ROBOX understands that in moving towards more sustainable oxidation processes applied in the manufacture of consumer products for societal user needs we need to develop and adopt new science and technology with new processes and equipment. The introduction of ROBOX bio-oxidation processes is expected to bring about substantial reductions in cost (up to -50%), energy use (-60%), chemical use (-16%) and GHG-emissions (-50%) in these processes that are essential to modern lifestyles in Europe.

We also recognise the need to pay close attention to other crucial areas including the training and education of future scientists, using tools and metrics to define improvements, all coupled to engagement activities so the public can clearly see the advantages of ROBOX technology. Also the overall chemical industry needs to work closely with academia to translate important new scientific discoveries to a point where they can be quickly adopted for manufacture. The ROBOX project contributes to all these objectives.

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### 1. Introduction

Welcome to the fourth newsletter of the ROBOX project that was formed to develop greener biooxidation methods for the manufacture of the chemicals needed by the chemical and pharmaceutical industries to manufacture the products required for virtually all consumer products.

This project targeted four biocatalytic oxidation methods and better ways for implementation and has plans to reach demonstration level so industry can have the confidence to implement these technologies.

Hence the ROBOX project was designed to develop green chemical manufacturing methods and improve methods for analysing and presenting metrics to stakeholders for these problems by implementing products at a scale suitable to analyse these metrics.
2. ROBOX AT UPCOMING CONFERENCES

At the Novel Enzymes 2018 conference in October this year there will be 4 speakers from ROBOX (PIs - Turner, Schwaneberg, Panke and Fraaije) and most aspects of ROBOX enzymes will be presented. We also have two of our PIs chairing sessions (Woodley, Gleider) – so contact us or speak to our people there for more information.

https://novelenzymes2018.eu/

C-Lecta GmbH will be at the Gordon Research Conference (Biocatalysis) and at the EMBO workshop - Enzymes, biocatalysis and chemical biology: At the new frontiers - to present on the development of ADH and NOX for robust, selective and efficient oxidation reactions suitable for industrial processes. Please contact us or c-Lecta if you are interested.

http://meetings.embo.org/event/18-biocatalysis

The ‘5th Winter Process Chemistry Conference’ will be held in Manchester at the MCC, December 11th – 13th 2018. Our partner UNIMAN will present on biocatalysis for use in industry.

https://www.scientificupdate.com/conference_events/5th-winter-process-chemistry-conference-exhibition/20181211/

UNIMAN will be at 9th International Congress on Biocatalysis Hamburg, Germany presenting on panels of Cytochrome P450 enzymes for drug metabolites.

http://www.biocat-conference.de/

UAB will present on cyclohexanone monooxygenase at Congress II Jornadas Españolas de Biocatalisis in Oviedo
http://jeb2018.uniovi.es/presentacion

RWTH will also be at the EMBO workshop “Enzymes, biocatalysis and chemical biology: The new frontiers” to present on their 96-multiplex capillary electrophoresis screening platform.

http://meetings.embo.org/event/18-biocatalysis

Please contact us if you would like to find out more about any of these technologies.

3. SOCIAL MEDIA

You can find out a lot more about ROBOX activities and biotechnology from our blogs and videos

You can also watch video background to our ROBOX project on the following youtube videos-
https://www.youtube.com/watch?v=ruifWn_7hVk
https://www.youtube.com/watch?v=MfDdZK8NB_A

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4. Some ROBOX Publications

The target of getting high impact publications by producing world class collaborative science and publishing in leading journals was seen as an important indicator of the quality of ROBOX outcomes.

ROBOX “Hot Paper” describes Characterization and Crystal Structure of a Robust Cyclohexanone Monoxygenase. This collaborative effort between RUG and UP describes that cyclohexanone monoxygenase (CHMO) is a promising biocatalyst for industrial reactions owing to its broad substrate spectrum and excellent regio-, chemo-, and enantioselectivity in reactions. Historically the low stability of many Baeyer–Villiger monoxygenases has been an obstacle for their exploitation in industry. Characterization and crystal structure determination of a robust CHMO from Thermocrispum municipale is reported in this paper. The enzyme efficiently converts a variety of aliphatic, aromatic, and cyclic ketones into their lactones as well as oxidizing prochiral sulfides into chiral sulfoxides. A compact substrate-binding cavity explains its preference for small rather than bulky substrates. Small-scale conversions with either purified enzyme or whole cells demonstrated the remarkable properties of this newly discovered CHMO. The exceptional solvent tolerance and thermostability make the enzyme very attractive for biotechnology. For these reasons this paper was awarded “Hot Topic” status by the prestigious journal Angewandte Chemie International Edition https://onlinelibrary.wiley.com/doi/abs/10.1002/anie.201608951.

Cover Picture was selected by the journal CHEMCATCHEM highlighting an enzymatic cascade developed in ROBOX and shows the chemical structure of ketoisophorone (KIP), a valuable building block used to make vitamins, pharmaceuticals and APIs. In this full paper M. Tavanti et al. describe the first biocatalytic cascade to access this key intermediate through the double allylic oxidation of the readily available α-isophorone employing a variant of P450cam-RhFRed and Cm-ADH10. The designed cascade has been demonstrated both as a one-pot two-step process and as a cascade process employing designer cells co-expressing the two biocatalysts, with a productivity of up to 1.4 g L⁻¹ d⁻¹. More information can be found in the Full Paper by M. Tavanti et al. on page 3338 in Issue 17, 2017 (DOI: 10.1002/cctc.201700620).


ROBOX have made a significant contribution to the review “Human Enzymes for Organic Synthesis https://onlinelibrary.wiley.com/doi/abs/10.1002/anie.201800678. This focuses on the application of human enzymes that catalyse chemical reactions in course of the metabolism of drugs and xenobiotics. Some of these reactions were explored on the preparative scale and are so of great interest to applied scientists. The major field of application of human enzymes is currently drug development, where they are applied for drug metabolite synthesis which are an essential part of drug safety.
5. THE BIOCATALYSTS

Biocatalysts are enzymes produced by and in micro-organisms and offer an attractive and sustainable alternative for stoichiometric chemistry and chemical catalysts, but need to be tailored to the chemical conditions in large scale required by the industries producing chemicals needed for our lifestyles. These chemicals are needed for all our consumer requirements such as pharmaceutical ingredients, fine and agro-chemicals, vitamins, flavours and fragrances and polymers which are vital components of our European lifestyle. Industry still mainly uses classical chemical oxidations using stoichiometric amounts of oxidants which tend to often involve toxic heavy metal reagents and chlorinated solvents which have a large environmental footprint or oxidants like bleach, bromine, peracids and hydrogen peroxide which are extremely hazardous. Therefore using biocatalytic oxidations for these processes is highly desirable from a safety, environmental and economic perspective. By using molecular oxygen (from air) under benign and mild conditions such as ambient temperature, moderate pH and low pressure we can therefore reduce the environmental footprint and improve the economics of processes to introduce and further oxidize alcohol functionalities in target molecules. Other advantages of biocatalytic oxidations are generally better selectivity in a regio-, enantio- and chemo-selective, manner. For example introduction of a hydroxyl group occurs only at specific positions of the target molecule in a defined orientation and without affecting oxidation prone functional groups such as amines or thiols.

To achieve the expanding use of industrial application of enzymatic biooxidation processes ROBOX is developing the techno-economic viability of biotransformations with four classes of oxidative enzymes:

- Cytochrome P450 monooxygenases (CYP450s)
- Baeyer-Villiger MonoOxygenase (BVMOs)
- Alcohol DeHydrogenases (ADHs)-NOX*
- Alcohol OXidases (AOXs)*

*Alcohol DeHydrogenases (ADHs)-NOX and Alcohol OXidases (AOXs) are equivalent chemical reactions

Cytochrome P450s (CYP450)

These are heme-containing monooxygenase enzymes that are able activate oxygen to introduce hydroxyl groups in a highly selective manner, producing chiral alcohols as precursors pharmaceuticals, flavour & fragrance compounds, nutraceuticals (such as vitamins) and drug metabolites for biomedical studies. This is an extremely challenging reaction from a synthetic chemical standpoint. Auxiliary redox proteins shuttles electron equivalents from NAD(P)H allowing the reaction to proceed as shown below.
Cytochromes P450s are heme-enzymes capable of catalysing the monoxygenation of a substrate assisted by redox partners.

Many inherent limitations of these enzymes have led to a few successful applications of CYP450s and only on a small scale. So engineering and improving these enzymes into robust biocatalysts using rational protein design and directed evolution has been applied in ROBOX with the aim of improving the catalytic efficiency, substrate scope, stability and solvent tolerance.

The availability of microbial CYP450s mimicking human CYP450s would be excellent biocatalytic tools given their broad substrate specificity.

**Baeyer-Villiger Monooxygenases (BVMO)**

These enzymes perform the insertion of oxygen into the carbon backbone of cheap starting materials to create value-added building blocks that can be polymerised into various materials and coatings or are flavour & fragrance molecules themselves. The Baeyer–Villiger oxidation forms an ester from a ketone or a lactone from a cyclic ketone without using chemically hazardous peroxycids or peroxides as the oxidant. A typical product made this way is caprolactone used in polycaprolactone products which are applied in many different market segments such as polyurethane elastomers, adhesives and sealants and industrial coatings and many other diverse (engineering) application areas.

In ROBOX the enzyme technology is being used to synthesize lactones for specialist polymers and fragrance products where the selectivity can offer unique products.

**Alcohol Oxidases (AOX)**

These enzymes are able to take readily-available alcohols and convert them into more reactive and synthetically-useful aldehyde or ketone building blocks in a controlled manner, producing only water as a waste product. A concomitant release of hydrogen peroxide occurs but this can easily be converted into oxygen and water by a second catalase enzyme which can then be consumed in further reactions of the AOX. ROBOX is investigating these for several specialist oxidations.

**Alcohol DeHydrogenase (ADH) and NAD(P)H Oxidase (NOX)**

By coupling ADH and NOX, oxygen can be used to oxidise alcohols into carbonyl groups and can be used as an alternative approach to the AOX. In ROBOX ADH enzymes were identified and optimized especially to show high enantioselectivity and activity in oxidative reactions. The robustness of the oxidative ADHs under process conditions were massively improved for oxidative reactions. Additionally, robust NOX enzymes for the efficient recycling of NAD⁺ and NADP⁺ were developed to show high activity and stability under process conditions. ROBOX will apply these enzymes in several oxidative processes to demonstrate their applicability and efficiency in industrial scale oxidative biotransformations.
6. **Drug Metabolite Synthesis**

One of the main targets of **ROBOX** was to develop and demonstration of drug metabolites to assist the pharmaceutical industry as they develop better and safer new drugs. Most drugs used by patients are metabolised in their liver by CYP450 enzymes and these metabolites have different activity profiles from the parent drug and play an important role in excretion of the active drug substance. Therefore for safety, dosage and activity studies the industry requires production of humanized CYP450s and/or microbial CYP450s mimicking human CYP450 activity modification patterns to produce these compounds. This has been a major focus of **ROBOX** activities and has resulted in several advances in this field.

**InnoSyn demonstration production of diclofenac metabolites by applying CYP450 TECHNOLOGY**

One focus of the **ROBOX** project is the use of CYP450 monooxygenases for the selective hydroxylation of diclofenac to 4’-hydroxy- and 5-hydroxy-diclofenac. After optimisation of expression and reaction conditions the production of the two metabolites on a multi hundred-gram scale was achieved (Chimica Oggi - Chemistry Today - vol. 35(6) November/December 2017)

**InnoSyn** is now planning to exploit this **ROBOX** technology of producing such API metabolites for pharmaceutical companies for biological activity and toxicity studies as well selling to regulatory entities as metabolite references.

**Partner TUG promotes ROBOX technology through partner Bisy.**

Our partner **TUG** are offering services through their spin-out company Bisy ([http://www.bisy.at/#/news](http://www.bisy.at/#/news)) which have delivered efficient screening services for CYP450s which have incorporated some **ROBOX** enzymes.

**Publication of a review “Human Enzymes for Organic Synthesis” by ACIB/TUG.**

These results are all part of the wider use of human enzymes that have been widely studied in various disciplines which have been reviewed with **ROBOX** inputs. This review shows that the number of reactions taking place in the human body is vast, and hence so is the number of potential catalysts for synthesis. In this the focus is on the application of human enzymes that catalyze chemical reactions in course of the metabolism of drugs and xenobiotics and some of these reactions were explored on the preparative scale.


7. **Education and Training**

**ROBOX** sees training and education will help create the next generation of biotechnologists and process bio – engineers who will use sustainable biotechnological methods by default and hence contribute to cleaner and safer chemical industries. Our overall aim is to demonstrate and promote the uptake of biotechnology based green and sustainable methodologies with a particular focus on the synthesis pharmaceutical and consumer chemicals using biooxidations.

As such we have contributed to numerous degree, masters and PhD training programs where **ROBOX** examples have been used to educate these young scientists.

Numerous masters and graduate projects have been based on **ROBOX** science and theses produced. We have also contributed to summer schools and international training events.
8. PROCESS DEVELOPMENT FOR BIOOXIDATIONS WORKSHOP

On the 18th to 20th of April at the Department of Chemical and Biochemical Engineering at our partner DTU located near Copenhagen had Professor John Woodley run this workshop to educate on new ways to think about biocatalytic process development for oxidation. Although it has long been recognised that biooxidation has great potential to make the chemical industry cleaner, safer and more economic there have been very few examples where this has come to fruition. One of the reasons for this is that the development of such routes requires a multi-disciplinary supply chain that individual academic groups and smaller companies do not have the full skill set to supply.

The aim of this workshop was to supply the skills to be able to assess biocatalytic reactions at a very early stage of development even before a process has been defined. Such assessment then not only guides protein engineering improvements of the enzymes but also enables a quick evaluation of process feasibility and allows identification of the real bottlenecks and allows efforts to be assigned to the true problems. This approach also offers the possibility of shortened implementation timelines that is another hurdle that causes these methods to be dismissed early on in industrial consideration.

The attendees gathered hands-on practice at using process metrics determined from laboratory scale experimental results and worked as groups to identify which improvement strategies would contribute most to a business case that can be then presented for development.

Improvement Strategies that could be used included -

**In-situ substrate supply (ISSS)**

Biocatalytic processes are often limited by the solubility of the substrate or its toxicity towards the biocatalyst both of which can reduce process productivity. Appropriate substrate feeding strategies such as
fed-batch, continuous process or using a multiphase approach (either liquid-liquid or solid-liquid systems) where the substrate is supplied continuously can give advantageous results. Estimates of potential improvements can clarify targets to meet cost requirements.

**In-situ product removal (ISPR)**

Many biocatalytic reactions are product inhibited, which creates a trade-off between product concentration and productivity. However, integrating downstream separation with the upstream reaction can allow the product to be recovered as it is produced. This reduces product inhibition by lowering the product concentration in the reaction and also lowers the cost of product isolation due to a higher product concentration downstream. Many separation techniques can be applied for ISPR, namely extraction, membrane separation, gas stripping or adsorption and others. ISPR is also useful for isolating unstable reaction intermediates before they degrade.

**Biocatalyst recycling and immobilization**

In a biocatalytic process, the cost contribution of the biocatalyst towards the overall cost of the process is often relatively high. Recovering and reusing the biocatalyst, by membrane separation or immobilization on a solid support for instance, can improve the biocatalyst yield and reduce the cost-contribution of the catalyst. However catalyst recycling is often limited by the stability of the biocatalyst and the cost of the immobilization support which must be taken into account.

**Process conditions and control**

As with traditional chemical reactions, biocatalytic reactions are sensitive to operating conditions, such as pH, temperature and substrate or product concentrations. As such, optimization of the operating conditions and control of these conditions at desired set points can significantly improve the process metrics.

**Improved oxygen supply**

Biooxidations are often limited by mass transfer of oxygen into the medium, which results in inefficient use of the biocatalyst. Oxygen transfer can be improved by increasing the area for gas-liquid mass transfer by sparging the reactor with air or oxygen for instance. Furthermore, the solubility of oxygen can be improved by raising its partial pressure, either by increasing the concentration of oxygen in the gas feed or by increasing the pressure of the system. Additionally scaling up a process generally allows better mass transfer.

**Biocatalyst formulation**

The use of resting whole-cell biocatalysts can limit a process as they consume oxygen and other nutrients and possibly even the substrate for cell maintenance. This can produce unwanted by-products. All this can lead to reduced yields of the desired product. Furthermore, mass transfer of bulky or non-natural substrates and products across the cell membrane can often be rate limiting. To overcome these limitations, isolated enzymes can instead be used as biocatalysts.

**Metabolic engineering**

Some biocatalytic processes take advantage of complex multistep biosynthetic pathways in whole-cell biocatalysts for the synthesis of a desired product. In these cases, metabolic engineering can be a powerful tool to insert additional reaction steps or remove unwanted metabolic pathways that compete for the substrate. Metabolic engineering can also be used to facilitate transport of the substrate into parts of the cell where the biosynthetic enzymes are situated or transport the product out of the cell for easier product
recovery without needing to disrupt the cells. Additionally, metabolic engineering can be used to increase the availability of cofactors, like NAD(P)H, that are often required by enzymes but are too expensive to feasibly supply to a reaction.

**Protein engineering**

Biocatalytic processes are frequently limited by the biocatalyst itself. In some cases, engineering of the process is sufficient to overcome these limitations and improve its economic feasibility. However, in many cases the enzyme itself needs to be engineered to operate at industrially relevant operating conditions using non-native substrates. Protein engineering can be used to improve the tolerance of enzymes towards organic solvents or toxic compounds, increase their stability at higher temperatures and increase their activities towards native or novel substrates. This can significantly improve the productivity and yield of the process. In P450-based processes, protein engineering is especially important to improve coupling efficiency. This ensures that available oxygen is used efficiently for production of the desired product and not unwanted by-products (such as hydrogen peroxide or reactive oxygen species) which can destabilize the enzyme.

**DTU found this workshop was very useful for the participants and would be interested in presenting it to others if interested –please contact us.**

9. **ROBOX Engagement with Wider Audiences**

We are always trying to engage a wide audience to generate interest in biotechnology and science as well as allowing people to be well informed about these crucial developments and the impacts on society.

Our partners at ACIB have run a series of demonstrations of introductory science aimed at younger scientists.

The kids were engaged in some simple experiments in the lab such as studying things under the microscope, watching yeast to fill a balloon with carbon dioxide, learning about biotechnology and enzymes, carrying out some pH experiments with red cabbage extract plus watching what happens if dry ice is added to soap water. Kids and teachers enjoyed the lab atmosphere.

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10. STAKEHOLDER ANALYSIS

ROBOX completed a deliverable Stakeholder Analysis [https://h2020robox.eu/wp-content/uploads/2017/02/D6.6.pdf](https://h2020robox.eu/wp-content/uploads/2017/02/D6.6.pdf) that was an interaction with groups of people outside of the ROBOX project to gather solid information on how the technology being developed was viewed and how the results obtained could be best exploited both in a technological sense and for informing and educating society in general.

The overall objective was to find out how the different stakeholder’s groups were able to contribute to the exploitation of ROBOX results, discover if stakeholders have a strategic interest in ROBOX results such as cooperation and where the positive attitudes towards ROBOX were. This meant we had to establish what knowledge do stakeholders have of bio-catalysis and bio-oxidations what knowledge they needed to become engaged.

We could then establish recommendations for stakeholder management based on the Stakeholder Analysis and what kind of information stakeholders would like to have to evaluate ROBOX results.

11. PARTNERS

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IF YOU WISH TO FIND OUT MORE ABOUT THE ROBOX PROJECT OR OUR TECHNOLOGY CONTACT US VIA OUR WEBSITE [HTTPS://H2020ROBOX.EU/](https://h2020robox.eu/)