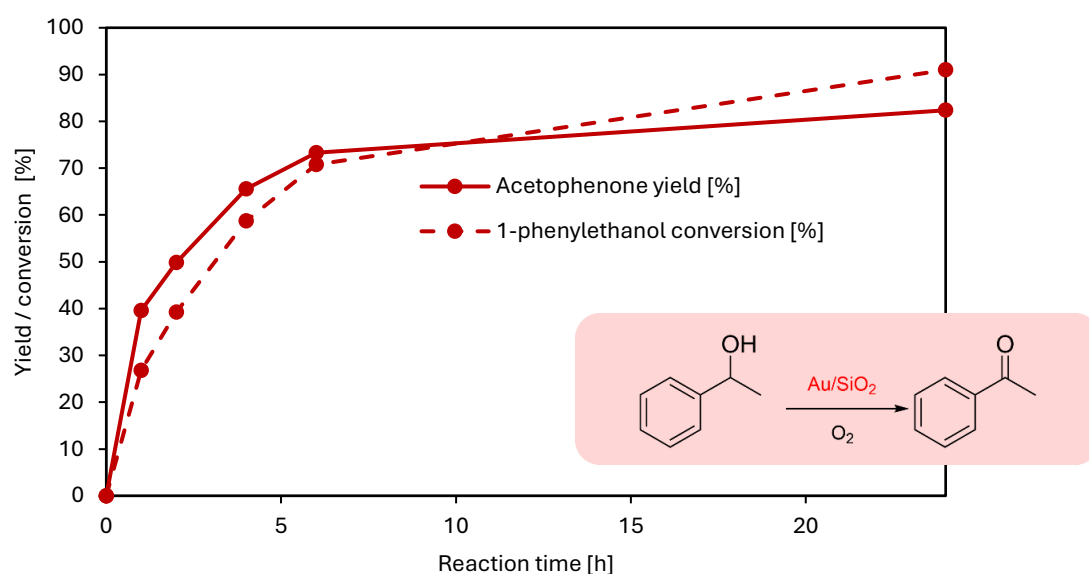


Figure 20. Acetophenone yield and 1-phenylethanol conversion over time using Au/SiO₂ catalyst
Comparison of 24 h product formation and substrate conversion kinetics using gold on silica (Au/SiO₂) catalyst.
Reaction conditions: 20 mM 1-phenylethanol, 100 mM buffer to reach final volume, and 20 mg/mL Au/SiO₂.

II.2. Characterization of Au/SiO₂ catalyst

XRD was performed to estimate the size of the gold nanoparticles on the support and the crystallinity of our sample. The diffractogram used for this analysis is shown in **Figure 21**. The average particle size was determined to be approximately 3.9 nm, based on the full width at half maximum of the Au (111) diffraction peak and using the Sherrer equation (Experimentals Section II.2.4. X-ray diffractometry (XRD)).

The XRD pattern of Au/SiO₂ displays a broad amorphous feature characteristic of the silica support. Four distinct diffraction peaks are attributed to metallic gold at $2\theta = 38^\circ$, 44° , 65° , and 78° , corresponding respectively to the (111), (200), (220), and (311) planes of face-centred cubic structure of metallic gold¹⁰⁵. Additional gold diffraction peaks exist beyond the Au (311) reflection, but they become too broad to be clearly observed.

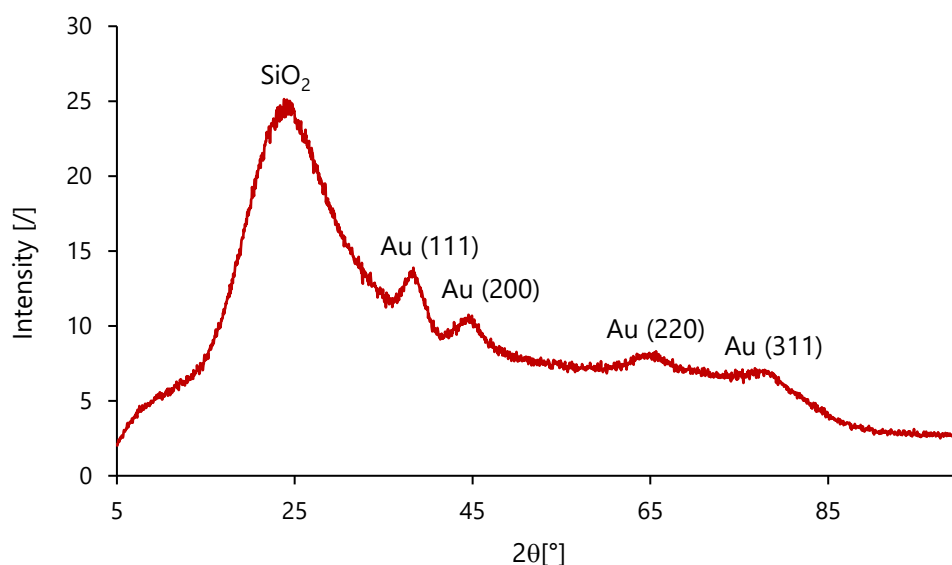


Figure 21. X-ray diffractogram of Au/SiO₂ catalyst

Crystalline structure characterization of the Au/SiO₂ catalyst, highlighting diffraction peaks associated with metallic gold and the amorphous nature of the silica support.

Surface composition was analyzed by X-ray Photoelectron Spectroscopy (XPS) mainly to verify the location of gold nanoparticles. As expected, strong signals corresponding to oxygen and silica were observed in the survey spectrum (**Figure 22**). A high resolution spectrum acquired on the spectral region characteristic of gold shows the two 4f peaks, confirming the presence of gold.

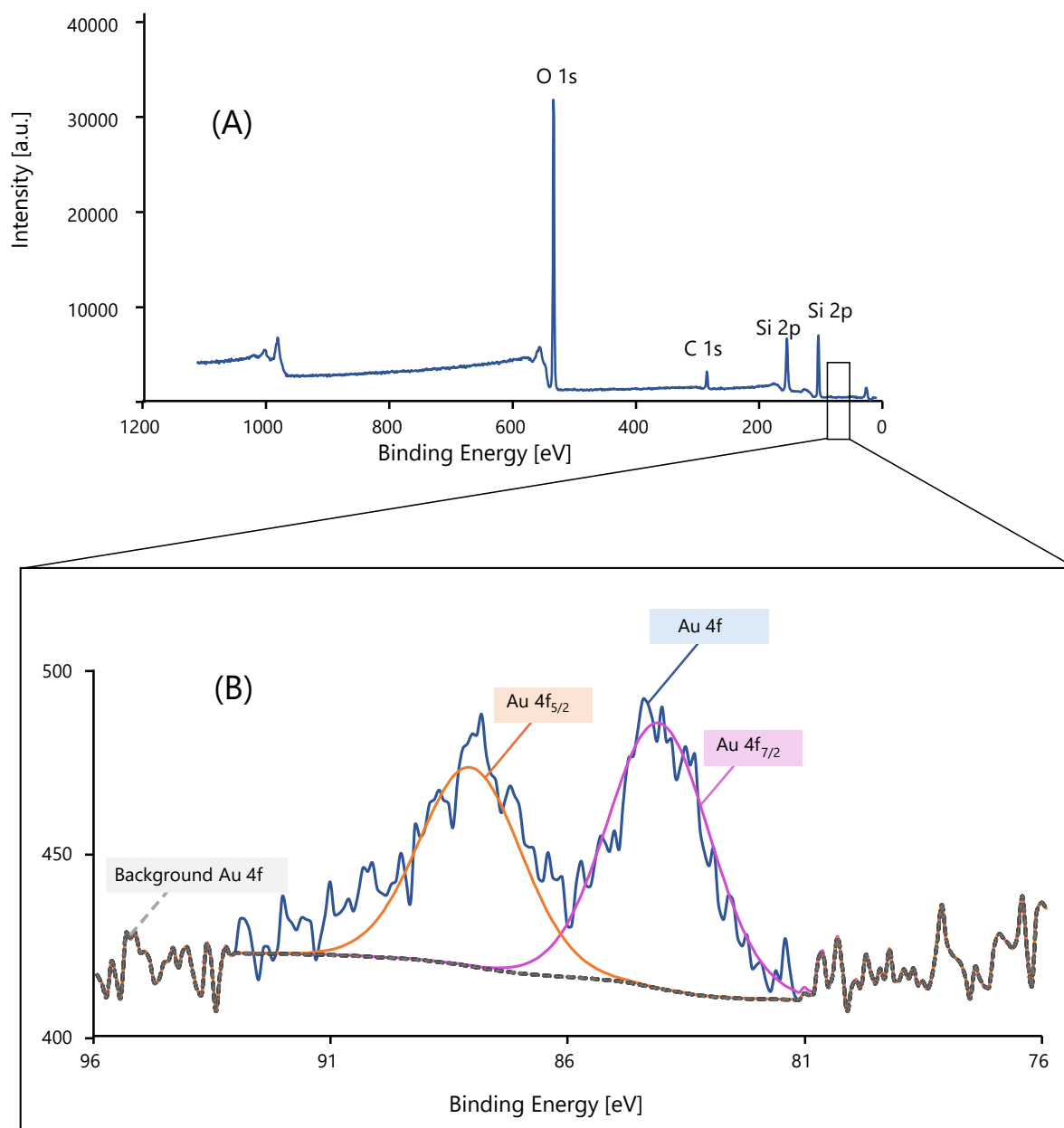


Figure 22. XPS survey and Au 4f region of Au/SiO₂ catalyst

(A) Wide-range XPS survey spectrum showing the elemental composition. (B) High-resolution scan of the Au 4f region highlighting the characteristic doublet peaks of metallic gold.

The atomic proportions obtained by XPS, corrected for carbon contamination, are revealed in the table below (**Table 1**). The measured O/Si atomic ratio of 1.7 is lower than expectations, considering that silica (SiO₂) has an ideal stoichiometry of two oxygen atoms per silicon atom. The lower observed ratio can be attributed to the formation of external silanol groups and the loss of water molecules during calcination, resulting in fewer oxygen atoms at the surface. The presence of chloride was investigated, but XPS analysis confirmed its absence on the catalyst surface.

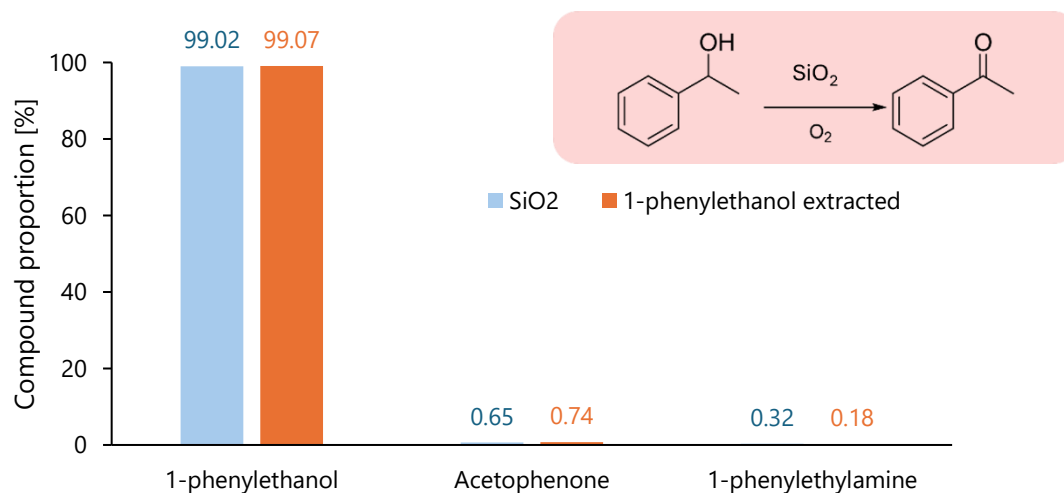
Table 1. Atomic proportions [%] by XPS after carbon correction

Elemental surface composition expressed as atomic percentages, adjusted to account for carbon contamination.

ATOM	O	Si	Au	Cl
Atomic proportion [%]	62.5	37.5	0.032	0

XPS analysis detected only 0.032% gold on the surface, whereas the nominal gold loading was 1 wt% (corresponding to 0.103 atomic%). This lower-than-expected surface signal is consistent with the synthesis strategy, which favours the dispersion of gold nanoparticles within the porous structure of the support, particularly along the internal pore surfaces⁹⁴. Given that XPS probes only the outermost few nanometers and may not access the internal surfaces, part of the gold remains undetected. Nevertheless, the observed catalytic activity confirms that the gold nanoparticles, although mostly located inside the pores, are accessible to the substrate. The weak XPS signal reflects the spatial distribution of gold. For further validation, ICP-AES could be employed to determine the total elemental composition.

A control experiment confirmed that the catalytic activity originates from gold, despite its low surface concentration, and that SiO₂ is inert. A silica material synthesized without the gold precursor was tested under identical conditions (**Figure 23**).

**Figure 23. Comparison between SiO₂-“catalyzed” reaction and standard 1-phenylethanol composition**

Overlay of chromatographic profiles showing the product mixture obtained from a reaction in the presence of SiO₂ alone, compared to the composition of pure 1-phenylethanol standard.

Only 0.15 mM of acetophenone was detected, corresponding to the ~0.7% impurity in the commercial 1-phenylethanol (98% purity). Additionally, 0.06 mM of 1-phenylethylamine was observed, likely due to reagent contamination, which will be accounted for in subsequent tests. No further conversion was detected, and the product profile was identical to that of the pure alcohol, confirming that silica alone is catalytically inactive. The oxidation reaction can thus be solely attributed to gold.

Scanning Electron Microscopy was used to visualize the morphology of the material at the microscopic level. The surface and 3D topography of the sample, shown in **Figure 24**, reveal that the synthesized catalyst does not exhibit the expected spherical shape but instead presents irregular structures with elongated, arm-like extensions.

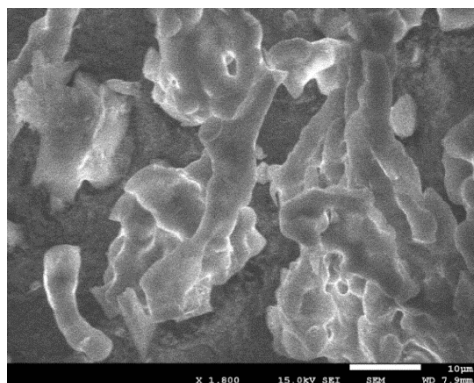


Figure 24. SEM image of Au/SiO₂ catalyst synthesized in-house for this master thesis

Surface morphology of the Au/SiO₂ catalyst observed by scanning electron microscopy. The sample was not ground prior to SEM analysis. The material was spray-dried at 175 °C, then calcined under static air at 550 °C with a heating ramp of 1 °C/min for 5 hours.

To enable comparison, the morphology of the reference catalyst, originally designed and synthesized by Margot Van der Verren following a slightly different synthesis protocol, was characterized by transmission electron microscopy (TEM), which reveals internal structural features (**Figure 25**).

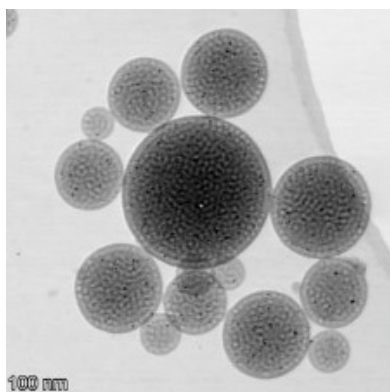


Figure 25. TEM image of Au/SiO₂ catalyst synthesized by Margot Van der Verren⁹⁴

Transmission electron microscopy image of the Au/SiO₂ catalyst synthesized by Margot van der Verren. The material was spray-dried at 350 °C, followed by overnight drying at 80 °C and calcination at 550 °C with a heating ramp of 1 °C/min for 6 hours.

The TEM image clearly shows perfectly spherical particles and, in addition, a well-dispersed distribution of gold nanoparticles. The morphological differences observed between the two catalysts are likely due to the modified synthesis conditions used here. The current catalyst was synthesized following a similar protocol, with a key modification: the use of a different spray-dryer operating at a faster drying rate. This variation seems to affect particle morphology. Rapid drying could lead to pore collapse or reduced structural definition. Although SEM and TEM differ in the type of information they provide, both

techniques offer insights into particle morphology, making a meaningful comparison between the two materials possible.

Textural properties of Au/SiO₂ were characterized by N₂ physisorption. The material exhibits a specific surface area of 386 m²/g, a total pore volume of 0.1 cm³/g, and an average pore diameter (BJH, adsorption branch) of 6.9 nm.

Compared to the reference material (Au/SiO₂-Margot, **Table 2**), the current catalyst exhibits a lower specific surface area and total pore volume, along with a larger average pore diameter. These differences are attributed to the faster drying conditions imposed by the alternative spray-dryer. As a result, our synthesis method appears to suffer from poorer pore definition.

Table 2. Textural properties of Au/SiO₂ catalysts synthesized for this thesis and by Margot van der Verren
Comparison of specific surface area, pore volume, and pore size for the Au/SiO₂ catalyst synthesized in-house during this master thesis and the one prepared by Margot van der Verren.

Catalyst	Specific surface area [m ² /g]	Total pore volume [cm ³ /g]	Average pore diameter [nm]
Au/SiO ₂	386	0.1	6.9
Au/SiO ₂ -Margot	440	0.5	5

Although these differences suggest that additional optimisation could be beneficial, the catalyst already delivers an activity that is fit for purpose. Because process optimisation is beyond the scope of this master's thesis, the material will be employed in its as-synthesised form.

The nitrogen adsorption and desorption isotherms are provided in **Figure 26**.

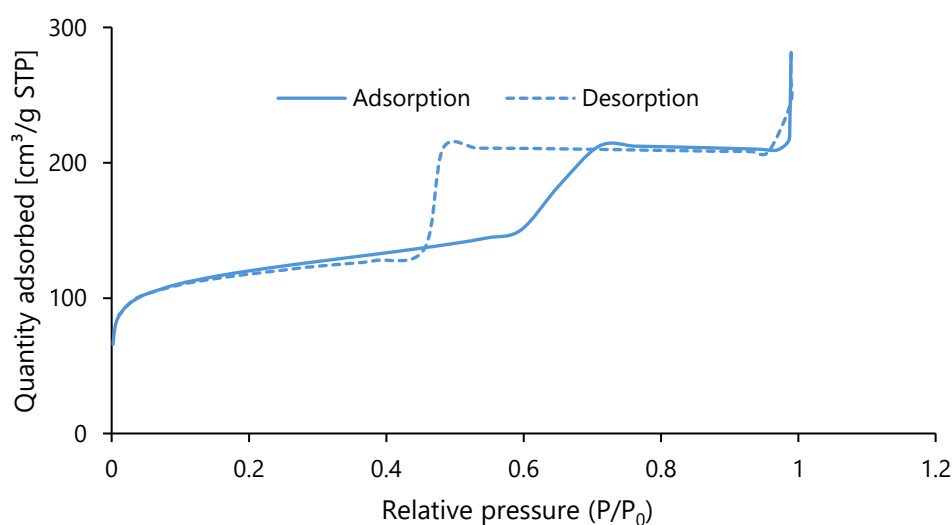


Figure 26. N₂ adsorption–desorption isotherms of the Au/SiO₂ catalyst
Physisorption profile used to assess surface area and pore structure, showing characteristic mesoporous behaviour.

The shape of these type IV isotherms is characteristic of mesoporous materials, with a distinct plateau at high relative pressure and a hysteresis loop. Initially, multilayer adsorption occurs, followed by capillary condensation as the pores are gradually filled¹⁰⁶. According to IUPAC classification, the observed pore sizes between 2 and 50 nm confirm the mesoporous nature of the material¹⁰⁷.

The non-parallelity between the two branches suggests a complex mesoporous structure, reminiscent of ink-bottle pores, characterized by large cavities (here, centred around 6.9 nm) connected to the gas phase through narrower pore mouths¹⁰⁶. The pore size distribution is relatively broad with smaller constrictions at the pore entrances. All mesopores empty at the same relative pressure ($P/P_0 \approx 0.45$), which is attributed to the tensile strength effect¹⁰⁸. This phenomenon involves cavitation: when the tension within the condensed liquid reaches its resistance limit, the liquid becomes unstable and undergoes sudden evaporation. This results in the abrupt drop observed in the desorption branch of the isotherm¹⁰⁸.

III. Towards cascade application: compatibility assessment and mitigation strategies

With the individual behaviours of the biocatalyst and chemo-catalyst established under their respective conditions, and optimal immobilization conditions identified, the next step was to evaluate their compatibility for a one-pot, one-step cascade. To this end, each catalyst was tested under the operating conditions of the other.

III.1. Compatibility between the catalysts

The potential impact of the gold catalyst on enzymatic transamination, and of the enzyme on alcohol oxidation, were assessed by adding the chemo-catalyst to the enzymatic reaction medium and the enzyme to the chemo-catalytic reaction medium, respectively. At this stage, each reaction was still monitored separately.

III.1.1. *Au/SiO₂* impact on transamination reaction

As shown in **Figure 27**, whether the transamination reaction is performed in presence or in absence of the chemo-catalyst does not impact the yield as the two curves are very close one to the other. The little difference that is observed is likely due to experimental variations.

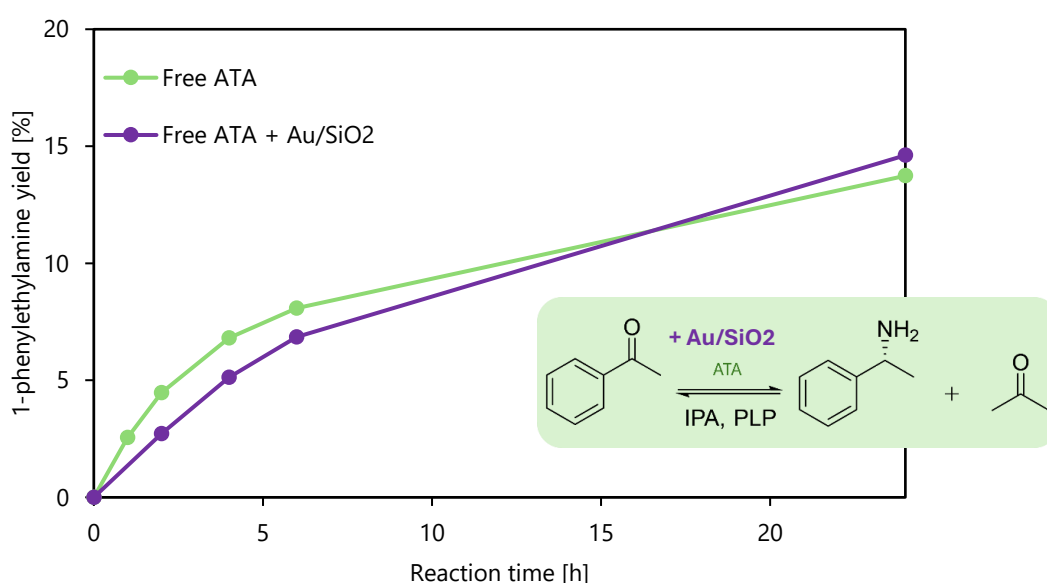


Figure 27. 1-phenylethylamine yield over time in the presence or absence of Au/SiO₂ in ATA_117 catalyzed transamination

Comparison of 24 h product formation kinetics using free ATA_117 with and without Au/SiO₂ catalyst to assess the effect of the gold catalyst on enzymatic activity. Reaction conditions: 20 mM acetophenone, 1 mM PLP, 500 mM IPA, 100 mM buffer added to reach final volume, 20 mg/mL Au/SiO₂ and 1 mg/mL free ATA_117.

The enzyme is not affected by the gold catalyst because, as shown by nitrogen physisorption, the diameter of the pores measures 5 nm. Since the enzyme itself is about 5 nm in size, it cannot enter the pores that contain gold nanoparticles. While this pore size

allows efficient diffusion of 1-phenylethanol and acetophenone, it prevents direct contact between the enzyme and the gold particles, thus avoiding biocatalyst deactivation.

III.1.2. ATA_117 impact on oxidation reaction

In contrast, the presence of the enzyme within the reactor was found to negatively affect the chemo-catalytic performance, as evidenced in **Figure 28**.

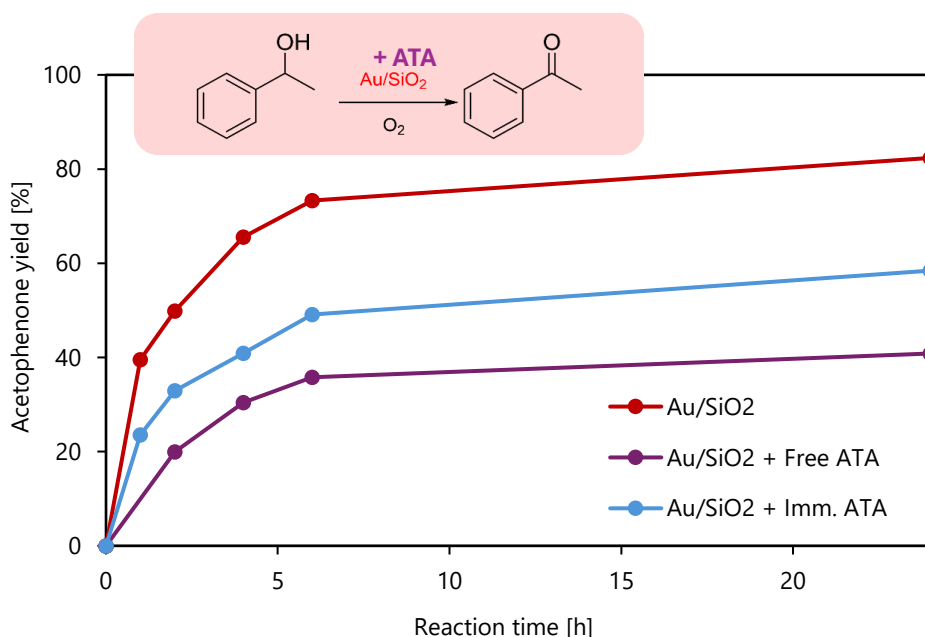


Figure 28. Acetophenone yield over time with free or immobilized ATA_117 in Au/SiO₂-catalyzed oxidation
Comparison of 24 h product formation kinetics using Au/SiO₂ catalyst in the presence of free or immobilized ATA_117, to assess the effect of enzyme form on the oxidation of 1-phenylethanol. Reaction conditions: 20 mM 1-phenylethanol, 100 mM buffer to reach final volume, 1 mg/mL free or immobilized ATA_117 and 20 mg/mL Au/SiO₂.

Introducing the pure free enzyme reduced the final product yield to 41% (instead of 82% when the gold catalyst was alone), whereas using the immobilized form limited the decline, reaching 58% yield. This might suggest that the enzyme (approximately 5 nm in size, matching the pore diameter) impairs the gold catalyst by blocking pore access. Immobilization appears to mitigate this inhibition by distancing the enzyme from the gold catalyst surface; yet if simple physical separation was sufficient, full recovery of gold activity would be expected.

However, control experiments employing the resin alone revealed an equivalent reduction in yield (-24%, **Figure 29**) with immobilized enzyme and with resin, suggesting that the observed loss in catalytic performance would be attributable solely to the resin component. This also suggests that the enzyme itself does not contribute to the inhibition under immobilized conditions. These findings strongly support the conclusion that immobilization effectively restricts the enzyme's access to the gold nanoparticles during the reaction, thereby preventing further catalytic deactivation. Nevertheless, the

functionalized amine groups of the resin may interfere with the activity of the gold, as discussed further in Section III.2. Impact of transamination reaction components on Au/SiO₂ catalyst, and the resin may contain impurities that could be released during the reaction. Indeed, it is common practice in biocatalysis to include a washing step when using resin for immobilization¹⁰⁹.

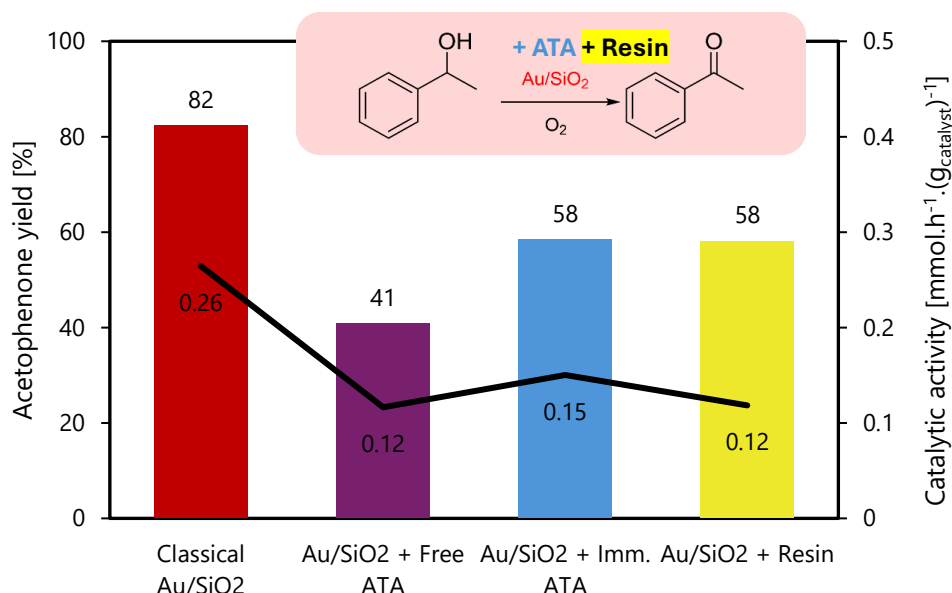


Figure 29. Acetophenone yield after 24 hours and initial catalytic activity (2h) in Au/SiO₂-catalyzed oxidation with free or immobilized enzyme or with resin

Comparison of catalytic activity and 24 h product formation using Au/SiO₂ catalyst in the presence of free or immobilized ATA_117 or resin alone, evaluating their respective influence on the oxidation of 1-phenylethanol. Reaction conditions: 20 mM 1-phenylethanol, 100 mM buffer to reach final volume, (1 mg/mL free or immobilized ATA_117), (125 mg/mL resin) and 20 mg/mL Au/SiO₂.

Figure 29 also reveals that whether the enzyme is free, or immobilized, or with simple resin, the effect on the initial activity is comparable. The slightly higher activity observed in the presence of the immobilized enzyme may be attributed to a lower release of impurities from the resin when enzyme is immobilized on it. This is likely due to the immobilization protocol itself, which involves an overnight incubation followed by washing steps that partially eliminate contaminants, unlike in the condition where the resin is used alone, without pre-treatment and directly added in the reaction medium. Entire kinetics are available in Appendix A.2. Oxidation kinetics with addition of free or immobilized ATA or resin

Since immobilization led to higher acetophenone yield through lower gold deactivation than the free enzyme and was shown to prevent direct interaction with gold nanoparticles, while also improving enzyme stability, this strategy was retained for subsequent applications.

III.2. Impact of transamination reaction components on Au/SiO₂ catalyst

In this chapter, two more parameters need to be evaluated, as the enzyme-catalyzed reaction involves the amine donor (IPA) and the enzyme cofactor (PLP) that could impact the chemo-catalyst's performance. Under standard enzymatic conditions, 1 mM PLP and 500 mM IPA are present. Their individual effects were tested separately under chemo-catalytic conditions (**Figure 30**).

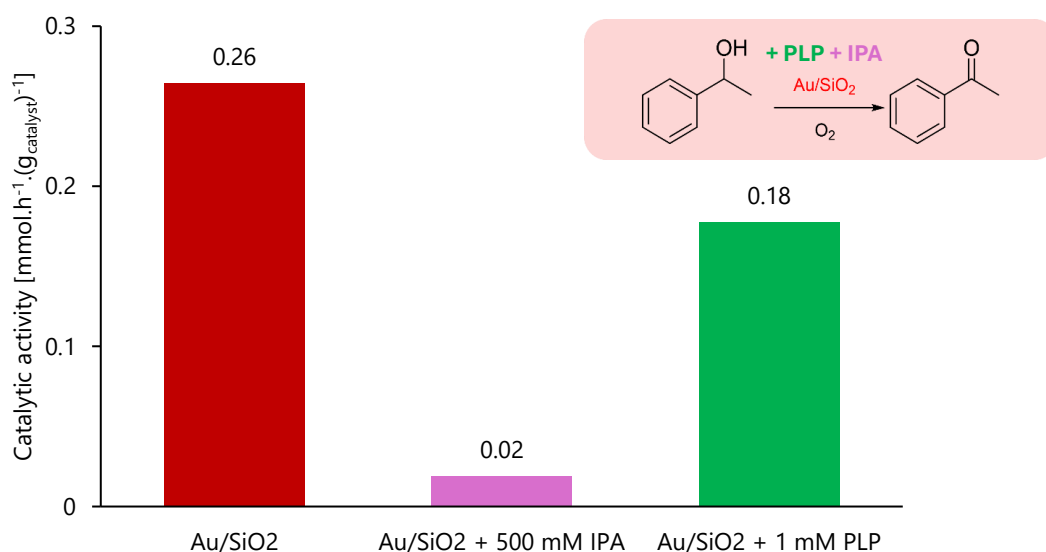


Figure 30. Effect of PLP and IPA on the initial catalytic activity of Au/SiO₂

Comparison of initial catalytic activity in the presence or absence of 1 mM PLP and 500 mM IPA, assessing their influence on the oxidation of 1-phenylethanol catalyzed by Au/SiO₂. Reaction conditions: 20 mM 1-phenylethanol, 100 mM buffer to reach final volume, (500 mM IPA), (1 mM PLP) and 20 mg/mL Au/SiO₂.

Catalytic activity decreased to 0.18 mmol.h⁻¹.(g_{catalyst})⁻¹ in the presence of 1 mM PLP and drastically dropped to 0.02 mmol.h⁻¹.(g_{catalyst})⁻¹ with 500 mM IPA, compared to 0.26 mmol.h⁻¹.(g_{catalyst})⁻¹ in the absence of these two components. These results demonstrate a significant detrimental effect of the IPA and PLP on the performance of the gold catalyst, indicating that mitigation strategies are needed.

Several hypotheses are proposed to explain the deactivation of the gold catalyst in the presence of high amine concentration. The first involves the aggregation of gold nanoparticles, resulting in lower dispersion. This would be particularly detrimental, as the catalytic activity of gold is known to be highly dependent on nanoparticle dispersion, as previously reported^{75,85}. However, such catalyst transformation is irreversible, and if aggregation was the main cause of deactivation, washing the catalyst would not restore its activity. To test this hypothesis, the catalyst was washed three times with 10 mL of water after exposure to 500 mM IPA, and was found to partially regain its catalytic performance when reused under optimal conditions in the absence of IPA (**Figure 31**). This result is an argument that argues against irreversible nanoparticles aggregation.

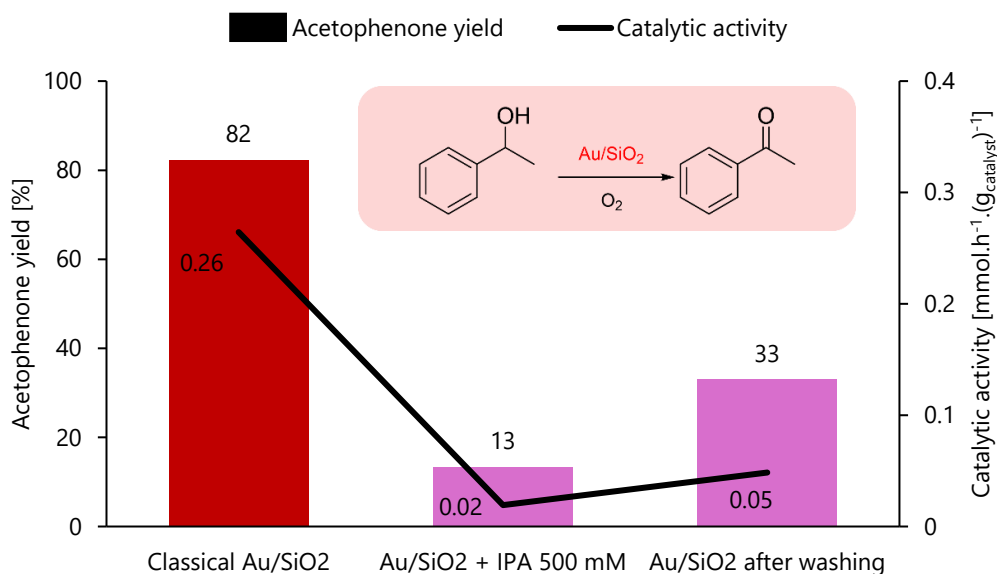


Figure 31. Catalytic performance of Au/SiO₂ after IPA incubation and water washing

Comparison of acetophenone yield after 24 h and initial catalytic activity using Au/SiO₂ catalyst under three conditions: untreated, after incubation in 500 mM IPA, and after incubation followed by three water washings. Reaction conditions: 20 mM 1-phenylethanol, (500 mM IPA), 100 mM buffer to reach final volume, and 20 mg/mL Au/SiO₂.

A second hypothesis involves the formation of weakly bound amines on the gold surface and on silica, leading to catalyst passivation. According to the literature, amines can coordinate to gold atoms due to their high affinity for noble metals^{110,111}. Aliphatic amines are known to form strong interactions with metal surfaces, and their adsorption can effectively block active sites, resulting in catalyst poisoning¹¹². This could justify the partial recovery of activity due to partial washing of remaining IPA as a layer.

To further investigate the passivation phenomenon, infrared (IR) spectroscopy was employed. A total of five samples were analyzed under various conditions:

- 1) SiO₂ support (untreated),
- 2) Au/SiO₂ catalyst (untreated),
- 3) SiO₂ immersed in IPA and then dried in open air for one night (3a) or 72 hours (3b),
- 4) Au/SiO₂ immersed in IPA and dried in open air for one night (4).

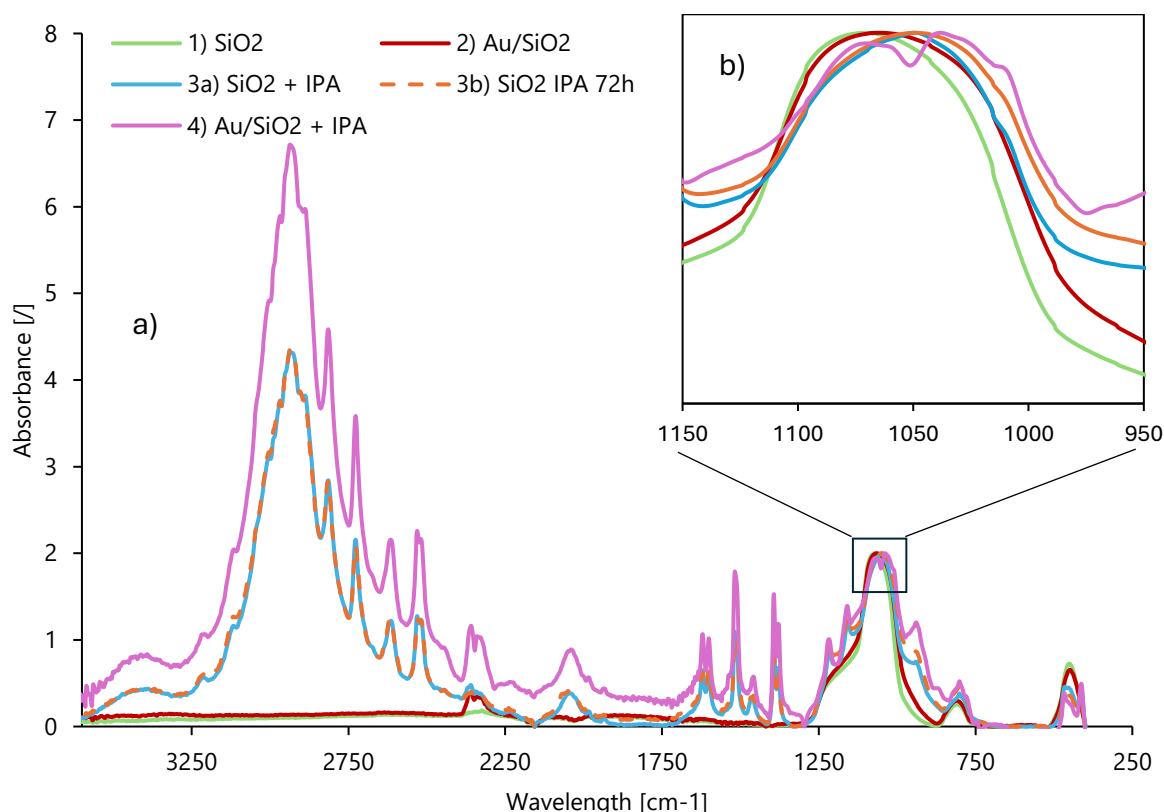


Figure 32. IR spectra of SiO₂ support and Au/SiO₂ catalyst under different treatments

(a) Full-range IR spectra comparing untreated SiO₂ and Au/SiO₂ catalyst subjected to various treatments. (b) Zoom on the 1150–950 cm⁻¹ region highlighting changes in the Si-O-Si stretching vibrations.

Figure 32a shows a broad peak around 1050 cm⁻¹, characteristic of the SiO₂ support. The IR spectra of samples 3 and 4 (SiO₂ + IPA and Au/SiO₂ + IPA, respectively) exhibit distinct shifts and new bands compared to their untreated counterparts (samples 1 and 2). These new features are attributed to the presence of IPA, as they appear consistently upon its addition and the peaks around 2950-2850 cm⁻¹ is identified as the aliphatic C-H stretching vibrations of IPA¹¹³.

Interestingly, the spectra of SiO₂ incubated with IPA for 1 night (3a) and 72 hours (3b) are nearly identical, suggesting a stable adsorption of IPA onto the SiO₂ surface, even in the absence of gold.

A key observation is the greater intensity of IPA-associated peaks for the Au/SiO₂ sample, despite identical evaporation conditions. This suggests that IPA either has a stronger affinity for the gold-containing surface, or that gold alters the vibrational environment, enhancing the IR response. This stronger interaction may play a role in the passivation phenomenon by forming a persistent organic layer on the catalyst surface, potentially hindering access to active sites.

Another notable feature is the doubling or splitting of the 1050 cm⁻¹ band in sample 4 (Au/SiO₂ + IPA), highlighted in the zoomed-in region (**Figure 32b**). This band typically

corresponds to Si-O-Si vibrations, but the alteration observed only when both gold and IPA are present suggests a specific Au-IPA interaction. Such interaction may induce shifts or reinforcement of vibrational modes, consistent with adsorption phenomena of IPA on gold or silica.

While these findings provide insight into possible surface interactions, definitive conclusions cannot yet be drawn. It is conceivable that further washing steps or the use of alternative solvents could remove this residual IPA layer, potentially restoring full catalytic activity.

Figure 33 illustrates inhibition by PLP through a lower gold catalyst stability. PLP likely deactivates the gold catalyst through its aldehyde function or its phosphate group, both of which can bind strongly to metallic surfaces. It has been reported that molecules containing carbonyl groups (such as 2-propanol) can occupy and poison up to 95 % of gold's active sites, even at low temperatures¹¹⁴. Because PLP is larger and more polar, its adsorption on gold could be even stronger. Furthermore, PLP degradation under light can exacerbate this deactivation^{48,115} (detailed in next Section).

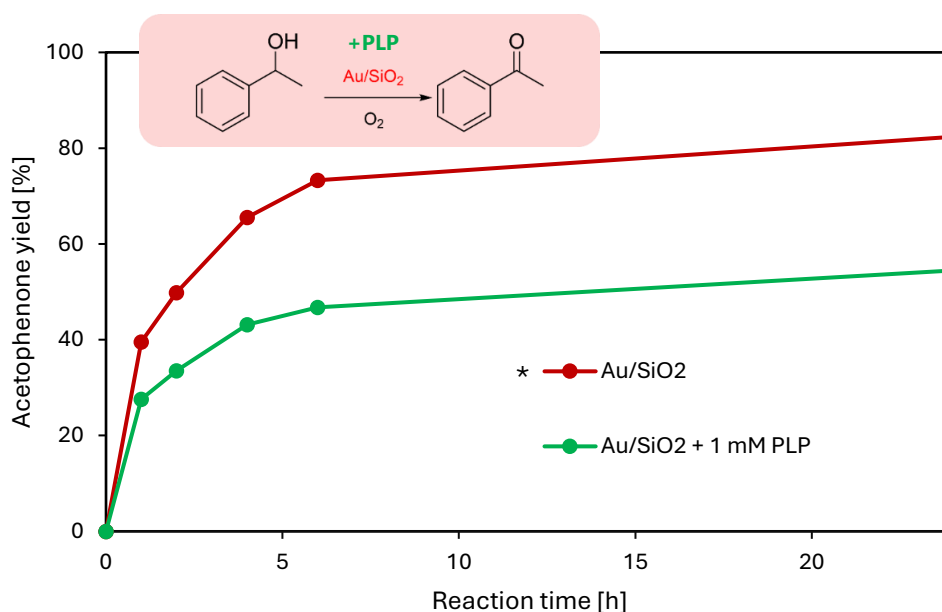


Figure 33. Effect of PLP on acetophenone yield over time

Comparison of 24 h product formation kinetics in the presence or absence of 1 mM PLP to assess its impact on the catalytic performance of Au/SiO₂. Reaction conditions: 20 mM 1-phenylethanol, (1 mM PLP), 100 mM buffer to reach final volume, and 20 mg/mL Au/SiO₂.

III.3. Deactivation mitigation strategies

All activity losses are reported as relative decreases from the yield reached with classical chemo catalytic test, taken as 100%.

First, enzyme immobilization was shown to mitigate gold catalyst deactivation compared to the free enzyme, leading to a 21% improvement in performance. On the other hand, it was demonstrated that both IPA and PLP contribute to the partial deactivation of the gold

catalyst, with activity losses of 84% and 34%, respectively. Given these incompatibilities, two main approaches were investigated to improve catalysts' compatibility.

III.3.1. Mitigating deactivation by amine donor

IPA was initially added in excess (500 mM) in order to stir the transamination reaction towards product formation as it is thermodynamically unfavoured. However, adding a lower concentration could potentially reduce gold-catalyst deactivation, unless the simple presence of an amine kills the gold catalyst.

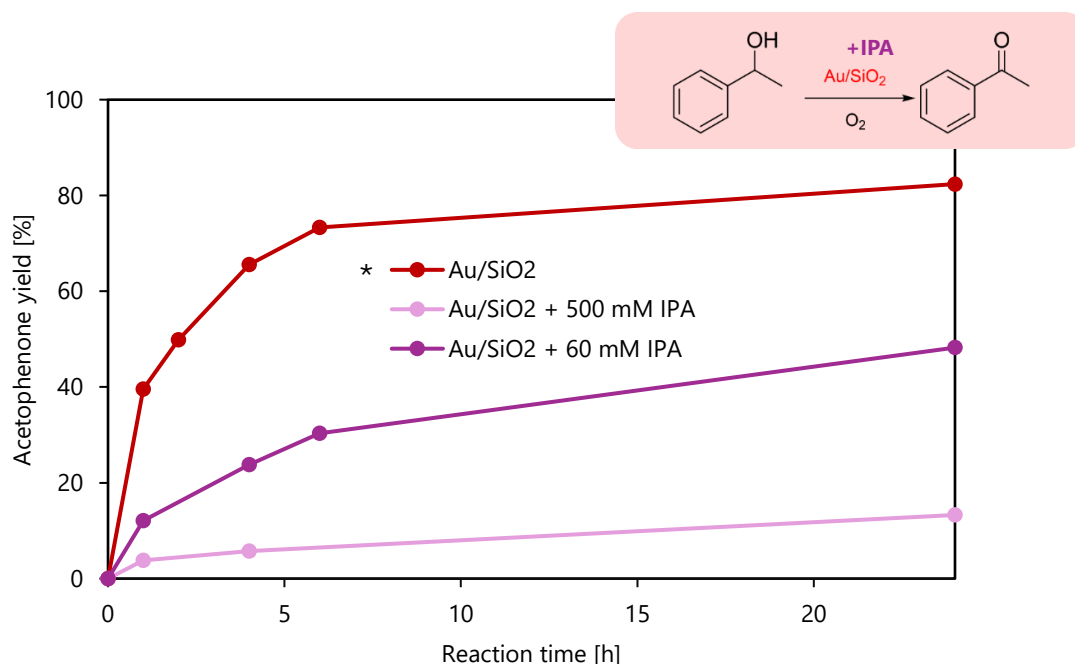


Figure 34. Effect of IPA concentration on acetophenone yield over time

Comparison of 24 h product formation kinetics using Au/SiO₂ catalyst in the presence of 60 or 500 mM IPA, evaluating the influence of IPA concentration on the oxidation of 1-phenylethanol. Reaction conditions: 20 mM 1-phenylethanol, (60/500 mM IPA), 100 mM buffer to reach final volume, and 20 mg/mL Au/SiO₂.

Reducing IPA concentration from 500 mM to 60 mM increased acetophenone yield to 48%, compared to only 14% at 500 mM (Figure 34.). Although this remains below the optimal 82% yield under ideal conditions, it nonetheless constitutes a significant improvement. However, while beneficial for enhancing the oxidation step, lowering the amine donor concentration could adversely affect the transamination rate, as the initial excess was intended to compensate for unfavourable reaction thermodynamics. Consequently, this reduction will inevitably shift the equilibrium position against product formation.

III.3.2. Mitigating deactivation by PLP

A PLP maturation protocol was attempted, inspired by López-Gallego *et al.*¹¹⁶ and already applied by Meersseman *et al.*³⁰ to preload PLP into the enzyme after immobilization, thus

avoiding PLP addition for the cascade. Immobilized ATA was incubated for 2 hours with a 10 mM PLP solution, followed by extensive washing to remove excess PLP.

a) Impact on enzymatic activity

Incorporating a PLP maturation step ensures the enzyme is fully loaded with cofactor, since only catalytic amounts (0.1–2 mM) are needed and PLP is regenerated during catalytic cycle. This preloading renders the biocatalyst self-sufficient, eliminating the need for exogenous PLP additions¹¹⁷. Furthermore, co-reusing the enzyme with its preloaded cofactor would enhance the cost-efficiency of the process.

Figure 35 compares different PLP addition strategies. A 2-hour PLP maturation provided a 23% yield after 24 hours, exceeding the 18% achieved by the classical addition method. Conversely, overnight maturation damaged the enzyme, with negligible activity after 6 h and no further increase until 24 hours.

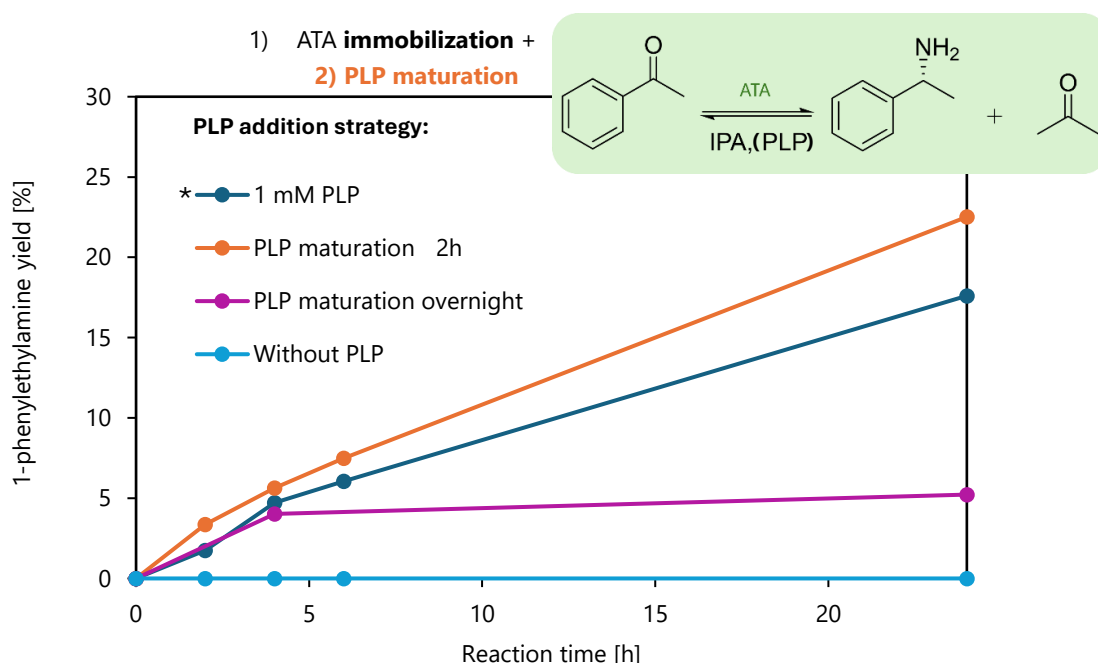


Figure 35. 1-phenylethylamine yield over time with different PLP addition strategies

Comparison of 24 h product formation kinetics using ATA_117 with varying PLP addition strategies, assessing their impact on transamination efficiency. Maturation step is performed between immobilization and reaction steps. Reaction conditions: 20 mM acetophenone, 1 mM PLP (when applicable), 500 mM IPA, 100 mM buffer to reach final volume, and 1 mg/mL ATA_117.

Prolonging the maturation step to approximately 16 h reduced activity. This inhibition is likely due to light exposure: PLP is highly photosensitive and degrades under normal laboratory or daylight conditions, forming species that oxidise amino-acid residues and irreversibly inactivate the enzyme^{48,115}. In our trial, approximately 6 hours of the 16 hours maturation were under light, compared with only 2 h in the standard protocol, resulting in accelerated enzyme poisoning and complete inactivation after just 4 hours of reaction.

Merz *et al.* reported that the inactivation rate rises with PLP concentration, with a pronounced effect at 10 mM PLP that also depends on the buffer¹¹⁵. Additional studies show that, in 100 mM phosphate buffer, PLP degradation is especially severe and the loss of activity under light is greater than in other buffers (TRIS or HEPES)⁴⁸.

The improvement of yield observed thanks to PLP maturation as compared to punctual addition of 1mM PLP before reaction can be discussed. The same paper¹¹⁵ proved the enhanced stability and initial activity for increased PLP concentration, which is in line with the observed results. At the start of the reaction, nearly all transaminases are activated by PLP due to maturation, accelerating the initial rate.

b) Impact on chemo-catalyst

Given the results obtained for the enzyme following PLP maturation, the impact of this treatment on the chemo-catalytic system was also evaluated. This was done by comparing the acetophenone yield in the standard gold-catalyzed reaction upon addition of either (i) the immobilized enzyme or (ii) the immobilized enzyme preincubated for PLP maturation. **Figure 36** shows that both curves overlap almost perfectly throughout the entire reaction kinetic. This indicates that the oxidation reaction proceeds identically in both cases, leading to the same acetophenone yield. Consequently, the deactivation observed in the system can be attributed solely to the presence of the resin, PLP maturation was thus systematically employed in all subsequent cascade reactions.

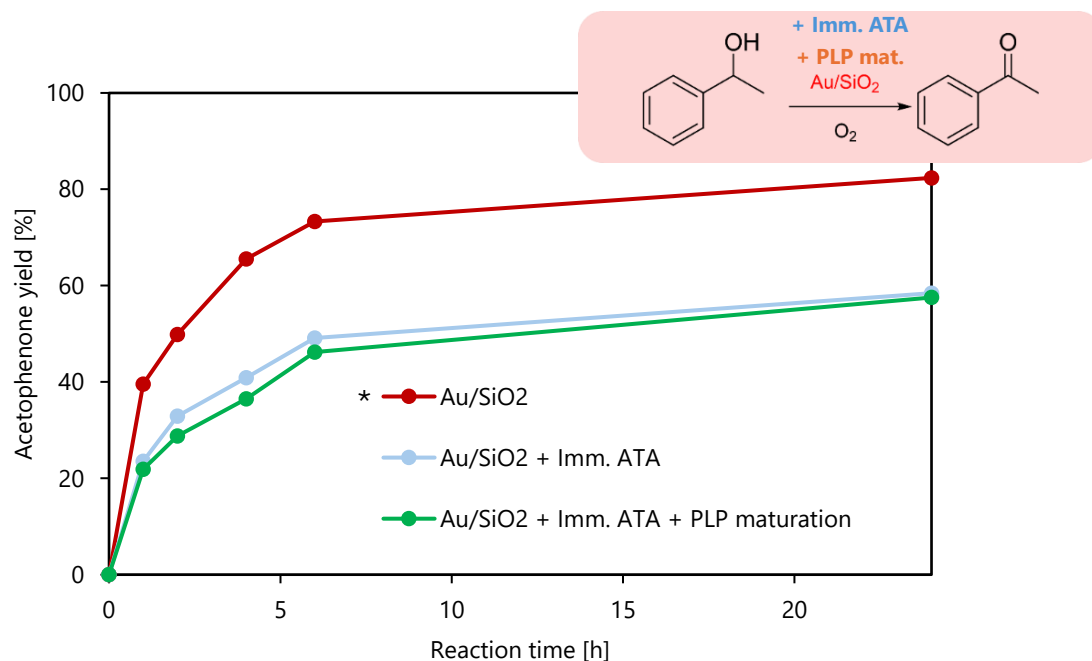


Figure 36. Acetophenone yield over time with or without PLP maturation of immobilized enzyme

Comparison of 24 h product formation kinetics in the Au/SiO₂-catalyzed oxidation of 1-phenylethanol in the presence of immobilized ATA_117, with or without a maturation step in the presence of PLP prior to reaction. Reaction conditions: 20 mM 1-phenylethanol, 100 mM buffer to reach final volume, and 20 mg/mL Au/SiO₂.

III.3.3. Overall mitigation strategies

Figure 37 summarizes the three mitigation strategies implemented to reduce gold catalyst deactivation: enzyme immobilization, PLP maturation, and lowering the IPA concentration to 60 mM.

Immobilization alone enhanced acetophenone yield by addressing enzyme-induced catalyst deactivation, recovering 17% yield. PLP maturation further contributed by preventing additional gold deactivation associated with PLP, without negatively affecting catalyst performance compared to immobilization alone. Although this approach did not significantly surpass the standard 1 mM PLP addition, it simultaneously tackled two sources of deactivation (enzyme and cofactor), reinforcing overall catalyst stability. Finally, decreasing IPA concentration led to a substantial improvement, allowing the gold catalyst to regain 48% of its activity and significantly boosting overall system efficiency.

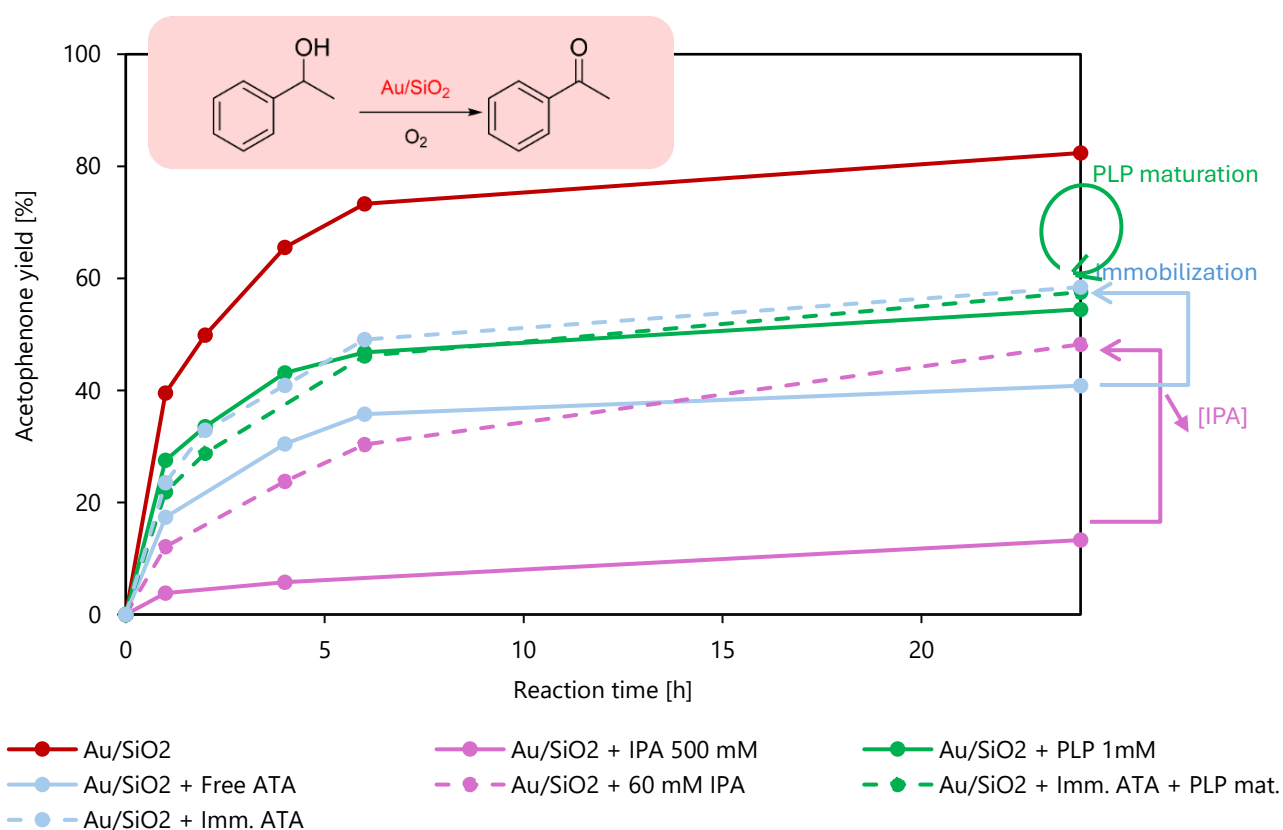


Figure 37. Acetophenone yield over time with different mitigation strategies

Comparison of 24 h product formation kinetics in the Au/SiO₂-catalyzed oxidation of 1-phenylethanol using various mitigation strategies: IPA concentration reduction, enzyme immobilization, and PLP maturation. Reaction conditions: 20 mM 1-phenylethanol, 100 mM buffer to reach final volume, and 20 mg/mL Au/SiO₂. Additional conditions applied as indicated.

Individually, the free enzyme and PLP caused activity losses of 50% and 34%, respectively. When combined independently, this corresponds to a residual activity of:

$$\text{Residual activity} = (1 - 0.5) \times (1 - 0.34) = 0.33$$

This corresponds to a global activity loss of 67%, which is significantly higher than the 30% loss observed when both mitigation strategies are applied together.

For the cascade experiments, a concentration of 60 mM IPA was selected. While lowering IPA concentration reduces the reaction's driving force, it significantly decreases the inhibitory effect on the gold catalyst, reducing the activity loss from 84% (with 500 mM IPA) to 41%.

When considering the two mitigation strategies independently: immobilization + PLP maturation (70% residual activity) and reduced IPA concentration (59%), the theoretical cumulative residual activity is:

$$\text{Residual activity} = 0.70 \times 0.59 = 0.41$$

Therefore, assuming no synergy between the mitigation strategies, the expected residual gold activity would be 41% of its maximum activity (in the absence of any biocatalytic component interfering with the reaction). Although this still represents a substantial loss, it is a striking improvement compared to the scenario where all components are combined without any mitigation, which would have resulted in complete deactivation, or theoretically, just 5% residual activity:

$$\text{Residual activity} = 0.66 \times 0.5 \times 0.16 = 0.05$$

58 when combining the effect of 1 mM PLP, free enzyme and 500 mM IPA, respectively.

IV. Chemo-enzymatic cascades

With the conditions now optimized, both reaction steps can be integrated and evaluated in a single batch cascade.

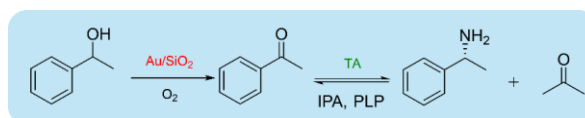
IV.1. One-pot, two-steps cascades

Before performing the cascade in a single uninterrupted step, several two-steps cascade experiments were carried out, in sequential mode. The strategy consisted in allowing the gold catalyst to operate under optimal/less deactivating conditions during the first 24 hours, before introducing the deactivating components associated with the transamination step.

Yield and concentration data should be treated with caution, as all samples were injected into the GC before vortexing and centrifugation. Unfortunately, this procedural error likely compromised the liquid-liquid extraction into ethyl acetate, leaving a portion of the analytes in the aqueous phase. Consequently, only general trends and relative comparisons are reliable as all data are probably biased the same way, while absolute values should not be trusted. As expected, the conversion of alcohol to ketone gradually declined upon the addition of deactivating agents during the oxidation step. Consequently, the formation of phenylethylamine also decreased, in line with the reduced availability of acetophenone. These results are available in Appendix A.3. Two-steps intermediate cascades

IV.2. One-pot, one-step cascades

IV.2.1. Configurations tested



The second part of these cascade experiments aims to establish a one-pot, one-step concurrent cascade.

Various cascade configurations were tested, including combining optimal immobilization conditions, varying the amount of chemo-catalyst, with or without PLP maturation, adjusting the amount of resin, using enzyme in its pure form or in cell-free extracts, and modifying the initial concentrations of reagents. Only the most relevant or promising outcomes are presented in this section.

In the cascade system, the mitigation strategies, namely enzyme immobilization, its preloading with PLP, and limiting IPA to 60 mM to protect the gold catalyst, must be taken into account when evaluating enzyme performance, since this reduced IPA concentration shifts the reaction equilibrium.

IV.2.2. Cascade with best immobilization conditions

Logically, the best cascade outcome was expected by combining all the individually optimized conditions: immobilization at 22 °C, 10 mM acetophenone, 50 mM buffer, and

post-immobilization PLP maturation. These conditions were tested both in the transamination reaction alone and in the full cascade. Surprisingly, none of these attempts were successful. Each time these conditions were combined, erratic behaviours were observed: substrate concentrations did not follow typical kinetic trends and the carbon balance was inconsistent. In cascade reactions, no amine product was formed.

This all-in-one strategy was abandoned after some tests. Instead, only PLP maturation was retained, as it proved to be an effective mitigation strategy compatible with both catalysts. The immobilization was performed at room temperature without acetophenone. Under these conditions, the cascades exhibited a much more consistent carbon balance, and the final amine product was successfully formed. **Table 3** presents a synthesis of the conditions that led to success or failure.

Table 3. Comparison between failing and successful conditions for immobilization, maturation, and reaction
Summary of experimental conditions tested across the immobilization, maturation, and reaction steps, highlighting those leading to failed or successful 1-phenylethylamine formation.

STEP	Failing conditions	Working conditions
<i>Immobilization</i>	buffer 50 mM, 10 mM Ac, 22°C	buffer 100 mM , no Ac, 22°C
<i>Maturation</i>	PLP 10 mM, 2h, 22°C	Same
<i>Reaction</i>	buffer 50 mM, IPA, 20 mM Ac	buffer 100 mM , IPA, 20 mM Ac

The issue appears to arise from the combination of PLP maturation, substrate-assisted immobilization, and lower ionic strength conditions.

Experimental evidence suggests that starting with PLP maturation followed by substrate addition does not disrupt the system. However, when these steps are reversed (i.e., acetophenone is present before PLP binding) the system fails entirely.

In the transaminase mechanism, PLP enters the active site of the enzyme and forms a Schiff base with the lysine residue of the enzyme, yielding the catalytically active enzyme-PLP complex. If acetophenone is introduced first, it may non-covalently bind to the active site, potentially inducing a closed conformation of the enzyme. This may either block PLP access to the lysine or alter the position of the lysine, preventing proper complex formation. **Figure 38** compares the two scenarios for PLP and acetophenone attachment.

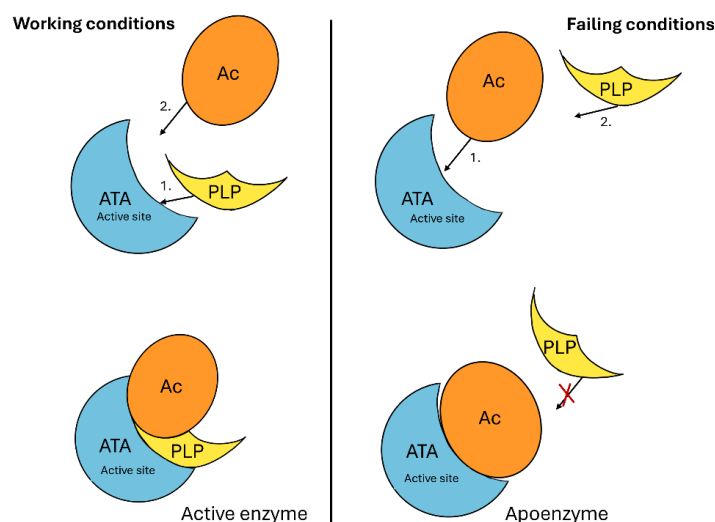


Figure 38. Representation of the impact of substrate and cofactor binding order on transaminase activity
Schematic depiction of different binding sequences between the substrate and PLP cofactor, illustrating their influence on ATA_117 catalytic performance.

As a result, PLP would fail to bind effectively, remaining in solution, and would ultimately be removed during washing. In this hypothesis the enzyme would stay in its inactive apo-form, with only a few correctly formed active sites, leading to very limited catalytic activity. This is consistent with the low yields observed (i.e., ~4% phenylethylamine formation).

Subsequent cascades were performed with PLP maturation but substrate-assisted immobilization was abandoned.

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IV.2.3. Process optimization

a) Impact of resin amount and mixing efficiency

As illustrated in **Figure 39**, a non-negligible amount of resin, mixed with the gold catalyst, as evidenced by its texture and colour, was found adhered to the reactor walls. This likely reduced the effective concentration of both catalysts in the reaction medium, ultimately leading to lower yields.

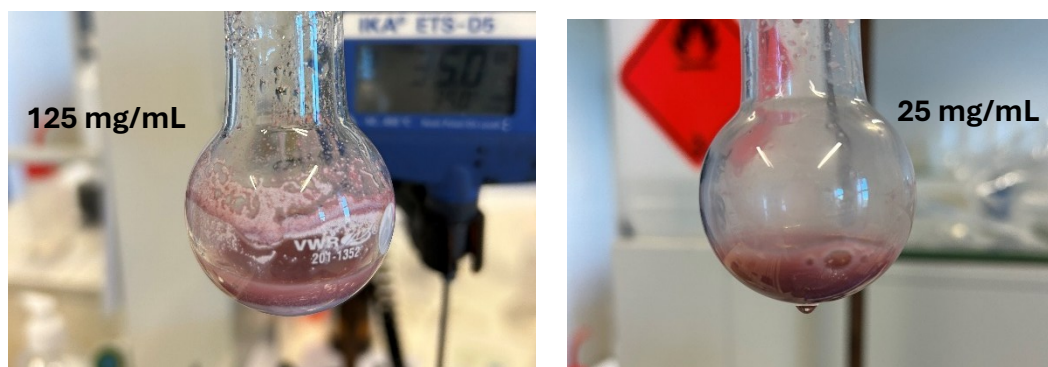


Figure 39. Visual comparison of resin distribution within the reactor at different concentrations
Formation of an inaccessible resin layer on the reactor walls at 125 mg/mL versus a homogeneous medium at 25 mg/mL, illustrating the impact of resin concentration on medium homogeneity.

To overcome this issue, stirring speed was reduced from 2500 to 1000 rpm, which ensured sufficient mixing to maintain a homogeneous reaction medium while preventing the projection of the solid catalyst into the upper part of the reactor, where it could no longer contribute to catalysis.

In parallel, the amount of resin used for immobilization was significantly reduced while maintaining the enzyme concentration at 1 mg/mL in the reaction (i.e. increasing the loading in the resin). Initially, 125 mg of resin per mL was employed (corresponding to a 0.8wt% enzyme loading), which was subsequently adjusted to 25 mg/mL (4wt% loading). This new loading value also ensures more efficient utilization of reactive epoxy groups, reducing the likelihood of stability issues caused by residual unreacted functionalities¹¹⁸. Immobilization efficiency was measured before and after this adjustment, yielding 91% initially and 94% after the change, confirming that the reduced amount of resin still enables highly efficient immobilization.

b) Effect of these improvements on cascade performance

The impact of these two modifications was assessed in the cascade reaction. After 144 hours, amine production was significantly higher when using the reduced amount of resin, likely due to improved substrate access to the enzyme and less material loss on the reactor walls. Globally, best achieved cascade was the one with less resin. It achieved 0,8 mM phenylethylamine production after 144 hours reaction (**Figure 40**). This corresponds to 27% of the possible reachable concentration before being at equilibrium (2.95 mM product).

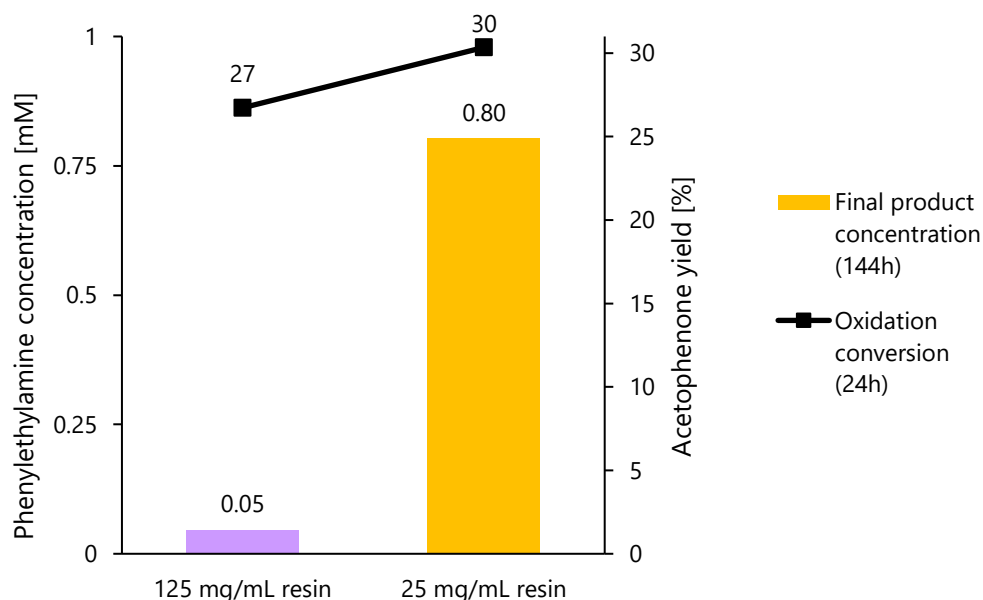


Figure 40. 1-phenylethylamine concentration after 144 hours and acetophenone yield after 24 hours in cascade reactions with different resin loadings

Comparison of 24 h product formation during the enzymatic cascade using 125 mg/mL and 25 mg/mL resin for immobilization, evaluating the influence of resin concentration on 1-phenylethylamine production. Reaction conditions: 20 mM 1-phenylethanol, PLP maturation, 60 mM IPA, 100 mM buffer to reach final volume, 20 mg/mL Au/SiO₂ and 1 mg/mL immobilized ATA₁₁₇.

Furthermore, as previously observed, the presence of excess resin interfered with the gold-catalyzed step. By limiting the resin to the minimum required, this negative effect can be mitigated, resulting in a better working first step, with oxidation yield rising from 27% to 30%. The lower resin concentration was therefore adopted for subsequent experiments. Evolution of each compound's concentrations over time is represented in Appendix A.4. Best cascade: "less resin" cascade

c) Stepwise analysis of the cascade reaction

Separately analyzing each step's performance in the cascade enables direct comparison with the expected results from individual step optimizations.

- Oxidation reaction

Oxidation reaction reaches a yield representing 37% of that obtained in standard catalytic test. It is in good agreement with the 41% residual activity predicted based on the combined deactivation effects of IPA and the immobilized, PLP-matured enzyme (Section III.3.3. Overall mitigation strategies). This indicates that, under the conditions selected as the optimal compromise for both the enzyme and the gold catalyst, no additional deactivation occurs beyond what was previously identified.

Because IPA is continuously converted into 1-phenylethylamine, the total amine concentration remains constant. Thus, product formation does not introduce additional deactivation of the gold catalyst, assuming that 1-phenylethylamine exerts an inhibitory effect comparable to IPA.

The match between the yields obtained in the cascade and in the individual gold catalytic tests confirms that PLP maturation is compatible with the gold system, even over extended durations, with minimal PLP leaching.

- Transamination reaction

To assess whether the amount of amine produced by the enzyme in the cascade is consistent with its normal activity, a control experiment was carried out under conditions mimicking those of the cascade at 0-24 hours. Acetophenone was used at 5.5 mM, corresponding to the average concentration in the cascade during that time frame. PLP maturation was applied, and IPA was added at 60 mM, as in the cascade.

Figure 41a shows that in the transamination reaction, acetophenone starts at 5.5 mM and logically decreases as it is consumed to produce phenylethylamine. In contrast, in the cascade, acetophenone starts at nearly zero and increases over time, since oxidation proceeds faster than transamination, leading to accumulation.

Interestingly, the amount of amine produced is similar in both setups, as well as the initial catalytic activity (**Figure 41b**), indicating that the enzyme performs comparably whether in

an isolated transamination or in a full cascade. This suggests that the enzyme is not significantly disturbed in the cascade conditions. A slight difference in yield after 24 hours is observed, which is consistent with the higher availability of acetophenone at the beginning of the isolated transamination reaction.

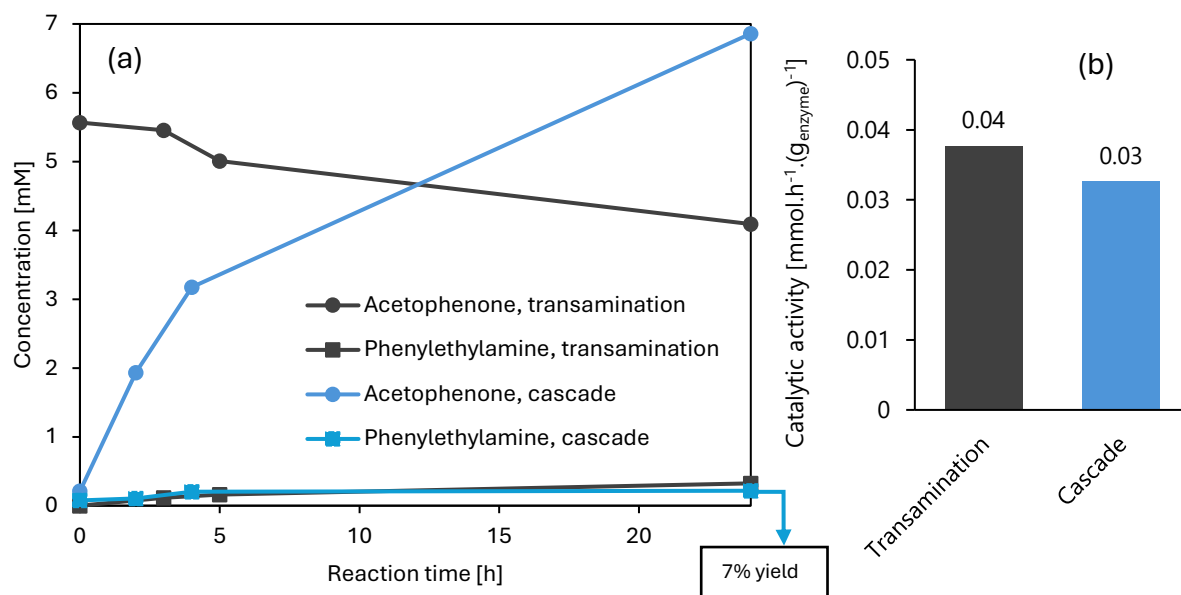


Figure 41. 1-phenylethanol and acetophenone concentrations and ATA_117 activity in transamination and cascade reactions

(a) Concentrations of 1-phenylethanol and acetophenone over time during transamination and cascade reactions performed under identical conditions, with transamination starting with 5.5 mM acetophenone, a representative acetophenone concentration in the cascade. (b) ATA_117 catalytic activity measured under both conditions to assess the impact of the cascade setup. Transamination conditions: 5.5 mM acetophenone, PLP maturation, 60 mM IPA, 100 mM buffer to reach final volume, and 1 mg/mL immobilized ATA_117. Cascade conditions: 20 mM 1-phenylethanol, PLP maturation, 60 mM IPA, 100 mM buffer to reach final volume, 20 mg/mL Au/SiO₂ and 1 mg/mL immobilized ATA_117. Amount of resin for immobilization: 25 mg/mL.

As a reminder, when immobilization and transamination were optimized independently from the oxidation step, the use of 500 mM IPA led to a 23% yield after 24 hours under PLP maturation conditions (Figure 35). This yield contrasts sharply with the less than 10% observed in the cascade (Figure 41a), where only 60 mM IPA is used.

This comparison highlights a key limitation of the system: an intermediate IPA concentration that is still too high for the gold catalyst, causing deactivation, but too low for efficient transamination, which requires excess amine donor to shift the equilibrium. This incompatibility between the two catalytic systems appears to be the main constraint of the cascade process.

Overall, Figure 41 highlights that at this IPA concentration, ATA displays a very low catalytic activity, resulting in limited product formation. Since it is well known that an excess of IPA is required to drive the reaction forward due to unfavourable thermodynamics ($K=0.0333$)¹¹⁹, the next step is to determine whether the system has already reached equilibrium, or if further conversion remains possible.

IV.2.4. Transamination thermodynamic equilibrium

A new cascade was initiated using 40 mM of the starting reagent, a concentration that would severely inhibit the transamination reaction if used alone, starting from 40 mM acetophenone. Starting the cascade at this level will allow us to verify the relevance of implementing a cascade to solve substrate inhibition problem.

In the resulting graph (Figure 42), the initial catalytic activity can be attributed to the low concentration of ketone available at the early stages of the reaction. Maximum activity is then reached between t_0 and t_4 , once a sufficient amount of ketone is produced by the first reaction.

The subsequent sharp drop in activity likely reflects either a progression toward thermodynamic equilibrium or partial enzyme deactivation or destabilization. In this case, substrate depletion can be excluded, as ketone is continuously produced by the oxidation step and remains below the inhibitory threshold of approximately 10 mM (see Section I.1. Acetophenone and 1-phenylethanol inhibition). Additionally, although Truppo *et al.* demonstrated possible inhibition by the amine product, it occurs only at concentrations around 50 mM phenylethylamine, significantly higher than in our current conditions⁹⁵.

To investigate whether the system had indeed reached equilibrium, 1 mg of free enzyme and 1 mM PLP were added to the reaction medium at the point indicated by the arrow. However, no increase in catalytic activity was observed following this addition, confirming that the reaction was no longer limited by enzyme availability, but rather by intrinsic thermodynamics or inhibitory constraints.

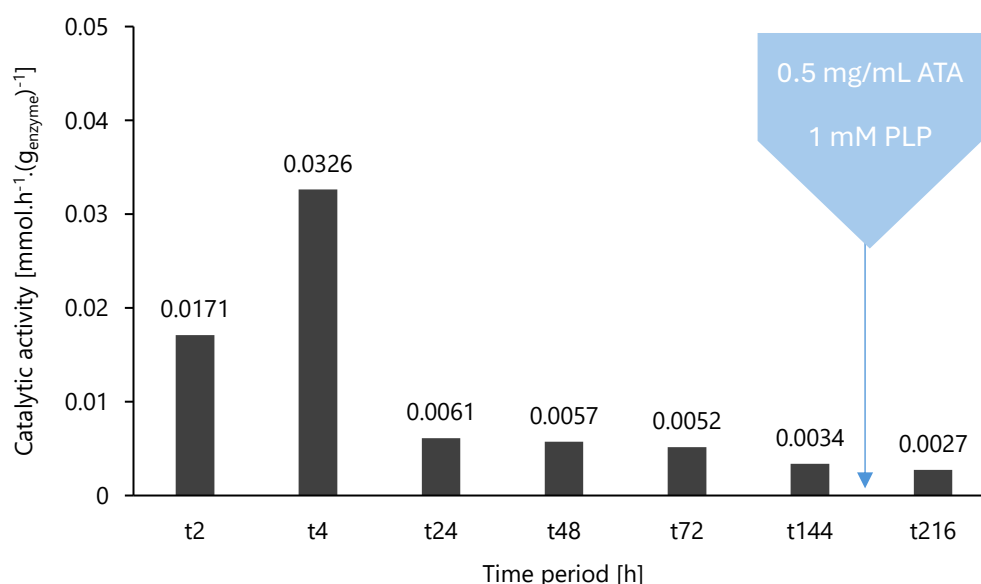


Figure 42. Enzyme catalytic activity over time in cascade reaction with late addition of enzyme and PLP

Catalytic activity measured over defined time intervals. The blue arrow indicates the addition of 0.5 mg/mL free ATA₁₁₇ and 1 mM PLP after t144 to assess whether the reaction could be reactivated. Reaction conditions: 20 mM 1-

phenylethanol, PLP maturation, 60 mM IPA, 100 mM buffer to reach final volume, 20 mg/mL Au/SiO₂ and 1 mg/mL immobilized ATA_117.

This test suggests that even more 1-phenylethylamine could form, as amine production continues past 216 hours and equilibrium has not yet been reached. However, enzyme activity appears to be hindered, possibly due to PLP degradation after 216 hours of light exposure. Another potential cause is sub-product inhibition⁴⁵, since acetone may not be evaporating effectively with openings only every 24 hours.

IV.3. Gold catalytic activity

When comparing the initial catalytic activity of the gold catalyst at different starting concentrations of the cascade-initiating reagent, a clear trend is observed (Figure 43).

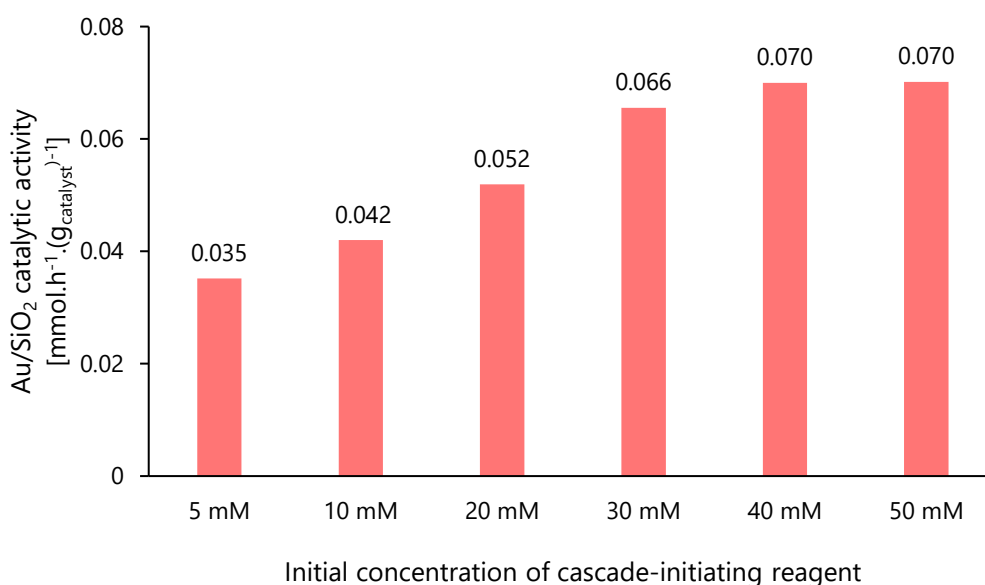


Figure 43. Au/SiO₂ catalytic activity as a function of 1-phenylethanol concentration to initiate the reaction

Initial catalytic activity measured with increasing concentrations of 1-phenylethanol (5–50 mM), assessing the influence of substrate loading on Au/SiO₂ performance. Reaction conditions: (5–50 mM) 1-phenylethanol, PLP maturation, 60 mM IPA, 100 mM buffer to reach final volume, 20 mg/mL Au/SiO₂ and 1 mg/mL immobilized ATA_117.

While increasing the concentration from 5 to 30 mM significantly enhances the initial activity, a plateau is reached beyond 30 mM, with only marginal increases in catalytic performance. This suggests that, although more substrate is available, the catalyst cannot convert more than a certain amount within the given timeframe as the system reaches a catalytic activity ceiling. Therefore, increasing the initial substrate concentration further does not result in higher intermediate formation, as the catalyst operates at or near its maximum capacity under the current conditions.

This limitation can be attributed either to the catalyst loading (20mg/mL) or to partial inhibition of the active sites. Increasing the catalyst amount or further mitigating its inhibition could help surpass this constraint and unlock higher productivity in the first step of the cascade. Furthermore, the current conditions (35 °C and limited oxygen availability) may be too demanding for the catalyst.

IV.4. Global relevance of cascade implementation

The primary objective of implementing a cascade reaction was to overcome the substrate inhibition typically observed for ATA at concentrations as low as 20 mM. In direct transamination using 40 mM acetophenone, severe inhibition occurred, resulting in a minimal catalytic activity representing only 24% of that achieved when starting with 10 mM acetophenone, a concentration that doesn't inhibit ATA.

As shown in **Table 4**, initiating the cascade with 40 mM 1-phenylethanol doesn't present any inhibitory effect for the enzyme. In fact, the initial catalytic activity at 40 mM is 117 % of that seen at 10 mM. This is logical, since a higher starting concentration provides more substrate early on as gold has already generated twice more acetophenone when beginning from 40 mM (**Figure 43**). Therefore, in the cascade context, starting at 40 mM is advantageous compared to 10 mM, whereas in a standalone transamination, increasing to 40 mM is detrimental.

This enhancement can be directly attributed to the controlled in situ generation of ketone during the oxidation step, which maintains the acetophenone concentration below the critical inhibition threshold (~10 mM), allowing the enzyme to operate under more favourable conditions.

Table 4. Enzymatic catalytic performance in transamination and cascade reactions with varying initiating reagent concentrations

Comparison of catalytic activity and relative activity (expressed as the activity at 40 mM relative to that at 10 mM) of ATA_117 in classical transamination and optimized cascade reactions starting from either 10 or 40 mM of initiating reagent (acetophenone for transamination, 1-phenylethanol for cascade). Catalytic activity in cascade in considered for period t2-t4. Classical transamination conditions: 10 or 40 mM acetophenone, 1 mM PLP, 500 mM IPA, 100 mM buffer to reach final volume, and 1 mg/mL free ATA_117. Optimized cascade conditions: 10 or 40 mM 1-phenylethanol, PLP maturation, 60 mM IPA, 100 mM buffer to reach final volume, 20 mg/mL Au/SiO₂ and 1 mg/mL immobilized ATA_117. Amount of resin for immobilization: 25 mg/mL.

Reaction	Activity [mmol.h ⁻¹ .(g _{enzyme}) ⁻¹], 10 mM initiating reagent	Activity [mmol.h ⁻¹ .(g _{enzyme}) ⁻¹], 40 mM initiating reagent	Relative activity [%]
Transamination	0.53	0.13	24
Cascade	0.04	0.05	117

Substrate inhibition is avoided. However, we were unable to improve the overall performance of ATA when starting from 40 mM 1-phenylethanol compared to 40 mM acetophenone. Although substrate inhibition is resolved, the enzyme must now work with a much lower initial IPA concentration, which limits product formation and reduces the equilibrium concentration of 1-phenylethylamine. To summarize, while resolving substrate inhibition, implementing this cascade introduced additional challenge for the enzyme.

Another interesting point is to compare the relative yield, that is, the actual production of 1-phenylethylamine versus its maximum equilibrium concentration, calculated in **Table 5**.

Table 5. Relative 1-phenylethylamine yields compared to equilibrium concentration in transamination and cascade reactions

Comparison of 1-phenylethylamine concentrations at 6 and 144 hours, expressed relative to the theoretical equilibrium value, for reactions catalyzed by ATA_117 alone (transamination) or in cascade configuration. Classical transamination conditions: 10 or 40 mM acetophenone, 1 mM PLP, 500 mM IPA, 100 mM buffer to reach final volume, and 1 mg/mL free ATA_117. Optimized cascade conditions: 10 or 40 mM 1-phenylethanol, PLP maturation, 60 mM IPA, 100 mM buffer to reach final volume, 20 mg/mL Au/SiO₂ and 1 mg/mL immobilized ATA_117. Amount of resin for immobilization: 25 mg/mL.

Reaction	Equilibrium	6 hours production		144 hours production	
	[mM]	[mM]	[%]	[mM]	[%]
Transamination	11.66	0.75	6	?	?
Cascade	2.95	0.13	4	0.80	27

In standalone transamination at 40 mM, 6 % of 1-phenylethylamine is produced after 6 hours, whereas under the best cascade conditions (“less resin”), only 4 % is reached in the same timeframe. However, by running the cascade for 144 hours, we achieve a 27 % relative yield. This very slow production is due to the lower concentrations of both transamination products (acetophenone and IPA) in the cascade, which slow down the reaction. In standalone transamination, the relative yield is higher at 6 hours, but if the reaction was continued to 144 hours, it likely would never reach the cascade’s 27 % because substrate inhibition would become too strong and stop production before then. These are hypotheses, and further research is needed to confirm this trend.

In summary, although current 1-phenylethylamine production remains modest, the cascade approach proves its relevance by avoiding substrate inhibition, laying a foundation for further optimization.

Conclusion

Transaminases and Au/SiO₂ were successfully combined in a single-pot system by optimizing enzyme immobilization, preloading the transaminase with PLP, and reducing IPA to 60 mM. Under these conditions, both catalysts retained a residual activity and 1-phenylethylamine was produced, achieving Objective 1 (combining both catalysts) and Objective 2 (establishing a working cascade). However, this required using a suboptimal IPA concentration and resulted in low amine yields.

Starting the reaction from 40 mM acetophenone reduced enzyme activity to 24 % of the 10 mM reference, whereas using 40 mM 1-phenylethanol in cascade mode increased activity to 117 % compared to basic 10 mM. This confirms that the cascade mitigates substrate inhibition. However, the low IPA and acetophenone concentrations were insufficient to drive significant 1-phenylethylamine formation. Additional factors, such as PLP degradation under light and limited enzyme stability over long reaction times, likely further constrained the reaction.

Consequently, Objective 3 (demonstrating that the cascade outperforms a process starting directly from the prochiral ketone) was only partly met. Substrate inhibition was solved, but overall amine yields remained low due to equilibrium limitations and other system constraints. These results nevertheless provide a clear foundation for future work.

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Perspectives

A critical issue is the intermediate concentration of IPA, too high for the gold catalyst and too low for the transamination reaction, reducing both catalytic activities. As this limitation currently affects both catalytic components, it represents the primary limiting factor in the system and thus the most relevant target for improvement. To address this, the gold surface could be protected with a thin self-assembled layer (e.g. hydrophobic polymer yolk-shell nanoreactors) by controlled access to active sites^{120,121}. Increasing IPA would improve the thermodynamic driving force for transamination while preventing poisoning of gold sites.

Additionally it is essential for the subsequent transamination step to maintain pace, ensuring a smooth catalytic cascade without undesirable accumulation of intermediate products. Indeed, this system is limited by the very slow activity of the enzyme and its low concentration (1 mg/mL). In comparison, concentrations around 5-10 mg/mL are commonly used in the literature for transaminases^{122,123}. Increasing the enzyme load would likely speed up the second step, helping it keep pace with the initial oxidation. This would, in turn, better justify using a cascade approach.

Additionally, a reaction with more favourable thermodynamics would probably enhance the system's overall effectiveness. Given that transaminases are naturally involved in amino acid metabolism, using substrates structurally similar to amino acids could boost reaction yields. Beyond that, a study showed that producing 4-phenyl-2-butylamine with isopopylamine benefits from less unfavourable thermodynamics, with an equilibrium constant K of 0.7¹¹⁹. This is significantly better than the K value of 0.033 observed in our current system.

Furthermore, utilizing a more robust enzyme variant should be considered. For example, the mutant enzyme ATA_117_11Rd demonstrates increased thermal stability¹²⁴, potentially providing conditions that support sustained gold catalyst activity and overall reaction robustness.

Moreover, PLP maturation should be carried out in the dark to prevent PLP dissociation and subsequent enzyme poisoning, and an appropriate buffer should be defined to limit photodegradation¹¹⁵.

Once the cascade system is well-implemented with better synchronization of the two reactions, an elegant perspective would be to functionalize the silica support with (3-aminopropyl)triethoxysilane (APTES)¹²⁵ or (3-glycidyloxypropyl)trimethoxysilane (GLYMO)¹²⁶ that introduce amine or epoxy groups, respectively. The transaminase can then be covalently attached directly to the particles that bear the gold nanoparticles. This co-immobilization strategy would ensure close proximity between enzyme and metal, simplify catalyst recovery and recycling, and benefit from a local proximity effect that may accelerate transfer of the intermediate from oxidation to transamination.

By pursuing these interrelated improvements (protecting the gold to raise IPA, increasing enzyme loading and selecting a more favourable substrate, using a more thermostable mutant, maturing PLP in the dark) remaining bottlenecks will be systematically addressed. These steps will lead to a more robust and productive hybrid chemo-enzymatic cascade.

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Appendix

A.1. Determination of experimental error

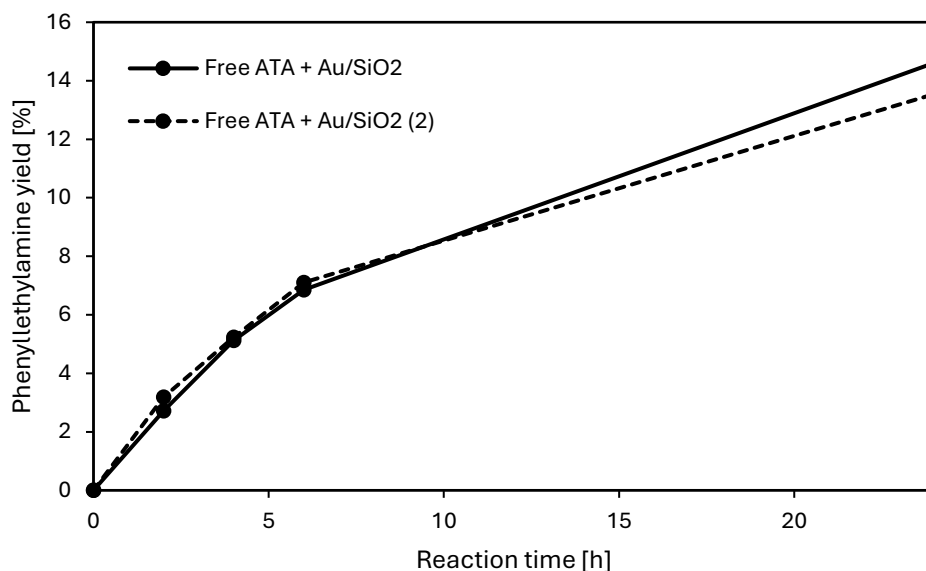


Figure 44. Reproducibility of ATA_117 activity in the presence of Au/SiO₂ catalyst

Two independent repetitions of the full experiment, including both the reaction and analytical measurements, using free ATA_117 in the presence of Au/SiO₂ to evaluate experimental variability and assess reproducibility. Reaction conditions: 20 mM acetophenone, 1 mM PLP, 500 mM IPA, 100 mM buffer to reach final volume, 20 mg/mL Au/SiO₂ and 1 mg/mL free ATA_117.

Table 6. Relative error across time points for repeated experiments with free ATA_117 in the presence of Au/SiO₂

Relative error calculated at each time point based on two independent repetitions of the complete experiment (reaction and analysis). The mean relative error derived from these measurements was used as the reference error throughout the master thesis.

Time [h]	0	2	4	6	24	Mean relative error
Relative error [%]	0	15.7	2.1	3.6	7.6	7.2

A.2. Oxidation kinetics with addition of free or immobilized ATA or resin

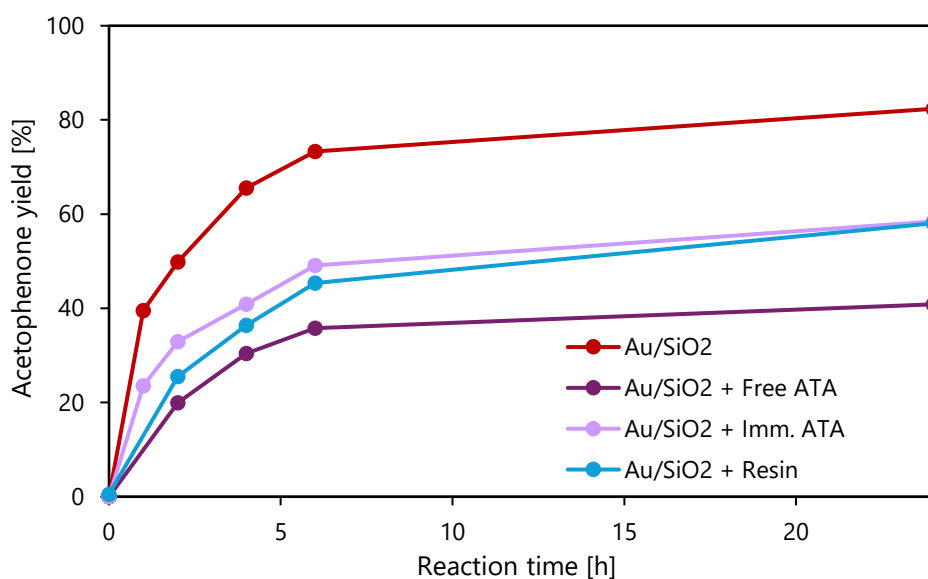


Figure 45. Impact of free enzyme, immobilized enzyme, and resin on acetophenone yield over time
Comparison of 24 h product formation kinetics in the presence of free ATA_117, immobilized ATA_117, or resin alone, assessing the respective influence of each component on the oxidation of 1-phenylethanol. Reaction conditions: 20 mM 1-phenylethanol, 100 mM buffer to reach final volume, and 20 mg/mL Au/SiO₂.

A.3. Two-steps intermediate cascades

In these experiments, IPA was added at a concentration of 500 mM to maximize the second reaction. When enzyme is used as immobilized enzyme, PLP was systematically supplied through enzyme maturation. **Table 7** presents three different configurations of two-step cascade reactions:

Table 7. Two-step cascade configurations with defined reaction components in each step
Overview of cascade setups in which the first step focuses on maximizing the oxidation of 1-phenylethanol to acetophenone using Au/SiO₂, followed by a second step involving the addition of transamination.

TEST	Au/SiO ₂	PhEtOH	Free ATA	PLP 1mM	Imm. ATA	IPA 500mM
(A)	Step 1	Step 1	Step 2	Step 2	/	Step 2
(B)	Step 1	Step 1	Step 1	Step 2	/	Step 2
(C)	Step 1	Step 1	/	/	Step 1	Step 2

(A) Step 1: Au/SiO₂, PhEtOH. Step 2: Free ATA + PLP + IPA.

Only the reagent and the catalyst are added to buffer for the first 24 hours, allowing the best 1-phenylethanol conversion. Free enzyme, 1 mM PLP and 500 mM IPA are then added in order to start transamination reaction from acetophenone produced in step 1 (**Figure 46**).

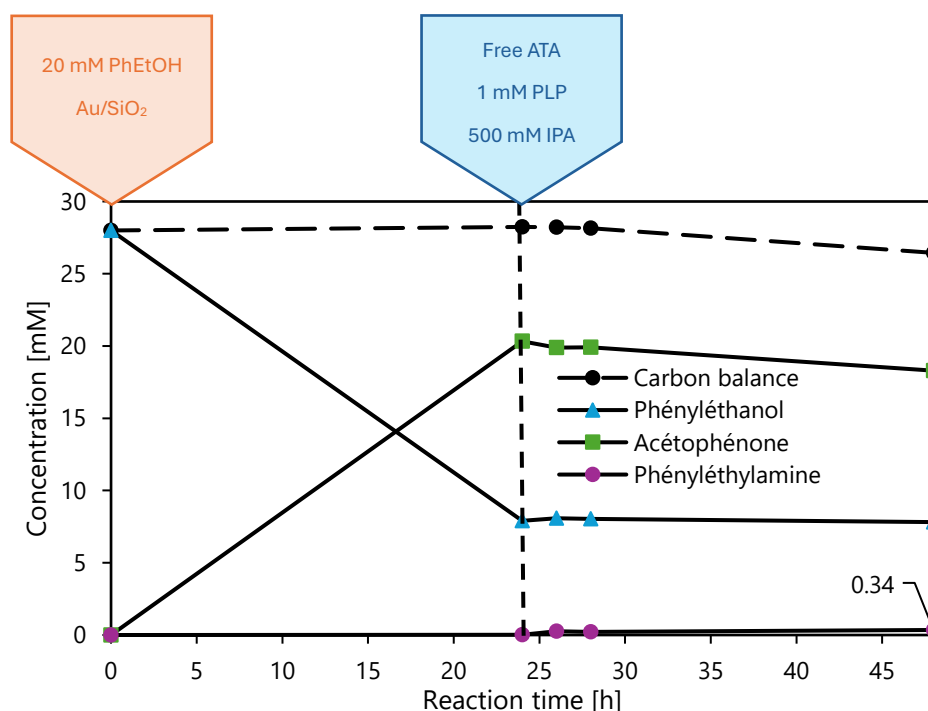


Figure 46. Evolution of 1-phenylethanol, acetophenone, and 1-phenylethylamine concentrations and carbon balance over time in a two-step cascade in configuration (A)

Time-course of reagent, intermediate, and product concentrations, along with carbon balance, for a cascade initiated with the oxidation of 20 mM 1-phenylethanol using 20 mg/mL Au/SiO₂. The second step, starting at t₂₄, involves transamination by 1 mg/mL free ATA₁₁₇ with 1 mM PLP and 500 mM IPA. Reaction medium contains 100 mM buffer to reach final volume.

Carbon balance is defined as the cumulative concentration of the three target compounds (1-phenylethanol, acetophenone, and 1-phenylethylamine) measured at each time point.

(B) Step 1: Au/SiO₂, PhEtOH, Free ATA. **Step 2:** IPA + PLP.

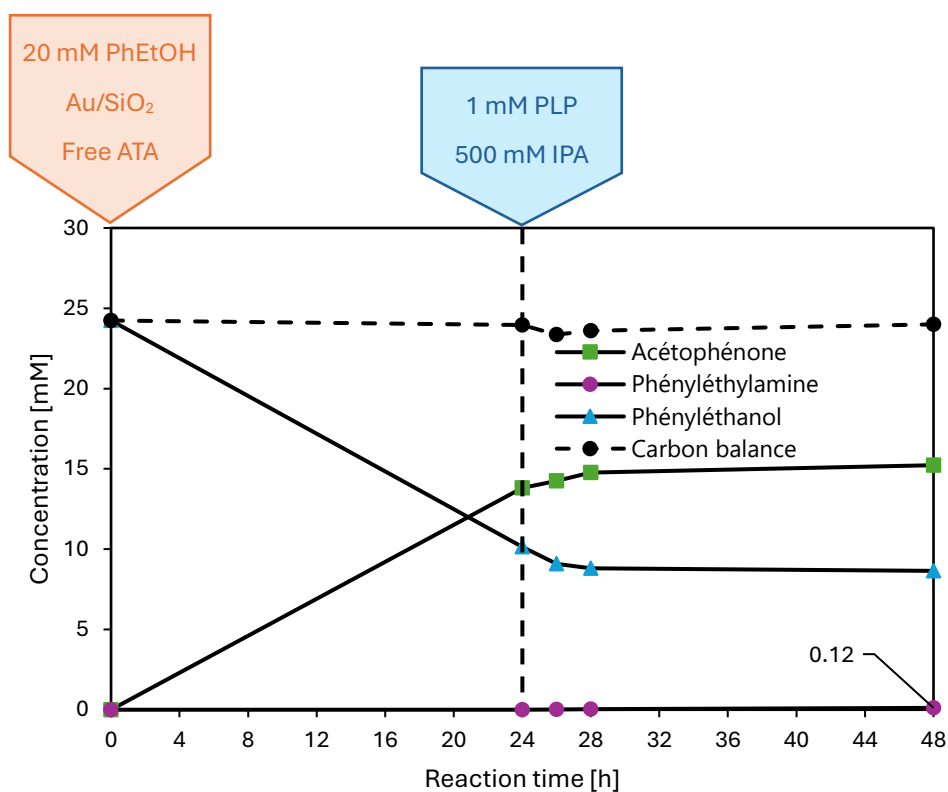


Figure 47. Evolution of 1-phenylethanol, acetophenone, and 1-phenylethylamine concentrations and carbon balance over time in a two-step cascade in configuration (B)

Time-course of reagent, intermediate, and product concentrations, along with carbon balance, for a cascade starting with the oxidation of 20 mM 1-phenylethanol catalyzed by 20 mg/mL Au/SiO₂ in the presence of 1 mg/mL free ATA₁₁₇.

The second step, initiated at t₂₄, involves the addition of 1 mM PLP and 500 mM IPA to trigger the transamination.

Reaction medium contains 100 mM buffer to reach final volume.

(C) Step 1: Au/SiO₂, PhEtOH, Immobilized and matured ATA. **Step 2:** IPA.

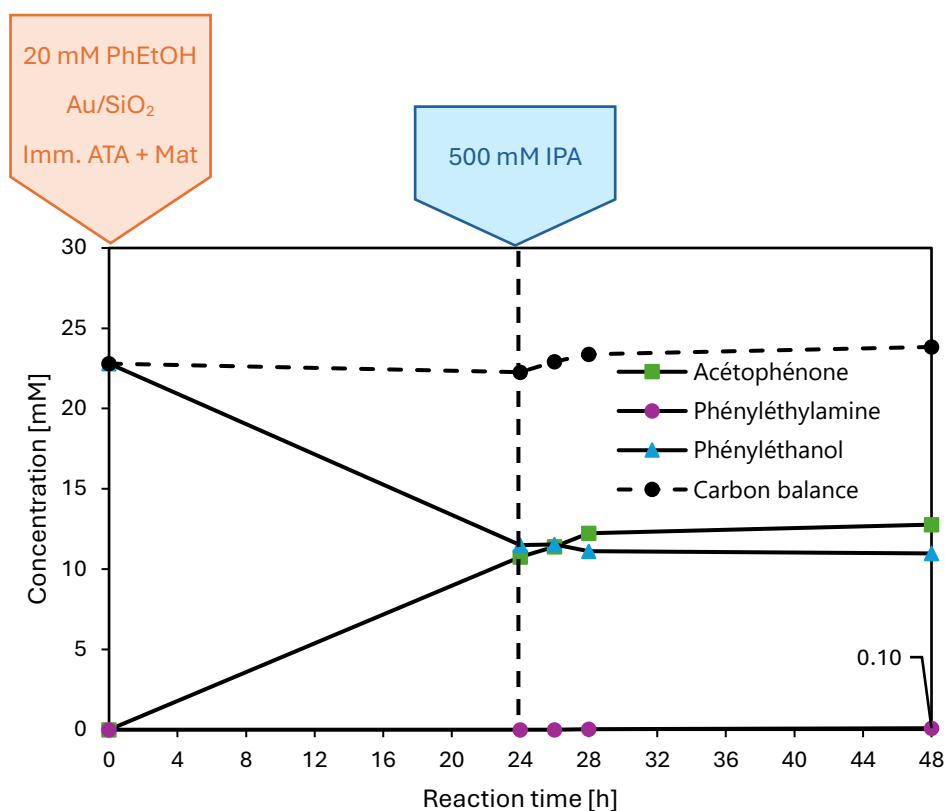


Figure 48. Evolution of 1-phenylethanol, acetophenone, and 1-phenylethylamine concentrations and carbon balance over time in a two-step cascade in configuration (C)

Time-course of reagent, intermediate, and product concentrations, along with carbon balance, for a cascade initiated with 20 mM 1-phenylethanol and 20 mg/mL Au/SiO₂ in the presence of 1 mg/mL ATA_117 (pre-immobilized and matured with PLP). The second step, starting at t₂₄, involves the addition of 500 mM IPA to initiate transamination. Reaction medium contains 100 mM buffer to reach final volume.

As expected, the conversion of alcohol to ketone progressively decreased with the addition of deactivating agents during the oxidation step. Similarly, phenylethylamine concentrations decreased from 0.34 mM in test (A) to 0.12 mM (B), and 0.10 mM (C).

A.4. Best cascade: “less resin” cascade

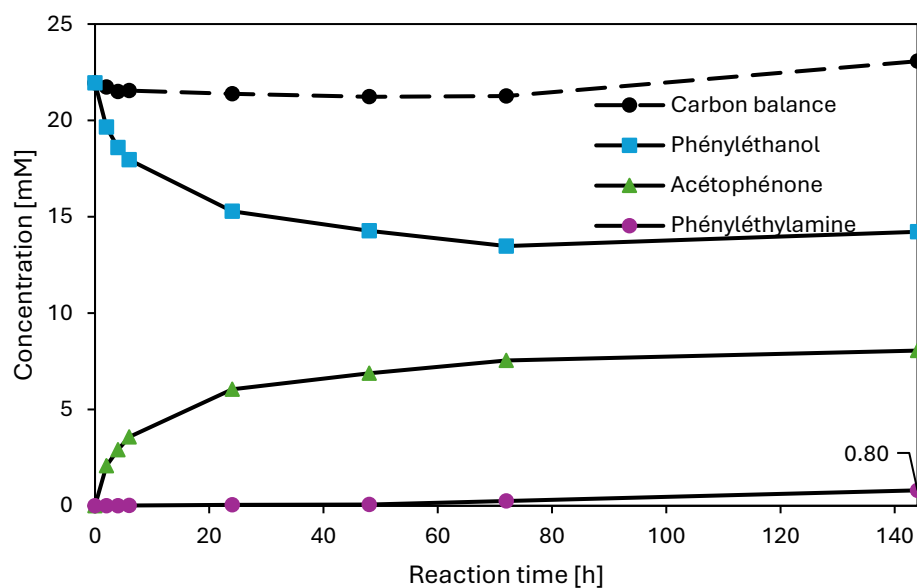


Figure 49. 1-phenylethanol, acetophenone, and 1-phenylethylamine concentrations and carbon balance over time for the “less resin” cascade

Time-course of reagent, intermediate, and product concentrations, along with carbon balance, for the cascade configuration using a reduced resin concentration during enzyme immobilization. Reaction conditions: 20 mM 1-phenylethanol, PLP maturation, 60 mM IPA, 100 mM buffer to reach final volume, 20 mg/mL Au/SiO₂ and 1 mg/mL immobilized ATA_117. Resin amount for immobilization: 25 mg/mL.

Biocatalytic chiral amine synthesis coupled with in situ chemocatalytic substrate production

Maela Claeys

Transaminases are promising biocatalysts that transfer an amine group to a ketone, enabling the synthesis of chiral amines; key components in over 40% of pharmaceuticals and other fine chemicals. Their high enantioselectivity and mild operating conditions help reduce the need for costly and environmentally intensive purification processes. However, the practical application and industrial-scale adoption of transaminases are hampered by substrate-induced enzyme inactivation.

This master thesis addresses this issue through the development of a cascade reaction in which another non-inhibiting compound is used as a starting substrate and converted in situ to the substrate of the amine, enabling the progressive and controlled synthesis of the inhibitory ketone. The model reaction involves the one-pot conversion of 1-phenylethanol into 1-phenylethylamine, via oxidation to acetophenone followed by the transamination step. The proposed approach couples transaminase activity with a selective oxidation step catalyzed by gold nanoparticles supported on silica (Au/SiO₂). This inorganic catalyst exhibits effectiveness under mild conditions compatible with enzymatic reactions.

A compatibility window between the enzyme and inorganic catalyst was identified, considering interference with the enzyme, the cofactor and the amine donor. To mitigate these interferences, the enzyme was immobilized at room temperature on a commercial resin to reduce catalyst inhibition. Additionally, the concentration of the amine donor was reduced from 500 mM to 60 mM. Immobilization was further improved by applying a step of incubation with the cofactor (PLP), contributing to improved enzyme stability and alleviating the need for high PLP concentration in the reaction (which help preserve the activity of the gold catalyst).

Although the implementation of the chemoenzymatic cascade introduced additional challenges for the enzyme, leading to a lower catalytic activity compared to transamination alone, it nevertheless effectively addressed the issue of substrate inhibition. This was confirmed by the comparable enzymatic activity observed when using 10 mM or 40 mM of 1-phenylethanol as a starting material, whereas similar concentrations of acetophenone would have resulted in a 75% loss of activity.