

BIOINTENSE Description of Main Results/Foregrounds

The main objective in **BIOINTENSE** was to **increase biocatalyst productivity and process intensity**. This will result in economically feasible processes by integration and intensification and also shorten the development times by developing optimized tools and protocols that can be widely applicable in industry. More than this, the lack of fundamental understanding on the interactions between reaction, biocatalyst and process characteristics will be addressed to minimize the uncertainties with respect to the cost of future biocatalytic processes. **BIOINTENSE** was also supposed to increase understanding about the factors contributing to the total cost and environmental impact. The project has been organized in 11 workpackages, of which one was targeted specifically at the dissemination of the results and one dealt with the management of the project. In **WP1 μ -fabrication** the design of small scale equipment with integrated separation and analysis for catalyst and process screening has been performed. **WP2 the Bioprocess with in situ product recovery and substrate supply** was investigated. Novel biocatalysts were evaluated and characterized using the microfluidic toolbox developed in the first WP. In order to successfully use microtechnology **WP3 worked with Monitoring and Control** of such processes. Here novel sensor materials were developed for pH, oxygen glucose, measurement and a tool for the analysis amines and ketones was developed and where possible integrated into the microfluidic devices. **WP4 Scale up & Scale Out** developed and investigated the potential of the microtechnology in relation other scale processes. **WP5 Fermentation development & production** was on the one hand supplying the consortium with enzymes while subsequently the proteins were improved with respect activity and solvent and temperature stability. **WP6** was targeting the **Multiobjective biocatalyst screening** designing screening protocols and methods adapted for process conditions and investigating temperature & solvent stability, substrate/product concentration, activity and selectivity. **WP7** studied intensively the **immobilization and stability** of the biocatalyst under process conditions. Here fundamental understanding was used for the design of surface immobilization and entrapment methods. **WP8** evaluated with input from two major European industrial partners the **Economic and environmental** aspects of potential biocatalytic processes. Finally **WP9** was interactively bringing many of the workpackages together by focusing at the process modelling and design of experiments as well as the design of new microfluidic structures. A summary with more detailed information about the findings of the workpackages are found in the following.

WP1: MICRO-FABRICATION

1. Microreactor Toolbox Established

Throughout the project different microreactors were developed, which were applied for rapid screening and characterization of biocatalysts and biochemical process options. After defining specifications for these micro reactors in close cooperation with the project partners, prototypes were fabricated and hydrodynamically characterized. Finally, fabrication processes for mass production of the most promising candidates were established. The microfluidic toolbox can be subdivided into several subgroups consisting of meander reactors, packed bed reactors, chips with integrated sensors (O_2 / pH/ glucose), screening devices with a high degree of parallelization (biocatalyst screening devices and 96 microchannel plates) and microfluidic chips for special applications (long residence time chips, Y-Y- and Ψ - Ψ -extraction units, scale-out devices and integrated valve systems). A detailed overview over the single modules, which have been developed within is given in *Figure 1*.

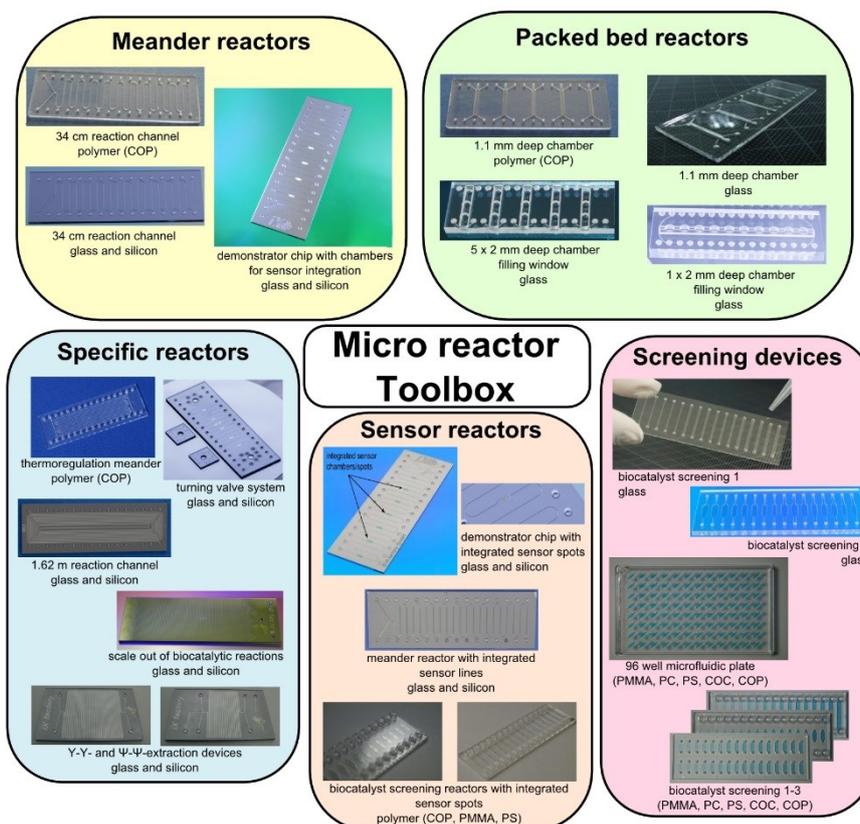


Figure 1: Microfluidic toolbox elements, which have been developed within the BioIntense project.

To sum up the development efforts, the toolbox provides single modules, which serve to perform all Micro Unit Operations necessary for biocatalytic process development, namely biocatalyst screening tools, process intensification and development tools and production tools. The most important features of the micro reactor toolbox are summarized here as follows:

- **Microreactor toolbox with application-specific choice of micro reactor material**

To allow maximum of flexibility for application of the microfluidic devices, a remarkable variety of new devices were fabricated in different materials. The two industrial project partners iX-factory and microfluidic ChipShop cover the whole range of thermoplastic polymers, glass and silicon, which are the most commonly used materials for commercial microfluidic chip production. Additionally, surface coatings can be applied during or after fabrication of the chips to tune the material properties for a given application. Ultimately this has resulted in the **design of 17 new microreactors, resulting in 24 different microreactor prototypes**

- **Compatibility with mass production techniques**

The adaptability with mass fabrication technologies enables further use of the developed micro reactors by research and industry. The principle of bearing the later production scale of the single modules in mind was strongly enforced by the involvement of companies, which cover the whole technology chain. That principle was maintained throughout the whole development process, starting already at the definition of requirement specifications, and allows further exploitation of the project's results such as implementation of new microfluidic building blocks into the online-Shop of ix-factory and the Lab-on-a-Chip Catalogue of microfluidic ChipShop. Ultimately this has resulted in the development of **24 mass fabrication compatible micro reactors for further commercial application**

- **Standardization of device formats: applicability with standard laboratory equipment**

A major focus for designing and fabricating the single modules has been the issue of using standardized device formats such as the microscopy slide format and the microtiter plate format. These formats enable the use of standard laboratory equipment like microscopes and instruments for laboratory automation and decrease the initial hurdle for researchers and industry to start with microfluidics technology. Consequently, **microscope slide and microtiter plate format has been established as international standards.**

- **Standardization of interfaces: the plug-and-play approach**

Standard fluidic interfaces such as through-holes and Mini Luers have been chosen for all microreactor modules to allow simple arrangement of modules for single Micro Unit Operations as components of a whole biocatalytic process. The use of O-ring connector modules and of commercial press tight fitting module from Upchurch have been established to connect all the different chip types via tubing from the outlet of one Micro Unit Operation module to the inlet of the next Micro Unit Operation module. Thereby, the plug-and-play-concept has been realized within Biointense. Ultimately this has resulted in using **standardized interfaces as international standards for plug-and-play approach.** By using the described principle of standardization we also follow the latest trend of standardization within the microfluidics community, which aims to facilitate the process of designing new microfluidic sensors, actuators, connectors etc. by providing guidelines for the seamless integration with other microfluidic components and systems [Design Guideline for Microfluidic Device and Component Interfaces, Henne van Heeren]. This trend of standardization is said to be among the most promising developments to overcome the challenge of reducing time and investment costs to move from a research prototype device to a production device.

2. Successful Integration of Optical Sensors into Microfluidic Devices

Integrated oxygen sensors represent valuable tools for online analysis of catalyst and process screening. While the development of sensor materials was part of WP 3, their integration into microfluidic devices is one of the highlights within WP 1 (**Figure 2**). Starting from simple investigations of material compatibility, the major outcomes of the project comprise the fabrication of micro reactors especially suited for sensor integration for oxygen or pH, new processes to integrate these sensors into microfluidic devices and the optimization of automated sensor integration regarding spot appearance and reproducibility of the fabricated oxygen sensors. Furthermore, assembling technologies were developed to maintain the functionality of integrated sensors. The sensor material developed within BIOINTENSE as well as the automatization of sensor integration directly into microfluidic channels make integrated oxygen sensors interesting candidates for product development. Therefore, the know-how generated within previous investigations was transferred to microfluidic ChipShop, where automated fabrication of oxygen sensors into polymer based devices was further optimized regarding spot appearance and reproducibility of the fabricated oxygen sensors, and iX-factory, which outsources their sensor integration to partner GUT.

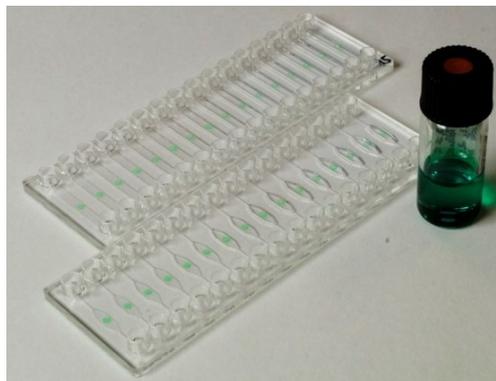
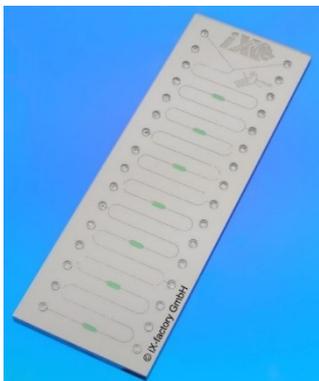


Figure 2: Integrated oxygen sensors into the demonstrator chip with integrated sensor chambers (ix-factory, glass and silicon) and polymer based biocatalyst screening reactors with integrated sensorspots (microfluidic ChipShop, COP)

3. New Developments in Process Methodology and Microfabrication Achieved

Several important improvements in process methodology and standard microfabrication techniques were achieved with respect to specific biocatalytic and enzymatic applications including monitoring tasks with integrated optical sensors. The most important improvements will be highlighted here:

- **Integration of optical sensor coatings into microfluidics**

New processes have been established to integrate optical sensor coatings into microfluidic devices. Automated sensor integration was optimized regarding material compatibility, spot appearance and reproducibility of the fabricated oxygen sensors.

For the integration of sensor coatings on the micro reactor surfaces of glass reactors a specific sequential process was developed. Due to the special treatment of the micro reactor surfaces and the patterning process sensor spots down to 100 μm diameter with very accurate alignment could be successfully integrated.

- **Low temperature anodic bonding as wafer-level packaging for sensor integration**

For integration of multifunctional coatings used in biomedical, pharmaceutical or chemical applications the technology of anodic bonding were improved to be applicable at low temperatures. Coatings to change locally the properties of surface areas, sensing e.g. pH, hydrogen or functionalization of different enzymes or DNA are especially critical to higher temperature. Due to the special treatment of materials and the specially optimized process the developed low temperature anodic bonding process enables hermetic bonding at temperatures below 180°C.

- **Development of surface functionalization strategies**

Procedures have been developed for efficient surface immobilization of biocatalysts on materials commonly used for micro reactor fabrication, including glass, polystyrene, poly (methyl methacrylate), polycarbonate, and olefin-based polymers, namely Zeonor® and Topas®. The knowledge on surface coatings has also been increased regarding general methods such as hydrophilization of silanization methods, which can be applied during or after fabrication of the chips.

These improvements in process methodology can and will be further used by SMEs ix-Factory and microfluidic ChipShop on a technology level to increase the performance of new microfluidic product development.

WP2: BIOPROCESS WITH IN-SITU PRODUCT RECOVERY AND SUBSTRATE SUPPLY

The aim of this work package was to design set-ups that can be used for characterisation of an individual unit operation as well as in combination. This is important because the core part of the reactor, namely the enzyme, will be altered through successive rounds of protein engineering. Process development must therefore be flexible and fast enough to accommodate this change. The work package has therefore delivered solutions to commonly experienced problems surrounding the development of amine transaminase (ATAs) processes. More specifically protocols for solvent selection and experiments to characterize the enzymes kinetics as well as to determine thermodynamic stability have been established. This has created a platform from which many biocatalytic processes can be developed.

4. New Kinetic Characterization Methodology Established

The key to unlocking process development is to identify the reactor performance. An experimental protocol was therefore created to efficiently characterise the kinetic profile of the candidate enzyme, see *Figure 3*.

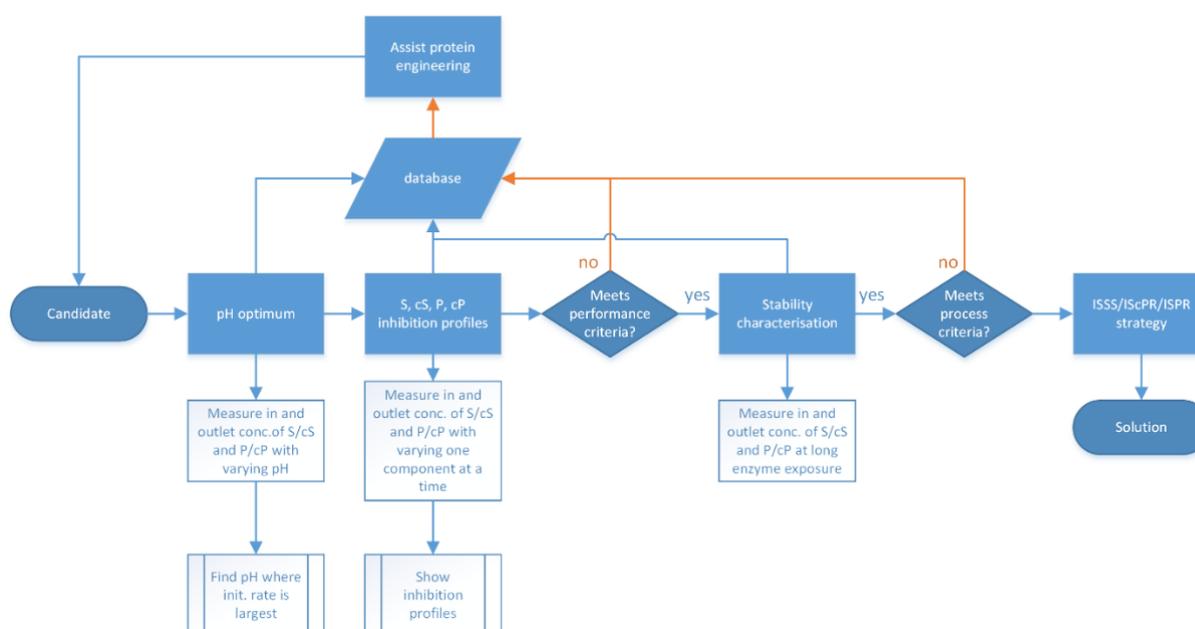


Figure 3: Experimental kinetic characterization methodology

The kinetic characterisation methodology will have to be carried out multiple times for a single process development campaign. Microfluidics offers a great solution for this since it delivers true reaction kinetics as it eliminates mass transfer limitations and can do this with minor consumption of material. A microfluidic set-up was therefore created that could make up desired substrate compositions, determine initial rates and be broadly applicable for reactions with UV active reagents. The set-up could be programmed to operate autonomously and deliver triplicates of a substrate inhibition profile overnight.

A model for the biocatalyst was developed where the fundamental properties were easily modified. From this it was possible to simulate different scenarios to illustrate the effects of varying biocatalyst properties on the so called 'Substrate and Inhibition plots' and the effect on reaction progression with time. Based on the observations the following general recommendations could be made:

- 1) If effective ISPR tools are available then V_{max} is the property to screen for. ISPR tools can later be used to optimize process performance.

2) If ISPR tools are unavailable or too costly to implement then the minimum time to achieve the desired yield at process conditions should be screened for.

The implementation of ISPR for thermodynamic limited reactions is a necessity. A protocol to investigate ISPR methods was therefore made, see **Figure 4**, right. Each possible ISPR method has been carefully evaluated in terms of input and output. This was carried out to identify what properties that was required to characterize the method, see **Figure 4**, left.

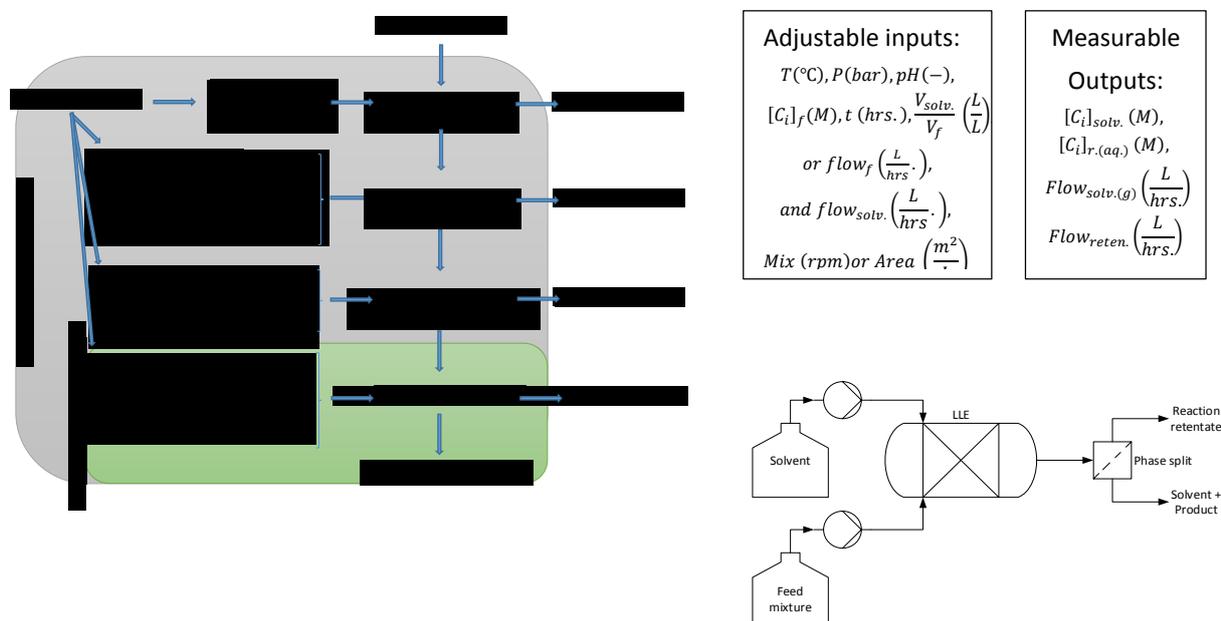


Figure 4: Left: General overview of the general ISPR selection and screening procedure, where economic evaluation and biocompatibility are taken into account. Right: Parameter analysis method for liquid-liquid extraction.

5. Solvent Selection Methodology Established

The reaction system that has been the focus of BIOINTENSE will for most cases have poorly soluble substrates and products. Solvents will therefore always have a role to play in processes carrying out this chemistry. To aid solvent selection the current selection guides from the pharmaceutical industry was inspected with three perspectives as illustrated in **Figure 5**. The goal is to use a solvent system that promotes the highest reactivity, combine with best downstream processing and which minimises the environmental impact and health and safety issues.



Figure 5: The goal set for solvent selection

A collection of properties of 112 solvents has been put together with rankings of four solvent selection guides. From this 24 solvents were selected and was hereafter reduced to 10, it is suggested to use the water miscible solvents: methanol, ethanol and DMSO. For water immiscible solvents it is suggested to use: MTBE, heptane, toluene, ethyl acetate, benzylacetone, rapeseed methylester and undecane. The solvents potential is evaluated by measuring the residual enzyme activity after various treatments of the enzyme with the solvents, e.g. different exposure times in the presence of the solvent before running a standard activity assay. The results are presented in the WP 7.

6. Integrated Process and ISPR Configuration Designed, Developed and Validated

Microfluidic modules were combined, in a plug-and-play manner, to test complex process options and process steps. 1. intensification of ATA processes by putting in place in-direct ISPR based on a two-step LLE strategy, see **Figure 6**. Intensification of ATA processes by direct ISPR via a two phase droplet system, see **Figure 7**. In both cases it is possible to successfully extract the product while conducting the reaction.

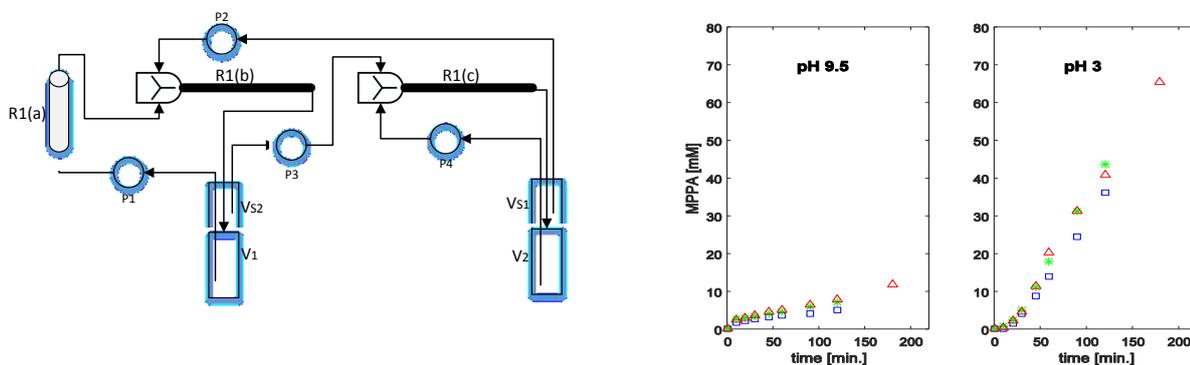


Figure 6: Microfluidic experimental setup. P1-P4 syringe pumps with 250 μ L syringes, V1 and V2 aqueous reservoirs 1 mL of pH 9.5 and pH 3, respectively, Vs1 and Vs2 solvent reservoirs 750 μ L. R1(a) PBR, R1(b) 1st LLE module, R1(c) 2nd LLE module.

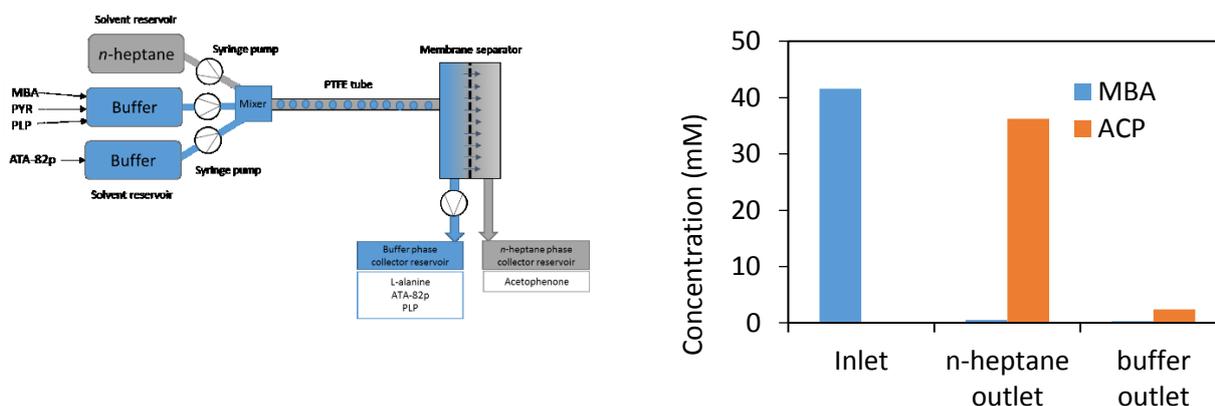


Figure 7: Microfluidic experimental set-up of transamination in n-heptane/buffer two phase system with in-situ product removal and integrated with membrane separator.

WP3: MONITORING AND CONTROL

In this workpackage advanced tools for monitoring and control of bio-catalytic processes were investigated. New optical chemical sensor technology was developed and applied. Transaminase

screening assays were elaborated employing oxygen and pH sensors and probes. Model bio-transformations in micro-reactors are performed and monitored and controlled online.

7. Luminescence Sensors for Application in Micro- and Large-scale Bioreactors Developed

• Oxygen sensors

We have synthesized new oxygen sensor materials. The indicator dye Pt(II) meso-tetraphenyltetraenzoporphyrin was covalently bound to a host polymer. The resulting polystyrene based sensor material exhibits a remarkably increased sensitivity and a better applicability on polymeric supports for microreactors compared to sensor materials with physically entrapped dyes. The material showed excellent resistance to steam sterilisation. In addition, a new oxygen sensor material for the use in glass based microfluidic devices was developed consisting of a polystyrene-silicone rubber composite matrix with an entrapped oxygen indicator dye.

• pH-sensors

New sensors enabling pH-sensing over the entire pH range from acidic to basic conditions are demonstrated. Novel BF₂-chelated tetraarylazadipyromethene pH-sensitive dyes (aza-BODIPYs) were synthesized. These indicator dyes are exceptionally photo-stable, show sharp absorption/emission bands in the near-infrared region (NIR). A full set of aza-BODIPY indicators is now available to enable pH-sensing over the entire pH range. We show that a combination of four of these pH dyes yields a pH sensor with an extended dynamic range from pH 2 to 9. The pH-sensor exhibit long-term stability, resistance towards x-ray sterilisation, were applied in micro-reactors (see **Figure 8**) and to monitor a fermentation process.

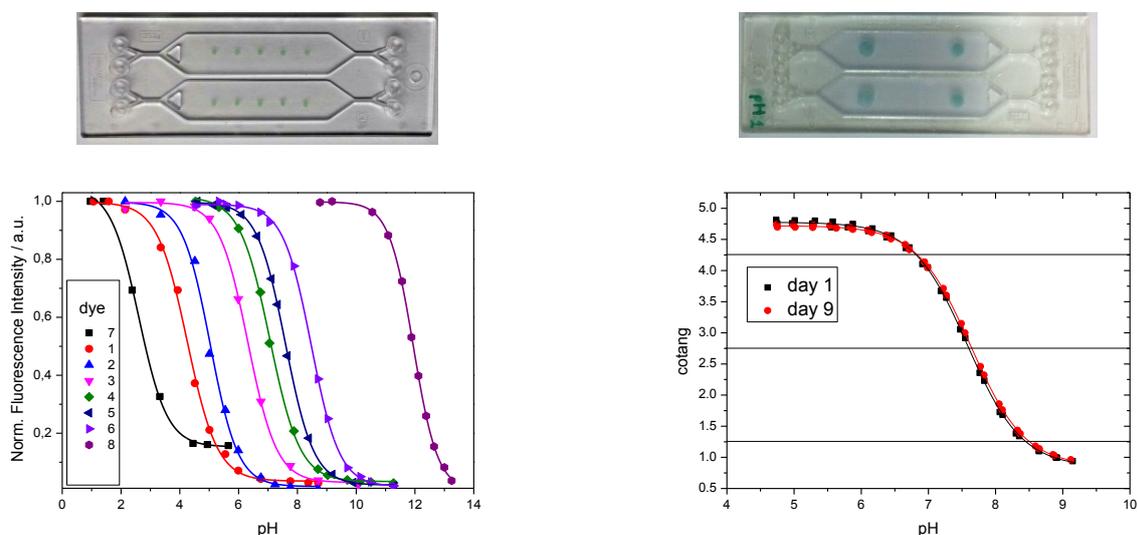


Figure 8: Top: Oxygen and pH sensor spots integrated into microfluidic chips. **Bottom:** Calibration curves of the set of pH-indicator dyes and calibration curves of a pH sensor over a period of nine days measured at 37 °C illustrating the long-term stability.

• Glucose sensors

The feasibility of an enzymatic glucose sensor for the application in μ -bioreactors and large-scale reactors is demonstrated. The three layer optical sensors were integrated in micro-reactors and investigated as dipping probes for large scale reactors.

• Nanosensor particles

The integration of sensors into microfluidic devices is not always feasible, therefore nanosensors were developed that can be added to the sample. The nano sensors allow the determination of the pH, oxygen or the simultaneous determination of both parameters. In addition, nanosensor particles with magnetic properties were investigated, which can be separated from the surrounding solution to form *in-situ* sensor spots within microfluidic channels.

8. Measurement Tools for On-line Biocatalytic Reaction Control Established

The simultaneous determination at various positions of analytes produced in the reaction is enabling a full online control over the entire process. Measurement set-ups for the parallel determination of analytes are developed employing a 4-channel oxygenmeter from Pyroscience or miniaturized USB-oxygenmeters (modified with a focusing lens), syringe pumps, micro-reactors and a chipholder. An overview about those solutions is given in **Figure 9**.

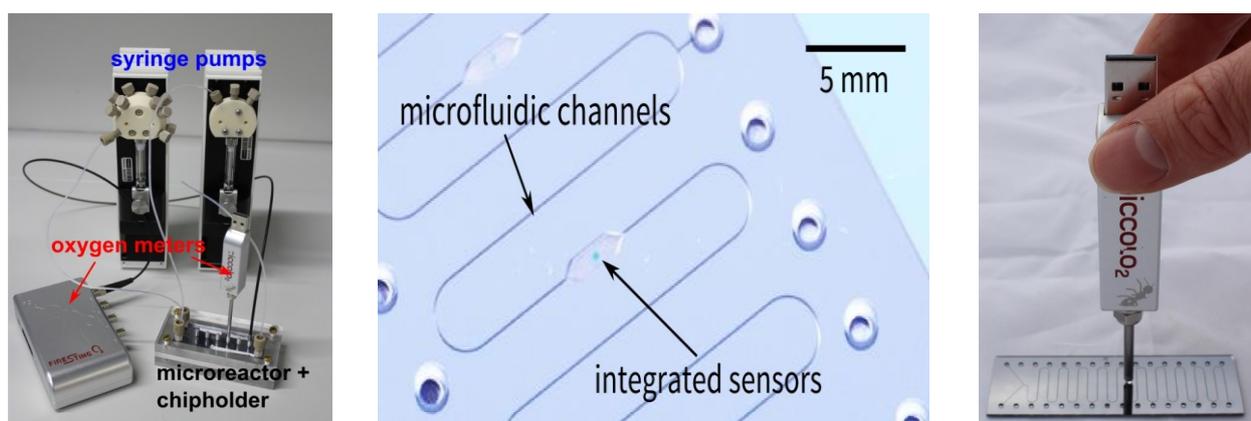


Figure 9: (Left): Measurement set-up consisting of the micro-reactor, chip holder, syringe pumps, FirestingO2 equipped with a 1 mm fibre and a Piccolo2. (Middle): Microreactor with integrated sensors and miniaturized read-out instrument. (Right): Magnified view of the glass micro-reactor with integrated sensor spot with a diameter of 200 μm .

9. Transaminase (ATA) Screening Assays Developed

Tools to facilitate the convenient measurement of Transaminase (ATA) activity are available from Luxcel. The assays developed use a range of water-soluble oxygen probes and sensors, with ATA activity monitored indirectly through measurement of a coupled oxygen-consuming amino acid oxidase (AAO) catalysed cascade reaction.

The measurement approach was further adapted to low-volume enzyme activity measurements to provide a 'bridge' between the oxygen-based microtitre plate screens and the on-reactor O_2 measurements facilitated by technology developed and integrated by partner TU Graz. These measurements were performed on glass chips custom designed by partner iX-Factroy and on polymeric chips custom designed by partner Microfluidic Chipshop and facilitated activity measurement in volumes as low as 5 μL . See **Figure 10**.



Figure 10: Picture of the biocatalyst screening microreactors.

10. On-line Control of Biocatalytic Reactions in Microreactors Achieved

Meander micro-reactors with integrated sensors prepared within the project were used to demonstrate the online monitoring and control of biotransformation. The oxidation of D-alanine and D-phenylalanine by D-amino acid oxidase, and glucose oxidation by glucose oxidase were used as model reaction as shown in **Figure 11**.

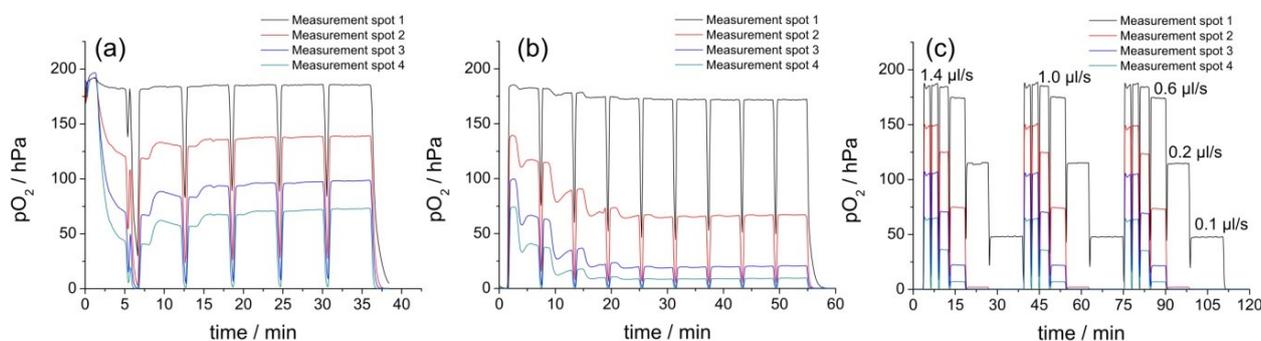


Figure 11: Oxidations of D-alanine (a) and D-phenylalanine (b) by D-amino acid oxidase at a flow rate of 0.6 $\mu\text{l/s}$ and oxidation of glucose with glucose oxidase to D-glucono- δ -lactone and hydrogen peroxide at 1.4 $\mu\text{l/s}$, 1.0 $\mu\text{l/s}$, 0.6 $\mu\text{l/s}$, 0.2 $\mu\text{l/s}$, 0.1 $\mu\text{l/s}$. All reactions were carried out at room temperature.

In another approach, microfluidic modules were combined with high sensitivity and low concentration measurement systems consisting of a UV-VIS detector or alternatively a HPLC detection system. The BIOINTENSE model reaction, the transamination of benzyl acetone to methylpropyl phenyl amine was characterized with the automated set-up by online measurements.

A significant advancement on integrated process control was achieved by the implementation of in-line pH monitoring and control in a two-step liquid-liquid (LLE) *in-situ* product removal (ISPR) process concept for amine transaminase (ATA) processes, which was developed in BIOINTENSE. The given process concept has been presented in MS7 (WP2). pH measurement technologies described above were employed for this purpose. The in-line optical pH sensors were used as soft sensors to predict final product concentration (see **Figure 12**) and as a way to perform titration in the product reservoir. The pH measurement and control strategy will make it possible to achieve high throughput data acquisition, process understanding, experimental repeatability, real-time control actions and troubleshooting with a minimum of manual labour, compared to labour-intensive analysis by off-line HPLC.

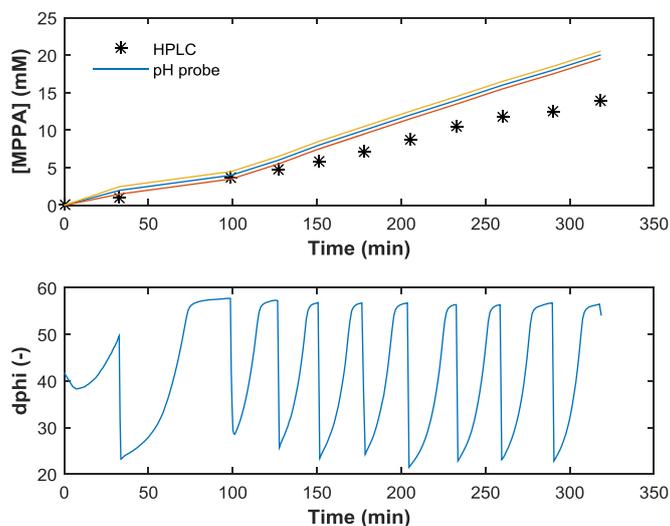


Figure 12: Experimental results from a performed experiment with in-line pH measurement and control in the proposed

two-step LLE ISPR process strategy. The top figure shows the MPPA concentration based on the HCl titration and HPLC determination. The bottom figure shows the signal (dhipi (-)) generated by the pH sensor as the pH in the reservoir changes

WP4: SCALE UP & SCALE OUT

In WP4, the performance of single and numbered up/scaled out microfluidic enzyme reactors were compared for the MBA + PYR reaction by UL, DTU, iX-Factory and ChipShop. In addition, UGent used a modelling approach to evaluate the performance of a numbered-up Microfluidic Enzyme Reactor (MER) system under several flow maldistribution scenarios. Finally, all this information was used to give an overview of parameters needed to decide if a process can better be scaled by scale up or numbering up of reactors and new experimental systems applying transaminase-catalyzed conversions of newly synthesized substrates into new products have been realized by Sigma Aldrich, whereby scale up effects of intensified biocatalytic processes, opportunities for scale out (numbering up) up ml or μ l scale reactor systems and experience for going to scale up vs scale out has been assembled.

11. Successful Numbering-up of Microfluidic Enzyme Reactors Completed

In a first step, the different techniques for numbering up and scale out were described as well as the different set-ups and materials that could be used for the scaling of these microreactors. iX Factory in collaboration with DTU developed scale out reactors with the following benefits: inert, long term stable, biocompatible materials, total hermetic sealing, free of adhesives, temperature and pressure resistant, easy to clean (acidic, basic or alcoholbasic) and to re-use, observation of reaction through glass cover possible. In addition, the standard microfabrication techniques for silicon and glass were further improved by triple stack bonding to enable industrial mass production fabrication of the scale out reactors.

12. Successful Comparison of the Performance of Single and Numbered-up/Scale-out Microfluidic Enzyme Reactors Completed

Several scaled microreactor set-ups were investigated to increase the productivity of the MBA + PYR to ACP + L-ALA reaction. UL set up a microfluidic system with a two-liquid phase (n-heptane/buffer) system enabling in situ product removal. As a consequence of improved mass transfer between both phases, the yield of the reaction could be increased several fold when the reaction was performed in a continuously operated tubular reactor with micro scale diameter instead of a vigorously mixed small reactor operated in a batch mode. In addition, by connecting two of these silicon/glass chips consecutively and immobilizing Z_{basic2} -tagged ω -transaminase ATA-82p (**Figure 13**), the volumetric productivity increased from 1.43 to 2.00 mmol/L min.

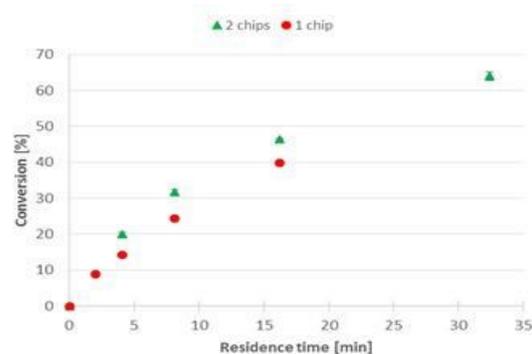
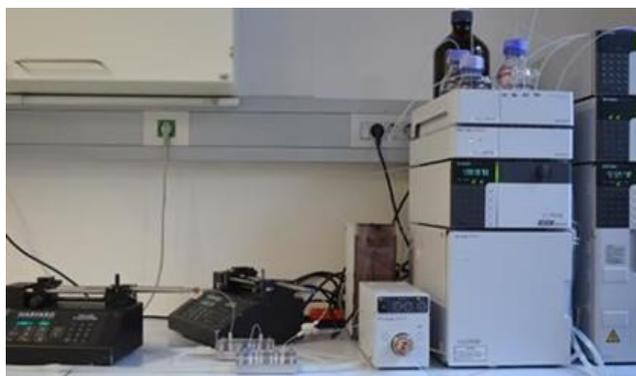


Figure 13: Experimental set up (left) and comparison of biotransformation conversion in single microreactor and two

microreactors connected consecutively

UL also scale-out this same reaction using a continuously operating home-made miniaturized packed bed reactor (MPBR), packed with LentiKats[®] PVA particles containing immobilized ω -transaminase ATA-v1. Under different retention time (i.e. flow rates ranging from 2 to 100 $\mu\text{L min}^{-1}$) and changing channel dimensions, the MPBRs retained favorable hydrodynamic conditions as well as linear proportionality of production rates. Besides, a MPBR showed a high operational stability over 21 days of continuous operation. DTU together with iX-factory also compared the performance of ATA-wt for this same reaction in a simple meander chip and a parallel channel micro reactor (**Figure 14**). The chip was designed to have very similar pressure drop across channels to have an even flow split between the two microreactors so that the performance of the microreactors will not decrease when the microreactors are numbered up. Based on residence time distribution experiments, the volumes of both set-ups were validated. Based on these volumes, the product formation as a function of residence time corresponded very well for the simple and parallel channel meander chips.

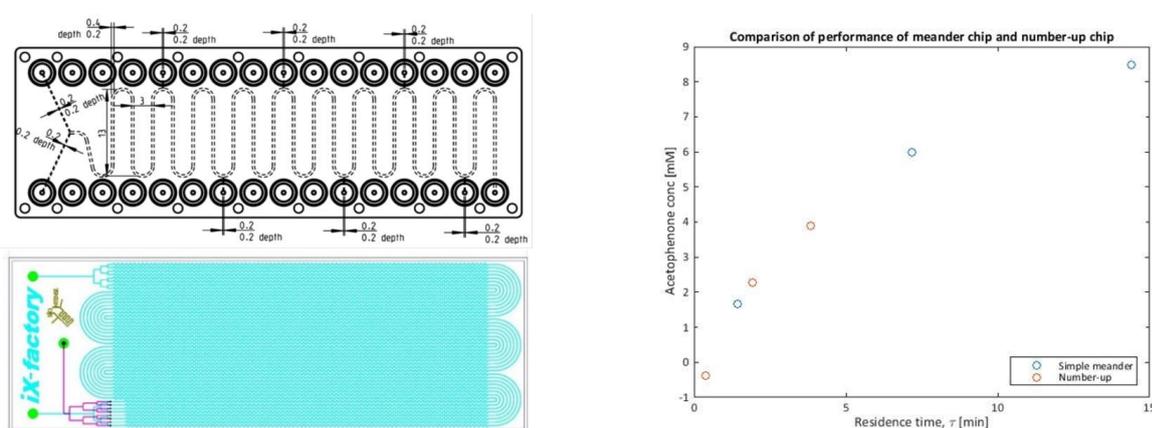


Figure 14: simple (top) and parallel channel micro reactor (bottom) meander chip design (left) and product formation as a function of residence time in the simple meander chip compared with the number-up chip, experimental conditions: 40 mM MBA, 40 mM sodium pyruvate, 0.1 mM PLP, 0.83 mg/mL ATA-50, 20 mM potassium phosphate buffer at room temperature (22° C).

13. Successful Modelling Studies Completed

In addition, a modelling approach was used by UGent to evaluate the performance of a numbered-up Microfluidic Enzyme Reactor (MER) system under several flow maldistribution scenarios. The linearity rule can be applied safely when flow maldistribution does not exceed a certain deviation parameter limit ($\approx 10\%$) (**Figure 15**). Flow maldistribution is not highly affecting the overall performance as long as the 8 microreactors receive inlet flow rate. This can be explained by the fact that the enzyme catalyses the substrates at a very slow rate and changes in flow rate are compensated by increases in residence time in the microreactor system. However, an uncertainty analysis of the kinetic parameters indicated that even under no flow maldistribution events and with parameter correlation, propagation of uncertainty on the key performance indicator total volumetric productivity needs to be taken into account.

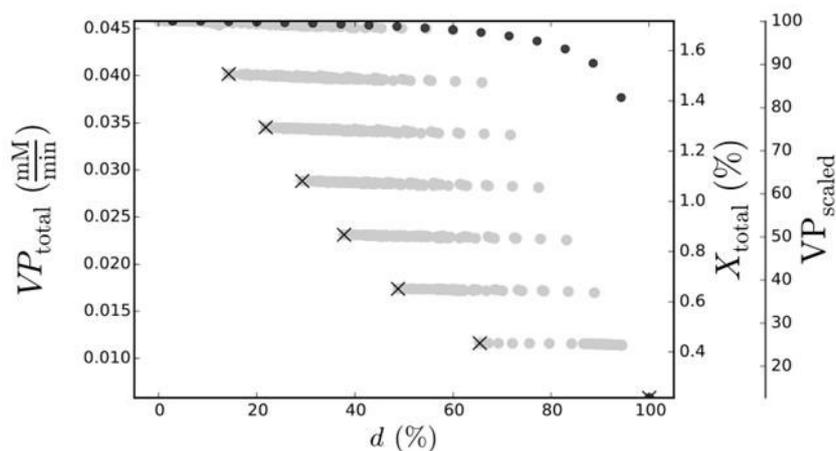


Figure 15: Flow maldistribution impact on total (VP_{total}) and scaled (VP_{scaled}) volumetric productivity in mM/min, and conversion (X_{total}) in % as a function of the deviation parameter d (%).

WP5: FERMENTATION DEVELOPMENT AND PRODUCTION

At the beginning of the project a suitable ω -transaminase was selected (ATA-wt) from the transaminase-collection at c-LEcta as a model enzyme for the BIOINTENSE project. The selection was based on high activity towards the model reaction (**Figure 16**).

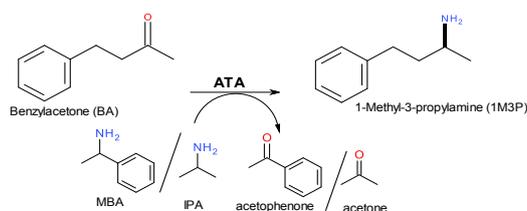


Figure 16: Model reaction used in BIOINTENSE

14. Scalable Fermentation Protocol Established

A scalable, antibiotic- and animal-free fermentation process was developed using c-LEcta's proprietary *E. coli* expression system. Initial fermentation process optimizations were performed in a 1L parallel fermentation unit, testing different conditions like induction time and feed-rates. The best protocol was transferred to 15 L fermentations scale.

15. Downstream Processing and Product Formulation Protocol Established

After successful development of an up-stream process (USP), also the down-stream process (DSP) was developed. A first 60 g batch of a dry powder formulation was obtained and provided to interested partners.

In WP6 the optimized variant ATA-v1 was developed showing much higher stability and activity under industrially relevant conditions. The USP of ATA-v1 was also optimized using c-LEcta's proprietary expression system, first in parallel 1L fermenters. The USP was then successfully transferred to our in-house pilot plant and up-scaled to 300 L fermentation volume.

The DSP protocol for ATA-v1 was optimized and adapted to the exceptionally high thermostability. The enzyme does not lose any activity after incubation for several hours at 70°C. This could be used for a heat-precipitation step that yielded nearly pure ATA-v1 and the DSP was significantly shortened (**Figure 17**). The yield of active enzyme after the DSP was increased to over 90%.

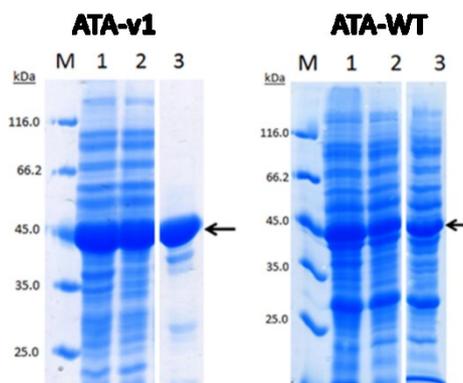


Figure 17: SDS-Page of DSP fractions of ATA-v1 and ATA-WT; M: Protein marker, Lane 1: 0.2 mg biomass, Lane 2: Homogenate (soluble fraction), Lane 3: sterile filtrate. 12% SDS-Page, coomassie staining.

Part of the biomass from the pilot-plant was processed and over 700 g of dry powder formulation of ATA-v1 was obtained.

Different formulations (liquid or dry) were tested and the dry formulation showed in long time (4 month) stability tests superior storage stability. Several tests were conducted analysing the precipitation of enzyme formulations in solution. It is critical for micro reactor applications that the enzyme does not precipitate and clog the channels. We could show that the highly pure ATA-v1 formulation shows much less turbidity in a two phase system of heptane:water.

The transaminases were tested under different industrially relevant conditions. In nearly all transaminase processes on industrial scale isopropylamine (IPA) is used as an amine donor. To increase the space-time-yield and to shift the equilibrium of the reaction to the product side, usually high IPA concentrations are used. It turned out that especially high concentrations of amine donor are critical for the transaminase stability. We could show that the engineered variant ATA-v1 (s. WP6) shows high stability and tolerance at presence of high IPA concentrations. An example is shown in **Figure 18**.

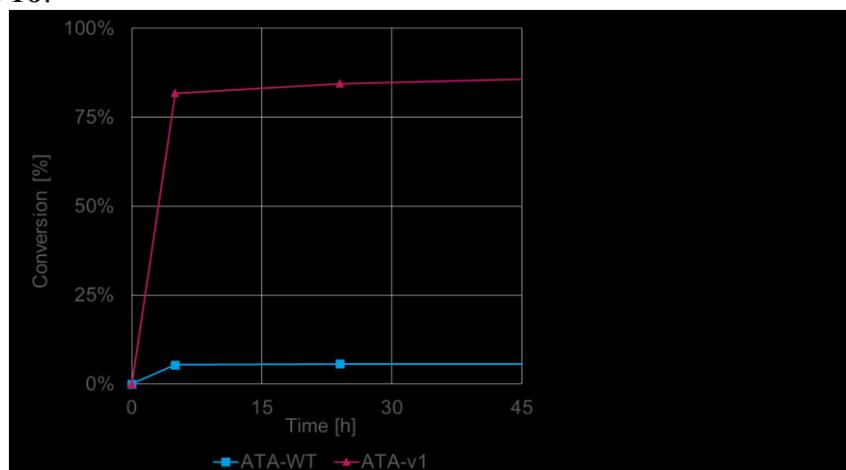


Figure 18: Comparison of ATA-WT (blue) and ATA-v1 (red) using significant amount of co-solvent and high IPA concentration at elevated temperature. Reaction conditions are indicated.

ATA-v1 shows also good conversions at 2M IPA, is very tolerant to co-solvents, high temperature and high substrate and product concentrations. We benchmarked ATA-v1 towards transaminases from competitors and found the ATA-v1 to be among the most stable and active transaminases.

Due to the USP and DSP development and the great performance of the ATA-v1, this enzyme and other optimized variants are now part of c-LEcta's Plug&Play Biocats™ products and complement c-LEcta's transaminase platform (see c-LEcta Newsletter: <http://www.c-lecta.com/newsroom/news/view/entry/prequalified-transaminase-platform-ready-for-industrial-use/>).

At TU Graz, novel oxygen sensors based on covalently linked NIR indicators in a matrix polymer were developed to monitor the oxygen concentration during fermentation processes. It could be shown that the sensors are stable enough to withstand 11 sterilization cycles of 30 min at 120°C (hot vapour sterilization). A performance test during a fermentation process showed a good agreement with an oxygen values of an electrochemical sensors. The new miniaturised format using a 1 mm optical fibre is attractive for the application in lab-scale fermenters of various sizes. This is a first part of the online processing tool which is developed in WP3 including sensors for the parameters pH and glucose that is used for the optimisation of fermentation processes, e.g. different feeding strategies.

WP6: MULTIOBJECTIVE BIOCATALYST SCREENING

16. Screening Methodologies Established

During the course of the ‘Biointense project’ UNIMAN has developed a solid-phase screening protocol for colonies expressing both (*R*)- and (*S*)-selective ω -transaminases (ATA). This high throughput assay was used to screen large variant libraries with enhanced substrate selectivity and enantioselectivities. An (*S*)-selective aminotransferase (ATA-wt) kindly provided from c-LEcta metagenomic library gave 70-75% *ee* for (*S*)-(+)-1-methyl-3-phenylpropylamine from benzylacetone and L-alanine as the amine donor. A random generated mutant library was screened using the sandwich screen solid phase assay to identify colonies with greater activity towards (*S*)-(+)-1-methyl-3-phenylpropylamine (see **Figure 19**). A colony was found containing a single point tyrosine mutation at a conserved residue Phe87 and gave the (*S*)-isomer in >98% *ee*.

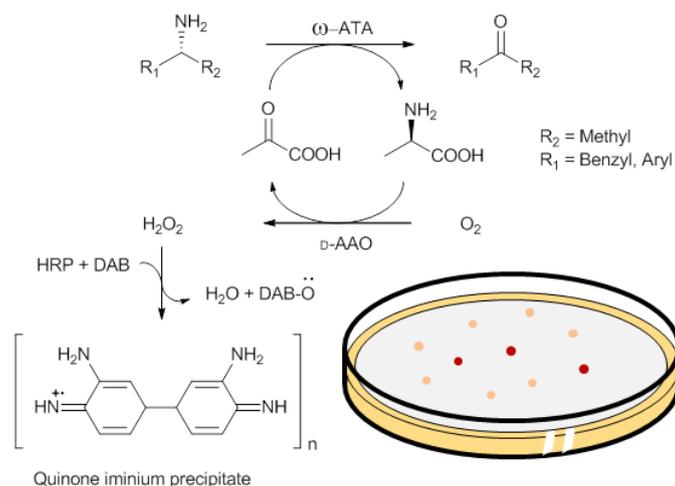


Figure 19: Schematic colorimetric assay screen for detecting D-alanine from the co-transformation of aminotransferase and D-amino acid oxidase (D-AAO) in *E. coli* and plated on a membrane.

Our collaborative partner c-LEcta worked on a complementary approach through high throughput screening of ω -ATAs based on enzyme structure and homology information used to create site-directed mutagenesis libraries - Approximately 300 positions were targeted with a restricted set of mutations for each position according to MDM (multi-dimensional-mutational). A microtiter plate (MTP) photometric assay was established to monitor the transaminase reaction with the detection of product formation via HPLC analytics this was shown to be a reliable and effective method for assessing protein stability, product formation and the quality of the mutant library.

17. Successful Biocatalyst Improvements Achieved

The thermostability of ATA-wt could be increased by +22°C with ATA-v1 being the most stable variant identified thus far (**Figure 20A**). This variant enzyme shows high performance using high concentration of isopropylamine IPA which gave 93.2% ee for the (*S*)-isomer (**Figure 20B**). In WP5 different reaction conditions were tested to evaluate the limits and to benchmark the enzyme performance and stability under extreme substrate concentrations, high temperatures and high amounts of co-solvents compared to a competitor's enzyme. It could be shown that ATA-v1 can be compared with “state of the art” enzymes showing comparable or better performance. ATA-v1 is one of the most stable transaminases known thus far.

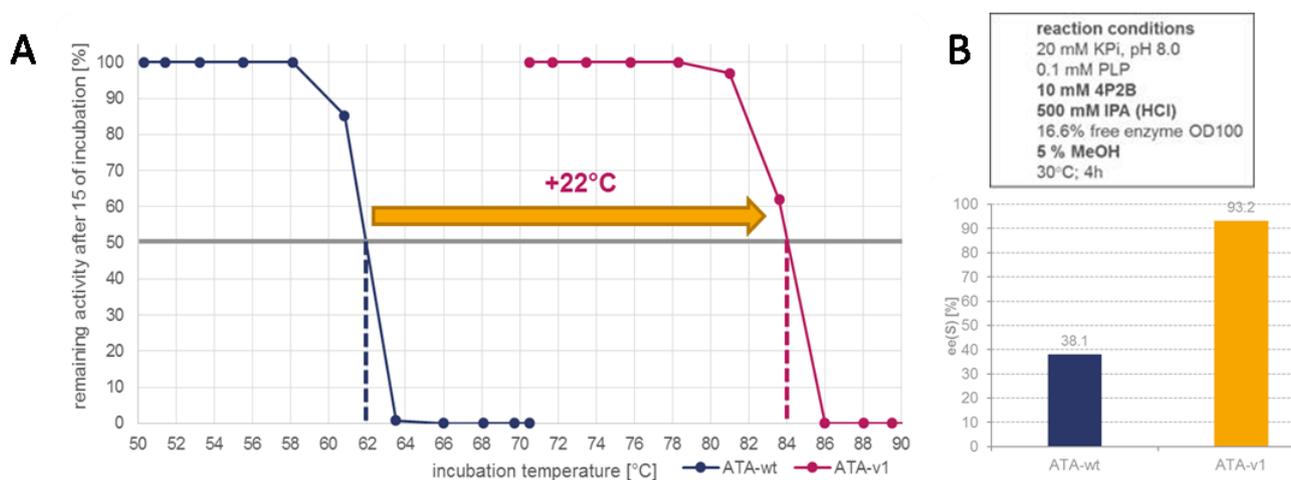


Figure 20: **A:** Thermostability of t ATA-wt and the variant ATA-v1. The enzyme was incubated at different temperatures for 15 min and the residual activity was plotted over the incubation temperature. The Tm-50%-value indicates the temperature at which 50% of the initial activity is still present. **B** The enantioselectivity of ATA-wt in comparison with the best variant ATA-v1.

Luxcel has developed a suite of tools to facilitate the convenient measurement of Transaminase (ATA) activity. The assays developed use a range of water-soluble oxygen probes and sensors. A 96 well plate based assay was developed to measure ATA activity by harnessing an oxygen consuming amino acid oxidase (AAO) based cascade reaction. The assay measured on a time-resolved fluorescence plate reader using sequential intensity measurements separated in the time domain to generate lifetime data. Successful coupling for the cascade reaction to the transamination reaction was demonstrated and data demonstrates that differences in activity are easily detected and good assay reproducibility is observed. ATA enzymes were measured at fixed concentration (mg/ml) and fixed reported activity (U/ml), which led to further investigation of enantioselectivity for the panel of ATA enzymes. A convenient means of determining enzyme enantioselectivity has also been achieved through the parallel analysis of *R*-selective and *S*-selective AAO and provides valuable additional information on the activity and selectivity of the enzymes under characterisation. These data demonstrate that the assay as currently configured is capable of screening ATA activity, conveniently characterising that activity, heat stability, substrate preferences, and enantioselectivity.

WP7: IMMOBILIZATION AND STABILITY

In this WP, inactivation mechanisms of amine transaminases (ATAs) were elucidated and this information was used to achieve dramatic stabilization using protein engineering and reaction engineering approaches. Furthermore, several highly efficient methods for immobilization of the enzymes were developed for use in microreactors and larger reactors.

18. Elucidation of Mechanisms for Inactivation of ATAs Established

In order to make it possible to improve the stability of ATAs in a rational way, mechanisms of their inactivation were elucidated. It was observed that the stability during incubation without substrates was much better than the operational stability during transamination reactions. This was true for the ATA in focus of Biointense (ATA-wt) as well as for other ATAs (**Figure 21**).

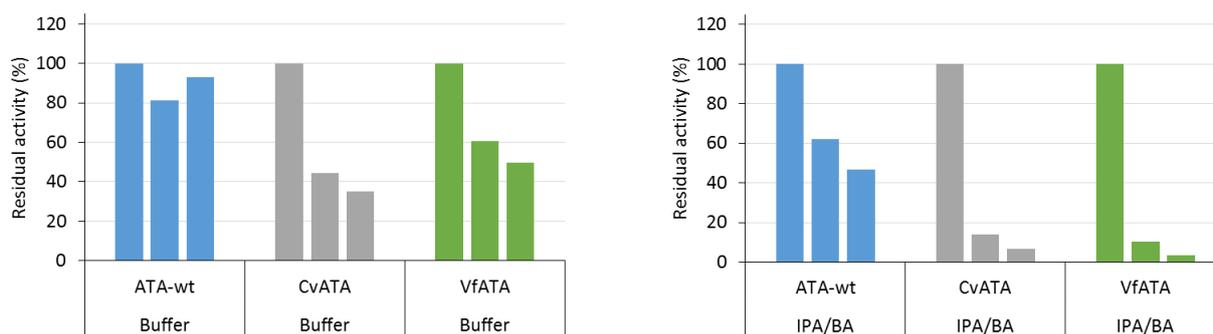


Figure 21: Operational stability of ATAs in the resting state (left) and during catalysis (right) converting the two substrates isopropylamine (IPA) and benzylacetone (BA) at 40 °C. Residual activity was measured at 0, 2 and 4 hrs. CvATA: ATA from *Chromobacterium violaceum*; VfATA: ATA from *Vibrio fluvialis*

Experimental and computational studies indicated that binding of the cofactor (PLP/PMP) and interactions between the enzyme subunits were of crucial importance for enzyme stability. During catalysis, the holo enzyme (E-PLP) is converted into the PMP state (E:PMP) (**Figure 22 left**). PMP dissociation leaves the enzyme in the vulnerable apo form (E⁰) which undergoes unfolding and irreversible aggregation and inactivation. It can, however, be rescued by excess of PLP or amine donor to re-establish the E-PLP form. Increased subunit interactions in the tetrameric ATA-wt compared to the dimeric CvATA and VfATA might be the reason for its higher stability (**Figure 22 right**).

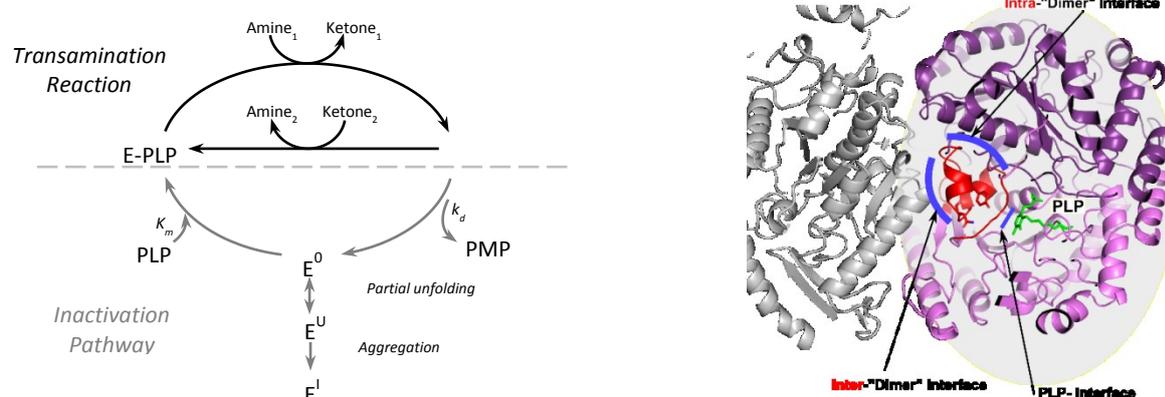


Figure 22: Postulated inactivation mechanism of amine transaminases (left). View on the active site of the tetrameric wild-type amine transaminase ATA-wt and its interface region between monomers (intra) and dimers (inter)(right). Structural figures were prepared using PyMol Molecular Graphics System

19. Protein Engineering as a Strategy for ATA Stabilisation Developed

The most successful strategy for stabilisation of ATA-wt was protein engineering. Using several rounds of directed evolution, c-LEcta produced mutants with greatly improved thermostability. The thermal inactivation of the mutant ATA-v1, with only 3 mutations, was shifted about 17 °C compared to the wild type enzyme (Fig. 7.20A). Interestingly, similar stabilisation was achieved for the resting state and for the operationally active enzyme. Furthermore, ATA-v1 was considerably more stable towards organic solvents (**Figure 23**). The beneficial mutations were located in the cofactor binding site and at the subunit interaction sites, which supports the postulated inactivation mechanism (**Figure 24**).

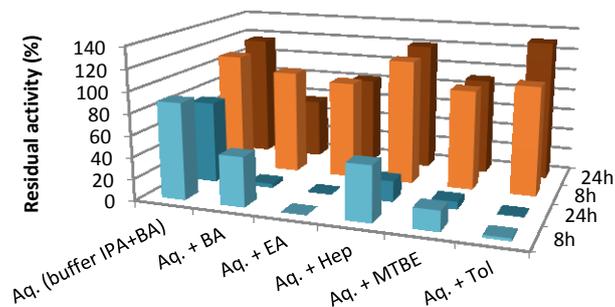


Figure 23: Residual activity of wild-type (*ATA-wt*, blue) and mutant (*ATA-v1*, red) enzyme after incubation in various organic/aqueous two-phase systems. Benzylacetone, BA; ethylacetate, EA; n-heptane, Hep; methyl-tert-butylether, MTBE; toluene, Tol. The aqueous buffer contained substrates (0.5 M IPA, 10 mM BA) and cofactor (0.1 mM PLP)

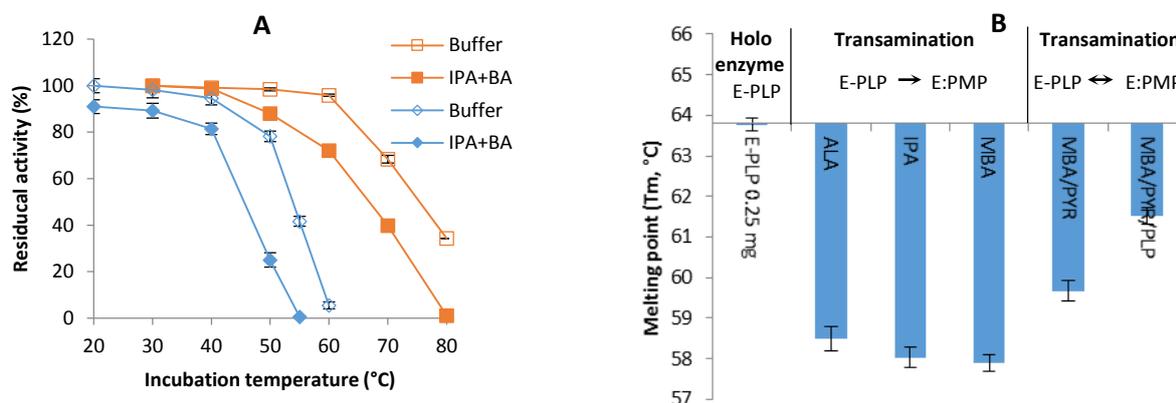


Figure 24: **A)** Remaining ATA activity of wild-type (*ATA-wt*, blue) and mutant (*ATA-v1*, red), either resting (buffer) or during catalysis (with substrates) after 2 h incubation at different temperatures. **B)** Thermodynamic stability (melting point) of *ATA-wt* at different conditions. L-alanine, ALA; methylbenzylamine, MBA; pyruvate, PYR

Measurement of the melting point using Differential Scanning Fluorimetry (DSF) was found to be a fast and reliable method to estimate effects of different conditions on *ATA* stability. The addition of any amine donor caused a dramatic drop in melting point of *ATA-wt* (**Figure 24B**) probably because a large part of the enzyme was converted to the sensitive PMP-enzyme form. Addition of an amine acceptor made the complete catalytic cycle possible and a part of the enzyme was thus converted back to the E-PLP form, causing the melting point to increase (**Figure 24B**). Thus, it is important to operate the *ATA* catalysed reactions in a way that the enzyme exists mainly in the E-PLP form. There should be a sufficient supply of amine acceptor present at all times, and high amine donor concentrations are to be avoided, if possible. This can be difficult, since high amine donor concentrations can be used to shift the unfavourable equilibrium position. However, more efficient methods to address both inhibition and equilibrium were developed in Biointense. *In situ* removal of the amine product through membrane extraction made it perfectly possible to operate with moderate amine donor concentrations and maintaining high reaction rates.

20. Successful Surface Immobilization of ATAs Achieved

Several different methods for immobilization of ATAs on surfaces in microreactors were developed. Covalent coupling of *ATA-wt* using silanisation and glutaraldehyde coupling resulted in low enzyme loadings; consequently, only low volumetric productivity was achieved (Table 1). The use of *ATA* tagged with a silica binding module (SBM) made it possible to reach high enzyme loadings by simple adsorption on the walls of a microreactor from iX-factory. This method afforded a volumetric productivity as high as 2 mmol/l min. Using covalent coupling of whole cells, a

similarly high volumetric productivity was obtained due to high biocatalyst loading. The most efficient reactor was the one containing adsorbed SBM-tagged ATA-wt in the microreactor from iX-factory. In this case the conversion was about 20 % in a residence time as short as 7 minutes.

Table 1: Immobilization of ATA catalysts on the surface of microreactors. Assay reaction: 40 mM methylbenzylamine and 40 mM pyruvate at 30 °C.

	Enzyme, covalent	Enzyme, adsorbed	Cells, covalent
Reactor type	Chip Shop	iX-factory	Chip Shop
Catalyst	ATA-wt	SBM-ATA-wt	ATA-wt in <i>E. coli</i>
Immobilization method	Covalent	Via N-SBM tag	Covalent
Biocatalyst conc (g/l)	0.027	0.864	74
Specific activity	0.771 U/mg	2.315 U/mg	22 U/g cell dry weight
Volumetric prod (mmol/l min)	0.021	2.00	1.59

21. Successful ATA Immobilization by Entrapment Achieved

Whole *E. coli* cells containing ATA were successfully immobilized in chitosan matrices. Widely different ratios between cells and chitosan can be used. When large amounts of chitosan are applied, immobilization is typical entrapment, but with higher cell to chitosan ratios chitosan acts more as a crosslinker and preparations with extremely high enzyme loadings can be obtained. Importantly, the specific activity remained unaffected, even at cell/chitosan ratios as high as 100.

Another successful method to entrap ATA was developed by LentiKats AS. Polyvinyl alcohol carrier was used for ATA entrapment and lentil-shaped particles of LentiKats Biocatalyst (LB) were prepared. The shape of the LB particles makes diffusion distances relatively short, thereby facilitating mass transfer of substrates and products. The LB with immobilized ATA was shown to be very stable (stability in different organic solvents) and useful in reactors ranging from less than 100 µl to 20 l. Similar activity for free and immobilized enzyme indicated that mass transfer limitations were negligible. No problems appeared during scaling up so similar conversions were obtained independent on scale at the fixed enzyme concentration of 5 mg per ml of reaction mixture (Table 2). However, the best performance was achieved in a microreactor, where controlled fluid flow enabled efficient biocatalyst accessibility. Advantageously, the immobilized preparation could be stored for 25 weeks with minimal loss of activity. Furthermore, good operational stability made it possible to use the immobilized preparation in 12 consecutive batches with less than 10 % decline in conversion, which is important for industrial application.

Table 2: Scale-up of the conversion of BA to 4-phenyl-2-butanamine catalysed by ATA-v1 (5 mg/ml reaction volume)

	Free	Immobilized (50 mg_{enzyme}/g_{Lentikats Biocatalyst})		
	TUBE (2 ml)	TUBE (15 ml)	BIOREACTOR (5 l)	BIOREACTOR (20 l)
Reaction volume	1 ml	5 ml	3 l	18 l
Lentikats Biocatalyst	–	0.5 g	300 g	1.8 kg
Specific activity (mU/mg _{enzyme})	7.6	10.0	9.4	8.8
Conversion after 24 h (%)	81.1	82.4	85.1	84.5

WP8: ECONOMIC AND ENVIRONMENTAL ASSESSMENT

The work carried out within WP8 has been focused on methods for early-stage assessment of process candidates based on biocatalysis, using the catalysts and reaction components employed within Biointense as case studies. The first part of the work provides a detailed analysis of the cost structures involved in the implementation of a biocatalytic process. Specific to the cases studied within Biointense, an analysis of the impact of the choice between different amine donors (isopropyl amine, IPA; phenyl ethylamine, PEA (or methyl benzylamine, MBA); and alanine, Ala) on the operating cost has been established. Next, an assessment of capital and operating costs has been made for these processes at different scales, together with an estimate of typical development costs for biocatalytic processes depending on the maturity of the catalyst. Finally, the potential target products have then been grouped according to value and market size - to gauge the available margin for implementation - to put the other aspects into context.

The second part of the work within WP8 has been focused on the evaluation of the technical performance potential of the different systems studied within biointense, focusing on the effect of implementing in situ product removal (ISPR) to achieve (partial) integration of the reaction and product work-up stages.

22. Successful Assessment of Biocatalytic Process Potential Achieved

The methodology developed within Biointense for the evaluation of biocatalytic processes is aimed at providing an assessment of the potential of a specific process at an early stage of process development. The gathered information is further meant to guide and support development efforts. The methodology relies on a combination of established concepts for costing of processes with know-how of the performance of different process technologies, as well as different aspects of biocatalysis – how they work, what they cost and challenges for development.

• Allowable cost

An important question to ask at an early stage of process development is related to allowable cost – how much can be spent to produce a certain amount of product? While it can be hard to answer this question exactly, some generalizations can be made. One aspect is the cost of competing technologies, with the following categories identified for fine chemicals and pharma production:

- 1) Competing with simple chemistry at conversion costs of 30€/kg
- 2) Competing with standard chemistry at conversion costs of 50€/kg
- 3) The process telescopes one or more existing chemical steps, or competes with difficult chemistry – allowed conversion cost 100€/kg
- 4) The process telescopes several existing chemical steps, Unique chemistry, or where implementation speed is the primary target – allowed conversion cost 500€/kg

The allowable production cost sets a limit for the operating cost (OpEx) of the process. A second aspect is the scale of the market, which to an extent defines the process scale, but also puts a limit to the amount of development efforts that can be put into the implementation of the process. In our view, implementation of a pre-existing suitable catalyst should not require more than 6 PM for a skilled process engineer and technician. However, longer times may be required when there is a need to improve the biocatalyst through protein engineering; 6-18 months of development time for a team of skilled scientists could be considered normal (about 120-600 PM). It can be easily understood from the above values that extensive development work (e.g. protein engineering) can only be considered for products that have a relatively large production volume. Even moderate development efforts add significantly to product costs if the sales volumes are small (in the range of 1 to a few tons per annum).

• Process design

The design of a complete process involves the identification of suitable reaction conditions (and reactor), and also the down-stream processing stages required to manufacture a product of the required purity. This requires knowledge of the characteristics of the biocatalytic reaction stage, as well as the physico-chemical properties of the reaction components. For initial costing the process

can be relatively simple, but it is often prudent, as was the case for the reaction systems studied within Biointense, to integrate continuous product separation with the reaction stage to achieve the processing efficiency required for industrial implementation.

- **Process costing**

Once a process candidate has been established the investment and operating costs (CapEx and OpEx) can be estimated using established methodologies. The cost of the biocatalyst will depend on several factors, including how efficiently it is produced, how it is formulated (whole cell, free enzyme, immobilized), if it requires co-factors and, in some cases, if the reaction relies on several different biocatalysts.

23. Process Potential for Alternative Scenarios in BIOINTENSE Established

The work on techno-economic assessment within Biointense has been focused on the comparison of three alternative reaction systems, using IPA, PEA and Ala as amine donors. The technical assessment of these processes indicates that, for small production volumes and fast implementation in a simple process, a two-phase aqueous-solvent reaction system with PEA as the donor can provide high product concentrations with limited excess of donor and relatively small waste generation (the E-factor, meaning waste generated for a given mass of product, is on the order of 25-50). However, for larger production volumes it would be more efficient to use the cheaper IPA as a donor and employ ISPR, either distillation or extraction, to achieve high yields. These option reduces both waste generation and material use at the expense of a slightly more complicated process. The alanine-based technology has great potential, but is currently deemed too immature to be considered for implementation.

These conclusions were substantiated by the economic analysis of the cases. As summarized in the table below, the total allowable processing cost was split (1:2:2) between amine donor, processing tasks and biocatalyst for the four identified cost levels mentioned above. To support the analysis, certain assumptions were made regarding the process: 24h reaction and 12h recovery; biocatalyst cost wet cells (WC) of 50€/kg and crude free enzyme (FE) or immobilized enzyme (Imm.) of 1000€/kg.

As can be seen in the table, the required process metrics in Scenario 1 (30 €/kg) and 2 (50 €/kg) are very challenging. As was reported in Deliverable report 8.1, where cost was plotted against process intensity, in order to achieve the targets, product concentrations need to exceed 150 g/L. Likewise, the catalyst needs to be very effective; a biocatalyst yield of 50-100 kg/kg would be required, corresponding to using less than 1-2 g/L biocatalyst (without recycle) and a cheap amine donor need to be used and in moderate excess. In all scenarios except 4 (500 €/kg) the use of L-alanine as the amine donor is not feasible due to the high cost for L-alanine and NAD⁺.

Table 3: Economic and process metrics for different process scenarios, modified from: Tufvesson P, Nordblad M, Krühne U, Schürmann M, Vogel A, Wohlgemuth R and Woodley JM (2015) Org. Process Res. Dev. 19 652.

Target cost	Max amine excess (fold)			Process intensity (g/L*) in 10 m ³	Biocatalyst yield (kg P/kg B)	
	IPA	Ala	Rac-MBA		WC (50€/kg)	FE/Imm. (1000€/kg)
30	<1-5	‡	<1	>150-200	>5	>100
50	<5	‡	<5	>80-125	>2.5	>50
100	<10	No excess	<10	>50-75	>1	>25
500	<10	<1-5	<10	>10-20	>0.25	>5

‡ No feasible options

* Given the reaction and recovery times above

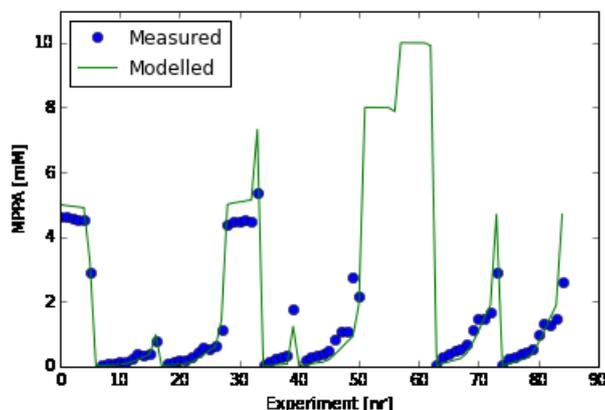
The use of optically pure R-(+)-MBA is not feasible in any of the cases due its high cost. Racemic MBA could be suitable in the cases where economic constraints are tighter; however it would depend on the cost of recovery. It should be noted that even where the economic constraints have been relaxed (scenario 3 and 4) the requirements on process intensity (>10g/L, i.e. 100mM) are still relatively high in relation to the majority of published reaction conditions used in transamination, which are often in the 10mM range (corresponding to 1 g/L if the molecular weight of the product is 100 g/mol). Even so, it is clear from the development work done by Codexis and Merck in the Sitagliptin case (uses IPA), that it is indeed possible to achieve the numbers required for scenario 1 and 2.

WP9: PROCESS MODELLING AND DESIGN OF EXPERIMENTS

In WP9 of the BIOINTENSE project the aim is to model the processes of interest (biocatalyst kinetics, separation and fluid dynamics) and apply the Optimal Experimental Design (OED) methodology. The first part consists of the application of OED to acquire models with high predictive power hereby reducing the number of required lab experiments needed for obtaining high-quality data for model calibration. The acquired data allowed to successfully calibrate and validate the model describing the enzyme kinetics of ATA-wt. The second part of this summary discusses the different microreactor configurations which were implemented and how these configurations can be optimised using topology optimisation. Moreover, the use of topology optimisation to optimise the distribution of enzyme in the reactor allowed to improve the reactor performance. The third part shortly discusses the productivity increases when applying process intensification strategies.

24. Kinetic Model of ATA Enzymes Developed

By using ω -transaminase (EC 2.6.1.X), optically pure chiral amines are produced by transferring the amine group from an amine donor, to a pro-chiral acceptor ketone, yielding a chiral amine and a ketone as co-product. The enzyme requires pyridoxal 5'-phosphate (PLP) as a cofactor to act as a shuttle to transfer the amine moiety between the molecules. As a model reaction, the synthesis of acetone (ACE) and 1-methyl-3-phenylpropylamine (MPPA) from isopropylamine (IPA) and benzylacetone (BA) by the ω -transaminase ω -TA was considered. In order to calibrate the kinetic model describing this reaction, the methodology proposed by Al-Haque et al. (2012) was followed¹. This methodology consist of three different parts: first the initial forward reaction rate is calibrated, second the initial backward reaction rate is calibrated. Finally, the remaining parameters are calibrated using progress curves. Using this procedure, the model was successfully calibrated (by yielding parameters with acceptable confidence intervals). A validation of the model was performed using progress curves (**Figure 25**), from which it can be concluded that the model is predicting the data well and thus the model can be regarded as validated.



¹ N. Al-Haque, P. A. Santacoloma, W. Neto, P. Tufvesson, R. Gani, J. M. Woodley, A robust methodology for kinetic model parameter estimation for biocatalytic reactions., *Biotechnol. Prog.* 28 (5) (2012) 1186–1196.

Figure 25: The predicted MPPA concentration (green line) is compared with the measured MPPA concentration (blue dots). A good resemblance between the calibrated model and the progress curve experiments is obtained

25. Modelling of Spatial Heterogeneities Achieved Using Computational Fluid Dynamics

The understanding of the basic principles and the mechanisms involved in the fluid flow characteristics at the microscale is essential since their behavior affects the transport phenomena and kinetics of microfluidic applications. In order to describe the reaction-convection-diffusion dynamics and to depict the governing transport characteristics in different types of enzymatic microreactors (**Figure 26**). Different modes of operation of the enzyme microreactor were investigated, e.g. slug flow, split flow, immobilised enzyme,..., and were modelled by using Computational Fluid Dynamics (CFD) based on the Navier-Stokes equations. Lattice-Boltzmann was also used to study flow and mass transfer phenomena based on the collision of particles, and from the results it was concluded that Navier-Stokes and Lattice-Boltzmann yield the same results for the microreactors under study.

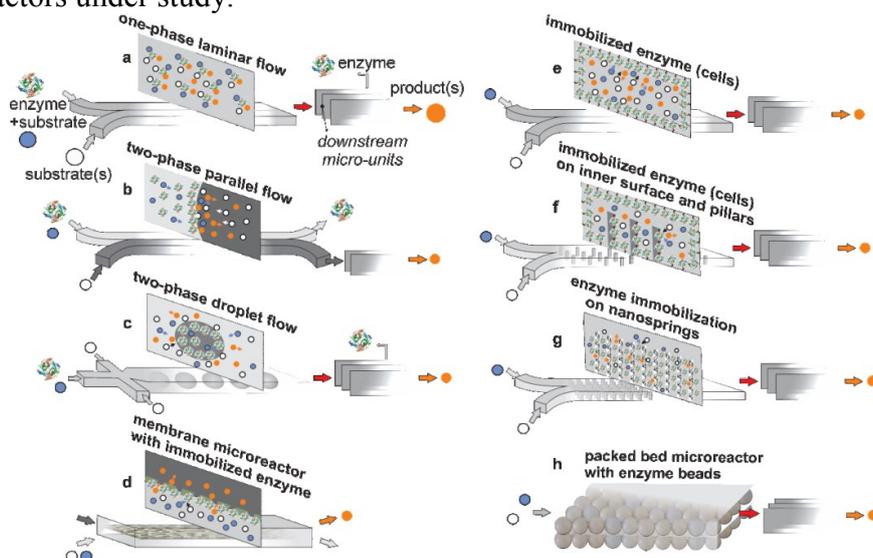


Figure 26: Schematic presentation of enzymatic microreactors (Reproduced with permission from Wohlgemuth R, Plazl I, Znidarsic-Plazl P, Gernaey KV and Woodley JM (2015) Trends Biotechnol **33**, 302): a) free enzyme in a two-phase parallel flow; b) free enzyme in a droplet flow; c) free enzyme in a homogeneous system; d) membrane microreactor with enzyme immobilized on the membrane; e) immobilized biocatalyst on the inner surface of a microchannel; f) immobilized biocatalyst on the inner surface and pillars; g) nanospring supports for increasing surface area and biocatalyst loading in the microchannel; h) packed bed microreactor with beads containing biocatalyst

26. Topology Optimisation Methodology Validated

The aim of topology optimisation is to identify the bottlenecks of the reactor configurations and make alterations to the enzyme layout in order to improve the product concentration at the outlet. In this way, the optimal, or at least an improved microreactor configuration will be found before testing it in the laboratory. The design variable for all the case studies is the enzyme concentration inside the microreactor. The cost function corresponds to maximizing the concentration of the product at the outlet of the microreactor.

By applying this methodology, it is found that the productivity can be increased up to 30% by placing the enzyme more wisely. In **Figure 27**, different topology configurations are compared for the same reaction and experimental conditions. The reaction under study, consists of the reduction of hydrogen peroxide by oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to its radical form catalyzed by horseradish peroxidase (E.C. 1.11.1.7). From **Figure 27**, it can be concluded that topology optimisation can increase the reactor productivity significantly.

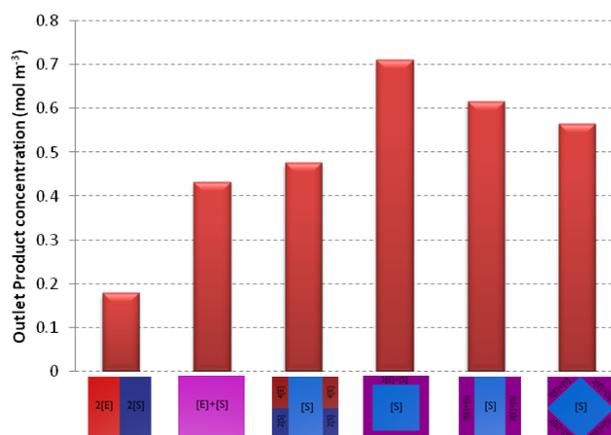


Figure 27: Productivity for different reactor configurations, illustrating the importance of topology optimisation.

27. Process Intensification Achieved

To increase the reactor productivity, the reaction can also be coupled with an in-situ product recovery (ISPR) technique. This approach was successfully modelled and showed comparable results with the experimental setup in WP2. The application of ISPR is promising and can potentially improve system performance by at least 30%. For the production of unstable intermediates, it was shown that microreactors are superior over CSTR reactors. As a model reaction, the conversion of N,N'-Dibenzyl-1,4-diaminobutane (DBDB) to putrescine was selected.² For this reaction, it was found that the maximum productivity of the CSTR is 27% lower compared to that of a microreactor. This shows that the use of microreactors for the production of unstable intermediates has great potential and should be further explored and demonstrated.

WP11: DISSEMINATION

28. Executed dissemination activities

An innovative project like the BIOINTENSE project deserves an appropriate level of scientific and public dissemination of the results. Accordingly to other EU projects a considerable effort has been made for the formulation of scientific peer reviewed articles, scientific presentations (including oral and poster presentations) as well as articles addressing the broader public, like e.g. in engineering journals going beyond the biotech industry. [Statistics: Scientific Peer Reviewed articles:31 Conference contributions: 163]. Besides of those classical activities, the University of Ljubljana has twice been organizing the International conference IMPLEMENTATION OF MICROREACTOR TECHNOLOGY IN BIOTECHNOLOGY – IMTB (2013 and 2015). The conference was held Cavtat and in Opatija, Croatia, and has been bringing together experts using microfluidic in biotechnological applications.

At the other side it was attempted to address a broader public audience by the production of an explanatory animated video, explaining main aspects of the BIOINTENSE project. Two example screenshots of this video are presented in **Figure 28**. This video is embedded into the homepage of the project, but a second “landing page” holding more information about the project partners, the videos and the making of the videos has been established. This homepage can be found here: <http://www.worldofmicrofluidics.com>. It should be mentioned that the synergies to a Marie Curie Actions Multi-ITN project about microbioreactors (EUROMBR Grant Agreement no 608104) is used. At this point of time the video has been seen by more than 850 visitors of the homepage, which is quite impressive, considering when the video was first published. The video is currently submitted to six international acknowledged film festivals in several countries (please see the homepage <http://www.worldofmicrofluidics.com> for more information).

² Roberts, K.M., Tormos, J.R., Fitzpatrick, P.F. 2014. Characterization of Unstable Products of Flavin- and Pterin-Dependent Enzymes by Continuous-Flow Mass Spectrometry. *Biochemistry*, 53, 2672-2679



Figure 28. Screenshots of the explanatory video. Please visit <http://www.worldofmicrofluidics.com> for further information.