DEVELOPING A VALIDATED TECHNOLOGY PLATFORM FOR THE APPLICATION OF OXYGEN DEPENDENT ENZYMES IN SYNTHESIS AND TRANSFORMATION OF ALCOHOLS

DESCRIPTION OF THE SCIENTIFIC AND TECHNICAL RESULTS

Work Package 1

BIOOX incorporated a fully integrated project structure in order to ensure that the process requirements are considered from the earliest stage of the project. This integration enabled efficient translation of research expertise into scalable process development, with outputs transferred directly to industrial partners for evaluation. A key early output from **WP1** was a *Vision for Success*, defining performance criteria for enzymes, productivity, yields, and reactor types for the selected oxidations studied in **BIOOX**. Progress beyond the initial state of the art was the result of an iterative process of planning and evaluation of research throughout the project, guided by the *Vision for Success*.

Enzyme discovery and characterisation in **BIOOX** was guided by the implementation of sophisticated computational techniques capable of analysing the huge wealth of published genetic and reaction data, much of which is often poorly curated or speculatively annotated. Without suitable bioinformatics tools, the huge volumes of information available in puplic databases would remain impenetrable and underutilised, and also potentially lead to the unnecessary duplication of previous research efforts. **Bio-Prodict BV** (hereafter **BPT**) developed a Cytochrome P450 (P450) protein superfamily information system at the start of the project and granted access to the partners. Called 3DM, this system collects, combines, and integrates many forms of protein-related data in order to facilitate the exploration of structure-function relations. The use of 3DM in **BIOOX** helped to guide enzyme discovery and engineering efforts by the other partners in **WP1-3**, and reciprocal feedback from partners also facilitated the development of new 3DM tools. All interested partners were trained in a three-day 3DM system course at **BPT's** site. An update of the 3DM system for cytochrome P450 enzymes was made available in 2016 at the request of project partners, providing a rich resource for the design of discovery and engineering strategies for this important target enzyme family.

Furthermore, **BPT** has developed a prototype patent landscape analysis system that can scan and integrate patent data for a complete protein superfamily. A web-tool was made that can be used by the project partners to analyse the patent landscape of the P450 3DM system, allowing evaluation of patented modifications in **BIOOX** systems where appropriate, but also enabling 3DM users to select novel enzymes with freedom to operate (FTO).

Table 1. Database statistics for the 2014 and 2016 version of the P450 3DM system.

Family	Structures	Alignment positions	Aligned proteins	Scanned articles	Mutations extracted
P450 (2014)	1,090	387	23,069	82,309	31,016
P450 (2016)	1,186	387	39,365	71,805	29,508

New enzyme discovery using bioinformatics approaches and studies of in-house enzymes, has enabled screening of hundreds of ODEs for activity towards substrates identified by the industrial partners, as well as the auxiliary enzymes for cofactor recycling. New metagenomic technologies have allowed identification and production of large panels of completely novel enzymes, including new classes of activity, with freedom to operate.

The **University of Manchester** (**UNIMAN**) used a 3DM database of alcohol oxidases to discover enzymes suitable for the oxidation of primary alcohol targets provided by **Firmenich SA** (**FIR**) for applications in the flavours and fragrances sector. One enzyme in particular showed activity towards many substrates and was chosen for further development. High-throughput screening of enzyme variant libraries targeted substitutions to the active site and in flexible loop regions. This work yielded a six-point variant of choline oxidase from *Arthrobacter chlorophenolicus* with improved activity towards the oxidation of hexanol; 20 fold increase in the turnover number (k_{cat}) from the wild type. 100mg of hexanol was oxidised to aldehyde with negligible production of the acid over-oxidation production, and was isolated with a yield of 72%. The enzyme was further improved in its tolerance for organic solvents and activity at higher temperatures. In a screen of 50 primary alcohols, this variant showed improved activity for 90% of the substrates compared to the wild type enzyme, thus yielding an enzyme that could be applied as a more general alcohol oxidase.

Prozomix Ltd (**PROZO)** used proprietary technologies to survey sequence space to achieve maximum diversity in the selection, cloning, and production of enzymes, rapidly creating panels of relevant target enzymes to supply the consortium. Panels were developed for three key enzyme activities required in **BIOOX**; NAD(P)H oxidases (NOXs), alcohol oxidases (KREDs) and P450s. **PROZO** developed in-house metagenomic libraries as a rich genetic resource allowing discovery of enzymes entirely unknown in public databases. This aspect gained additional importance following the implementation of new legal frameworks by the EU and member states for Access and Benefit Sharing (ABS), governing the research and commercial use of "genetic resources", i.e. the *Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity* and EU Regulation 511/2014. These frameworks aim to ensure fair and equitable sharing of benefits arising from the utilisation of genetic resources, thereby contributing to the conservation and sustainable use of genetic resources (such as the genes encoding enzymes). Development of metagenomes sampled from local environments rather than third party nations not only ensured legal compliance, but is also consistent with the principles of responsible research and innovation. RRI is an increasing prominent aspect of research and development, and embodiment of those principles in the technology developed through the project serves both to raise the awareness of such issues amongst particularly the **BIOOX** early career scientists, and also distinguishes the technologies in the marketplace. The metagenomic approach to enzyme discovery created the opportunity to ensure freedom to operate for all **BIOOX** enzymes by avoiding patented sequence space, with the additional commercial advantages to **PROZO** of avoiding known sequences commonly duplicated across the commercial enzyme panels of competitors and hardening the technology against reverse engineering.

A total of 698 putative KRED targets were identified from **PROZO**'s in-house strain collection. Over 180 of these were rapidly cloned by **PROZO**'s proprietary GRASP™ cloning protocol and of these, 145 yielded soluble protein. The first 96 enzymes were arrayed in kREDy-to-go™ format, a flagship colorimetric screening plate system which allows rapid and facile identification of enzymes with activity towards test substrates. Further enzymes were discovered to complete **PROZO**'s second such plate, i.e. the kREDy-to-go™ plate 2.

UNIMAN screened kREDy-to-go™ plates 1 and 2 against a panel of 21 primary alcohols from **FIR** and identified hits for all targets. In particular, KRED(020) and KRED(150) were selected for further study in **BIOOX**.

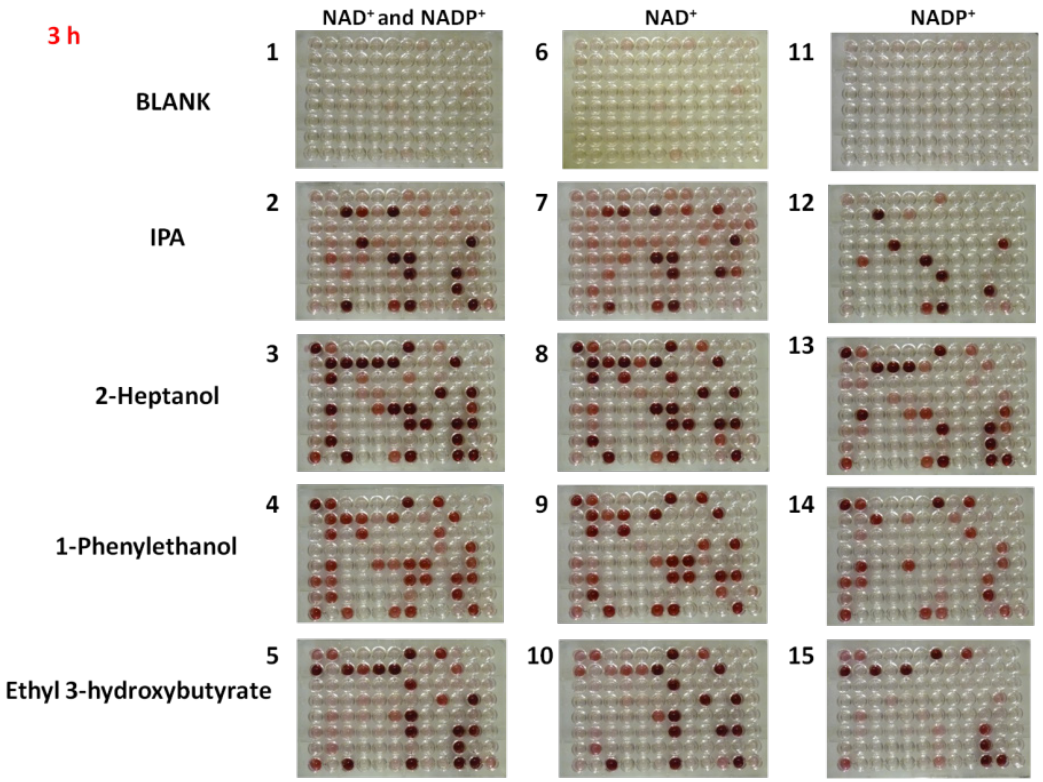

Figure 1. kREDy-to-go™ characterisation of the first 96 **BIOOX** novel / diverse KREDs (097-192) discovered and produced by **PROZO** during M1-12**.** Plates 1-5 contained both cofactors, while 6-10 and 11-15 contained either NAD^+ or $NADP^+$, as indicated, respectively. The blank plates contained no alcohol, whereas a 1 % (v/v) substrate concentration was employed with respect to the 4 model alcohols as detailed.

A total of 29 highly diverse putative NOXs were targeted for development as part of **WP1**. These targets were distant homologues of known *L. brevis* (NADH specific) and *L. sanfranciscensis* (dual cofactor specific) enzymes. The former of these was the subject of a then current, but now lapsed, patent (US2005064570). However, the patent only claimed primary sequence space of >80 % identity to the *L. brevis* enzyme. Careful selection of the panel using bioinformatics tools meant that the closest expressed and active homologue developed (NOX 2) was at only 62% identity, and all four novel NOXs of this type therefore had FTO in any case. Six constructs containing genes from a known nitroreductase family yielded five soluble novel NOX recombinant proteins, one of which - NOX 25 - exhibited significant NOX activity and all of which demonstrated strong preference for NADH as cofactor. All of the NOX enzymes were produced by **PROZO** and supplied to the rest of consortium on demand, with a number explored by the **Technical University of Denmark** (**DTU**) and **UNIMAN** as lead candidates in combination with KRED(020) and KRED(150) for the oxidation of hexanol.

Table 2. Protein family (Pfam) architectures identified and evenly sampled towards novel NAD(P)H oxidases.

Pfam architecture	Targets selected	Cloned	Expressed	Activity
PF00070 / PF07992 / PF02852	11 genomics 1 metagenomics	NOX 1 NOX 2 NOX 3 NOX 5 NOX 6 NOX 10	- NOX 2 NOX 3 NOX 5 - NOX 10	- Y Y Y - Y
PF00258 / PF00753	2 genomics + 1 via synthesis 1 metagenomics	NOX 12 NOX 13 NOX 26	- NOX 13 -	- - -
PF00724 / PF00070 / PF07992	3 genomics + 1 via synthesis	NOX 15 NOX 16 -	- - -	- - -
PF00881	9 genomics	NOX 18 NOX 19 NOX 21 NOX 23 NOX 24 NOX 25	NOX 18 - NOX 21 NOX 23 NOX 24 NOX 25	- - - - - Y
Total	**29**	**17**	**10**	**5**

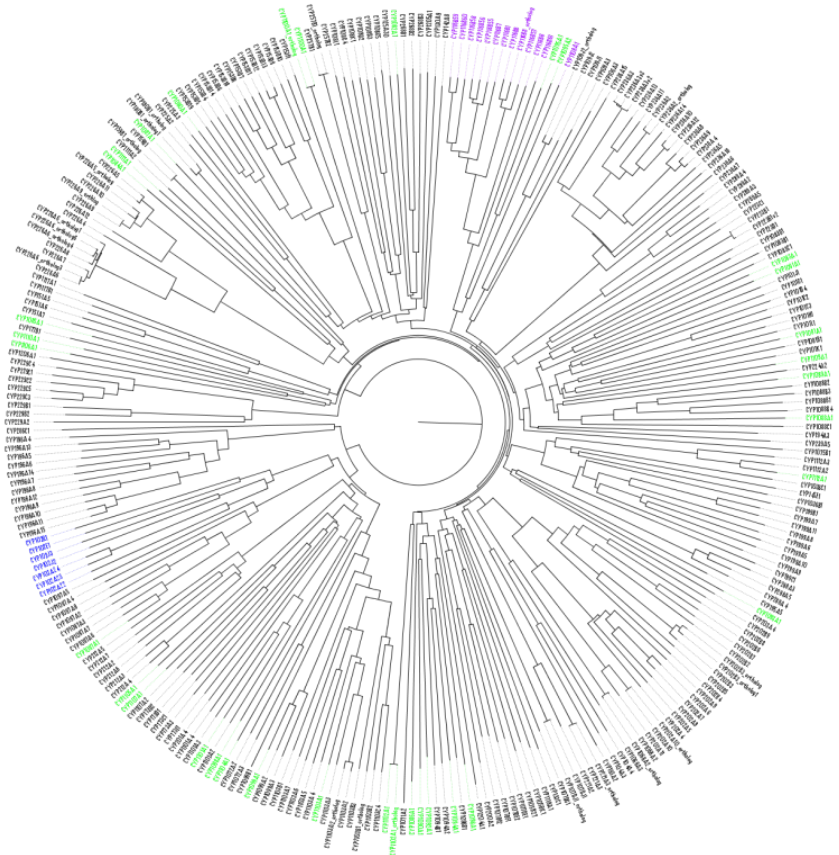

Figure 2. Phylogenetic tree of all P450s identified in the PROZO Metagenomes. Newly identified families are highlighted green, the Class VII P450s purple and the Class VIII P450s blue.

P450s available from **PROZO**'s in-house metagenomes cover a number of architectures, including Class I, VII and VIII, and were used to further develop panels of greater interest right to the end of the project. The number of targets that were available for harvesting was large and covered huge diversity. Interestingly, of those studied in greatest detail as part of **BIOOX,** the Class VII and Class VIII P450s form distinct clades, demonstrating that there is much more work to be done with this resource beyond the end of the project.

The Class VII enzymes were pursued with the greatest urgency, since the literature suggested activities of significant commercial interest both within and outside the consortium, including towards compounds such as diclofenac (a nonsteroidal anti-inflammatory drug) and 7-ethoxycoumarin (a fragrance molecule). Of 14 metagenomic Class VII members cloned four produced soluble enzymes, which were supplied to partner **UNIMAN**; CYP116B59, CYP116B60, CYP116B1 and CYP116B1_ortholog. Preliminary data also demonstrated that each soluble Class VII candidate had some degree of activity towards either diclofenac or 7-ethoxycoumarin. A panel of Class VIII enzymes were supplied to partners **UNIMAN** and the **University of Stuttgart** (**USTUTT**) and after screening, a number of lead enzymes were then utilised in further deliverables and transferred to **FIR** for further application development and evaluation in fermentation processes.

Table 3. All reductases utilised in the study, including cloning vector and expression data

Reductase	Vector	Expression
Putidaredoxin reductase (65a)	pET-28a	Soluble
Spinach ferredoxin reductase	pET-28a	Soluble
Acinobacter reductase	pET-28a	Soluble
Spin_red_91% homolog	pET-28a	Insoluble
Q1_90% homolog	pET-28a	Insoluble
Put. Red. 70% homolog	pET-28a	Soluble
Cyp153A reductase 1	pET-28a	Soluble
Cyp153A reductase 2	pET-28a	Insoluble
E. coli Reductase 353	pET-28a	Soluble (weak)
E. coli Reductase 354	pET-28a	Soluble
E. coli Reductase 355	pET-28a	Soluble
Metagenome Reductase 1	pET-28a	Soluble
Metagenome Reductase 3	pET-28a	Soluble
Human P450 Reductase	pET-28a	Soluble

Class I P450 enzymes were further studied, collecting data for use in combination with non-native ferredoxin and ferredoxin reductase partner proteins. **PROZO** demonstrated that there is no necessity to find native electron transfer protein pairs to activate Class I P450 enzymes. Indeed, functional assays demonstrated no fewer than five non-native ferredoxin/ferredoxin reductase pairs to be more active than the most utilised system in the literature, that of $P450_{cam}$. Further data was collected from an expanded panel of ferredoxin reductases which were assayed against a panel of approximately 25 ferredoxins. The reductases are now part of a **PROZO** panel which can be screened for simple identification of auxiliary partners for use in P450 systems.

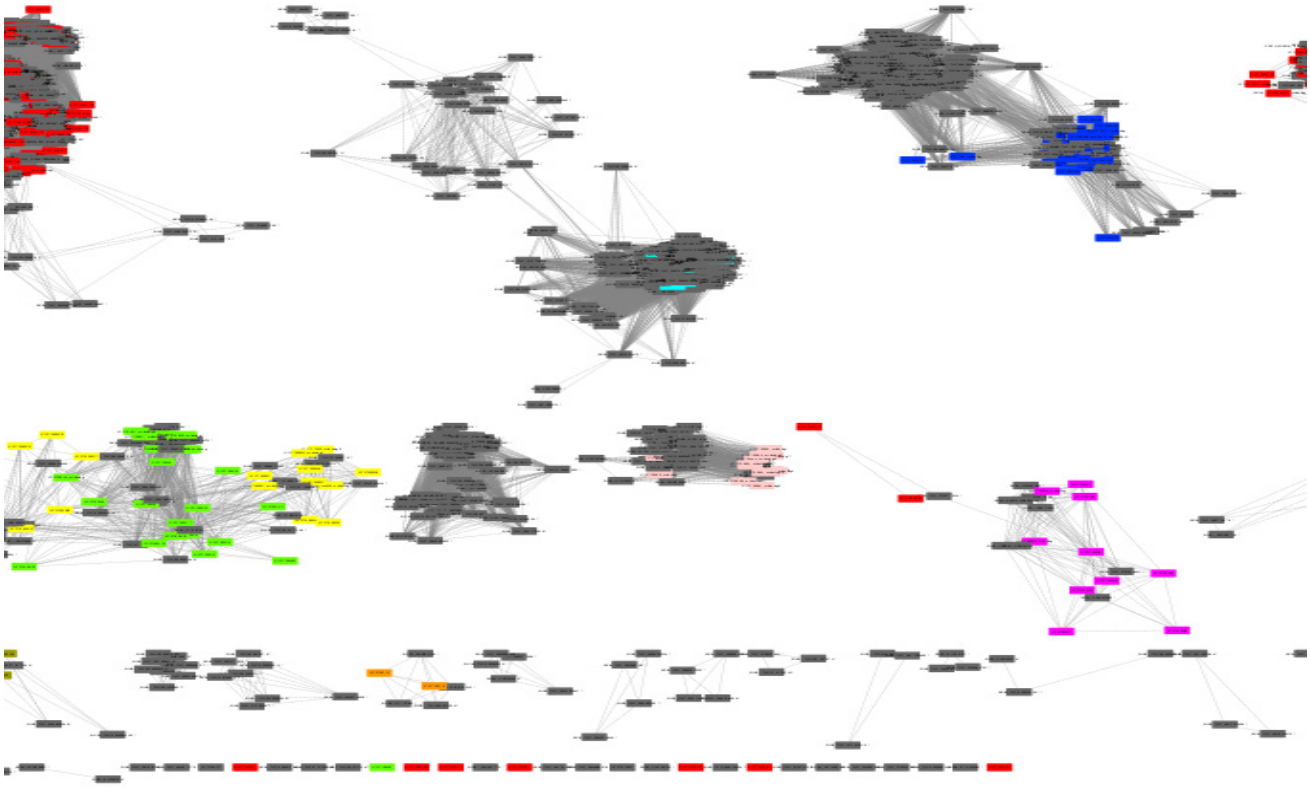

Figure 3. Network of Class VIII P450 sequence space generated by pairwise-alignments of full-length sequences at an edge-cut off value of 55% sequence identity. Node colour denotes subfamily of CYP name assigned P450s, unnamed P450 sequences coloured dark grey. Visualised in Cytoscape v3.5.1.

Ultimately, **PROZO** developed a novel panel of 48 catalytically self-sufficient Class VII and VIII P450s, each with known activity. Catalytically self-sufficient P450s are natural (or engineered) fusion proteins where the CYP and redox domains exist as a single protein. These are attractive systems for application because they do not require the auxiliary partner proteins essential for the activity of other P450 classes. Use of self-sufficient P450s simplifies development and application, and can also help to reduce the cost of the biocatalyst. Moreover, members of these classes have been identified with activities suitable for key **BIOOX** target biotransformations, thus demonstrating the biocatalytic utility of the panel. Subsequent to this successful evaluation by the consortium, the novel panel was produced in prototype 96-well commercial (freeze-dried) format and made available for screening by potential customers, thus significantly contributing to the **PROZO** Biocatalysis Enzyme Toolkit. Continued development beyond the project will see a final panel of 96 self-sufficient P450s fully commercialised in 2018, enabling new biocatalytic syntheses and drug metabolism studies, where P450s possess crucial utility. The new panel will be signposted in an imminent publication reporting the large networks of novel self-sufficient P450s discovered and annotated by **PROZO** as part of **WP1**.

USTUTT found approximately 20 putative monooxygenases by shotgun sequencing of two arene-degrading strains (*Arthobacter* sp. and *Phenylobacterium immobile*). While *P. immobile*

did not grow on sesquiterpene substrates, *Arthrobacter* sp. was able to grow using the target molecules bisabolene and premnaspirodiene as sole carbon sources. This behaviour is typical of strains producing P450s with activity towards the target as part of the metabolitic pathway of the orgsanism. In addition to *Arthrobacter* sp., *Beauveria* sp. was investigated by proteomic analysis, since it was able to convert sesquiterpene substrates in initial investigations. Seven putative CYPs were identified along with one bifunctional reductase as promising candidates for further studies. The proteomic analysis data as well as the corresponding P450-sequences were shared with **FIR** and **BiCT srl** (**BiCT**) to support work towards functional enzyme production in *Saccharomyces cerevisiae* and *Aspergillus oryzae*.

To further increase the panel of potential ODEs the organism *Chondromyces apiculatus* was investigated, which is closely related to *Sorangium cellulosum* a strain known for P450s active towards sesquiterpenes. Putative P450 genes were cloned and the enzymes produced for activity screening. Hydroxylation activities were detected for a number of enzymes: a putative steroid hydroxylase CYP125E; a member of the CYP264 family; and a member of the CYP109 family. The redox partners from *P. putida* (Cam A and Cam B) were determined to be the most efficient electron transport system. The P450 from the CYP109C family demonstrated broad substrate scope, ranging from mono- and sesquiterpenes, to nonsteroidal anti-inflammatory drugs like diclofenac, and steroids.

In the course of this project, **FIR** has collaborated with **USTUTT**, **PROZO** and **UNIMAN** on the discovery and development of P450 monoxygenases capable of transforming selected terpene compounds to produce flavor and fragrance molecules. Candidate P450 monooxygenases were screened and the positive hits functionally characterised by **USTUTT** and **UNIMAN**, prior to enzyme engineering to develop activities towards the prototypical terpene hydrocarbons produced by **FIR** using submerged fermentation of engineered bacterial cells. **FIR** produced and transferred approximately 10g of each of the sesquiterpene substrates to the academic partners enabling this initial P450 screening.

Work Package 2

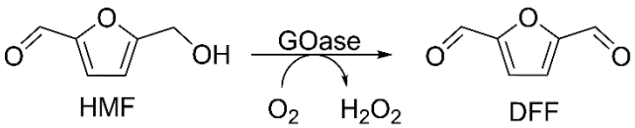

Figure 4. The oxidation of 5-hydroxymethylfufural to 2,5-diformylfuran.

2,5-diformylfuran (DFF) is an attractive intermediate with potential applications in a variety of polymer formulations as a bio-based alternative to petroleum based plastics and other materials. The oxidation of hydroxymethylfurfural (HMF) to produce DFF was a high priority target identified by **BASF** during the development of the *Vision for Success*. Engineering galactose oxidase (GOase) for production of DFF from 5- HMF was a primary target for **UNIMAN** within **BIOOX**. **UNIMAN** developed new GOase variants from two separate engineering experiments that showed improved conversion in biotransformations at high substrate loading 100mg/mL (~800mM) semi-crude HMF. A screen targeting active site residues was performed

at reduced oxygen concentration to identify variants with greater oxygen reactivity suitable for use in the relatively low oxygen environments of the reactors developed in **WP5-7**. The other library targeted non-active site residues and was performed against low activity substrates to identify variants with generically improved alcohol oxidation activity. A generation of variants created through combining the unique mutations in these top hits resulted in further productivity improvements at this high substrate loading, with the best yield to date of 76% conversion to DFF within 6 hours.

Continued optimisation of the reaction for oxidation of HMF by GOase G2 variants has identified industrially applicable solvents that are more suitable for application in biphasic reactions. Equally importantly, some of these new solvents lead to enhanced production of DFF in biotransformations scaled up to 100mg/ml of crude HMF substrate loading. Extensive GOase reaction optimisation and process development performed during **BIOOX** have resulted in joint patent applications by **BASF SE** (**BASF**) with **UNIMAN** and **DTU**.

Further characterization by **UNIMAN** of the aldehyde oxidase activity of M_{3-5} towards a panel of substrates identified trends in the characteristics of substrates that can be oxidized by the enzyme. Notably, several of the nitrogen-containing heteroaromatic alcohols could be sequentially oxidized twice to produce carboxylic acids via the aldehydes in high conversions. This unique activity has potential applications in production of heteroaromatic carboxylic acids which are often used in the synthesis of pharmaceuticals.

USTUTT developed enzymes identified for the hydroxylation of **BIOOX** target substrates including sesquiterpenes, norisoprenoids, and fatty acids. A number of examples from various enzyme classes, including Rieske non-heme iron dioxygenases (RO) and several P450 monooxygenases from the **USTUTT** in-house library, along with those discovered in **WP1**, were chosen as biocatalysts. Additionally, seventeen natural P450 fusion enzymes were provided as lyophilized Cell Free Extract (CFE) from **PROZO** for the screening of hydroxylation activity.

USTUTT pursued engineering of one enzyme provided by **PROZO**, generating enzyme variant libraries with active site substitutions which were screened for improved activity toward **BIOOX** targets, including the flavour and fragrance molecule targets of **FIR**. The most active new variant showed 18-fold higher activity toward regioselective hydroxylation of β-ionone than the wild type enzyme, and others from the full panel of active enzymes provided access to new regioselectivities for hydroxylation of several sesquiterpene substrates. Similarly, **UNIMAN** has made and screened libraries of **PROZO** enzyme variants, resulting in an enzyme with 16-fold higher activity and greater product specificity in hydroxylation of β-bisabolene. Reaction optimisation through addition of an immiscible cosolvent further improved performance and reaction efficiency of this enzyme.

Through enzyme engineering, **USTUTT** generated a panel of variants of the RO naphthalene dioxygenase with expanded substrate scope for mono- and di-hydroxylation of arene substrates. A P450 variant with increased activity for C12 fatty acid ω-hydroxylation was also engineered. Omega hydroxylation of fatty acids is an important reaction both in the study of

physiological processes, but also in industrial synthesis. Alongside this several variants of a novel P450 enzyme were created with significantly improved conversion and entirely new product profiles for production of hydroxylated sesquiterpenes and β-ionone. The P450 variants developed within **BIOOX** have enabled production of both target and unanticipated, novel flavour and fragrance compounds at greater titres than were previously accessible.

Critical to the industrial application of biocatalysis is the ability to produce enzymes in a choice of host organisms that provide sufficient activity with suitable handling characteristics of the production organism. Additionally, freedom to operate (FTO) is a key requirement in order to avoid costly licencing of production vectors and host strains. **BiCT** constructed a new class of modular expression plasmids for the FTO production of enzymes in industrial strains of *Aspergillus* fungus species. These *Aspergillus* sp./*E. coli* shuttle plasmids enable facile integrative transformation of different strains of *Aspergillus*. Transformation protocols were defined for a number of industrially relevant species, including *A. oryzae, A. niger* and *A. niger var. awamorii*. *Aspergillus* expression vectors were assembled employing modular design in order to allow the rapid replacement of one module when new sequences, either synthetic or cloned directly from the genome, become available. Moreover, **BiCT** have continued to expand their sequence database focusing on both constitutive and inducible promoter sequences, export signals, and the marker genes that can be used to rapidly test different combinations of both, in order to achieve efficient production of the proteins of interest. These provide a suite of genetic tools offering multiple options to tune biocatalyst production with fine control.

The genetic constructs were optimised for production of bifunctional P450s CYP153/CPR from *Marinobacter aquaeolei* and CYP52A10 from *Beauveria bassiana* in *Aspergillus* sp. and cloned in a suitable vector. The galactose oxidase G2 variant was produced in *Aspergillus oryzae* and transferred to **BIOOX** partners for comparison with enzyme produced in the alternative expression systems available within the project (**PROZO's** production in *E. coli*, and **BASF's** production in *Saccharomyces cerevisiae***)**. Additionally, two challenging ODEs were successfully produced in soluble and active forms using the **BiCT** FTO *Aspergillus* system: the unspecific peroxygenase UPO1 from *Agrocybe aegerita* and the laccase LCC1 from *Coprinopsis cinereus*. Unspecific peroxygenases are an exciting class of enzymes offering industrially important activities similar to those of P450s but with simplified implementation compared to those enzymes. However, unspecific peroxygenases are extremely limited in application due to their notoriously difficult production; they are largely know fungi including mushrooms, which are impractical for industrial use. Development of heterologous production strains and protocols for UPO1 were therefore a key target for **BiCT**. Unspecific peroxygenase were effectively produced as concentrated, spray-dried material from *Aspergillus*, and these were made available to other **BIOOX** partners in multiple formulations.

As part of **WP1**, a number of putative enzyme panels were developed by **PROZO** targeted towards **BIOOX** objectives and supplied to the consortium. As part of **WP2**, further effort was made to improve upon the panels, largely via homologue sampling. In the central panel, a total of 27 new NOX homologues were identified, 12 of which were produced in soluble form and shown to be active.

Pre-commercial industrial prototyping of the KREDs discovered and produced in colorimetric screening format during **BIOOX** were validated by both **PROZO** customers and consortium partners. This demonstrated the utility of large panels of this commonly employed class of biocatalysts, and thus showed the need to expand the panels during **WP2**. Despite the fact that KREDs were already industrially applied, primarily for chiral secondary alcohol synthesis, there was still demand for further expanded panels offering enhanced properties and broader substrate scopes to access a wider range of products. The results in **BIOOX** from both academic partners and large end-users were of great fundamental and strategic value to **PROZO**, as it indicated that a much larger panel was actually required to meet ultimate industrial appetite, where the identification of off-the-shelf, process-ready biocatalysts saves incredibly valuable process development time. **WP2** efforts therefore focused on mining natural three-dimensional sequence space from in-house **PROZO** metagenomes, rather than mutating a small collection of earlier hits to create diversity as was previously proposed. This metagenome mining led to development of kREDy-to-go™ plates 2, 3 and 4. Furthermore, moving entirely to mining of UK derived metagenomic libraries protects against potentially huge future liabilities for **PROZO** under the Nagoya Protocol and provides significant commercial advantages. The utility of these panels was further demonstrated in **WP6** by providing screening materials to potential R&D partners and customers both within and external to the **BIOOX** project.

BPT has developed several 3DM protein information systems for the partners in **WP2** and trained these partners in the use of such 3DM systems. **BPT** hosted a 3-day course for the project partners to train them in using the generated systems in smart library design. This course started with training in visualizing protein structures and continued with an introduction to the 3DM systems technology. The participants were taught how to analyse data and how to answer biological questions using the 3DM system. Alongside maintenance of 3DM systems to enable enzyme discovery and engineering, **BPT** performed product development to launch the new 3DM patent analysis tool developed within **BIOOX**. The ability to mine patent literature represents an extremely valuable addition to the software package. Although it remains in early development, it is a novel tool with high potential to deliver value for the field, and has already attracted considerable attention through product validation activities performed in **WP6**. With further development planned, this tool will increase the value and appeal of the 3DM systems, giving clients a greater incentive to purchase a software license.

Over the course of **BIOOX**, **FIR** has collaborated with **USTUTT** and **UNIMAN** on the discovery and development of P450 monoxygenases active on selected terpene compounds. Candidate P450 monooxygenases have been screened, eventually engineered and functionally characterized by **USTUTT** and **UNIMAN** against prototypical terpene hydrocarbons produced by **FIR** using submerged fermentation of engineered bacterial cells.

The best candidate P450s identified by **USTUTT**, **UNIMAN**, and **PROZO** from approximately 20 different CYPs tested were transferred to **FIR** for evaluation in fermentative production strains. **FIR** functionally expressed these P450s in bacterial cells engineered to overproduce the monooxygenase terpene substrate from a simple, sustainable, and cost-effective carbon source. Performance of the engineered bacterial strains was initially assessed in small-scale functional assays, and the activity of these biocatalysts was optimised by

systematically testing different expression constructs, strain genetic backgrounds and production conditions. In order to further evaluate the performance of these more productive *E. coli* strains at scale, two strains producing the P450 susbtrates humulene and bisabolene were moved to fed-batch, biphasic fermentations in laboratory-scale bioreactors. Under optimised conditions, the strains achieved peak titres of humulene- and bisabolene-epoxides of >300mg/L. A downstream process was then implemented to recover about 4g of product for analytical characterisation of the product molecules and organoleptic evaluation by an **FIR** Master Perfumer in **WP 3** and **5**.

Work Package 3

Biocatalysts offer many advantages over chemical routes, principally inherent selectivity and relatively benign reaction conditions, however they also often require expensive and unstable cofactors and can themselves become unstable when isolated from cells. Moreover, the cost of enzyme purification may be prohibitive in many cases where the overall economy of the process does not support it. Although enzymes can often be deployed as cell free extracts which can be relatively cheaply produced requiring only that cells are disrupted to release a crude mixture of enzyme and other cellular debris, this may not result in the required enzyme or cofactor stability. In such cases, production of whole cell biocatalysts may be appropriate, where the enzyme is retained in either metabolically active growing cells or inactive resting cells. Additionally, whole cell biocatalysts can incorporate a number of enzymes acting in sequence to perform longer biosyntheses, deriving complex products from simple and sustainable feedstocks by exploiting combinations of natural and synthetic anabolic pathways to build molecules. The overall objective of **WP3** within the **BIOOX** project was development and delivery of suitable whole cell biocatalysts for the syntheses of industrial relevant omega hydroxylated fatty acids, alkenes and oxy-functionalized terpenes. To achieve these objectives Rieske non-heme dioxygenases and catalytically self-sufficient P450 monooxygenases were selected in **WP2** for initial studies (CYP116B3 from *Rhodococcus ruber* DSM 44319 and CYP153A from *Marinobacter aquaeolei*, fused to the non-natural redox partners CPR from *Bacillus megaterium* or Pfor from *Rhodococcus ruber* DSM 44319).

Early work by **USTUTT** focused on the optimisation of expression protocols for production of the P450 enzymes and ROs. For the RO, naphthalene dioxygenase (NDO) a fractional factorial screening design was employed using MODDE software. This design of experiment (DOE) approach allows efficient use of time and resources by enabling smaller numbers of individual experiments to be conducted in order to gather the data required to define specific operating parameters. Thereby, measures in four areas were considered for the optimisation of expression: expression temperature, expression time, aeration rate, and agitation speed required for efficient mixing. The results suggested the following conditions as optimal for expression: expression temperature of 18°C, expression duration of 20h, aeration rate of 1.6vvm, and agitation speed of 300 rpm. The calculated optimal conditions for expression were verified experimentally by performing biotransformations on two substrates, naphthalene and α-methylstyrene. In both cases, the highest conversion was achieved using the calculated optimised expression parameters. Complete conversion of naphthalene to the corresponding

dihydridiol was observed, whereas the conversion of α-methylstyrene resulted in a product mixture containing both 2-phenyl-1,2-propanediol and the allylic monohydroxylated 3-hydroxy-2-phenylpropene.

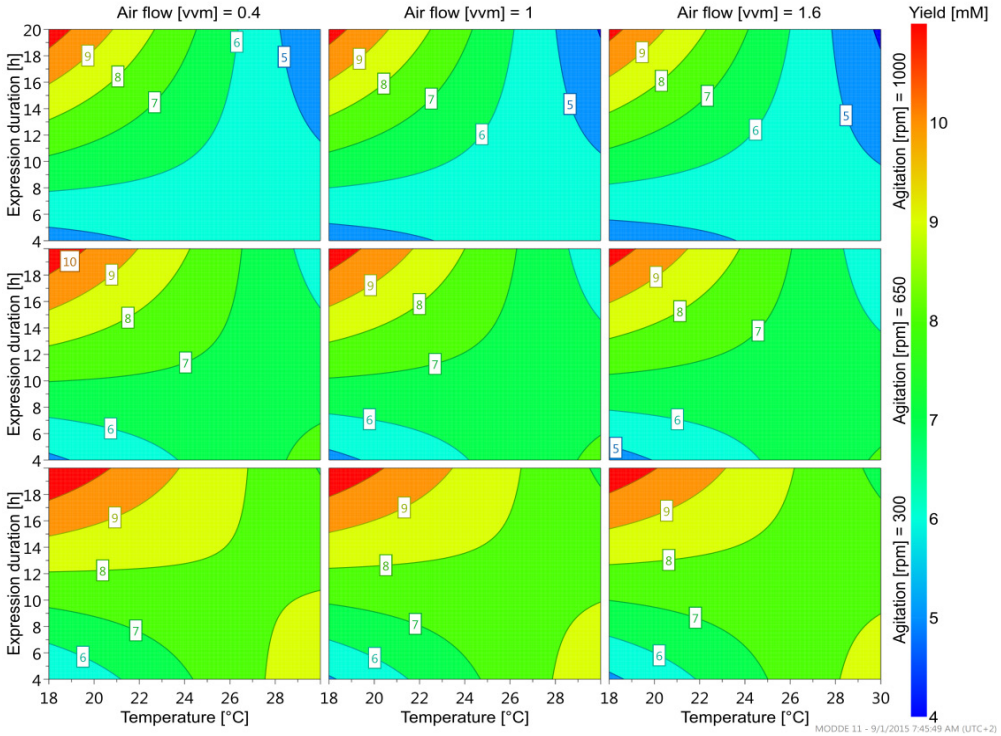

Figure 5. Fractional factorial screening design based on a design of experiment approach calculated in MODDE software.

Optimised expression protocols to produce the P450 enzymes were also established by **USTUTT**, and in particular the choice of expression vector was discovered to be critical to protein levels, rather than expression conditions. P450 enzymes are capable of catalyzing a broad range of reactions with excellent region- and stereo-selectivity, but their industrial use remains limited in no small part due to the genetic instability of constructs and low enzyme yields with often poor activity. Therefore, development of optimized expression strains and protocols represents significant progress beyond current applications in drug metabolism screening, towards wider use of P450s for biocatalysis. High level, reproducible expression of CYP153A$_{M.aq}$(G307A)-PFOR L2 was achieved using the strictly regulated pBAD vector system. A whole cell biotransformation using this biocatalyst achieved conversion of palmitic acid to the ω-hydroxylated fatty acid, with fivefold improvement over the previous systems. However, scale-up applying the same conditions for aeration, feeding and induction reported in previous studies in order to produce the biocatalyst in 5L fermentations was unsuccessful, resulting in inclusion body formation.

USTUTT studied the stability of artificial P450 fusion constructs, noting the genetic instability of previously reported biocatalysts used for whole cell biotransformations. By using the arabinose-inducible pBAD vector system the integrity of the fusion construct, and therefore the enzyme activity retained over time, could be enhanced significantly compared to other vector systems. During whole cell biotransformations with palmitic acid decreased product formation was observed due to the depletion of the product by the host. To overcome this issue the β-

oxidation pathway, which is part of the catabolic pathway through which molecules are broken down by bacteria, was inhibited by incorporation of the ΔfadD deletion into the arabinose-catabolism deficient *E. coli* BW25113 strain required for pBAD. Furthermore, the transporter system FadL was co-expressed to enhance the transport efficiency to achieve higher product yields. The *E. coli* host strain along with the plasmids pBAD33 CYP153A$_{M.aq}$(G307A)-PFOR L2 and pBAD18 FadL were transferred to **DTU** for optimisation of the biocatalyst fermentation at 5L scale.

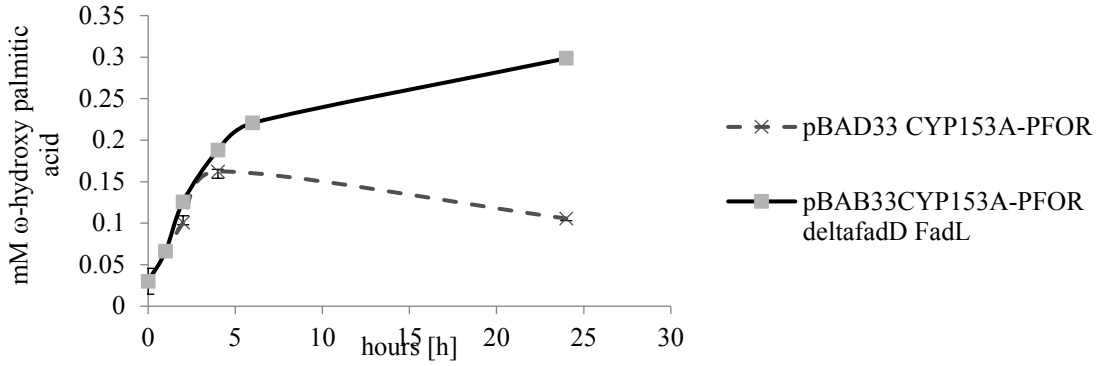

Figure 6. Conversion of palmitic acid to 16-hydroxy palmitic acid by CYP153A6$_{M.aq}$G307A-PFOR L2 in pBAD33 expressed in *E. coli* BW25113 with (boxes) and without (crosses, dashed) the transport system FadL and the deletion fadD.

Biocatalyst improvements from **WP2** were transferred directly into whole cells experiments performed by **USTUTT** in **WP3**. A possible anchor position for fatty acid substrates was identified in the crystal structure of CYP153A$_{M.aq}$, which by applying semi-rational design strategies, led to discovery of an improved variant Q129R. Significant yields of ω-hydroxy dodecanoic acid were obtained using this biocatalyst, comparing favourably with the previous best variant identified G307A. Indeed, once normalized for P450 protein content, the new variant displayed twofold enhanced performance in whole cell biotransformations. The strain *E. coli* BW25113 ΔfadD and the plasmids encoding the variant CYP153A$_{M.aq}$(Q129R)-PFOR L2 were transferred from **USTUTT** to **BASF** for further optimisation and evaluation in fermentation.

Table 4. Whole cell biotransformation towards dodecanoic acid using *E. coli* BW25113 ΔfadD harboring pBAD33 CYP153A$_{M.aq}$(G307A)-PFOR L2 respective pBAD33 CYP153A$_{M.aq}$(Q129R)-PFOR L2. Product (ω-hydroxy dodecanoic acid) after 8 h detected.

Variant	P450 content [μM]	ω-hydroxy dodecanoic acid [μM]
CYP153A$_{M.aq}$ (G307A)-PFOR L2	2.33	495
CYP153A$_{M.aq}$ (Q129R)-PFOR L2	1.47	530

The RO libraries of naphthalene dioxygenase viariants generated in **WP2** were screened *in vivo*. Nearly all variants could achieve 80% or higher conversion of the natural substrate naphthalene. The library was next screened for activity towards the unnatural substrates α-methylstyrene and *R*-limonene. Biotransformation of α-methylstyrene, an important drug precursor molecule, gave two detectable products in modest yield; the monohydroxylated

alcohol 3-hydroxy-phenylpropene, and the dihydroxylated 2-phenylpropan-1,2-diol. The stereoselectivity was also determined and reached up to 88% enantiomeric excess for the *S*-Diol with the single variant V260I. In contrast, the fragrance molecule *R*-limonene, was converted to two monohydroxylated alcohols; carveol and menthe-1,8-dien-10-ol, once again in modest yields. Notably, variants were identified which displayed complementary product distributions; single variant H295A showed a high selectivity for production the monoterpene carveol, whereas the variant A206G was more active but showed a higher selectivity towards production of mentha-1,8-dien-10-ol.

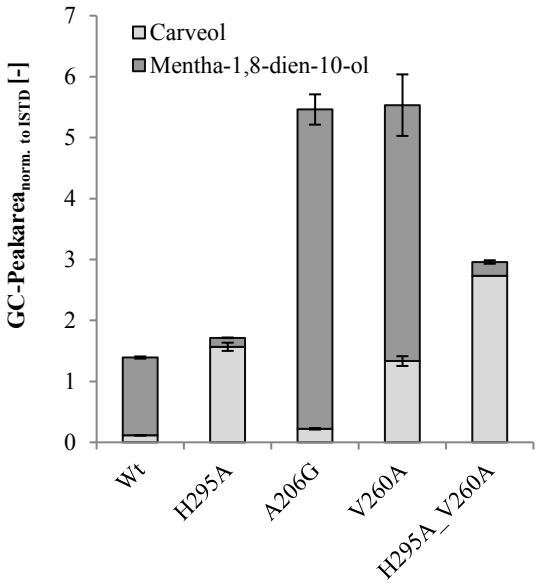

Figure 7. *In vivo* biotransformation of 10 mM *R*-limonene resulting in carveol and mentha-1,8-dien-10-ol with NDO_{Wt} and variants thereof at 30 °C for 20 h.

USTUTT further focussed on the oxy-functionalisation of a set of terpenes for application by **FIR**. Activities in **WP1**, **2** and **3** encompassed a full work flow where ODEs were identified and improved, before implementation in whole cell processes for the fermentation of valuable flavour and fragrance products. In collaboration with **PROZO** a set of 17 natural P450 catalytically self-sufficient fusion enzymes was screened for hydroxylation activity towards the **BIOOX** target substrate set. One candidate in particular was identified with the required activity, and further enhancements were attained using the 3DM database of **BPT** to implement semi-ration design strategies for protein engineering. The *in vivo* conversion of β-ionone to the allylic 4-hydroxy-β-ionone was improved 17-fold in some of the variants, however two single variants of enzyme P450_014 F95A and F95G exhibited interesting changes in substrate specificity compared to that of the wild type. Hydroxylated products of the **BIOOX** target substrates humulene, premnaspirodiene and longifolene could be identified in experiments using the two variants *in vivo*. These best candidate enzymes were transferred to **FIR** for further characterization of the products in the terpene producing strain.

In parallel with the development of the fusion enzymes, novel enzymes from the Class I and III P450 systems for the oxy-functionalisation of terpene substrates were investigated by **USTUTT**. Whereas Class VII and VIII P450s incorporate the CYP and redox domains in a single protein, Class I and III systems consist of three separate protein components; the CYP

and two auxiliary proteins required for electron transfer and therefore the overall the activity of the system. Although the use of fusion protein has the potential to simplify processes and applications, Class I and III systems remain of interest due to their important substrate scopes and activities.

Efficient P450 whole cell biocatalysts based on the pBAD18/pBAD33 system were developed in **WP1** and **2**. Following expression optimisation, the *Arthrobacter* sp. P450 system identified in **WP1** was tested as whole cell biocatalysts in **WP3**. Biotransformations of the natural substrate homovanillic acid yielded high product titres of up to 1.77 g L^{-1} of demethylated product, representing good productivity for a system of this type. Moreover, significant product titres of 0.5g/L of the **BIOOX** target 4-hydroxy-β-ionone were achieved in biotransformations using the *in vivo* system. CYP109C from *Chondromyces apiculatus* was produced using a similar whole cell pBAD system, incorporating the non-physiological CamA/B redox partners from *P. putida*. Once again, titres of approximately 0.5 g L^{-1} of hydroxylated β-ionone products were observed. A double variant of this enzyme T229L, A280V was of interest due to good activity and selectivity towards norisoprenoids. Three hydroxylated products were identified, and notably both non-allylic positions could be addressed, a useful activity that is rarely observed in biocatalysis. Alongside the main product, allylic 4-hydroxy-β-ionone, the formation of 3-hydroxy-β-ionone and 2-hydroxy-β-ionone was also observed, with a product distribution of 74%, 7%, and 19% of the total respectively.

The successful creation of an efficient system for whole cell biocatalysis also enabled the investigation of further substrate biotransformations. The CYP109C wild type enzyme accepted all **BIOOX** targets, and variants with increased activity or selectivity could be generated in each case. However, process development was also required in order to ensure that the biocatalyst could be implemented in realistic applications. Taking advantage of cyclodextrin as co-solvent for *in vivo* enabled biotransformation and analysis of sesquiterpenoid products. Cyclodextrin was used to both increase the solubility of the substrates, thereby enhancing mass transfer in the reaction, and to prevent evaporation of volatile products. Valuable targets including solavetivol and longifolene-aldehyde were produced by *in vivo* biotransformation with the most active variant V80A, A280I. Longifolene is an important fragrance compound found naturally in plant sources such as pine resins and certain types of tea. The pBAD constructs and best candidate enzyme variants were transferred to **FIR** for validation though application evaluation in the terpene-producing *E. coli* strains in **WP6**.

USTUTT in collaboration with the **UNIMAN** attempted to scale up the selective hydroxylation by the natural fusion enzyme P450 RhF of diclofenac to 5-hydroxy-diclofenac. 5-hydroxy-diclofenac is a metabolite of this important nonsteroidal anti-inflammatory drug, and therefore P450 systems capable of producing the compound synthetically find utility in toxicology screening and pharmacokinetic assays for drug development and formulation. Additonally, the use of this well characterised system as a model for application of P450s at preparative scale allowed identification of critical parameters and process improvements to be implemented in the fermentation protocols assessed. The fermentations incorporate two stages; i) growth of the

E. coli (biomass) and production of the enzyme, *the expression phase* and; ii) the *biotransformation phase* using either growing or resting cells. Based on an optimised expression procedure in M9 medium, a total concentration in the biomass of 33mg active P450 RhF enzyme per gram of cell dry weight could be achieved reproducibly, representing useful activity yields from the fermentation.

In the subsequent biotransformation phase, an average space-time yield was achieved of 0.54mg of hydroxylated diclofenac product per litre of fermentation volume, per hour, per milligram of enzyme, per gram (dry weight) of resting cells. This was much higher that the yields achieved with comparable growing cells, an observation that could not be easily rationalised since the enzyme activity for corresponding CFE biotransformations showed similar activities *in vitro*. The probable cause of this difference was that NADPH cofactor availability presented a bottleneck in the P450 reaction. The concentration of NADPH, a cofactor used in many cellular processes, is much higher in non-growing or glucose-limited cells compared to metabolically active growing cells. The use of resting cells was therefore favoured for production of 5-hydroxy-diclofenac using P450-RhF. Additionally, the non-enzymatically catalysed formation of an over-oxidation product (quinoneimine) was observed. Quinoneimine production was dependent on the salt concentration and temperature of the fermentation, and could therefore be controlled by optimising process parameters to avoid formation of this unwanted side product. Final fermentations performed by **USTUTT** using optimised reaction conditions, reached significantly improved product titres of 0.36g/L and an average productivities of 11.7mg L^{-1} h^{-1}.

Work Package 4

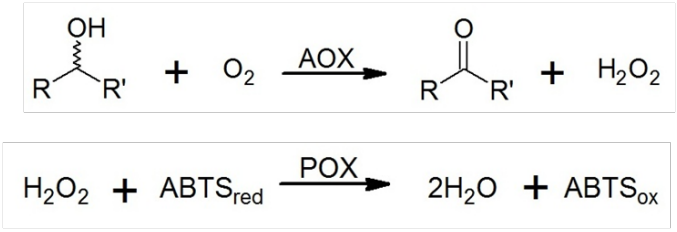

Figure 8. Alcohol oxidase assay reaction scheme.

Biocatalyst cost can limit the application of enzyme technologies in manufacturing, particularly where products have low market value. Even when applied for the production of high value products, use of enzymes can complicate downstream processing, requiring laborious sample isolation to achieve product specification. Thus, economically viable application of biocatalysts requires process options that address these challenges, and one such possibility is to employ immobilised enzymes which can be easily recovered from reactions either to be recycled or in order to simplify DSP. **WP4** was dedicated to development of immobilisation technologies for simplified and economical application of the enzymes developed in **WP1** and **2**. In **WP4**, **CLEA Technologies BV** (**CLEA**) focussed on two enzymatic oxidations assigned high priority in the *Vision for Success*. The first route was the GOase catalysed oxidation of HMF to produce DFF, and the second route combined alcohol dehydrogenase (ADH) for

primary alcohol oxidation with NAD(P)H-selective NOX for cofactor recycling as a platform technology.

Two methods for the aerobic oxidation of alcohols were developed using enzymes previously developed in **WP1** and **2** immobilised in the form of cross-linked enzyme aggregates (CLEAs). CLEAs are created using chemical treatments to form covalently cross linked aggregates of enzymes with good control over the size and composition of the final biocatalyst particles. Efficient application of CLEAs in biocatalysis requires that the reaction components can successfully penetrate the CLEA particles in order to fully access the enzymes therein. The first oxidation method developed by **CLEA** employed combi-CLEAs containing both GOase and catalase. This route was tested successfully for the oxidations of both the model substrate benzyl alcohol, and the priority target HMF, using a fed-batch hydrogen peroxide as the oxygen donor. The second method used a combi-CLEA containing ADH, NOX, and also catalase, and was successfully tested for oxidation of benzyl alcohol.

In order to produce a GOase CLEA with high recyclability, irreversible deactivation of the active site by hydrogen peroxide had to be prevented. For this reason it was decided to co-immobilise GOase with catalase, an enzyme capable of breaking down the peroxide, in one single CLEA. The activity of this combi-CLEA allowed lower global hydrogen peroxide concentrations within the CLEA and therefore increased GOase stability. Additionally, by using catalase in the GOase combi-CLEA, it was possible to generate high local oxygen concentrations, through hydrogen peroxide breakdown, only in proximity to the GOase where it was required. Catalase in combination with hydrogen peroxide therefore offered the potential to address the high K_M for oxygen of the GOase, one of the factors limiting the activity of GOase, due to the relatively low solubility of oxygen. In HMF oxidation reactions, activity recoveries and conversions observed for the combi-CLEA were substantially higher than those in a comparable system where soluble catalase was used in combination with a GOase CLEA.

It was found that at concentrations of HMF higher than 50mM the reaction could not reach completion due to product inhibition. Hydrogen peroxide addition rate was also identified as a critical parameter for the stability of the GOase/catalase combi CLEA.

BiCT focussed on developing carrier beads, procedures and protocols for the immobilisation of oxidase enzymes. In order to measure the activity of alcohol oxidase, a coupled colorimetric assay was optimised and tested with seven different commercial enzymes. Commercially available short chain (AOX2) and a long chain (AOX6) alcohol oxidases were used as model enzymes in **BiCT's** high-throughput immobilisation platform, which enables simultaneous screening of multiple variables. The activity of the immobilised biocatalyst was measured using a standardised alcohol oxidase assay, with excellent results.

In order to identify optimal immobilisation conditions for the ODE target enzyme GOase M3-5, **BiCT** carried out full factorial screening of parameters that can influence the immobilisation. Although the immobilisation is a rather empirical process, the optimised immobilisation conditions were rapidly determined for the GOase M3-5 using **BiCT's** high-throughput platform. Several carriers were tested in parallel, offering various different properties which can enhance performance: the functional groups that interact with the enzymes (the type and

concentration of reactive groups); granulometry; bead composition ("medium"); pore size; surface hydrophobicity.

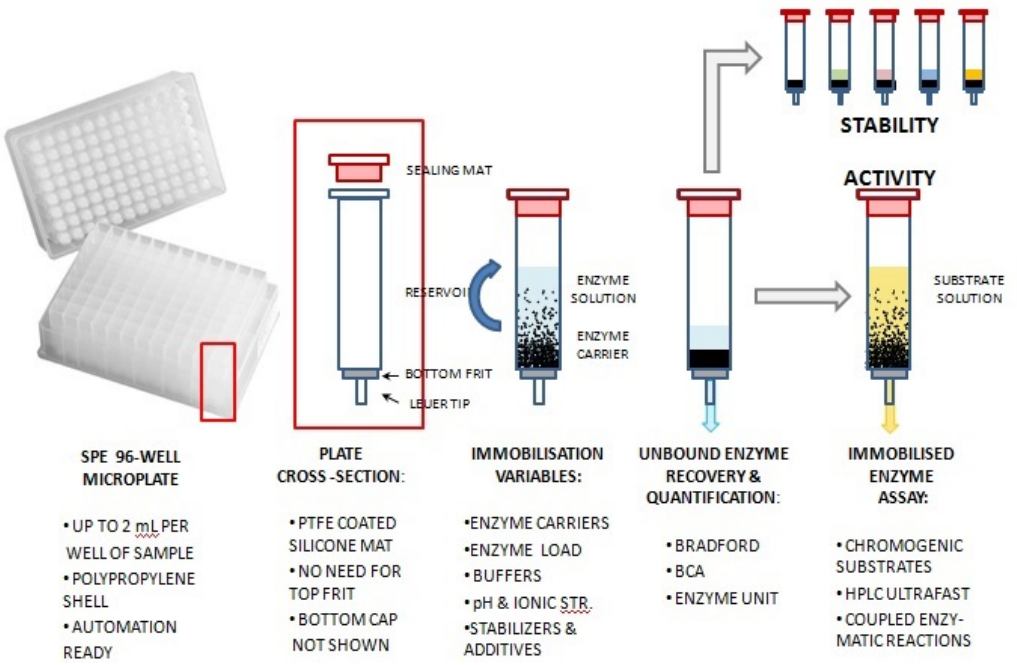

Figure 9. High-throughput immobilization platform scheme.

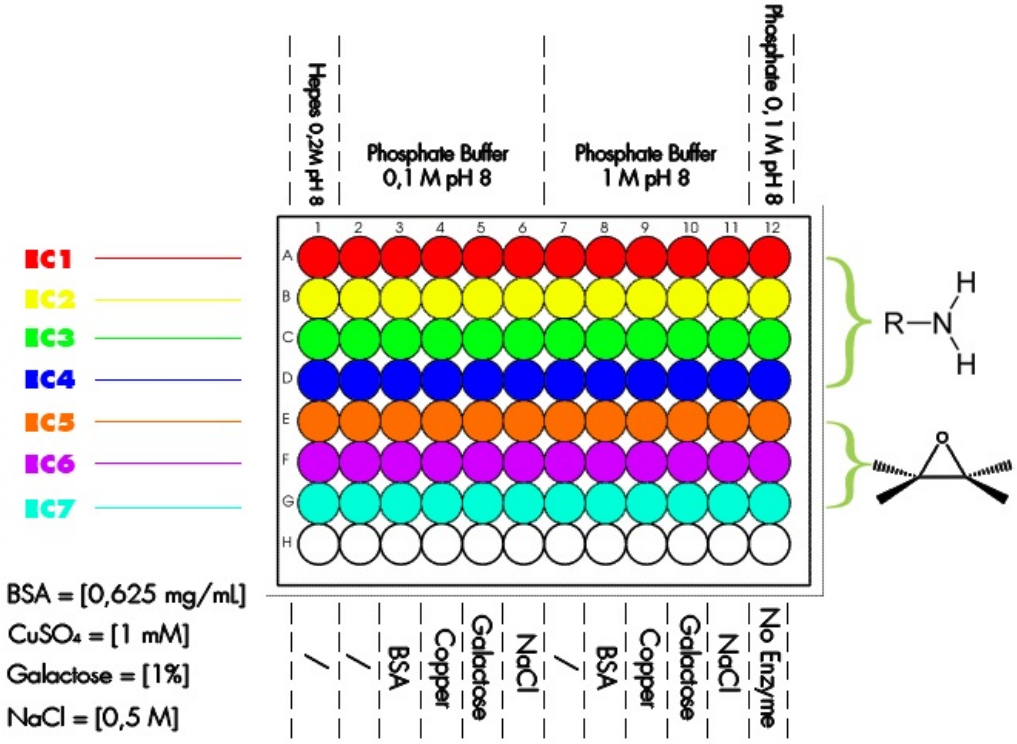

Figure 10. GOase M3-5 immobilization: full factorial experimental design. EC1-4: amino carriers activated with glutaraldehyde; EC5-7: epoxidic carriers. EC8: negative control (no carrier). Enzyme loading 2.5 $mg_{protein}/g_{carrier}$

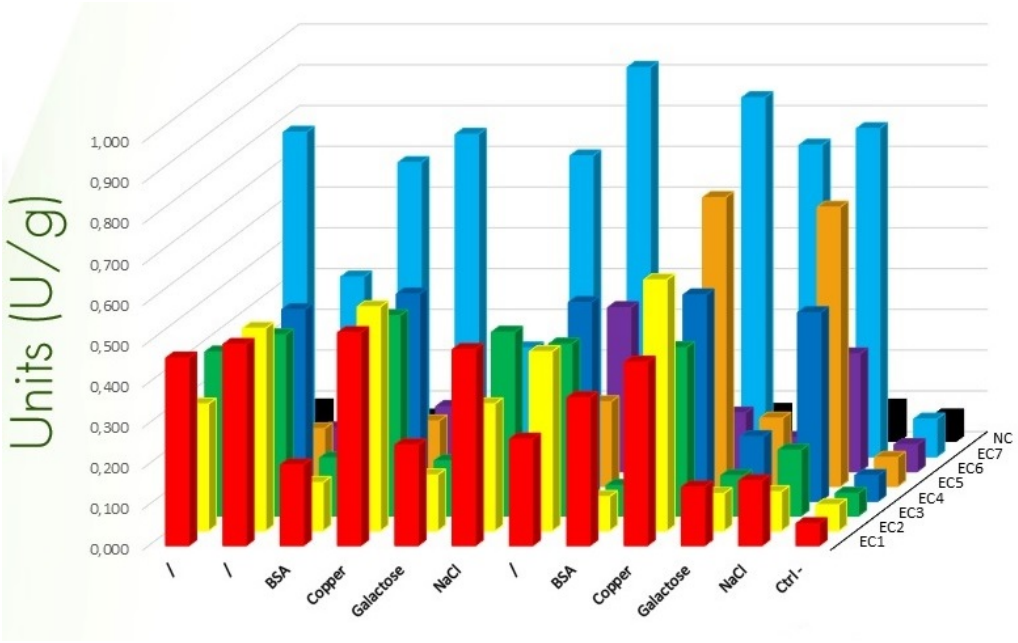

Figure 11. GOase M3-5 immobilization: activity measurements. Assay conditions: 10 mM 1-butanol, 0.25 mg/ml HRP, 0.2 mg/ml ABTS in sodium phosphate buffer 20 mM pH7.4 at 25°C. 1U corresponds to the amount of enzyme able to convert 1 μmol of 1-butanol in butyraldehyde in the reaction conditions.

C-TECH Innovation Ltd (**CTECH**) tested supported oxidase formulations in a pressurised continuous flow reactor, the Coflore Agitated Cell Reactor (ACR). Continuous flow reactors such as the ACR offer many potential advantages over traditional batch reactors, including precise reaction control, easy scalability, and reduced catalyst usage. The ACR consists of ten cells interconnected by small channels. An agitator is placed in each cell, which mixes the contents of the cell when the reactor body is shaken by lateral movement. Agitators can take a number forms. Where catalysts are used in solution, springs can be employed for efficient agitation. If immobilised catalysts are used, then they can be introduced in mesh-walled containment units ("baskets") which also perform agitation by moving within the reactor.

The rate of reaction for the oxidase catalysed process is primarily determined by the rate of oxygen uptake, which in turn is dependent on mixing efficiency and homogeneity of gas/liquid dispersion. Therefore, the use of a dynamically mixed continuous flow reactor with transverse mixing in preference over traditional rotational mixing had the potential for improved mass transfer under flow conditions, resulting in shorter reaction times and better enzyme consumption.

Following initial testing by **CTECH** of immobilised GOase G2 provided by **BiCT**, the basket containment units for the ACR had to be reengineered to provide adequate seals and prevent biocatalyst loss into the reactor. New stainless steel mesh inserts were manufactured to fit the existing baskets in order to contain the small 100-300 micron diameter beads. 100 micron mesh did not permit sufficient hydration of the beads so a 150 micron mesh was chosen, allowing sufficient hydration of the beads when under agitation. A small amount of the enzyme was lost into the system due to the compromise on mesh size, but this could be easily filtered from the system.

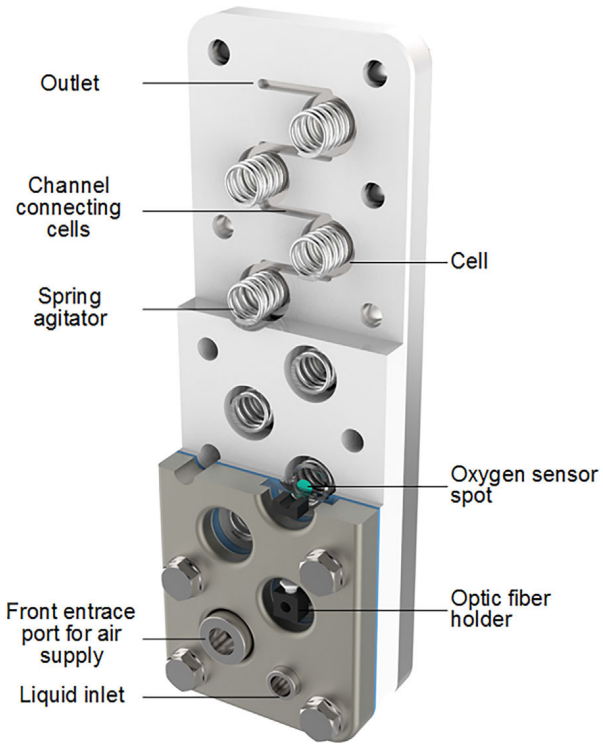

Figure 12. Cutaway illustrating the flow path of an Agitated cell reactor (ACR) with spring agitators.

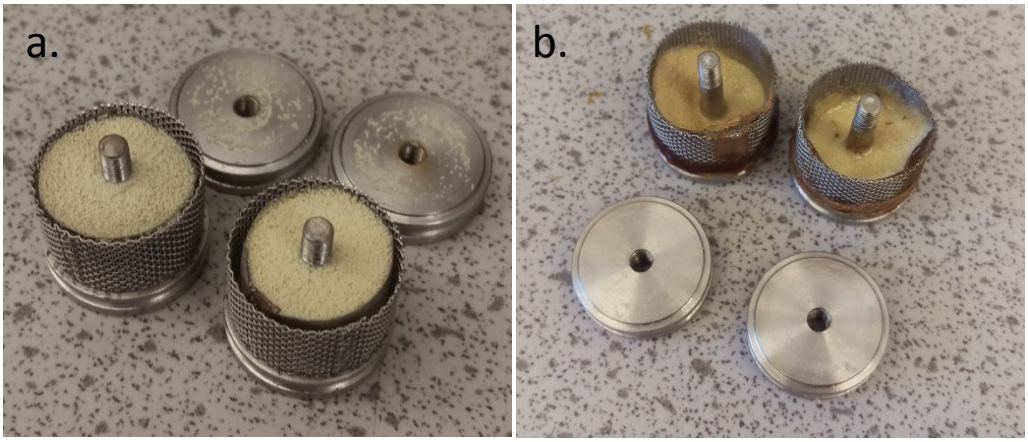

Figure 13. Basket containment agitators with immobilised GOase G2: **a.** immobilised enzyme after 4 hours in filled baskets; b) immobilised enzyme after 1 hour with half-filled baskets. Note poor hydration of (a) and complete hydration of (b).

Tests of the immobilised enzyme supplied by **BiCT** were conducted by **CTECH** using two out of the ten baskets available in the ACR, in order to enable shorter testing times and more prudent enzyme usage during testing. During initial reactions, the baskets were completely filled with the immobilised enzyme. This resulted in poor conversion to DFF, with little or no improvement even over extended residence times. This was due to poor hydration of the beads by the reaction mixture. Therefore, in subsequent operation the baskets were only half filled, resulting in much better hydration of the beads. The improved bead hydration resulted in better conversions to DFF, and in a more linear fashion.

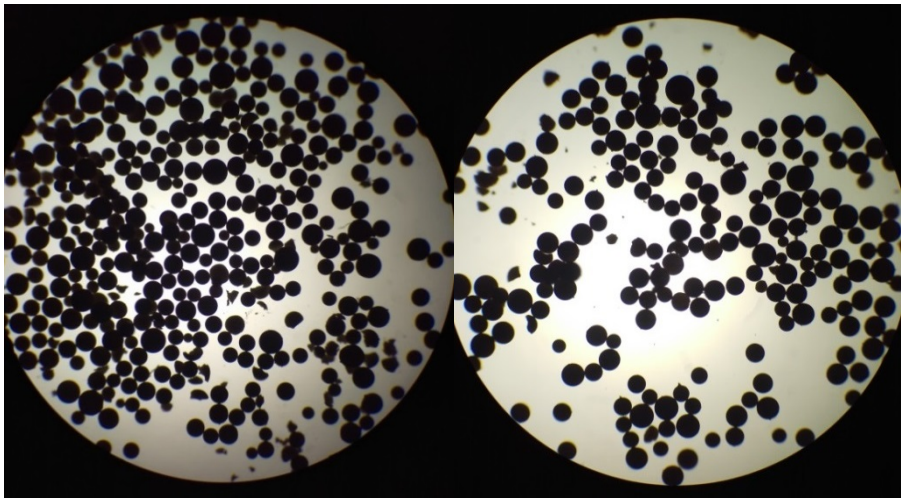

Figure 14. Visual integrity check of enzyme immobilisation carriers after extended use in the ACR: (left) 4 hours; (right) 24 hours.

After successful conversions to DFF over short residence times, extended twenty four hour runs were conducted to determine the effective lifetime of the immobilised GOase enzyme. Consistent conversion was anticipated over the time course, however the activity of the immobilised enzyme quickly decrease first five hour period. **CTECH** and **BiCT** were able to determine that the DFF product was adsorbed onto the immobilised enzyme carrier, which in turn inhibits the enzyme. It is therefore unlikely that this immobilisation system could be applied for the oxidation of HMF, however work in **WP4** provided excellent proof of principle for both **BiCT's** enzyme immobilisation technologies and the reactor operation protocols developed by **CLEA**. The operational stability of the immobilised support was good, and more than 99% of the beads remained intact and retained within the baskets during twenty four hours of continuous operation. Alternative reactor configurations for HMF oxidation were identified and evaluated in **WP5-7**.

Work Package 5

This work package focused on the evaluation of reactor configurations and oxygen feeding strategies, and how these influence the stability and kinetics of oxygen dependent enzymes. The evaluation was conducted based on isolated enzymes, immobilized enzymes, cell free extracts, and whole-cell biocatalysts, representing each of the principal methods for preparation of biocatalysts. This allowed direct comparison between different biocatalyst options for a reaction, or of the optimised formulation where the biocatalyst was only available in a single form.

The work has focussed on identifying suitable reactor configuration options based on batch reactors and continuous flow reactors, and three strategies for oxygen supply. Two primary model reaction systems were considered; a fast reaction catalysed by GOase enzyme, and a relatively slow reaction with a P450 whole-cell catalyst. These reaction systems enabled study of the effects of different reactor configurations and oxygen supply on the biocatalyst

operational stability and the overall reaction rate. These data provided inputs to WP7 to enable **DTU** to perform economic and environmental evaluations of the biooxidation processes.

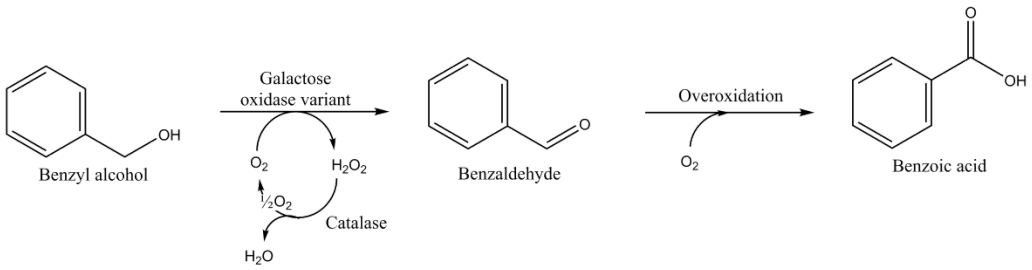

Figure 15. Fast model reaction system: oxidation of benzyl alcohol to benzaldehyde catalysed by GOase.

Stability under process relevant conditions is crucial for the development of a biocatalytic oxidation process. Furthermore, the performance of **BIOOX** model systems was compared where different biocatlaysts were capable of performing similar chemical transformations. Therefore, enzyme stability was investigated by **DTU** for two biooxidation systems developed in WP1 and 2, using the conversion of benzyl alcohol to benzaldehyde as a model reaction system. The influence of the aeration, as well as substrates and products was investigated, and particularly the effects on each of the individual enzymatic components in both systems: i) GOase variants supplied by **UNIMAN** and the enzyme required for its reactivation, horseradish peroxidase (HRP); ii) KRED and its cofactor regeneration enzyme NOX from **PROZO**.

Supply of oxygen is critical in to the application of ODEs, since oxygen limitation can severely restrict productivity, and the choice of oxygen supply method can drastically effect the safety, economy, and overall viability of the process. Three different methods of oxygen supply in reactors were evaluated by **DTU**: i) bubble aeration (sparging); ii) membrane contactors and; iii) *in situ* oxygen supply by hydrogen peroxide degradation using catalase. A comparison of the performance of three different membrane contactors, where oxygen permeates a membrane in order to supply the reaction. In each case, relatively similar performance was observed for the different modules, but practical implementation at large scale was found to be complex and expensive. The feasibility of feeding hydrogen peroxide and catalase was evaluated, but was impractical due to the inefficiency of mixing in large scale reactors, the tendency of highly reactive peroxides to deactivate enzymes at even low concentrations, and the large increases in reaction volumes caused by feeding liquid hydrogen peroxide at even the maximum safe concentrations for operation. It was therefore shown that bubble aeration is the primary choice based on the comparatively higher oxygen transfer rate and simpler operation.

However, the final choice of oxygen supply method is ultimately determined by matching process conditions to the biocatalyst or vice versa. Further testing was performed by **DTU** to determine the impact of the oxygen supply method on the stability of two oxidases, and the bubbling of gas into the reactor was found to be a feasible alternative for GOase, whereas it clearly damaged the NOX.

Oxygen supply is often a limiting factor in bioxidation reactions, due to the relatively poor solubility of oxygen in unpressurised reactor systems. It is therefore critical to be able to experimentally determine the kinetics of oxygen dependent enzymatic reactions in order to

enable rapid screening or reengineering of biocatalyst variants. Kinetic characterization of oxygen dependent enzymes is a time consuming and material intensive task due to the difficulties involved with setting and maintaining multiple different concentrations of poorly water-soluble oxygen. **DTU** developed a novel laboratory microscale reactor designed to obtain initial rates for oxygen dependent enzymes at a large range of different oxygen concentrations. A constant oxygen concentration was obtained by utilising a tube-in-tube micro reactor where oxygen is transferred from an oxygen-nitrogen mixture over a membrane with high oxygen permeability to the liquid reaction medium. The reactor can be pressurised to increase the solubility of oxygen in order to fully saturate even enzymes with low oxygen affinity. An online spectrophotometric detector then enables quantification of reaction substrates and products in the outlet of the reactor. The system is fully automated and can fully characterize an enzyme in less than 12 hours using a minimum amount of material. This novel system is an important innovation to enable rapid determination of enzyme kinetics, and will find further applications with other reaction systems and enzyme classes. The reactor is currently the subject of a patent application by **DTU**.

Galactose oxidase variants generated in **WP1** and **2** were kinetically characterised by **DTU** using the tube-in-tube reactor. An enzyme variant discovered by **UNIMAN** using reduced oxygen screening was determined to have a 2.8-fold higher reactivity with molecular oxygen; an important validation of the reactor technology, and a critical step towards viable application of this biocatalyst. This makes the variant a much better choice for industrial application, because due to the higher reactivity at the low oxygen concentrations experienced in an industrial reactor, the amount of enzyme required can be reduced by more than 50%. This is an important cost saving for large scale commodity product manufacturing.

Biocatalytic oxidation reactions employing molecular oxygen are difficult to conduct in a continuous flow reactor because of the requirement for high oxygen transfer rates. **DTU** selected the oxidation of glucose to glucono-1,5-lactone by glucose oxidase as a model reaction to study the ACR reactor system. In this case, the enzyme was used in solution with spring agitators. Based on tracer experiments, a hydrodynamic model for the ACR was developed. The model consisted of ten tanks-in-series with backmixing, where the reacted stream mixes with unreacted components, occurring within and between each cell. The backmixing was a necessary addition to the model in order to explain the observed phenomenon that the ACR behaved as two continuous stirred tank reactors (CSTRs) at low flowrates, while it at high flow rates behaved as the expected ten CSTRs in series. The performance of the ACR was evaluated by comparing the steady state conversion at varying residence times with the conversion observed in a stirred batch reactor of comparable size. It was found that the ACR could more than double the overall reaction rate, which was solely due to an increased oxygen transfer rate in the ACR caused by the intense mixing as a result of the spring agitators.

In **WP5**, **CTECH** applied the ACR for the conversion HMF to DFF, measuring performance parameters as one of the inputs to **WP7**. Immobilized galactose oxidase was used successfully to partially convert HMF, however, significant stability issues with the biocatalyst were identified. Adsorption of the product onto the beads led to inactivation of the enzyme, and would additionally complicate downstream processing. These challenges would need to be

addressed to enable industrial application of immobilized galactose oxidase for this type of reaction. Moreover, the ACR were used to convert HMF to DFF using free galactose oxidase in a biphasic reactor system using pure oxygen for aeration. In this setup 250 mM of HMF could be fully converted with a residence time of only three hours, significantly improved from reaction times taken to achieve similar conversions in small scale batch reactions. Alternative reactor configurations, including alternative and novel batch type reactions, were modelled in **WP7** in order to evaluate the feasibility of different process options for HMF biooxidation.

Work Package 6

The goals of **WP6** were aimed at demonstration activities for **BIOOX** technologies. Demonstration activities completed included a workshop on the use of flow reactors in ODE biocatalysis; presentation of 3DM tools for analysis of FTO; production of demonstration scale batches of enzymes/enzyme formulations, and testing of **BIOOX** materials and methodologies in industrially-relevant processes and at scale.

BPT demonstrated the patent analysis tool to five large Biotechnology companies, four large pharmaceutical companies and **BPT** has presented the patent analysis tool at two prestigious drug design conferences (Drug Discovery Chemistry, and Discovery on Target) in San Diego and Boston, respectively. This has already resulted in a follow up with two biotech companies and with several pharmaceutical companies. Continued development on the patent analysis tool will be required to accommodate the different use cases of each interested company. **BPT** also presented the patent analysis tool at the BIOTRANS 2017 symposium in Budapest. A presentation was given in the industrial session to illustrate the capabilities of the patent analysis tool for FTO analysis.

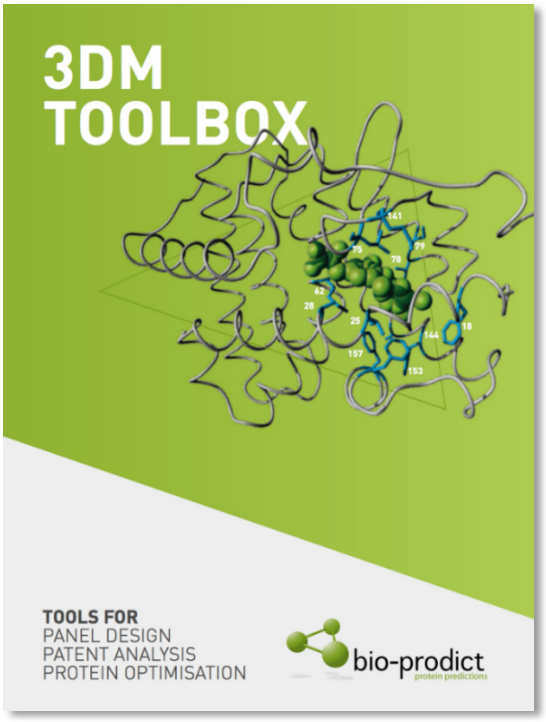

Figure 16. Promotional material for BPT 3DM Toolbox, now including the patent analysis tool.

Offering large collections of diverse enzymes in colorimetric screening format is an ideal proposition for **PROZO's** large pharmaceutical industry ("big pharma") customers, as the probability of identifying a process-ready enzyme off the shelf is maximised, potentially saving a great deal of process and/or enzyme development time, which is critical to competitiveness. Traditionally, the ability to rapid develop new syntheses was limited by the time taken to discover or engineer suitable biocatalysts, and is a key reason for the historically poor uptake of biocatalytic technologies by industries adapted to the short timelines required for development of chemical routes.

The utility of the metagenomic plates was demonstrated as part of **WP6** by offering the screening kits to the **BIOOX** consortium and within another EU grant consortium – the **H2020** project **CARBAZYMES** – where multiple KREDs were found to be suitable in native form for key biotransformations of interest. Beyond the consortium, one big pharma customer that expressed initial scepticism about whether any useful hits would be obtained through this approach reported 24 good hits against a small collection of challenging compounds during prototype screening in-house. In this way, and given that all prototype KREDs were produced at the 1-2 g scale, 100mg quantities of any hit suggested by kREDy-to-go™ can be immediately provided to the customer for in-house prototype testing against their prospective application, thereby maximising the potential for new business for **PROZO**, and delivers strong impact for the work performed during **BIOOX**.

Significantly, after evaluation of the **BIOOX** prototype NOX enzyme panel, one of **PROZO**'s biggest customers disclosed to the community that they identified a NOX enzyme for use in a major new application. While NOX enzymes have been known for some years, an enzyme suitable for the demanding application in question was only found by mining diverse homologues from relevant three-dimensional sequence space using **PROZO** GRASP™ technology. That process has now been scaled-up to 6kg as part of product validation activities in **WP6,** enabling **PROZO** to demonstrate the challenging fermentation of the NOX at industrial scale. This was a crucial demonstration of the feasibility for application of this enzyme, because production by scale-out using fermenter-in-a-flask was not suitable, and the scale-up of production was unprecedented. Providing that demonstration has enabled the big pharma customer to negotiate production by **PROZO** beyond the **BIOOX** project.

Galactose oxidase variant G2 was selected as the candidate biocatalyst for evaluation by **BASF** in the oxidation of HMF. In **WP2** GOase G2 was developed to produce extremely high activity towards HMF for oxidation to the commercially relevant intermediate chemical DFF. Optimisation of enzyme properties and reaction conditions for HMF oxidation were performed by **UNIMAN** to improve the suitability of the biocatalyst for application at a large scale. **PROZO** supplied GOase variants in hundreds of grams quantities to other **BIOOX** partners for experimental testing and evaluation in processes.

CTECH performed scale up in flow of HMF oxidations previously developed as shake flask or stirred vessel reactions at laboratory scale by **UNIMAN** and **DTU** in **WP1**/**3**. The HMF reaction was scaled up by **CTECH** to both 250ml and 1L, and the conditions used for a successful 3 hour run were then used to perform a continuous twenty-four hour run. This run

enabled production of a larger batch, including downstream processing and product isolation, allowing calculation of the overall yield for the process and characterisation of the product. Overall conversion of 80% to DFF was achieved over the course of twenty-four hours of continuous operation and approximately 10% of the HMF remained unconverted. After isolation of the product, this resulted in an overall isolated product yield of 52% with a purity of 89% DFF. The remaining 11% was mainly HMF, with less than 0.1% FFCA/FDCA. DSP was not further optimised in this case, but significant potential exists for improvement of product isolation methodologies.

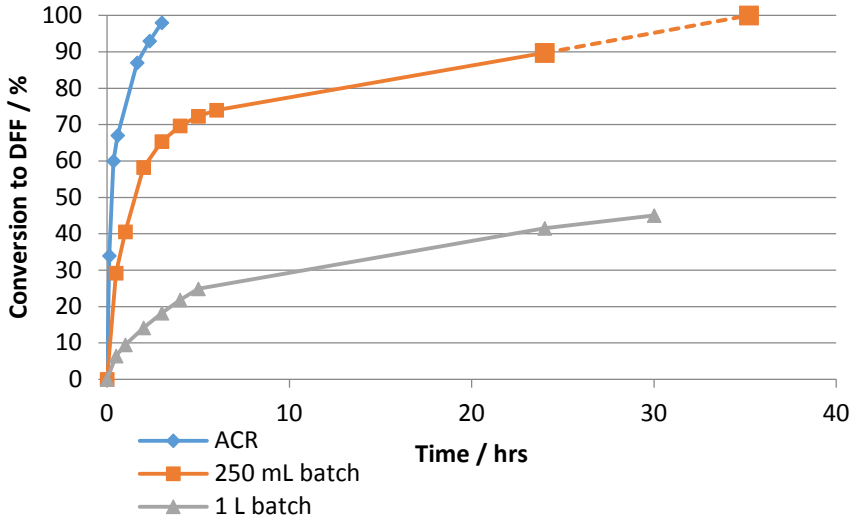

Figure 17. Comparison of HMF bioxidations carried out in stirred batch and ACR.

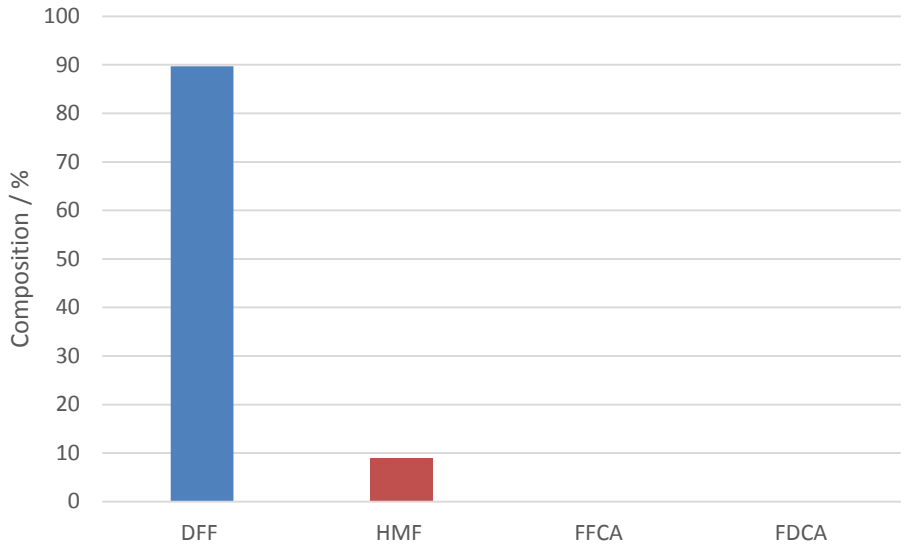

Figure 18. Product composition isolated from HMF biooxidation performed in the ACR continuous flow reactor.

Biooxidation reactions were performed by **BASF** at scale using two different reaction systems: i) GOase to produce DFF, and; ii) using xanthine oxidase (XOD) for production of an agrochemical active ingredient. For demonstration purposes this material was sent to **BASF** internal customers for further assessment. Several field trials with sample material based on a biooxidation process implemented by **BASF** showed poor performance of the products, and further development work (to be undertaken outside of **BIOOX)** will be required to achieve the required product specification.

Although the quality of DFF from galactose-oxidase catalysed biooxidation was sufficient if not excellent, there remained severe issues concerning its further chemical conversion to downstream products. Further work to derivatise DFF (performed outside the scope of **BIOOX**) is not yet finished, and additionally there are ideas to replace these chemical syntheses with enzymatic alternatives. The biooxidation of HMF remains an important target for **BASF** and patent applications have been submitted for two process variants developed in **BIOOX**.

As part of **WP6**, **CTECH** developed a workshop on the application of plug flow reactors for gas fed ODE catalysed reactions. This workshop was held as a satellite event to the Organic Process Research & Development Conference held in Stockholm, Sweden in September 2017. Alongside the workshop presented by **CTECH**, other **BIOOX** partners delivered presentations on the selection use of ODE in processes developed using plug flow reactors. An overview of the project was provided by **UNIMAN** (***BIOOX*** *project summary and objectives*), with additional presentations from **DTU** on novel tube-in-tube reactor systems (*Continuous reactors for biocatalytic applications*), and from **PROZO** on discovery and screening for selection of ODEs (*Screening methodologies for the selection of oxygen dependent enzymes*). A targeted audience was reached, including representatives from organisations such as Merck, Boehringer Ingelheim, AstraZeneca, University of Cambridge, GSK and Givaudan. Coupled to the workshop activities of **CTECH**, this created a high impact event delivering an overview of **BIOOX** technologies to potential end users.

Work Package 7

WP7 was focussed on economic and environmental process evaluation. The overall objectives were to develop economic and environmental models as well as green and sustainability metrics for the evaluation of oxidase bio-processes. This was a critical aspect of the project, representing the culmination of much of the biocatalysts optimisation and process development work undertaken in the other **WP**. Uptake of biocatalysis and other IB processes requires that they can compete on quality, performance, and economy, with alternative chemical technologies. Performance metrics could be compared against the targets established in the *Vision for Success*, allowing the project consortium to understand the gains made through development of technologies, and importantly to benchmark those technologies against the requirements of industrial processes where they find potential application.

Initial work by **DTU** was directed towards developing flowsheets for biocatalytic oxidations processes selected in **WP5**. Two important process aspects were prioritized. Firstly, different biocatalyst preparations, i.e. growing cells, resting cells or isolated enzyme, were compared in

terms of the expected oxygen requirement. The work also indicated that at least some processes may be sufficiently aerated using membrane technology, which may prove beneficial for the stability of some biocatalysts developed in the project. Others may be feasibly operated using bubbled aeration, which is a well-established method in industry. Secondly, several different co-factor regeneration alternatives for the biooxidation of alcohols were selected for further investigation. During the project, flowsheets have been developed based on the reactor configurations evaluated in other work packages. This resulted in process models that allow direct comparison of cost and environmental impact of different process alternatives, and enables the identification of bottlenecks which is useful for further process development.

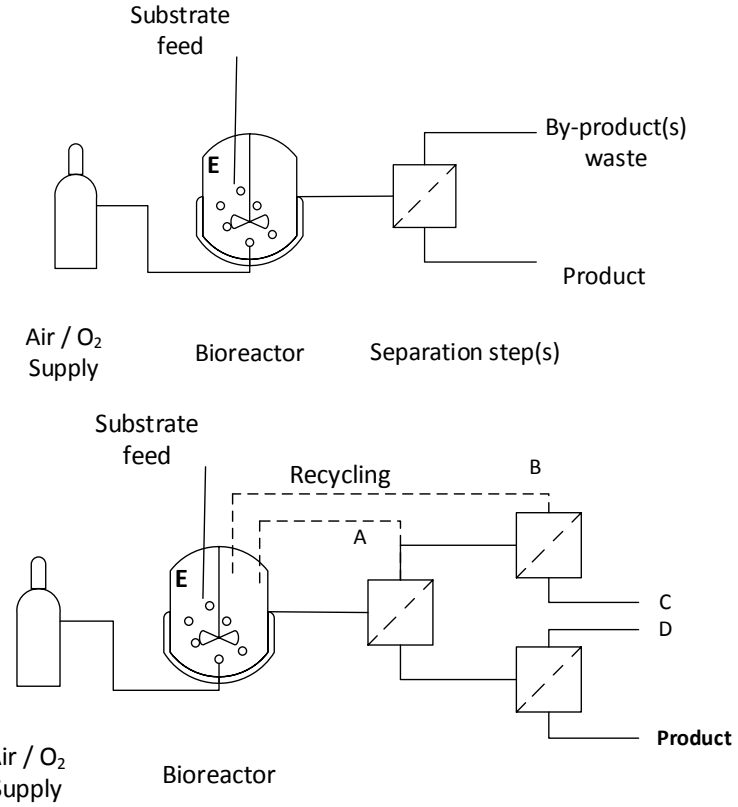

Figure 19. (Top) Example of a flow-sheet for a simple reaction with only one separation step. (Bottom) Example of a more complex flow-sheet for a process requiring several different separations for recycling and product recovery.

The design of a process flow-sheet is dependent on a number of factors, such as the reactor type, oxygen supply method, biocatalyst format, need for substrate feeding and/or product removal and the downstream processing for recovery of the product. Flow-sheets were constructed , including a simple process with only one separation step and a more complex process in which several separations are required both for recycling of e.g. biocatalyst and a reactant and for the recovery of the product.

The flow-sheet illustrates a process using a whole cell catalyst in a resting state. In such a process the biocatalyst is first produced through a fermentation step and subsequently separated and used to catalyse the synthetic reaction.

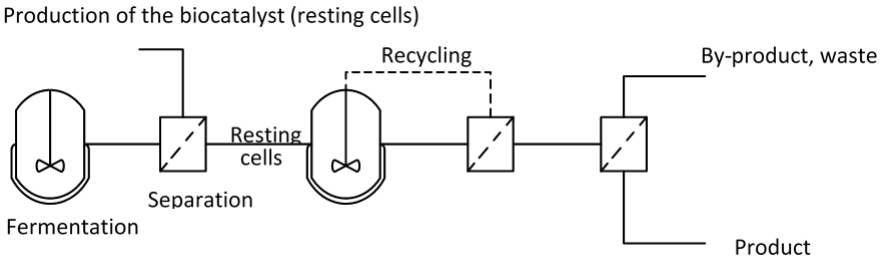

Figure 22. Example of a flow-sheet for a process using a resting cell biocatalyst.

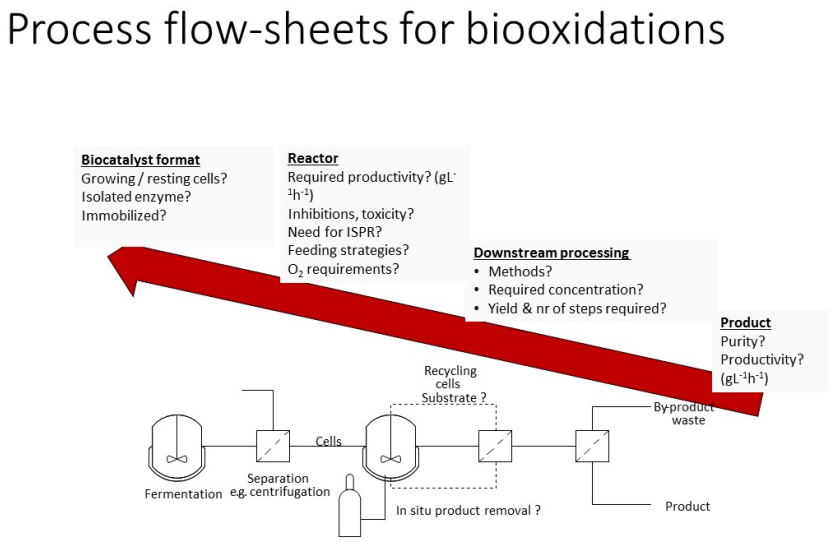

Figure 22. Description of the workflow for establishing a flowsheet.

For the work on flowsheet analysis conducted in **WP7**, the oxidation of HMF to DFF is considered as an example of what can be achieved. It was a carefully chosen system in order to reflect the challenges of oxidation for the synthesis of low-value products. At an early stage potential alternative process flow sheets for performing the GOase-catalysed oxidation HMF were considered. Based on a comparison of the physio-chemical properties of HMF and DFF it was concluded that an *ex situ* product removal strategy based on crystallization of DFF was the best alternative. The crude DFF crystal can be further purified via a series of recrystallization steps to obtain DFF crystals with sufficient purity to be used as starting material for further chemical modification or polymer synthesis. Nevertheless for practical operation in the demonstration activities, operation with the alternative two-phase system was assessed at **BASF** as part of **WP6**.

In addition using the flowsheet, the material and energy balance was also developed to give the basis for scaling and costing. Based on the current knowledge of the reaction conditions and a number of assumptions the energy and material consumption for a plant producing 100,000 tons of DFF per year was calculated. Based on the process simulation it was found that 1.14 kg of crude HMF (95% purity) and 3.3 g of enzyme were required per kg of pure DFF. The energy requirements without taking electricity for pumping into account was found to be

4.1kWh for heating and 4.3kWh for cooling per kg product. The current process is based on multiple assumptions that need to be investigated experimentally, but in general many assumptions are set conservatively.

The *Vision for Success* in **BIOOX**, has played a central role for establishing a process model, stating the process targets in terms of e.g. productivity ($gL^{-1}h^{-1}$) and product purity. With this information as a reference, the required performance in the preceding process steps could be assessed. This can then be used for evaluating performance realized in **DTU** model systems. In the final stage of the work an economic assessment of this exemplar system was carried out.

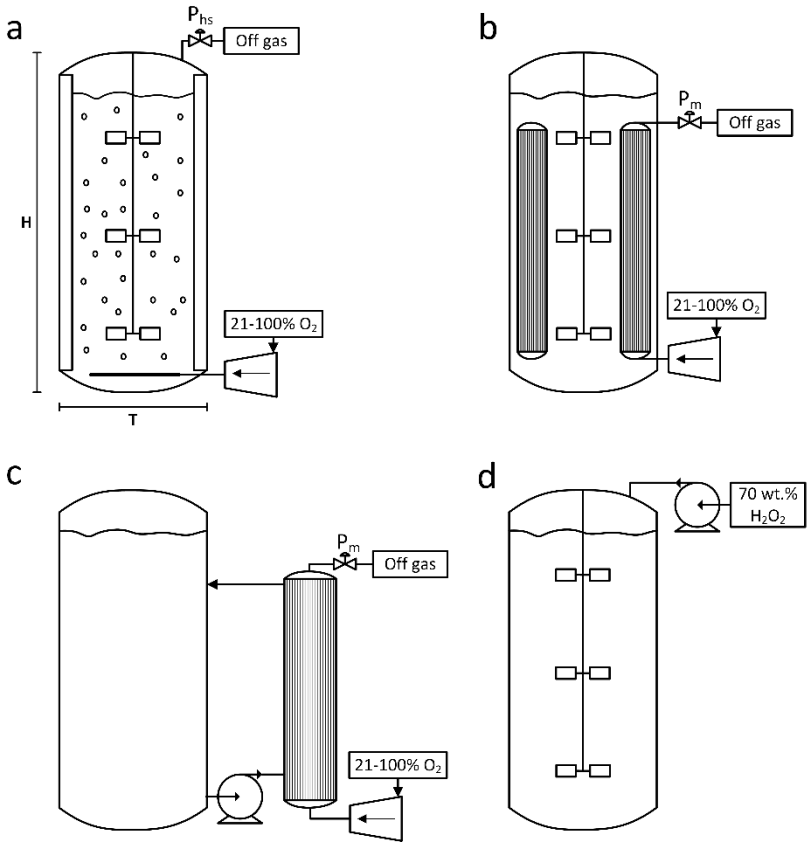

Figure 23. Investigated oxygen supply methods. a) stirred tank reactor (STR) with possibility of sparging enriched air and increasing head-space pressure. b) membrane reactor with submerged membrane modules (SMR). c) membrane reactor with external membrane module (EMR). d) generation of oxygen by decomposition of hydrogen peroxide.

Oxygen supply to fermentations has been a widely studied field of research for many decades. With the recent developments in oxidative biocatalysis, the supply of oxygen to biocatalytic reactions has never been more important. Although biocatalytic oxidations and aerobic fermentations share many similarities, a number of differences are critical when evaluating oxygen supply methods, such as instability of certain enzymes in the presence of gas-liquid interfaces. Likewise biocatalytic reactions can potentially be much faster, than their fermentative counterparts. To circumvent the problems associated with the traditional method of supplying oxygen via bubble aeration of stirred tanks, a number of alternative oxygen supply methods have been proposed in the scientific literature. In this **WP** the most promising alternatives were evaluated by **DTU** based on their technical feasibility and cost, and compared

to traditional methods of supplying oxygen via bubble aeration of stirred tanks. The methods investigated included enriched air aeration and pressurization of stirred tanks, membrane aeration with a submerged and an external membrane configuration, and *in situ* oxygen generation by hydrogen peroxide decomposition. In reality little difference in the price per kg based on the method of oxygen supply was found. This is the first investigation of its type and this is an important conclusion.

Summary

Overall, the aims of **BIOOX** have been achieved, and the technologies developed during the lifetime will find applications in industrial context. Key early successes beyond the project are likely to be through the commercialisation of tools and methods for ODE discovery and production. Equally, the body of research literature and laboratory technologies has been greatly enhanced over the course of the project, with new experimental approaches and capabilities available, alongside tools for decision making which will inform future research and development in the area. Further development will be required for the application of **BIOOX** processes in commercial chemical manufacturing, but planning for those efforts is already underway, and the desire to continue that development is reflected in patent applications from within the project, and further process development and evaluation activities being undertaken by the industrial partners beyond **BIOOX**.