Enzymatic oxidation/oxyfunctionalization

New enzymatic oxidation/oxyfunctionalization technologies for added value bio-based products

Mid-term project results summary

The overall objective of **EnzOx2** is to develop new bio-chemical technologies based on oxidative enzymes, for the production of some added value compounds from biomass components to substitute others of petrochemical origin. The potential of oxidative enzymes in such biotransformations has been shown in previous projects, including several oxidation and oxyfunctionalization reactions catalyzed by fungal oxidoreductases (oxidases and peroxygenases). In the above context, the **EnzOx2** project brings together three highly-specialized SMEs in the areas of fungal enzymes (**JenaBios**), 5-hydroxymethylfurfural (HMF) production (**AVA Biochem**) and chiral chemicals and active pharmaceutical ingredients (API; **Chiracon**); two world-leading companies in the sectors of industrial enzymes (**Novozymes**) and flavour & fragrance ingredients (**Firmenich**); one technological centre dedicated to the plastics sector (**AIMPLAS**); and six research/academic partners with high expertise in oxidoreductase structure-function and engineering (**CIB** and **ICP**), their application in different biotransformations (**TUDresden** and **IRNAS**), the design and optimization of enzyme-based bioprocesses (**TUDelft**), and biocatalyst immobilization (together with other partners) and life cycle assessment (LCA) analysis (**USC**).

To attain the above objective, the **EnzOx2** partners are taking advantage from the largely unexploited diversity of oxidoreductases in fungi from special habitats, and sequenced fungal genomes, to obtain new enzymes of interest. Moreover, the catalytic performance, selectivity and/or stability of the best candidates are being adapted, when needed, to the required reaction conditions using different protein engineering tools. Several concepts such as substrate loading, co-factor addition, biocatalyst stability and downstream processing, among others, are also being considered to further optimize the enzymatic reactions. Finally, preparatory work for LCA analyses of the enzymatic processes, compared with chemical processes for the production of the same or similar compounds, was initiated during this first period to prepare the final evaluation of their technical, economic and environmental feasibility.

Although the selection of new enzymes was largely based on activity detection in fungal cultures, and sequences available in databases and already sequenced genomes, two new genomes representing groups of fungi scarcely investigated for enzymes of interest (the ascomycetes *Lecytophora hoffmanii* and *Kretzschmaria deusta*) were sequenced (Büttner et al. Genome Announc 5, 2017; Leonhardt et al. Genome Announc. 6, 2018). Cultures and genomes were screened for new types of unspecific peroxygenases (UPO), the main enzyme type in **EnzOx2**, whose industrial applicability is also being improved by enzyme engineering methods (Gómez de Santos et al. ACS Catal. 8, 4789-4799, 2018, Martín-Díaz et al. Appl. Environ. Microbiol. doi: 10.1128/AEM.00808-18, 2018).

Oxidases were largely investigated in the project to catalyze reactions of interest or as a source of the hydrogen peroxide required by UPOs. Aryl-alcohol oxidase (AAO) was selected as a model for these enzymes, and its reaction mechanism with both reducing (benzylic alcohols) and

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oxidizing (molecular oxygen) substrates was in-depth investigated (Carro et al. Phys. Chem. Chem. Phys. 19, 28666-28675, 2017, Sci. Rep. 8:8121, 2018, Biochemistry 57, 1790-1797, 2018) for its application as an enzymatic catalyst. These applications include new enzymatic technologies for both sugar and lipid derived building blocks and other added value compounds, as described below for the first reporting period.

Concerning production of sugar (furfural) based building blocks, during these months we optimized the previously described two-enzyme cascade for HMF conversion into FDCA, and proposed a new cascade involving AAO, UPO and galactose oxidase with additional properties of interest (Karich et al. Microorganisms 6, 2018). Since future large-scale production of FDCA-based bioplastics (by the joint venture of companies BASF and Avantium) will be most probably based on 5-methoxymethylfurfural (MMF) instead of HMF, the former compound was also included in **EnzOx2**. A self-sustained enzymatic cascade for FDCA production from MMF was developed and patented (Carro et al. Biotechnol. Biofuels 11:86, 2018, ES Patent P201730805, 2017), where the action of UPO is fueled by hydrogen peroxide provided by both AAO and a second oxidase acting on the methanol release from the demethoxylation of MMF by UPO.

For the production of lipid "sensu lato" added value compounds, the action of UPOs on different compound types has been investigated. The enzyme from *Marasmius rotula* catalyzes unique reactions on fatty acids including terminal hydroxylation (first reported in 2016) and chain shortening. The latter has been fully investigated from enzymatic and mechanistic points of view (Olmedo et al. Chem. -Eur. J. 23, 16985-16989, 2017) and a patent has been deposited for the controlled one-carbon shortening of fatty acids (Gutiérrez et al. Patent EP17382211.5, 2017), while terminal hydroxylation, of interest for homopolyester production, is being investigated with engineered enzymes. Among steroid transformations, the enzyme from the basidiomycete *Marasmius rotula* (and a second *Marasmius* species) was also able to remove the side chain of cortisone for production of APIs (Ullrich et al. J. Inorg. Biochem. 183, 84-93, 2018) in a reaction reminiscent of the chain shortening mentioned above. A second steroid transformation of pharmaceutical interest was demonstrated for the UPO of the ascomycete *Chaetomium globosum*, which catalyzes the selective oxygenation of testosterone (Kiebist et al. ChemBioChem 18, 563-569, 2017). This reaction, already performed at the 100 mg scale yielding 90% testosterone epoxide (isolated with 96% purity) will be one of the oxyfunctionalization reactions to be further up-scaled and evaluated for industrial implementation during the second part of the project. Another reaction of pharmaceutical interest catalyzed by several UPOs is the hydroxylation of stilbene (and several stilbenoids) for the selective synthesis of resveratrol analogues (Aranda et al. Catal. Sci. Technol. 8, 2394-2401, 2018). Finally, challenging selective oxyfunctionalization of four model terpenes is being actively investigated by the above and other UPOs.

The results from optimization and feasibility evaluation of the above enzymatic reactions will be presented in the second report, since the former formally started a few months ago and the latter will be concentrated in the third year of the project. The impact of the above enzymatic transformations is related to: i) the use of environmentally-friendly enzymatic technologies in the manufacture of bio-based bulk chemicals such as plastic building blocks; and ii) the selectivity of UPOs catalyzing specific oxygenation reactions of pharmaceuticals, and other speciality chemicals, that are difficult or very complicated (expensive) to be obtained using chemical methods.

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