EUROPEAN SYNCHROTRON RADIATION FACILITY

INSTALLATION EUROPEENNE DE RAYONNEMENT SYNCHROTRON

Experiment Report Form

	Experiment title: Hard X-ray Fluorescence mapping to decipher the deactivation processes of immobilised metallo-enzyme systems	**Experiment number:** MA-4866
Beamline: ID16A	**Date of experiment:** from: 29/06/2021 to: 02/07/2021	**Date of report:** 22/11/2021
Shifts: 6	**Local contact(s): Murielle Salome**	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):
Dr. Carlos Sanchez-Cano*, CIC BiomaGUNE, Spain
Javier Santiago-Arcos*, CIC BiomaGUNE, Spain
Nicol Zeballos Lema*, CIC BiomaGUNE, Spain
Prof. Fernando Lopez Gallego, CIC BiomaGUNE, Spain

Report:

1. Abstract: Immobilisation of enzymatic cascades in porous materials is ideal to perform multi-enzymatic biotransformations in industrial settings.[1] Yet, they suffer from exhaustion processes that hampers their use for long operational periods. The exact mechanism of inactivation is unknown until now, but spatial reorganisation of enzymes across the material or loss of metal ions from their active centers seems to be relevant. Unfortunately, optical microscopy is not ideal to study those events, as labelling enzymes with fluorescent tags can alter their catalytic properties. ***We intend to acquire X-Ray Fluorescence maps at ID16A on methacrylate beads carrying a cascade formed by Zn and Cu-dependent enzymes used to transform diols to relevant hydroxyaldehydes,*** [2] ***both before and after their operational run.*** This will provide valuable information on the importance of enzymatic re-organisation and metal leaching for the mechanisms of deactivation of immobilised multi-enzyme systems upon operation in chemical manufacturing.

2. Experimental Details: Zn-dependent Alcohol dehydrogenase from *Bacillus stearothermophilus* (BsADH) and Cu-dependent Lacasse from *Aspergillus sp* (AsLAC) were immobilized into porous 50 µm diameter agarose beads to mimic natural compartmentalisation of multi-enzymatic systems. This allowed establishing an enzymatic cascade to achieve the asymmetric oxidation of diols to hydroxy aldehydes. Initially, agarose beads were functionalised with Epychloridirne overnight. After several washes, the carriers were incubated with Iminodiacetic acid (0.5 M, pH 11, 1 hour), ethylenediamine (2 M, overnight), and finally cobalt chloride (30 mg x ml^{-1}, 1 h). Afterwards, the multi-enzymatic system was immobilized using two complementary methods (Fig. 1): 1) first, AsLAC was attached to the beads by incubating in sodium acetate buffer (5 mM, pH 5, 1 hour) to form electrostatic interactions with amine groups from Ethylenediamine, and then, 2) histidine tags from BsADH were used to bind the enzyme to Co coordinated to carboxylic residues within the carrier (Sodium phosphate buffer 5 mM, pH 7, 1 h).

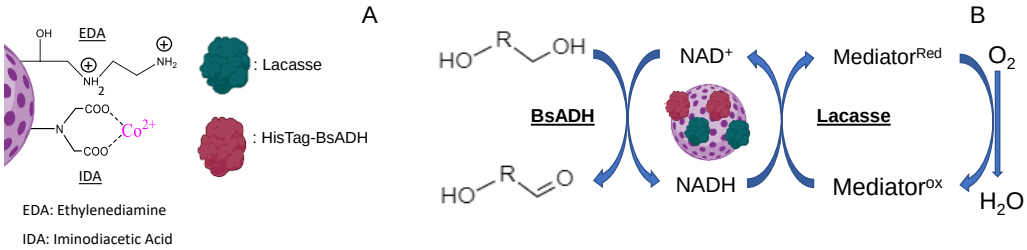

Figure 1. A: Scheme of the functionalization of the agarose carrier. B: Enzymatic cascade

All batch reactions were carried out by placing the biocatalyst in 1.5 ml centrifuge columns and incubating it with 1 mL of mix reaction (20 mM of 1,5-Pentanediol, 1 mM of NAD^+, 1 mM of Acetosyringone in buffer Sodium Phosphate 5 mM, pH 7, 30°C, 24 h). After each batch point, the reaction mix was removed by centrifugation (250 rpm), and a few mg of biocatalyst were collected for XRF analysis. Equally, thermal inactivation controls were generated by incubating 1:10 suspension of the biocatalyst in water at 70°C for 2 h.

The different beads were immobilised on silicon nitride windows prepared by using glow discharge. Then, the membranes were immersed in water, blotted with filter paper and manually plunge-freezed in liquid ethane.[3] The frozen membranes were transferred to holders that were 3D-printed inhouse for storage and transport, and kept under cryogenic conditions in liquid nitrogen until measurements were performed. XRF maps at ID16A were acquired under cryogenic conditions using two six elements silicon drift diode detectors. The beam energy was set to 17 keV, focused at 50 x 50 nm^2, $1.55*10^{11}$ phs/s. Coarse scans to detect areas of interest were done using 400x400 nm^2 steps (50 ms dwell time), and fine mapping of the beads was performed using a step size of 100x100 nm^2 (50 ms dwell time).

3. Results: XRF imaging at ID16A allowed us to visualize the spatial distribution of BsADH and AsLAC inside porous agarose beads supports, before and after performing operational runs for the transformation of diols into hydroxyaldehydes (Fig. 1). Cu and Zn maps obtained during the experiments showed different immobilization of both enzymes in beads before they were used for the transformation of diols. Zn-dependent BsADH was mainly found in cluster areas (probably) within the surface of the bead (Fig. 1A). Instead, Cu-dependent AsLAC was found across the whole agarose particle, following a distribution pattern that suggested internal localisation of the enzyme (Fig. 1B)[4]. Such distribution had been previously observed on intact beads carrying BsADH enzyme labelled with fluorophore (Rhodamine B).[4] However, the aggrupation of Zn from BsADH into clusters structures was gradually lost upon use of the beads for enzymatic reactions, and were no longer observed after the second batch reaction was performed. This suggested a change in the distribution of BsADH along the particle (from clusters within the outer surface to a more homogenous immobilization). On the contrary, AsLAC did not show any changes in its spatial distribution after any number of operational runs. Equally, study of the localisation of Zn and Cu-dependent enzymes within agarose beads after thermal inactivation showed a similar enzymatic distribution to the one observed inn beads used for batch reactions, with BsADH moving from a cluster to more homogeneous patterns, and AsLAC presenting no relevant changes in its spatial distribution (Fig. 1C).

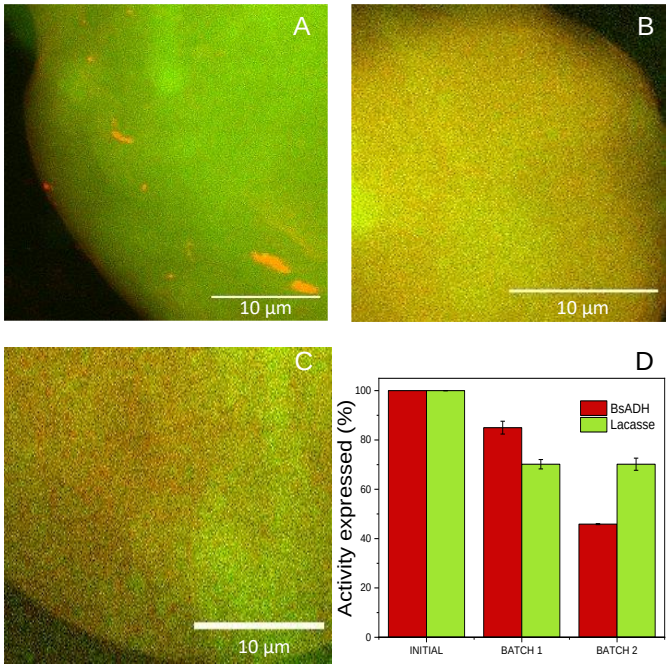

Figure 2: XRF maps showing the spatial distribution of the multienzymatic system (BsADH in red and AcLAC in green) under operational conditions. A: spatial distribution of Initial biocatalyst after co-immobilization. B: Spatial distribution of Initial biocatalyst after two sequentially batch reactions. C: spatial distribution of Initial biocatalyst after 2 hour of incubation at 70ºC. D: recover activity expressed of each immobilized enzymes under operational batch conditions

Remarkably, we have observed that there is a clear correlation between the changes in the enzymatic distribution of BsADH and the decrease of the enzymatic activity of the beads (Fig. 1D). Thus, the presence of BsADH in cluster structures may play an important role in the performance of our immobilised enzymatic cascade during its enzymatic reaction.

4. Conclusion and future work: XRF mapping at ID16A shows the possibility of following the spatial distribution of the metal co-factors from different metalloenzymes co-immobilized by different chemistry in porous materials, and capable of performing cascade reactions. The overall analysis supports the hypothesis that there is a change in the distribution of one of the co-factors during operational conditions or thermal inactivation, which is responsible for the loss of activity of the immobilized cascade. However, we do not know yet if such change in distribution is due to a release of the metal ions from the active sites of the enzymes or is the whole enzymes the ones that are moving within the beads. Currently we are performing a number of experiments to explore both of these possibilities, which will complete our study and allow us publishing this work.

5. References

1) W.D. Fessner, *N. Biotechnol.*, **2015**, 32, 658.
2) L.J. Sweetlove, *Annu. Rev. Plant Biol.*, **2013**, *64*, 723.
3) C. Bissardon, et al, *J. Vis. Exp.* **2019**, *2019*, 2.
4) J. Santiago-Arcos, et al, *Front.Catal*, ***2020***, 1:715075.