Plasma-driven biocatalysis with the unspecific peroxygenase from *Collariella virescens*

S. Klopsch[1], T. Dirks[1], S. Schüttler[2], J. Golda[2], J. E. Bandow[1]

[1]*Applied Microbiology, Faculty of Biology and Biotechnology, Ruhr University Bochum, Germany*
[2]*Plasma Interphase Physics, Faculty of Physics and Astronomy, Ruhr University Bochum, Germany*

Abstract: Biocatalysis represents a promising, green alternative to conventional catalysis. Peroxygenases carry out one-electron oxidation reactions using H_2O_2 and are not yet used as biocatalysts on an industrial scale. Plasma could serve the *in situ* H_2O_2 production to fuel peroxygenase reactions, preventing enzyme inactivation by accumulating of H_2O_2. Plasma-driven biocatalysis was established using the model enzyme *Aae*UPO. Here we present the expansion of plasma-driven biocatalysis to *Cvi*UPO produced heterologously in *E. coli.*

Keynotes: cold atmospheric pressure plasma, plasma-driven biocatalysis, peroxygenases

Introduction

Plasma is a complex mixture of many components, including electrons, ions, radicals, and excited species. Especially, reactive oxygen and nitrogen species (RONS) are of interest for biological applications. RONS can be distinguished based on their lifetimes. One of the long-living species is hydrogen peroxide (H_2O_2), which we utilize in biocatalysis applications to convert substrates into more valuable products using suitable enzymes (Fig. 1). Enzymes are of great importance as catalysts in different industries, converting organic and biological substances. Biocatalysis is thought to present an environmentally friendly and sustainable alternative to chemical catalysis. The enzyme class of peroxygenases carries out one-electron oxidation reactions and stereoselective oxyfunctionalizations using H_2O_2, which results in various chemical groups. Their industrial application and the use of hydrogen peroxide in biocatalysis is very challenging, since high concentrations of hydrogen peroxide result in enzyme inactivation (suicide inactivation).

We previously reported a non-invasive approach for *in situ* H_2O_2 production for biocatalysis using non-thermal atmospheric pressure plasma [1]. Plasma-driven biocatalysis enables the tuning of H_2O_2 formation to meet the specific needs of the respective enzyme without a requirement for additional components. It has already been shown that plasma-driven biocatalysis with unspecific peroxygenase from *Agrocybe aegerita* (*Aae*UPO) is possible using the microscale atmospheric pressure plasma jet (µAPPJ) as plasma source and immobilization of the enzyme as a protection strategy. The immobilization of enzymes describes the attachment of enzymes to functional groups of a carrier material. This allows to place the enzymes at a distance from the liquid surface, creating a buffer zone in which the short-living species react. This protection strategy at the same time offers the advantage of collecting the enzyme for reuse [3]. The *Aae*UPO catalyzes the conversion of the substrate ethylbenzene to the product R-1-phenylethanol using H_2O_2.

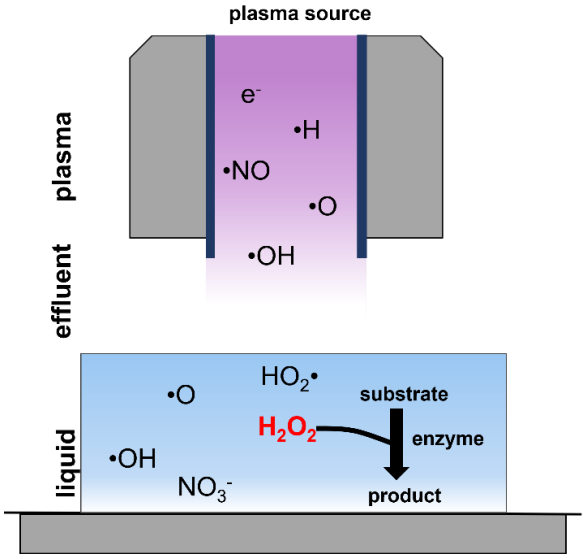

Fig. 1: In plasma-driven biocatalysis hydrogen peroxide is produced by non-thermal plasma and is subsequently used by suitable enzymes like peroxygenases to convert substrates to more valuable compounds.

For biotechnological use, it is necessary to produce enzymes in large quantities in a cost-effective manner. *Escherichia coli* (*E. coli*) is a commonly used host organism for overproduction since it can be handled safely and multiplies quickly. At this time, *Aae*UPO cannot be produced in *E. coli*. Therefore, it appears attractive to employ other enzymes to perform such oxidation reactions. The unspecific peroxygenase from *Collariella virescents* (*Cvi*UPO) can be overproduced in *E. coli* [4] and could thus present an alternative. We investigated the utility of the *Cvi*UPO for plasma-driven biocatalysis. Enzyme stability during plasma treatment was analyzed for the dissolved and immobilized enzyme. Enzymes are inactivated by the chemical modification of critical amino acid residues or degraded [1,2]. Immobilization on hydrophobic and hydrophilic carriers can offer protection

against premature inactivation by plasma treatment by creating a buffer zone between the treated enzyme and plasma-generated toxic species (Fig. 2).

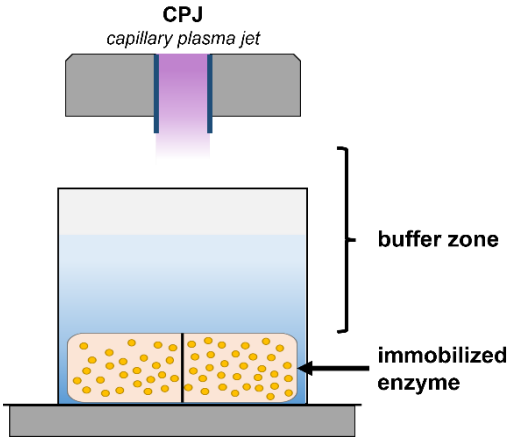

Fig. 2: Enzyme immobilization on different carriers generates an enzyme-free buffer zone between the plasma effluent and the immobilized enzyme, allowing the most reactive species to recombine.

Results

To investigate the efficiency of immobilization in protecting *Cvi*UPO from plasma-mediated inactivation, different immobilization types were tested for their performance under dielectric barrier discharge (PlasmaDerm, Cinogy, Germany) treatment. After immobilization, enzyme-loaded beads in aqueous solution were treated with plasma for 15 min (Fig. 3).

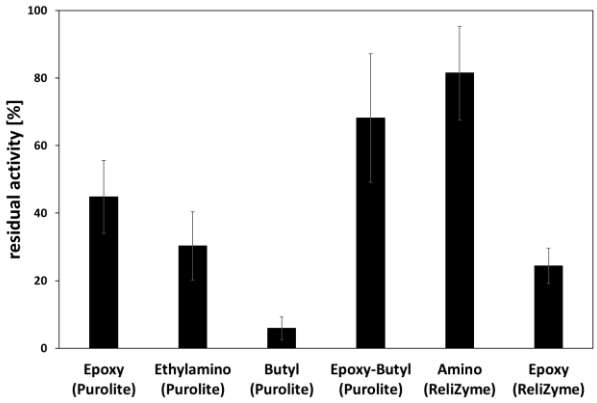

Fig. 3: Residual activity of immobilized unspecific peroxygenase from *Collariella virescens* on different solid supports after 15 min plasma treatment with the dielectric barrier discharge (PlasmaDerm, Cinogy, Germany). Untreated immobilized enzyme was set to 100% activity.

The residual activity after 15 minutes ranged from 5% to 80% depending on the type of carrier used. Residual activity was higher with hydrophobic carriers that bind enzymes covalently, like the amino carrier (ReliZyme).

To evaluate reusability of the immobilized enzyme and substrate conversion rates tested over several cycles of plasma treatment, *Cvi*UPO on ReliZyme amino beads, which showed the highest residual activity after plasma treatment with the DBD, was treated repeatedly with the full replacement of reaction solution between cycles. A capillary plasma jet (CPJ) driven at 6 W with 2 slm helium and 6400 ppm water vapor as feed gas was used to generate H_2O_2. Concentration of H_2O_2 was linear over 5 min. Enzyme activity was maintained over an hour of plasma treatment (Fig. 4), while the free (not immobilized) enzyme is inactivated completely after a few minutes (data not shown). After 6 cycles (60 min total operation) product was still formed, indicating that the enzyme was still active.

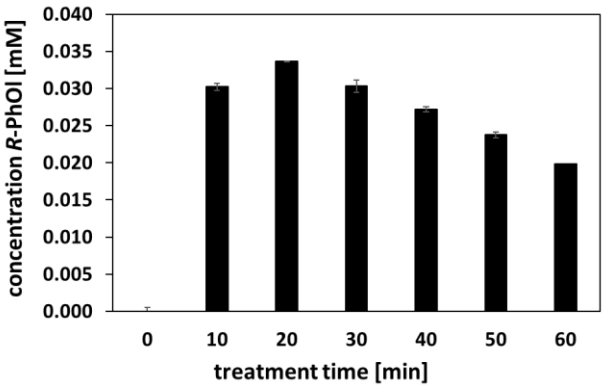

Fig. 4: Plasma-driven biocatalysis with the unspecific peroxygenase from *Collariella virescens* and direct plasma treatment with the capillary plasma jet. The enzyme was immobilized on amino beads (ReliZyme) and placed in a rotating bed reactor in a container filled with 5 ml buffer containing the substrate ethylbenzene. Every 10 min a complete buffer exchange was carried out and the product R-1-phenylethanol was measured with gas chromatography after extraction of the reaction solution.

Summary

*Cvi*UPO was produced in *E. coli* and plasma-driven biocatalysis performed with purified *Cvi*UPO. Like for the previously used model enzyme *Aae*UPO enzyme stability was improved by immobilization of the enzyme. The enzyme remained active for 60 min when the reaction solution was exchanged every 10 min.

Experimental section

Enzyme preparation

The gene *cviUPO* was overexpressed using a pET21a(+) expression plasmid. Overexpression was performed at 16°C and 120 rpm for 5 days. Cells were harvested by centrifugation and lysed by ultrasonication. Proteins were then purified using a nickel agarose-column with the ÄKTA pure system. The heme loading of the enzyme was measured with UV-Vis spectroscopy.

Immobilization
For immobilization, 200 mg of ReliZyme beads (ReliZyme HA403/M, Resindion, Italy) or Purolite beads (Purolite, United states) were washed thrice with 5 ml of 100 mM KPi buffer (pH 7.0). To preactivate the ReliZyme amino carrier, 5 ml 100 mM KPi buffer and 0.5% of glutaraldehyde (Merck, Germany) were mixed and ReliZyme beads incubated for 3 h at 25°C with constant overhead shaking. Afterwards, ReliZyme beads were washed thrice with 5 ml of KPi buffer before 50 µM of enzyme (*Cvi*UPO) were added to a total volume of 5 ml. The reaction mixture was incubated overnight at 8°C with constant overhead shaking to allow complete enzyme binding.

Plasma treatment
The capillary plasma jet was operated at 6 W with 2 slm helium and 6400 ppm water vapor as feed gas. To control water admixture, the feed gas was split and partially routed through a bubbler containing deionized water at ~1°C.
For plasma treatment, a rotating bed reactor filled with 50 µM immobilized enzyme was transferred into a 5 ml container filled with 5 ml of 100 mM KPi buffer (pH 7.0) containing 50 mM of ethylbenzene. Every 10 min the reaction solution was exchanged, samples were taken and extracted for GC measurement.

Product measurement
For analysis, a GC-FID (GC-2010 Plus, Shimadzu, Japan) was equipped with a Hydrodex β 6TBDM capillary column (25 m × 0.25 mm I.D and 25 µm film thickness, Macherey-Nagel). Hydrogen was used as the carrier gas and the flow rate was set to 3 ml min^{-1}. Samples (4 µl injection volume) were injected with a split of 1:50. The injector and detector temperatures were set to 250°C. The GC oven and column temperature was isothermally set to 120°C and held for 13 min. Final concentrations of converted products after biocatalysis were calculated from the measured area under the curve values of the corresponding peaks in the chromatogram normalizing against an internal standard.

Acknowledgements:
The German Research Foundation is kindly acknowledged for providing funding within CRC1316 and RTG2341.

References:
[1] A. Yayci, Á. G. Baraibar, M. Krewing, E. F. Fueyo, F. Hollmann, M. Alcalde, R. Kourist and J. E. Bandow, *ChemSusChem*, 13, 2072 (2020).
[2] M. Krewing, B. Schubert and J. E. Bandow, *Plasma Chem Plasma Process*, 40, 685 (2020).
[3] A. Yayci, T. Dirks, F. Kogelheide, M. Alcalde, F. Hollmann, P. Awakowicz and J. E. Bandow, *J Phys D: Appl Phys*, 54, 035204 (2021).
[4] D. Linde, E. Santillana, E. Fernández-Fueyo, A. González-Benjumea, J. Carro, A. Gutiérrez, A. T. Martínez and A. Romero, *Antioxidants*, 11, 891 (2022).