Sabine Schelch, BSc

Coimmobilized D-amino acid oxidase and catalase: An efficient partnership for bubble-free oxidations

MASTERARBEIT

zur Erlangung des akademischen Grades
Diplom-Ingenieurin
Masterstudium Biotechnologie

eingereicht an der

Technischen Universität Graz

Betreuer

Univ.-Prof. Dipl.-Ing. Dr. tech. Bernd Nidetzky

Dr. Juan M. Bolivar

Institut für Biotechnologie und Bioprozesstechnik

Graz, April 2016

Abstract

In modern chemical technology oxidases like D-amino acid oxidase (DAAO) have become an important resource used in a various processes. DAAO is mostly used in immobilized form on porous particles, increasing its stability and enabling it's recycling in industrial applications. For high reactor productivity essentials are a high active catalyst and especially for oxidases a distinguished oxygen delivery. However, independent on the oxygen transfer towards the liquid phase, another transport step exists when immobilizates of enzymes on porous carriers are utilized. Recently published studies show that immobilizates on porous particles indicate an oxygen diffusion limitation between particle and aqueous environment. The use of oxidases in a process requires in most cases addressing another major issue, protection against or removal of inactivating by-product H_2O_2. For some enzymes the coimmobilization with catalase has been shown to be very beneficial, as catalase is catalyzing the formation of O_2 and H_2O from H_2O_2. A proper designed catalase coimmobilizate may be useful not only to degrade hydrogen peroxide but also produce oxygen in situ. A strategy based in the use of H_2O_2 as unique oxygen source was developed in this thesis. The goal here was developing a feeding strategy of H_2O_2 that allows a high O_2 concentration in the particle, but low enough concentration of H_2O_2 to avoid additional inactivation of either of the two enzymes. As main tool for the development of the feeding strategy, oxygen inside the particle was monitored allowing a better understanding of oxygen production and consumption inside the particle.

After having established a stable preparation with a proper feeding strategy possibilities for reaction intensification were tested by increasing the particle amounts used in the reactor and by increase of H_2O_2 addition rates. DAAO productivity could in the end increased to a two-fold compared to productivity at air saturation under oxygen limited conditions.

Zusammenfassung

In der modernen chemischen Technologie sind Oxidasen wie die D-Amino Acid Oxidase (DAAO) zu einem wichtigen Biokatalysator geworden, der in vielen unterschiedlichen Prozessen angewendet wird. DAAO wird meist in immobilisierter Form auf porösen Partikeln angewandt, wodurch die Stabilität des Enzyms erhöht wird, außerdem ermöglicht die Immobilisierung die wiederholte Verwendung in industriellen Prozessen. Für eine hohe Produktivität im Reaktor sind ein sehr aktiver Katalysator und speziell für Oxidasen eine ausgezeichnete Sauerstoffversorgung ausschlaggebend. Allerdings existiert unabhängig vom Sauerstofftransport in die flüssige Phase ein weiterer Transportweg bei der Anwendung von Immobilisaten auf porösen Partikeln. Kürzlich veröffentliche Studien enthüllen dass sich bei Immobilisaten auf porösen Partikeln eine Diffusionslimitierung von Sauerstoff zwischen Partikeln und wässriger Umgebung zeigt. Die Anwendung von Oxidasen in einem Prozess verlangt in den meisten Fällen auf eine weitere wichtige Frage einzugehen, nämlich den Schutz vor oder die Entfernung des inaktivierenden Nebenprodukts H_2O_2. Für einige Enzyme zeigte sich, dass die Coimmobilisierung mit Catalase sehr hilfreich ist, aufgrund dessen dass Catalase die Bildung von O_2 und H_2O aus H_2O_2 katalysiert. Ein gut entwickeltes Coimmobilisat mit Catalase kann nicht nur zweckdienlich für den Abbau von H_2O_2 sein, sondern auch in situ Sauerstoff produzieren. Eine Strategie basierend auf der Verwendung von H_2O_2 als einzigartige Sauerstoffquelle wurde in dieser Arbeit entwickelt. Das Ziel war die Entwicklung einer Zuführungsstrategie für H_2O_2 welche eine hohe O_2-Konzentration im Partikel ermöglicht, gleichzeitig aber sollte die H_2O_2 Konzentration niedrig genug sein um eine Inaktivierung der beiden Enzyme zu vermeiden. Wichtigstes Hilfsmittel für die Entwicklung der Zuführungsstrategie war die Überwachung des Sauerstoff in den Partikeln, welche ein besseres Verständnis für die Sauerstoffproduktion und- verbrauch in den Partikeln erlaubte. Nach der Entwicklung eines stabilen Coimmobilisats mit einer funktionierenden Zuführungsstrategie, wurden Möglichkeiten für Intensivierungen der Reaktion durch

Erhöhung der Partikelmenge im Reaktor und Erhöhung der Zuführungsraten von H_2O_2 getestet. Die Produktivität von DAAO konnte am Ende um ein zweifaches, im Vergleich zur Produktivität wenn die Reaktion unter Sauerstofflimitierung durchgeführt wurde, erhöht werden.

Acknowledgements

I am very grateful to my supervisor Univ.-Prof. DI Dr. techn. Bernd Nidetzky for his ongoing support on my work with the enzyme DAAO in these past years and especially for the supervision of my master thesis.

I furthermore would like to thank my other supervisor Dr. Juan M. Bolivar for giving me an interesting and demanding research object, for constant support and for helping me become a better scientist.

I thank my colleagues at the Institute of Biotechnology and Biochemical Engineering, without their help I would not have been able to complete this work. I want to thank especially Tanja Consolati, Christina Krämer, Marco Tribulato, Donya Valikhani, Anna Trummer, Karin Longus, Margret Schiller and Natascha Loppitsch.

I would like to thank my family for their support and everything they have done for me.

Mareike Monschein and Bernhard Lauß, thank you for your friendship. Without you I wouldn't have made it through the past two years.

Fritz Pump, thank you for your patience and understanding every time I had to work late at the lab. Thank you for being my partner in life.

Table of contents

Part 1: Highly Active Solid-Supported Catalase Design Enables High Effective H_2O_2 Decomposition and O_2 Production

1. Introduction

Hydrogen peroxide as problem and opportunity in biocatalysis. Hydrogen peroxide is one of the most problematic side products in enzyme catalysed reactions, in particular for oxidase catalysed reactions. Hydrogen peroxide is not only a major inactivating agent for some enzymes, e.g. D-amino acid oxidase; it also has the potential causing unwanted chemical modification on the reaction product [1].

Removal of hydrogen peroxide is preferably done with enzyme catalase. Other possibilities such as use of metals usually tend causing unwanted side effects [1], while catalase is only producing O_2 and H_2O from H_2O_2. Since in most reactions producing H_2O_2 these two products are present from the start no disturbance of the reaction or no further modification of the product is expected.

One major concern about the use of catalase for hydrogen peroxide decomposition is the inactivation of catalase by its substrate hydrogen peroxide [2]. Besides the possibility of catalase inactivation by hydrogen peroxide another issue for inactivation is the tetrameric structure of catalase [2]. As a solution for these issues various studies [1, 2, and 3] suggest immobilization of the enzyme on a rigid surface. Immobilization has also been observed as beneficial against thermal inactivation and allowing an easier handling. Another beneficial effect would be that many oxidases are used in immobilized form, a well-designed approach of catalase immobilization is therefore eligible. Catalase could be immobilized on another carrier, allowing an easier handling and reuse in a reactor or another feasible suggestion is coimmobilization directly with oxidase for a more efficient removal of hydrogen peroxide from direct environment of the immobilized oxidase.

While there are many papers published on different approaches showing an improvement in stability and activity of the immobilized catalase for example immobilization via multi-subunit immobilization with crosslinking of the beads [2] or covalent attachment [3], we tried

a different approach focused on the generation of highly active catalase biocatalysts. In the last years we very successfully modified enzymes with a Z_{basic2}_tag [4, 5] allowing an oriented ionic adsorption on porous carriers. One of these enzymes was a D-amino acid oxidase (*Tv*DAAO) from *Trigonopsis variabilis* [4]. The modified *Tv*DAAO showed an increased stability immobilized to porous carriers [6, 7], however it is also a possible candidate to be used in combination with catalase as it is strongly inactivated over time by its by-product H_2O_2 even when the enzyme is immobilized on a carrier. Therefore we decided modifying a catalase with a Z_{basic2}_tag that could be used for simple and possibly stable immobilization and furthermore feasible coimmobilization with *Tv*DAAO on a porous carrier.

Besides direct decomposition of the inactivating agent hydrogen peroxide a coimmobilization of an oxidase and catalase can have another beneficial effect, namely an increase of oxygen in solution. Oxidases are at high concentrations oxygen limited in solution [8] or in immobilized form [6]. It could therefore be possible to recycle the unwanted by-product H_2O_2 and increase productivity of the oxidase reaction. In recent years developed monitoring of intraparticle oxygen [6, 7] should furthermore show if this recycling is possible and also gain a better understanding of the internal environment of immobilized catalase.

For intraparticle oxygen monitoring compatibility of labeling with luminescent dye $Ru(dpp)_3$ (Dichloride (4, 7-diphenyl-1, 10-phenantroline) ruthenium (II)) for catalase should be tested. This dye allows in combination with a fiber-optic cable monitoring of average oxygen concentration inside the particle.

The modification with Z_{basic2} of catalases from different microorganisms was previously described elsewhere [9]. In this work further characterization of a modified catalase from *Bordetella pertussis* (Z_{basic2}*_BP_KatA*) is reported. Furthermore the compatibility of catalase with luminescent dye $Ru(dpp)_3$ is studied and observation of intraparticle oxygen courses after addition of hydrogen peroxide gave a new insight into immobilized catalase.

2. Materials and Methods

2.1. Materials

Z_{basic2}_BP_KatA was expressed in E. coli [for details see 9]. Carriers Sepabeads sulfonic and Relisorb 400 were kind gifts of Resindion (Milano, Italy). Dichloride (4, 7-diphenyl-1, 10-phenantroline) ruthenium (II), $Ru(dpp)_3$, was from ABCR GmbH (Karlsruhe, Germany). Unless stated otherwise, all chemicals were of highest purity available from Sigma-Aldrich (Vienna, Austria) or Roth (Karlsruhe, Germany).

2.2. Assays

Activity of soluble or immobilized *Z_{basic2}_BP_KatA* was measured spectrophotometrically by observing the consumption of H_2O_2 by catalase at 240 nm [2]. Activity was determined with this assay for a pH range between 6.0 and 9.0 (Further details see section 6.2.1 in 6.2 Supporting Methodology). Activity of Glucose oxidase was determined by measuring oxygen consumption during oxidation of Glucose with a bulk oxygen sensor [6]. Measurement was performed at 30°C in 50 mM air-saturated potassium phosphate buffer, pH 8.0, 500 mM Glucose was used as substrate. Rates of O_2 consumption were measured with a fiber-optic oxygen microoptode (PreSens - Precision Sensing GmbH, Regensburg, Germany) connected via a fiber optic oxygen meter (model microx TX3, PreSens). O_2 concentration was monitored continuously (one measurement per 1 sec). One activity unit is the amount of *Z_{basic2}_BP_KatA* that consumes 1 µmol/min H_2O_2 at the conditions used.

2.3. *Z_{basic2}_BP_KatA* Immobilization

Z_{basic2}_BP_KatA was immobilized on carriers Sepabeads sulfonic in a previous work [9] and Relisorb 400 in this work. After the carrier was rinsed with buffer (Potassium Phosphate Buffer PPB, pH 7.0, 0.25 M NaCl), an appropriately diluted amount of crude extract (activity of *Z_{basic2}_BP_KatA* between 100 and 10 000 U/mL) was loaded on the carrier and incubated for an hour. The carrier was afterwards washed with buffer (PPB, pH 7.0, 0.25 M NaCl). Immobilization yield and catalytic effectiveness factor were determined by hydrogen peroxide assay.

Catalytic effectiveness factor (η) of immobilized *Z_{basic2}_BP_KatA* was defined as ratio given in percent of measured activity of the immobilizate (U/g_carrier) to the theoretical immobilized activity (U/g_carrier).

2.4. Bulk and Internal Oxygen Measurements of Decomposition of H_2O_2 by Immobilized *Z_{basic2}_BP_KatA*

Oxygen measurements in bulk and in particle were used for measuring oxygen production by catalase from a defined amount of H_2O_2.

Overall set-up for a bulk and internal oxygen measurement is described in detail elsewhere [6]. External oxygen was measured by using a fiber-optic oxygen microoptode (PreSens - Precision Sensing GmbH, Regensburg, Germany) connected to a fiber-optic oxygen meter (model microx TX3, PreSens).

Labeling of immobilizate was performed after immobilization. Labeling mixture contained 0.25 mg of oxygen responsive dye $Ru(dpp)_3$ per g of carrier and 5 % (v/v) of ethanol (98 %).

A general procedure for intraparticle oxygen measurement was published previously [SI of 6]. Signal changes of $Ru(dpp)_3$ on immobilizate are measured for intraparticle oxygen calculation by a fiber-optic cable connected to a fiber optic oxygen meter (model pH-1 mini, PreSens). Free *Z_{basic2}_BP_KatA* / immobilizate of *Z_{basic2}_BP_KatA* and Glucose (Stock 1 M) was added

to a flask containing buffer (PPB, 50 mM, pH 8.0) before the measurement was started. The final volume was 4 mL and particle concentration was 5 mg/mL, if not noted otherwise. Measurement was always performed at 30 °C. Temperature was kept constant by using a water bath.

Measurement itself can be divided into two parts: A first were oxygen is consumed by GOX and a second were oxygen is produced by free or immobilized catalase.

Measurement was started by adding GOX after bulk sensor and fiber optic cable (for detection of oxygen quenching from $Ru(dpp)_3$ on carrier; not added when only free Z_{basic2}*_BP_KatA* was measured) showed a stable signal. Oxygen measurement is continued until nearly all oxygen is consumed in bulk and no change in oxygen course occurs. Afterwards H_2O_2 is added (1.25 – 5 mM). Depending on purpose of experiment H_2O_2 concentration was varied and addition could be repeated at specific time points. Measurement is usually stopped after all by Z_{basic2}*_BP_KatA* produced oxygen is consumed again by GOX.

3. Results and Discussion

3.1. Functional Expression and Characterization of *Z_{basic2}_BP_KatA*

Screening and selection of potential candidates for the intention of highly active Z_{basic2} fused catalases was previously done [9]. Of all strains tested the fusion protein derived from *Bordetella pertussis* (*Z_{basic2}_Bp_KatA*) was showing the best results and was therefore used for further characterization. Data from a previously [9] done purification of cell lysate via cation exchange chromatography showed an activity of 111 943 U/mL at pH 7. Specific activity of catalase in the cell extract was 5331 U/mg protein and protein content was 21 mg/mL.

Activity of *Z_{basic2}_Bp_KatA* in cell extract (protein 8 mg/mL) was determined with a hydrogen peroxide assay in different buffers for a pH range between 6.0 and 9.0 in this work. In Figure 1 it can be seen that activity appeared to be highest at pH 6 (~ 36 500 U/mL); however noise of measurements was also considerably higher. The lowest activity was seen at pH 9 (~ 20 300 U/mL). The lower activity for cell extract in this work can be explained by using a higher amount of buffer for cell disruption. Results of studies about storage stability of the enzyme, its long-term stability at mild conditions and its inactivation under strongly diluted conditions are again described in another work [9].

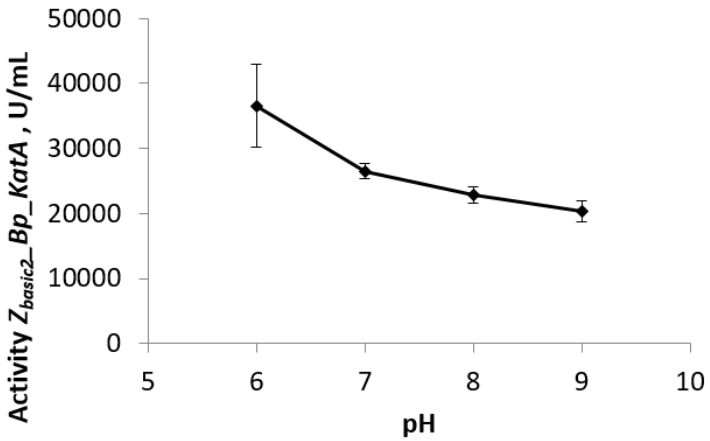


Figure 1. *Activity of crude extract (protein 8 mg/mL) from* Z_{basic2}*_BP_KatA measured by* H_2O_2 *consumption in a spectrophotometer at different pH (50 mM sodium citrate for pH 6; 50 mM potassium phosphate for pH 7 and 8; 50 mM sodium carbonate for pH 9). While activity at pH 6 was highest, measurement also showed the highest noise.*

3.2. Design of Solid-supported *Z_{basic2}_BP_KatA* on Sulfonate Activated Carriers

Z_{basic2}_BP_KatA is bound onto carrier Relisorb 400 resulting in a high yield and high selectivity of immobilization. Relisorb 400 are polymethacrylate porous carriers harboring a sulfonate active group and thus allowing a reversible ionic immobilization. Immobilization progress is very fast and a variable amount of activity can be bound with high yield. Between and 1 000 and 100 000 U were offered per g of carrier. The immobilization seems to progress quickly and in a previous work [9] more than 90 % were immobilized in the first 10 minutes, when previously purified *Z_{basic2}_BP_KatA* was used. When crude extract was used the amount the final yield was lower, around 70 %, when incubation time remained the same. Probably this was due to the high protein concentration in crude extract. The yield of immobilization was between 95 and 99 % after 45 min of incubation time. The incubation time in this work was 1h ensuring a complete immobilization. Immobilization yield was above 95 % for the whole range used. The apparent activity of the carrier was about 18 % of offered activity for a lower loading (3000 U/g carrier) and around 2 % for a higher one (100 000 U/g carrier, see Figure 2)). The catalytic effectiveness of the immobilized enzyme showed therefore an enzyme loading dependency. It should also be noted that apparent activity observed was very similar for both carrier (Sepabeads sulfonic and Relisorb 400).

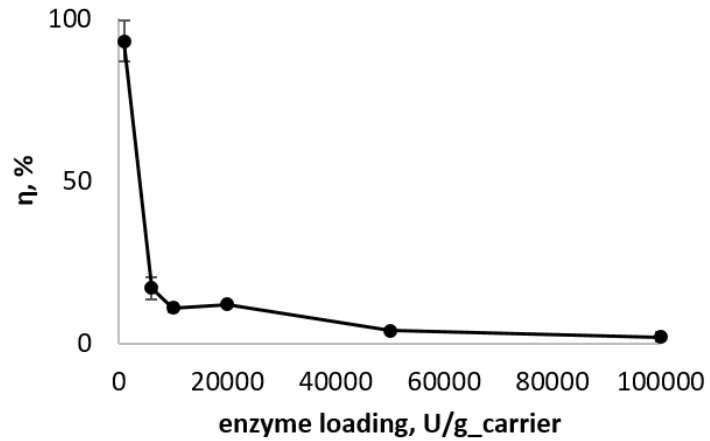


Figure 2. *Catalytic effectiveness factor of Z_{basic2}_BP_KatA immobilized on Relisorb 400. Activity of immobilized enzyme was measured from the rate of H_2O_2 consumption observed in a spectrophotometer, and η is the ratio of measured and theoretical activity. Theoretical activity was determined from the difference in soluble enzyme activity before and after the immobilization.*

To reveal the reason of low catalytic effectiveness, the enzyme was eluted from carrier and recovered activity was measured previously [9]. When the enzyme was eluted from the carrier, 70 -100 % of the activity could be recovered. This is indicating that the observed decrease of activity was not due to inactivation of the enzyme during immobilization. A possible explanation for this apparent loss in enzyme activity in its immobilized form is a diffusion limitation of substrate from bulk into the particle. This explanation is supported by the observed decrease in percentage activity with the increase of enzyme loading onto the carrier. Immobilization yield of immobilizates with crude extract of Z_{basic2}*_Bp_KatA* crude started decreasing after 3-4 weeks of storage at 4 °C. No apparent activity loss of crude extract could be detected by hydrogen peroxide assay during this time. A precipitation of protein could be observed and after removal of precipitate via centrifugation a loss of activity of Z_{basic2}*_BP_KatA* could be observed. Increase of ionic strength did not have a stabilizing effect on the crude extract.

3.3. Insight into the Catalase Performance of Immobilized *Z_{basic2}_BP_KatA* via Oxidation Coupled Reaction

As a standard procedure for catalase activity measurements the spectrophotometrically measured H_2O_2 assay as described above in the methodology section is a useful tool. However, it might be more useful gaining a better understanding of the activity of *Z_{basic2}_BP_KatA* when its product O_2 can be monitored for example when catalase is used in an oxidation coupled reaction. It is therefore of vital importance monitoring oxygen generation directly. On-line oxygen measurements could possibly be used for activity calculation of *Z_{basic2}_BP_KatA*. However, there are restrictions of oxygen solubility in aqueous solution at mild conditions (25-30 °C, atmospheric pressure). The issue is that oxygen production is very fast even for low amounts of *Z_{basic2}_BP_KatA* used, therefore the net oxygen production should be slowed down using a coupled reaction of oxygen consumption. Above a concentration of 1000 µmol/L bubble formation in solution will occur in solution, causing an inhomogeneous distribution in solution and thus hindering correct monitoring of oxygen generation. It is therefore advisable to couple oxygen generation with a reaction with oxygen consumption. For this purpose soluble glucose oxidase and glucose were used.

The final oxygen concentration reached, when GOX is consuming and *Z_{basic2}_BP_KatA* is producing O_2, is dependent on GOX activity (under the condition that available Glucose is not limited), H_2O_2 amount added and *Z_{basic2}_BP_KatA* activity (See Figure S1). Increased GOX activity when *Z_{basic2}_BP_KatA* activity and H_2O_2 addition are kept constant leads to a decreased final oxygen concentration. Repeated addition of H_2O_2 showed that equilibrium is always reached after each addition; however the oxygen concentration at this equilibrium is increasing with each addition (Figure 3). This is understandable considering that at this H_2O_2 concentration *Z_{basic2}_BP_KatA* is substrate limited.

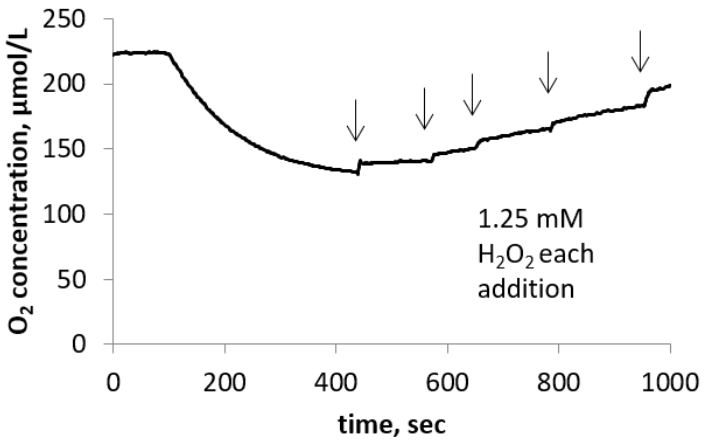


Figure 3. *Measurement of bulk oxygen consumption by free GOX (0.38 U/mL solution in flask) and bulk oxygen production by immobilized Z_{basic2}_BP_KatA from H_2O_2 addition (1.25 mM each time, indicated by arrows). Reaction is started by adding GOX to Glucose (500 mM) and immobilized Z_{basic2}_BP_KatA (10 000 U/ g_carrier; 5 mg carrier were added per mL of solution) in a stirred solution.*

Due to the fact that H_2O_2 is a limited substrate in above described set-up, it is not possible to calculate free or immobilized catalase activity in this way. When the concentration of free GOX used is known, it can be determined if the oxygen production by catalase is matching the consumption by GOX. For the first 2 addition of H2O2 in Figure 3 oxygen consumption and production seem to match as there is no noticeable increase or decrease in oxygen concentration, after the third addition however oxygen is slightly increasing, indicating that more oxygen is produced than consumed. These observations are useful to situate the order of magnitude of catalase activity. These findings are quite intriguing, the next step was therefore internal oxygen sensing.

3.4. Insight into Local O_2 Generation of Catalase: Design of Oxygen Responsive Catalase

Bulk monitoring is simply not enough for characterizing *Z_{basic2}_BP_KatA* immobilizates due to the fact that there is proof that oxygen has a diffusion limitation from bulk to particle and it seems reasonable assuming that this is true vice versa. Recent developments in intraparticle oxygen monitoring [2, 8, and 10] allow internal monitoring. Important for this monitoring is a labeling compatibility of the enzyme with $Ru(dpp)_3$. Activity measurements of *Z_{basic2}_BP_KatA* immobilizates showed hardly any observable inactivation of the enzyme after labeling with the dye was performed. Coimmobilization yield of dye on the carrier was 100 % for enzyme loadings between 1 000 and 50 000 U/g_carrier, however for higher enzyme loadings (100 000 U/ g_carrier) yield was only between 70 and 80 %. A possible explanation for this is that the high protein content is affecting the coimmobilization of dye onto the carrier. Labeled *Z_{basic2}_BP_KatA* immobilizates were used for calibration of dynamic range of oxygen response. For calibration between 0 and 250 µmol/L of oxygen concentration all oxygen in the system was first depleted with N_2 and then the system was monitored while oxygen diffused from the surrounding air into bulk and particle. This experiments showed a linear correlation between phase and oxygen concentration between 0 and 250 µmol/L, just the highest loading (100 000 U/ g_carrier) showed a nonlinear response in the lower range between 0 and 50 µmol/L. However, calibration in this range is not sufficient; *Z_{basic2}_BP_KatA* can increase oxygen in solution up to 1000 µmol/L at 30 °C (above this value a high bubble formation is occurring, hindering oxygen measurement in bulk). Study of the oxygen response in the complete range of oxygen solubility in the reaction medium is therefore necessary. Several methods were applied (See supporting information). The method allowing a controlled reaction over the range of 0 to 1000 µmol/L was a combination of saturating buffer with pure oxygen and a controlled decrease to 0 µmol/L by consuming oxygen with GOX and Glucose as substrate. This method showed a near linear response of the oxygen dye for an oxygen concentration up to 1000 µmol/L (Figure S2). Influence of

hydrogen peroxide (5 mM) on phase shift of oxygen responsive dye $Ru(dpp)_3$ were studied at 100 % and 0 % air saturation, ensuring no undesired effects occurred during monitoring of oxygen generation in particle with immobilized Z_{basic2}*_BP_KatA*. For control experiments labeled particles without immobilized enzyme were used. Bulk and intraparticle oxygen at 100 % air saturation was monitored and after recording a stable signal hydrogen peroxide was added to the solution. No change in phase could be observed after the addition of H_2O_2 for several minutes. The same experiment was done at 0 % air saturation. All oxygen was removed with N_2 before oxygen monitoring was started. Addition of hydrogen peroxide showed again no change of the phase shift for several minutes (See Figure S3). It can therefore be concluded that H_2O_2 is not affecting the dye labeled on the carrier nor is it interfering with phase shift.

3.5. Identification and Quantification of Inverse Oxygen Gradients into Immobilized Catalase. High Effectiveness for Local H_2O_2 Decomposition and O_2 Generation

After establishing intraparticle oxygen measurements for *Z_{basic2}_BP_KatA* immobilized over a wide range and proving that H_2O_2 has no effect on the measurement, the next logical step is the application of the method and monitoring of intraparticle oxygen gradients. Main goal of these experiments is gaining a better understanding of oxygen generation inside the particle and the effect of oxygen generation inside the particle on oxygen concentration in bulk. Intraparticle oxygen measurements of *Z_{basic2}_BP_KatA* immobilizates are performed in the same way as above described oxygen bulk measurements only intraparticle oxygen is monitored in parallel to bulk oxygen. For intraparticle measurements avoiding bubble formation is even more crucial, a homogeneous distribution of particles in solution gives the most accurate signal for phase measurement. Avoiding high accumulation of oxygen in bulk can be achieved by using a GOX activity that is comparable to catalase activity (previously measured with H_2O_2 assay), by adding enough glucose avoiding substrate limitation (mostly a stock of 200-500 mM Glucose was used) and by controlling the addition of H_2O_2.

Experiments were started by monitoring both bulk and intraparticle oxygen courses of a solution containing labeled *Z_{basic2}_BP_KatA* immobilizates and Glucose. Reaction was started by adding free Glucose oxidase (activity needed was calculated from H_2O_2 measurement for immobilized *Z_{basic2}_BP_KatA* and after nearly all oxygen was consumed in bulk and particle a defined amount of H_2O_2 (1.25 mM) was added. While monitoring both, bulk and intraparticle oxygen, it was apparent, that oxygen concentration was fast rising inside the particle and whereas there was no increase in oxygen concentration in bulk (Figure 4). Explanation for this phenomenon is a fast production of oxygen from hydrogen peroxide by *Z_{basic2}_BP_KatA* inside the particle and a slower diffusion of oxygen from the particle to the bulk, where oxygen is consumed by GOX. This phenomenon can be compared to oxygen diffusion limitation for oxidases immobilized on porous carrier with a high loading [6].

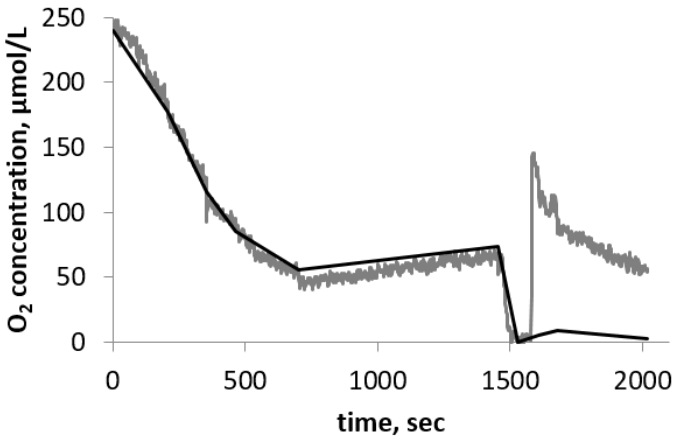


Figure 4. *Measurement of oxygen consumption by free GOX and oxygen production from H_2O_2 addition (1.25 mM, indicated by arrow) by immobilized Z_{basic2}_BP_KatA in bulk (black line, continuous measurement) and particle (grey line, continuous measurement). Differences in bulk and intraparticle oxygen after the addition of H_2O_2 indicate a fast intraparticle oxygen production and a slower transport of oxygen from particle to bulk (inverse oxygen gradient).*

Oxygen diffusion limitation is occurring, when oxygen inside the particle is consumed much faster than oxygen can diffuse from bulk into the particle. For *Z_{basic2}_BP_KatA* immobilizates this is reversed: Reaction producing oxygen inside the particle is much faster than diffusion of oxygen from particle to bulk. Therefore this oxygen gradient between bulk and intraparticle oxygen can be called an inverse oxygen gradient.

Observation of the inverse oxygen gradient raised also the question if there is a delay in response between both sensors when O_2 is generated inside the particle. Different amounts of *Z_{basic2}_BP_KatA* were used to show that the maximum reached O_2 concentration in bulk and particle is dependent on *Z_{basic2}_BP_KatA* concentration as well as on H_2O_2 concentration (Figure 5).

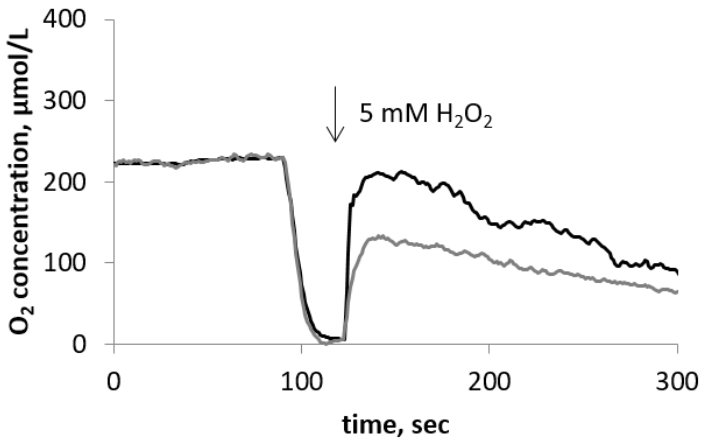


Figure 5. *Measurement of oxygen consumption by free GOX and oxygen production from H_2O_2 addition (5 mM, indicated by arrow) by free Z_{basic2}_BP_KatA in presence of particles labeled with $Ru(dpp)_3$ in bulk (black line, continuous measurement) and particle (grey line, continuous measurement). Experiment showed that oxygen produced in bulk needs a certain amount of time to diffuse into the particle (O_2 in bulk is initially higher than in particle) and thus undermines the finding of an inverse oxygen gradient when oxygen is produced in particle.*

Experiments did show that after the addition of glucose and after the addition of H_2O_2 the signals of bulk and intraparticle were the same, indicating no delay in response of both sensors. In all experiments a lower maximum O_2 concentration was reached in particle as compared to bulk, however this can be explained by O_2 diffusion limitation. This is fitting since O_2 is generated in bulk and has to migrate into the particle to be detected by the intraparticle O_2 sensor.

Inverse oxygen gradients were further studied with higher amounts of H_2O_2. Figure 6 shows the oxygen courses of two experiments done as described above. For these experiments the amounts of H_2O_2 added were 2.5 and 3.75 mM. Due to this higher concentration of H_2O_2 the inverse oxygen gradient is more difficult to observe.

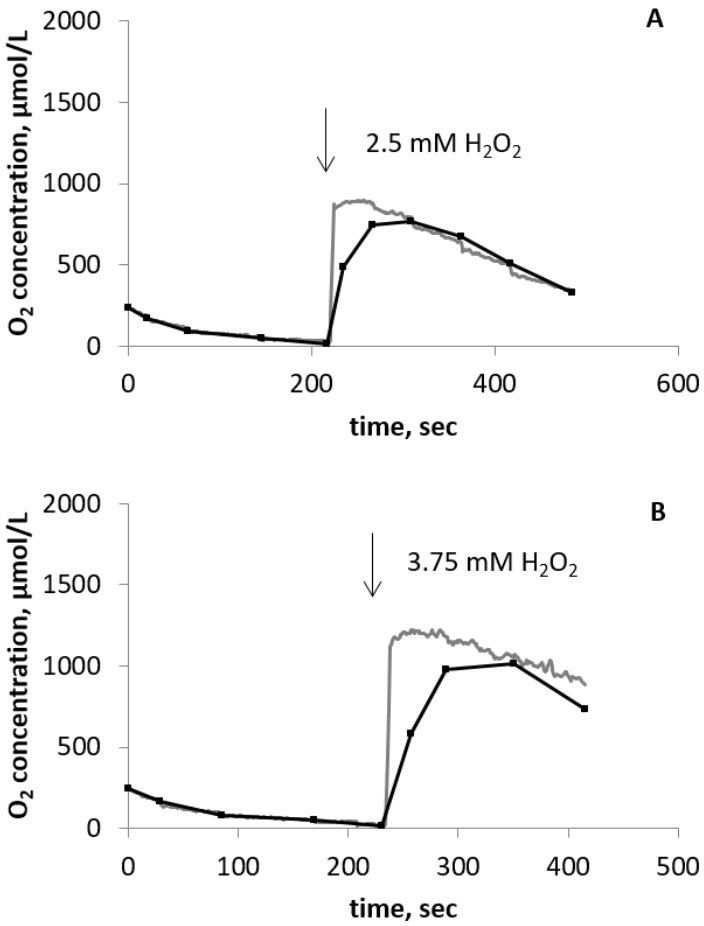


Figure 6. *Measurement of oxygen consumption by free GOX and oxygen production from H_2O_2 addition (Addition of 2.5 mM H_2O_2 (A) and 3.75 mM (B); indicated by arrow) by immobilized Z_{basic2}_BP_KatA in bulk (black line, continuous measurement) and particle (grey line, continuous measurement). Differences in bulk and intraparticle oxygen after the addition of H_2O_2 indicate a fast intraparticle oxygen production and a slower transport of oxygen from particle to bulk (inverse oxygen gradient).*

It can be clearly seen that oxygen is first generated in particle, however due to the high oxygen concentration quickly generated; oxygen is also accumulating in bulk, since the activity of the enzyme is the same as in previous experiments.

4. Conclusions

In this work a further characterization and development of recently established *Z_{basic2}_BP_KatA* confirmed that this catalase is in its immobilized form highly active and operationally stable. Activity measurement at different pH showed a reasonable high activity in a broader range of pH. Immobilization was performed over a wide range of loading (100 – 100 000 U/ g carrier) and over the whole range immobilization yield was above 95 % in 1h thus proving that this method of immobilization is applicable over a wide range for the enzyme.

However, effectiveness factor was decreasing with increased loading indicating a diffusion limitation into the particle. The confirmation of this was achieved by focusing on oxygen monitoring of the immobilized catalase. At first in combination with oxidase GOX bulk oxygen was monitored allowing observation of a fast oxygen production from hydrogen peroxide. Following this studies internal oxygen monitoring gave an insight in internal hydrogen peroxide consumption and oxygen production. After confirming a compatibility with luminescent dye $Ru(dpp)_3$, intraparticle oxygen courses gave proof of accumulation of produced oxygen in the particle due to oxygen diffusion limitation from particle to bulk, an inverse oxygen gradient. Inverse oxygen gradients are especially interesting for oxygen diffusion limited immobilized oxidases. Future studies with *Z_{basic2}_BP_KatA* should therefore focus on designing a coimmobilizate with an oxidase enhancing stability and activity of an immobilized preparation of an oxidase.

5. References

[1] Hernandez, K., Berenguer-Murcia, A., Rodrigues, R.C., Fernandez-Lafuente, R.

`Hydrogen Peroxide in Biocatalysis. A Dangerous Liaison´.

Current Organic Chemistry, 2012, 16, 2652-2672

[2] Lorena Betancor, Aurelio Hidalgo, Gloria Fernandez-Lorente, Cesar Mateo, Roberto Fernandez-Lafuente, and Jose M. Guisan

`Preparation of a Stable Biocatalyst of Bovine Liver Catalase Using Immobilization and Postimmobilization Techniques´.

Biotechnol. Prog. 2003, 19, 763−767

[3] Tukel, S.S., Alptekin, O.

`Immobilization and Kinetics of Catalase onto Magnesium Silicate´.

Process Biochemistry, 2004, 12, 2149-2155

[4] Wiesbauer, J., Bolivar, J. M., Mueller, M., Schiller, M., Nidetzky, B.

`Oriented Immobilization of Enzymes Made Fit for Applied Biocatalysis: Non-Covalent Attachment to Anionic Supports using Z_{basic2} Module´.

ChemCatChem, 2011, 3, 1299–1303.

[5] Bolivar, J.M., Eisl, I., Nidetzky, B.

`Advanced Characterization of Immobilized Enzymes as Heterogeneous Biocatalysts´.

Catalysis Today, 2015, 259, 66-80

[6] Bolivar, J.M., Schelch, S., Mayr, T., Nidetzky, B.

`Dissecting Physical and Biochemical Factors of Catalytic Effectiveness in Immobilized D-Amino Acid Oxidase by Real-Time Sensing of O_2 Availability Inside Porous Carriers´.

ChemCatChem, 6 (4): 981-986

[7] Bolivar, J.M., Schelch, S., Mayr, T., Nidetzky, B.

`Mesoporous Silica Materials Labeled for Optical Oxygen Sensing and Their Application to Development of a Silica-Supported Oxidoreductase Biocatalyst´.

ACS Catalysis, 2015, 5, 5984-5993

[8] Schneider, K., Dorscheid, S., Witte, K., Giffhorn, F., Heinzle, E.

`Controlled feeding of hydrogen peroxide as oxygen source improves production of 5-ketofructose From L-sorbose using engineered pyranose 2-oxidase from *Peniophora gigantea*´.

Biotechnology and Bioengineering, 2012, 109, 2941-2945

[9] Pfeiffer, M.

`Controlled Oriented Immobilization of Catalase to Overcome Oxygen Diffusional Limitations. From Gene to Biocatalyst´.

Project Laboratory performed at the Institute of biotechnology and biochemical engineering of TU Graz under supervision by Univ.-Prof. Dipl.-Ing. Dr. techn. Bernd Nidetzky, 2013

[10] Bolivar Bolivar, J. M.; Consolati, T.; Mayr, T.; Nidetzky, B.:

`Shine a Light on Immobilized Enzymes: Real-Time Sensing in Solid Supported Biocatalysts´.

Trends in biotechnology, 2013, 31, 194 - 203

6. Supporting Information

6.1. Supporting Figures

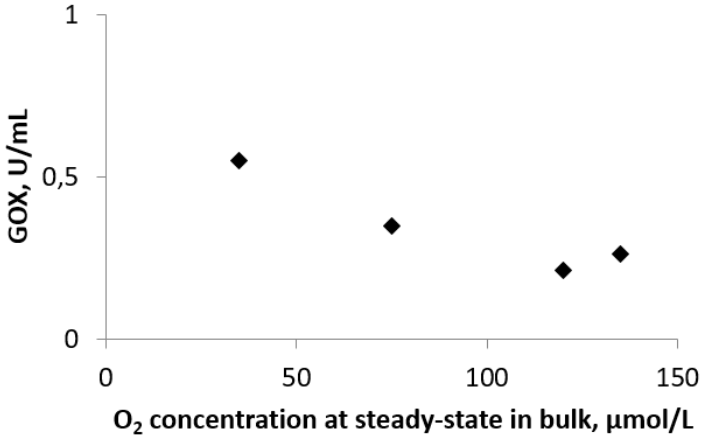


Figure S1. *Bulk oxygen concentration of immobilized Z_{basic2}_BP_KatA (10 000 U/ g carrier) on Relisorb 400, after addition of same amount of H_2O_2 in presence of different concentrations of GOX (and an unlimited concentration [500 mM] of Glucose). Increased GOX activity leads to a lower oxygen concentration.*

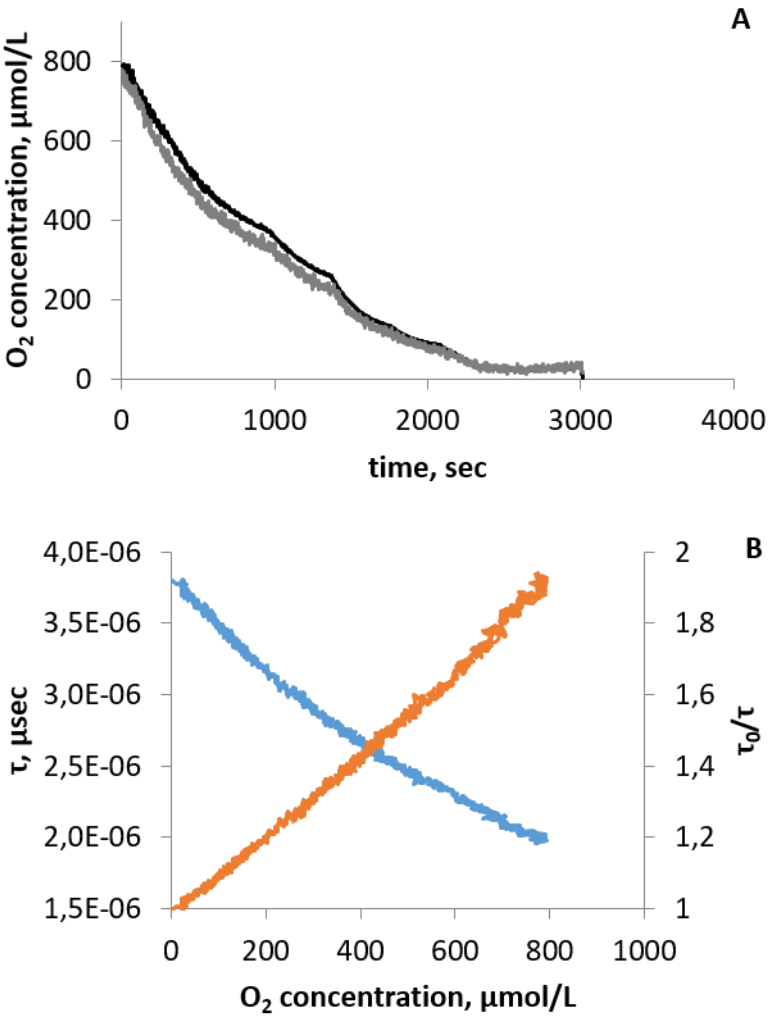


Figure S2. *Calibration of Ru(dpp)$_3$ labeled on immobilizate Z$_{basic2}$_BP_KatA (10 000 U/ g carrier). (A) After oxygen increased with pure O$_2$, O$_2$ was decreased again with Glucose oxidase and Glucose; graph is showing the controlled decrease of O$_2$. Bulk oxygen (black line, continuous measurement); Intraparticle oxygen (grey line, continuous measurement). (B) Calculated life time (recorded τ values, blue; τ ratios, red) for Ru(dpp)$_3$ over a range between 0 and 800 µmol/L O$_2$.*

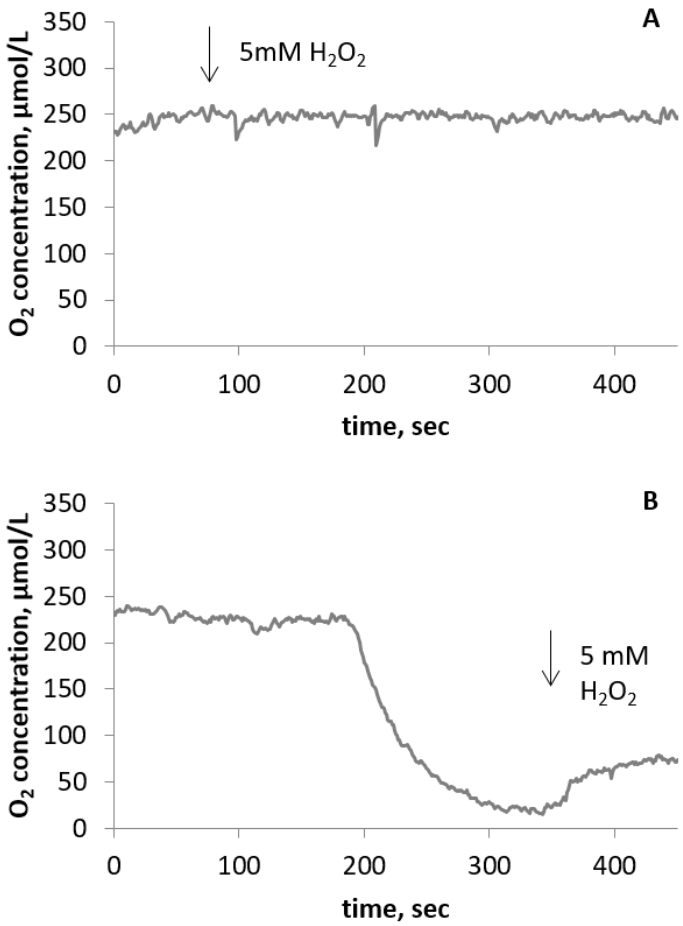


Figure S3. *Influence of H_2O_2 on oxygen response of immobilized $Ru(dpp)_3$ on a carrier Relisorb 400. (A) shows an intraparticle oxygen measurement when H_2O_2 is added at 100 % air saturation (~250 µmol/L O_2). (B) shows an intraparticle oxygen measurement when H_2O_2 is added at 0 % air saturation (0 µmol/L O_2).*

6.2. Supporting Methodology

6.2.1 Hydrogen peroxide assay

This assay was reported previously [1] and further adapted.

A spectrophotometer with a cuvette holder allowing stirring was used. Stirring proved necessary for a homogeneous distribution of hydrogen peroxide added directly to buffer (potassium phosphate buffer 50 mM, pH 8.0) after blanking. Stirring also allowed direct measurements of activity for Z_{basic2}*_BP_KatA* immobilizates. Different concentration of hydrogen peroxide were tested, the concentration giving the best signal under these conditions was 20 mM in the cuvette.

After getting a stable signal for around one minute of hydrogen peroxide in the cuvette adequately diluted sample of catalase was added. This assay is usually performed at pH 7.0; however a possible combination of Z_{basic2}*_BP_KatA* with an oxidase might require another pH optimum. Therefore hydrogen peroxide assay was tested for Z_{basic2}*_BP_KatA* using different buffers with pH from 6 to 9. Buffer for pH 6 contained sodium citrate, for pH 7 and 8 potassium phosphate and for pH 9 sodium carbonate. Concentration of each buffer was 50 mM. pH profile for Z_{basic2}*_BP_KatA* activity can be seen in Figure 1.

6.2.2 Calibration of oxygen response

Calibration of oxygen response of coimmobilized $Ru(dpp)_3$ for a range between 0 and 250 µmol/L O_2 in solution (~100% air saturation) was performed in a stirred vessel containing buffer and labeled carrier. O_2 was depleted by bubbling in N_2 until all O_2 was removed. Bulk and intraparticle O_2 were monitored while O_2 increased slowly through exchange of N_2 with O_2 available from air surrounding the open vessel. Point measurements with the bulk oxygen sensor were taken every few minutes, while intraparticle was constantly monitored with fiber-optic cable.

For a calibration for a broader range (0 – 1000 µmol/L O_2 in solution) several methods were explored. Oxygen increase up to 1000 µmol/L was achieved by using free Z_{basic2}*_BP_KatA* and H_2O_2 or bubbling in of pure O_2. The decrease of O_2 to 0 µmol/L was performed by bubbling in of N_2 or using Glucose oxidase with Glucose as a substrate to consume O_2. Oxygen increase and decrease were combined in every possible way. The use of enzymes with their respective substrate allowed the monitoring of bulk while O_2 was increased or decreased, while use of bubbling in required the measurement performed after stopping gas flow. The risk of breaking the bulk oxygen sensor was too high to measure while bubbles were present.

Also an alternative oxygen increase was tested, by adding potassium iodide to a stirred vessel containing buffer and labeled immobilizate.

6.2.3 Controls and blanks

The influence of H_2O_2 on $Ru(dpp)_3$ while measuring the intraparticle oxygen gradient was tested at 100 % (~250 µmol/L) and at 0 % (0 µmol/L) air saturation.

For testing at 100 % labeled Z_{basic2}*_BP_KatA* immobilizate was added to a stirred vessel with buffer (PPB, pH 8.0) and 5 mM H_2O_2 were added while intraparticle oxygen was measured. The same was performed for testing at 0 % air saturation, only O_2 was depleted with N_2 and then 5 mM H_2O_2 were added.

6.3. Supporting References

[1] Betancor, L., Hidalgo, A., Fernandez-Lorente, G., Mateo, C., Fernandez-Lafuente, R., Guisan, J.M.
`Preparation of a Stable Biocatalyst of Bovine Liver Catalase Using Immobilization and Postimmobilization Techniques´.
Biotechnol. Prog. 2003, 19, 763−767

Part 2: Bubble-free Oxidation Design: Controlled and Effective Supply of O_2 into Solid-supported Oxidases via H_2O_2

1. Introduction

In modern technology oxygen-dependent enzymes (oxidases) have become an important element for chemical processes [1, 2, and 3]. A major limitation in the use of oxidases is that operational stability and high reactor productivity are challenging to achieve concurrently. Improving operational stability of enzymes can be realized by immobilizing enzymes on a solid-support. Immobilization of enzymes further allows easier handling, enzyme reuse and might even improve storage of the enzyme.

For high reactor productivity essentials are a high active catalyst and especially for oxidases an outstanding oxygen delivery. High activity of the catalyst is dependent on the amount of protein incorporated in solid-support and the resulting catalytic effectiveness given by minimizing internal oxygen diffusion limitations. An effective oxygen delivery is generally achieved in gas-liquid contactors where the mass transfer of oxygen to the liquid phase is the limiting step, the most common use systems are bubble air reactors. However, independent on the oxygen delivery towards the liquid phase, another transport step exists when immobilizates of enzymes on porous carriers are used. Recently published studies [4, 5] show that immobilizates on porous particles display an oxygen diffusion limitation between particle and aqueous environment. The use of oxidases in a process requires in most cases addressing another major issue, protection against or removal of inactivating by-product H_2O_2. Protecting the enzyme against H_2O_2 has been attempted by immobilization on porous particles [6], however in some cases no protective effect could be observed. For some enzymes the coimmobilization with catalase has been shown to be very beneficial [3, 6, 7, 8, 9], as catalase is catalyzing the formation of O_2 and H_2O from H_2O_2. A proper designed catalase coimmobilizate may be useful not only to degrade hydrogen peroxide but also produce oxygen in situ. Feeding of additional H_2O_2 generating O_2 by catalase is by no means a new idea and it has been reported super-saturating a aqueous solution to over 100 % of O_2 [10]. Ideally H_2O_2 could be used as a sole tightly controlled oxygen source.

In the scope of this work a coimmobilizate of D-amino acid oxidase and catalase was designed and a feeding strategy for H_2O_2 was explored. Further studied were the effect of using H_2O_2 as additional oxygen source on oxygen diffusion limitation and a possible inactivation effect of H_2O_2 on both enzymes.

Recently a catalase from *Bordetella pertussis* (*Z_{basic2}_BP_KatA*) was modified with a Z_{basic2}-tag [Part 1]. This strongly positive charged protein tag (Z_{basic2}) allows a reversible oriented ionic adsorption on porous particles providing a high preservation of enzyme activity. In the same study intraparticle oxygen concentration of an immobilized *Z_{basic2}_BP_KatA* on a porous carrier was studied, revealing the existence of a reverse intraparticle gradient when H_2O_2 is added. A reverse intraparticle oxygen gradient is indicating an accumulation of O_2 inside the particle, where it is generated by *Z_{basic2}_BP_KatA* and a much slower diffusion into the surrounding bulk.

In this work the enzyme D- amino acid oxidase (DAAO) was chosen for designing a coimmobilizate with the above mentioned catalase (*Z_{basic2}_BP_KatA*) on porous particles and studying the possibility of decreasing oxygen limitation inside the particle.

DAAO is an enzyme industrially used for deracemization of D, L-amino acids [1, 11] and is an important catalyst for the production of cephem antibiotics [2, 11, and 12]. It is preferably used in immobilized form due to the fact that soluble DAAO shows a rapid decline in activity over several days even stored at low temperature (4°C). DAAO from *Trigonopsis variabilis (*Z_{basic2}*_Tv_DAAO)* was previously tagged with Z_{basic2} [4, 13], showing a high preservation of enzyme activity. In a previous paper we could prove that for *Z_{basic2}_Tv_DAAO* as for other oxygen dependent enzymes an oxygen diffusion limitation is effecting preparation of the enzyme immobilized on porous carriers [4]. Also above mentioned inactivation by H_2O_2 is a major concern for this enzyme. Especially *Tv_DAAO* is not protected rigidification trough immobilization from inactivation through chemical modification by H_2O_2 [6, 14]. Therefore this enzyme appears to be an excellent candidate for coimmobilization with catalase.

Relisorb 400 from Resindion containing sulfonate groups on its surface was chosen as carrier for coimmobilizate design as it had shown previously good immobilization yields [4, Part 1] for both Z_{basic2}-tagged enzymes as well as it was proven that oxygen diffusion limitation occurs for Z_{basic2}*_Tv_DAAO* at higher loadings [4]. Another important attribute was its compatibility of labeling with luminescent dye $Ru(dpp)_3$ and thus allowing the application of intraparticle oxygen measurements for the coimmobilizate. Monitoring bulk and intraparticle oxygen with or without feeding hydrogen peroxide is an integral part of coimmobilizate design. It gives vital information about ratio between loadings of both enzymes and about effect of H_2O_2 concentration added. Product concentration was confirmed by a specific assay for α-keto acids and by HPLC analysis. In this paper results of coimmobilization of both enzymes and its application with a semi-continuous and a continuous H_2O_2 supply are discussed. A special focus here is on using information from oxygen measurements for development of a feeding strategy for H_2O_2, when the reactor is run in fed-batch mode. Experiments focused at first on retaining high stability of the preparation over several hours and were later shifted to reaction intensification by modifying H_2O_2 addition rate and particle concentration in reactor. Ideally increased H_2O_2 addition rates used for oxygen generation in coimmobilizate should allow reaction intensification, observable by a higher production rate (µmol product/mL/min). The same is to be expected when a higher particle concentration was used.

2. Material and Methods

2.1. Materials

D-amino acid oxidase (*Z_{basic2}_Tv_DAAO*; from *Trigonopsis variabilis*) containing Z_{basic2} - fused to N-terminus of enzyme was used. Construction, isolation and characterization of *Z_{basic2}_Tv_DAAO* were reported previously [13]. *Z_{basic2}_BP_KatA* was expressed in E. coli [for details see Part 1]. D-Methionine (D - Met), 4-aminoantipyrine, N, N-dimethylaniline, 2, 4-dinitrophenylhydrazine, peroxidase from horseradish and Glucose oxidase (Type II-S, 15,000-50,000 units/g solid) from *Aspergillus niger* were purchased from Sigma Aldrich GmbH (Vienna, Austria). Carrier Relisorb 400 was a kind gift of Resindion (Milano, Italy). Dichloride (4, 7-diphenyl-1, 10-phenantroline) ruthenium (II), $Ru(dpp)_3$, was from ABCR GmbH (Karlsruhe, Germany). Unless otherwise mentioned, all chemicals were from Sigma (Vienna, Austria) in analytical grade.

2.2. Assays

Roti®-Quant assay (Roth) referenced against known concentrations of BSA was used for protein quantitation. Activity of soluble and immobilized *Z_{basic2}_Tv_DAAO* was determined with both reported coupled peroxidase assay (CPA) [4] and measuring the oxygen consumption rate. Activity of soluble and immobilized *Z_{basic2}_BP_KatA* was measured spectrophotometrically via monitoring of the H_2O_2 consumption [15]. One activity unit is the amount of Z_{basic2}_DAAO *Z_{basic2}_Tv_DAAO* that consumes 1 µmol/min O_2 or the amount of *Z_{basic2}_BP_KatA* that consumes 1 µmol/min H_2O_2 at the conditions used.

2.3. Product Concentration Determination (DNP assay and HPLC analysis)

Product concentration of α-Keto-γ-(methylthio)butyric acid from conversion of D - Met by *Z_{basic2}_Tv_DAAO* was determined using a photometric assay at 440 nm with 2,4-dinitrophenylhydrazine. Samples from experiments were measured with ion pair chromatography (Chromolith® RP-18e column) and substrate concentration (D - Met) and product concentration of α-Keto-γ-(methylthio)butyric acid were analyzed. Concentration of tetrabutylammonium phosphate used as running buffer was 6 mM with a flow rate of 2 mL/min. Peaks were detected at 210 nm (UV-Detection). Commercially available highly pure D - Met and α-Keto-γ-(methylthio)butyric acid were used as standards.

2.4. Enzyme Immobilization

The general immobilization protocol involved incubation (1 h) of 100 mg of carrier (Relisorb 400) with a loading mixture containing 100 – 300 µl of enzyme (*Z_{basic2}_Tv_DAAO* 20-80 U/mL; *Z_{basic2}_BP_KatA* 1 000 – 10 000 U/mL) in crude extract (depending on enzyme activity measured in crude extract) diluted with buffer (Potassium phosphate buffer PPB, 50 mM, pH 7.0) and a final concentration of 0.25 M of NaCl. For both enzymes immobilization protocols have been previously established [4, Part 1]. When immobilization occurred in several steps a washing step was performed for 15 min between immobilization steps. Washing buffer (PPB, 50 mM, pH 7.0) contained 0.25 M NaCl. Coimmobilization of both enzymes on the same carrier was performed in different strategies. Either enzyme were added in the same immobilization step (simultaneous immobilization) or one was immobilized first and after a washing step the second enzyme was immobilized (consecutive immobilization). After immobilization was finished, another washing step was performed and immobilizate was finally stored in buffer (PPB, 50 mM, pH 7.0).

2.5. O_2 Measurement via Microoptode Optical Sensor

General practical set-up for these measurements can be found in SI of [4].Oxygen in bulk was determined by using a fiber-optic oxygen microoptode (PreSens - Precision Sensing GmbH, Regensburg, Germany) connected to a fiber optic oxygen meter (model microx TX3, PreSens).

2.6. Luminescence Labeling

Labeling of coimmobilizate with 0.25 mg of oxygen responsive dye $Ru(dpp)_3$ per g of carrier was performed after coimmobilization was completed (SI of 4).

2.7. Intraparticle O_2 Measurement

A general procedure for intraparticle oxygen measurement was established recently [SI of 4]. Luminescence lifetime (τ) of coimmobilized dye $Ru(dpp)_3$ is decreased by dynamic quenching effects of oxygen present, and the phase modulation technique is used measuring τ [5]. Changes of phase of $Ru(dpp)_3$ on coimmobilizate are collected for intraparticle oxygen calculation by a fiber-optic cable connected to a fiber optic oxygen meter (model pH-1 mini, PreSens). Calibration of $Ru(dpp)_3$ immobilized on coimmobilizate over the range of 0 to 1000 µmol/L was performed by saturating buffer with pure oxygen and decreasing O_2 to 0 µmol/L by consumption with GOX and Glucose as substrate. Before starting the measurement coimmobilizate was added to a flask containing buffer (PPB, 50 mM, pH 8.0). The final volume before starting was 4 mL and particle concentration was 5 mg/mL, if not mentioned otherwise. Measurement was performed at 30 °C. A water bath was used keeping temperature of buffer in flask constant over course of reaction. Measurement was started by adding substrate D - Met (200 mM, pH 8.0) after bulk sensor and fiber optic cable (for detection of oxygen quenching from $Ru(dpp)_3$ on carrier) showed a stable signal.

2.8. Intraparticle Oxygen Measurement in the Presence of H_2O_2

Applying this methodology on coimmobilizates supplied with additional H_2O_2 required dividing the measurements into two parts. In the first part O_2 measurement is performed as described above. Oxygen measurement is continued until nearly all oxygen is consumed in bulk and no change in oxygen course occurs. In the second part of the measurement H_2O_2 is added (1.25 – 5 mM). Depending on purpose of experiment H_2O_2 concentration was varied and addition could be repeated at specific time points. Measurement is usually stopped after all by catalase produced oxygen is consumed again by Z_{basic2}*_Tv_DAAO*.

2.9. Bubble-free Reactor Design with Continuous H_2O_2 Feeding

Set-up was for a fed-batch reaction (Figure 7), feeding continuously H_2O_2 with an HPLC [100 mL buffer (PPB, 50 mM, pH 8.0) containing coimmobilizate (5 - 15 mg particles per mL buffer). Coimmobilization for Scale-up was performed as for the smaller scale (see section Enzyme immobilization).

A jacketed flask (~ 200 mL) with 3 openings coupled with an external water bath was used keeping temperature constantly at 30 °C. Magnetic stirrer with a magnetic stir bar inside the reactor was used (300 rpm). Microoptode and fiber optic cable for oxygen measurements were fixed in the middle opening of the flask.

Oxygen measurements were started and 15-25 mL (amount of 200 mM D - Met that will be added to start the reaction later) buffer were removed. Reaction was started by adding D - Met. After nearly all oxygen in bulk was consumed and oxygen courses were stable, HPLC pump was switched on. In most experiments H_2O_2 addition was started with a lower flow (0.1 -0.2 mL/min) and increased every 2-3 min (up to 1 mL/min). 1 mL samples from the reaction mixtures were taken before the H_2O_2 addition and afterwards every 15 -20 min.

Samples were analyzed with DNP assay for product concentration. Samples from several experiments were analyzed with HPLC for substrate and product concentrations.

Reactions were run continuously between 1 and 4 h. Reactions were stopped by stopping H_2O_2 addition and recording of oxygen measurements were continued until all oxygen was consumed again in bulk.

3. Results and Discussion

3.1. Preparation and Characterization of Coimmobilization of *Z_{basic2}_Tv_DAAO* and *Z_{basic2}_BP_KatA*

Z_{basic2}_Tv_DAAO loadings of 200, 400, 600 and 800 U/ g carrier were immobilized and oxygen availability inside the particle and the resulting oxygen diffusion limitation was analyzed. Intraparticle oxygen measurements revealed the expected increase of oxygen diffusion limitation up until 600 U/g carrier (Figure S1). Loading 800 U/g carrier showed a very similar oxygen diffusion limitation as 600 U/g carrier (around 30 % of oxygen availability after initial drop of oxygen intraparticle).

600 U/ g carrier were chosen for further experiments, this loading is affected highly by oxygen diffusion limitation in the particle and another important criterion is a lower loading of protein on the carrier enabling the coimmobilization of the second enzyme as well as luminescent dye $Ru(dpp)_3$. Especially $Ru(dpp)_3$ labeling can be affected by high protein loading on the carrier. This has been observed for high *Z_{basic2}_BP_KatA* loadings (100 000 U/ g carrier, Part 1). With the amount of catalyst used, total oxygen depletion was observed after 150 seconds (Figure S2a), allowing a short-term experimental approach design for following re-oxygenation with H_2O_2 addition.

The use of 600 U/ g carrier loading therefore met both criterions, an observable oxygen diffusion limitation and a protein loading that is not hindering the absorption of $Ru(dpp)_3$ on the carrier.

Previous studies of immobilizates of *Z_{basic2}_BP_KatA* [Part 1] mostly focused on loadings of 10 000 and 100 000 U/ g carrier. The theoretical activity in the reactor was with these loadings between 50 and 500 µmol/min/min and the hydrogen peroxide addition rate was never above 1 µmol/min/mL. Both loadings in this studies [Part 1] showed a good H_2O_2 decomposition (H_2O_2 assay) and O_2 generation intraparticle (bulk and intraparticle oxygen measurement), however 100 000 U/g carrier loading could not be fully labeled. In respect to

the high protein loading of *Z_{basic2}_Tv_DAAO* on the coimmobilizate, the first coimmobilizates generated had a loading of 10 000 U/ g carrier. For optimization of oxygen generation this amount was elevated to 50 000 U/ g carrier. Subsequent experiments were focusing on effects of coimmobilization on enzyme activity. Coimmobilization of both enzymes was performed in different orders (in parallel and consecutively). Furthermore immobilizates with just one of the two enzymes were also analyzed in parallel with coimmobilizates, eliminating any possible effects on immobilization by different batches of crude extract. Measurement of activity and calculation of effectiveness factors showed that coimmobilization had no observable effect on activity of either enzyme coimmobilized (Table S1). Small differences in effectiveness factor can be explained by small differences in protein content in applied crude extract. Parallel immobilization was dismissed due to the massive protein loading being applied all at once making light-binding of unspecific protein more likely and more difficult to remove. A consecutive immobilization strategy was approached including one or two washing steps with buffer in between further removing excess protein. *Z_{basic2}_BP_KatA* was finally chosen as the first enzyme immobilized due to its bigger size (~260 kDa for *Z_{basic2}_BP_KatA* Tetramer [16] compared to *Z_{basic2}_Tv_DAAO* Dimer with ~76 kDa [17]), ensuring a more even distribution within the carrier.

The final choice for immobilization protocol was first immobilizing *Z_{basic2}_BP_KatA* (theoretical enzyme loading: 50 000 U/g carrier; measured catalytic activity: ~ 4500 U/g carrier) and afterwards *Z_{basic2}_Tv_DAAO* (theoretical enzyme loading: 600 U/ g carrier; measured catalytic activity: 25-30 U/g carrier).

3.2. O_2 Responsive Coimmobilizate and Method Development for Intraparticle O_2 Concentration Determination

Intraparticle Oxygen gradient is a key analytical variable for monitoring oxygen while developing a feeding strategy for H_2O_2 as it delivers on-line information about oxygen consumption and production inside the particle. Calibration of oxygen responsive $Ru(dpp)_3$ incorporated on coimmobilizate showed a near linear response of dye (0.25 mg dye/100 mg particle) for an oxygen concentration up to 1000 µmol/L (Figure S5). Intraparticle oxygen was measured for immobilizates of only DAAO and compared to measurement of a coimmobilizate of *Z_{basic2}_Tv_DAAO* and *Z_{basic2}_BP_KatA*.

No significant difference in oxygen consumption after addition of D - Met could be observed intraparticle and in bulk (Figure S2). Coimmobilizates and *Z_{basic2}_Tv_DAAO* immobilizates show very similar oxygen consumption rates, only for the coimmobilizate the effect of partial oxygen regeneration can be observed. For both internal oxygen can be monitored and they show oxygen limitation.

In another experiment different H_2O_2 concentration were used for oxygen production with a coimmobilizate looking for a concentration where enough oxygen for *Z_{basic2}_Tv_DAAO* is produced while keeping the H_2O_2 concentration as low as possible. Figure 1 shows oxygen production after a single addition of hydrogen peroxide with different hydrogen peroxide concentrations. An increase of maximum intraparticle oxygen concentration reached can be observed with an increased concentration, indicating substrate (H_2O_2) limitation and also a fast conversion of H_2O_2 added. It can also be seen that O_2 is produced inside the particle due to the fact that O_2 intraparticle is increasing first and afterwards bulk O_2 is increasing much slower. This observation also shows that oxygen is produced faster inside the particle than it is diffusing from particle into bulk.

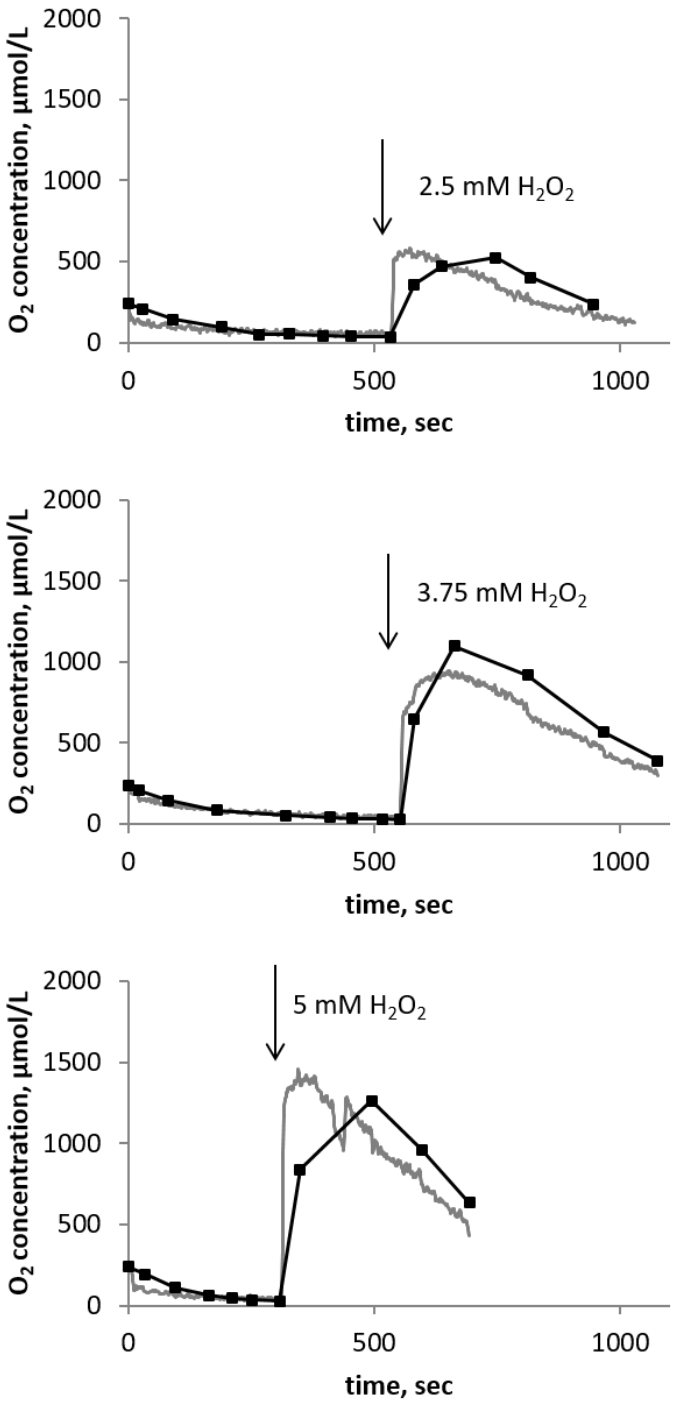


Figure 1. *Oxygen measurement for monitoring of effect of different H_2O_2 concentrations on the same coimmobilizate (Z_{basic2}_Tv_DAAO: 600 U/ g carrier; Z_{basic2}_BP_KatA: 50 000 U/ g carrier). Initially O_2 is depleted by addition of 200 mM D-Met, H_2O_2 is added after depletion. Bulk oxygen (black line with dots, point measurement at each dot); Intraparticle oxygen (grey line, continuous measurement); H_2O_2 addition (arrow).*

Decrease after reaching a maximum concentration of O_2 in particle and in bulk is indicating a consumption of O_2 by *Z_{basic2}_Tv_DAAO*. Prove of this consumption could be established by conducting an experiment (Figure S6) with the same set-up with an immobilizate of *Z_{basic2}_BP_KatA*, depleting initial oxygen in bulk and particle with N_2 gas and adding H_2O_2 after no O_2 was present in flask anymore. Maximum O_2 reached with the same amount of H_2O_2 was much higher and maximum slowly decreased after a few minutes, there was no fast decrease after maximum O_2 was reached thus proving a considerable consumption of O_2 by the coimmobilized *Z_{basic2}_Tv_DAAO*.

Monitoring of intraparticle oxygen proved that inside the coimmobilizate oxygen generation and consumption is occurring and thus showing a promising starting point for finding a solution for decreasing oxygen limitation inside the particle.

3.3. Design Internal O_2 Supply via Multiple H_2O_2 Addition

In previous section onetime additions of H_2O_2 were monitored gaining an understanding of occurrences inside the particle and thereby proven that coimmobilizates with H_2O_2 feeding have the potential to be a solution for oxygen limitation. However a onetime addition is not practical for a longer reaction, because the exposure of both enzymes to H_2O_2 and therefore an increased risk of inactivation is too likely. Ideally H_2O_2 is continuously added under controlled conditions allowing a controlled reaction with minimum risk of inactivation of both enzymes. A balanced H_2O_2 addition is a crucial point for developing a continuous hydrogen peroxide supply allowing a higher conversion of D - Met with immobilized *Z_{basic2}_Tv_DAAO*. Time and punctual concentration of hydrogen peroxide were key variables in development of a semi-continuous approach of H_2O_2 addition.

Figure 1 is showing monitoring of bulk and intraparticle oxygen, when different concentrations of H_2O_2 (2.5 – 5mM) are added to a system with a coimmobilizate, where all oxygen was previously consumed. Results indicated that the lowest concentration (2.5 mM)

lead to the lowest maximum oxygen concentration in bulk and particle (~ 500 µmol/L) and the highest concentration of H_2O_2 (5mM) showed the highest oxygen concentration (below 1500 µmol/L).

While these results are not surprising they prove that these amounts of H_2O_2 are quickly consumed and H_2O_2 is in this case a limited substrate. Since H_2O_2 is also a source of inactivation for both enzymes as mentioned in the introduction, it is not possible to be used in unlimited amounts. The use of H_2O_2 as a limited substrate is an important factor for designing a controlled supply of H_2O_2 for oxygen production in the particle.

Purpose of this design is reaching with every addition of the same amount of hydrogen peroxide the same or similar oxygen concentration intraparticle. Manual repeated additions of H_2O_2 lead very quickly to a few basic observations (Figure 2).

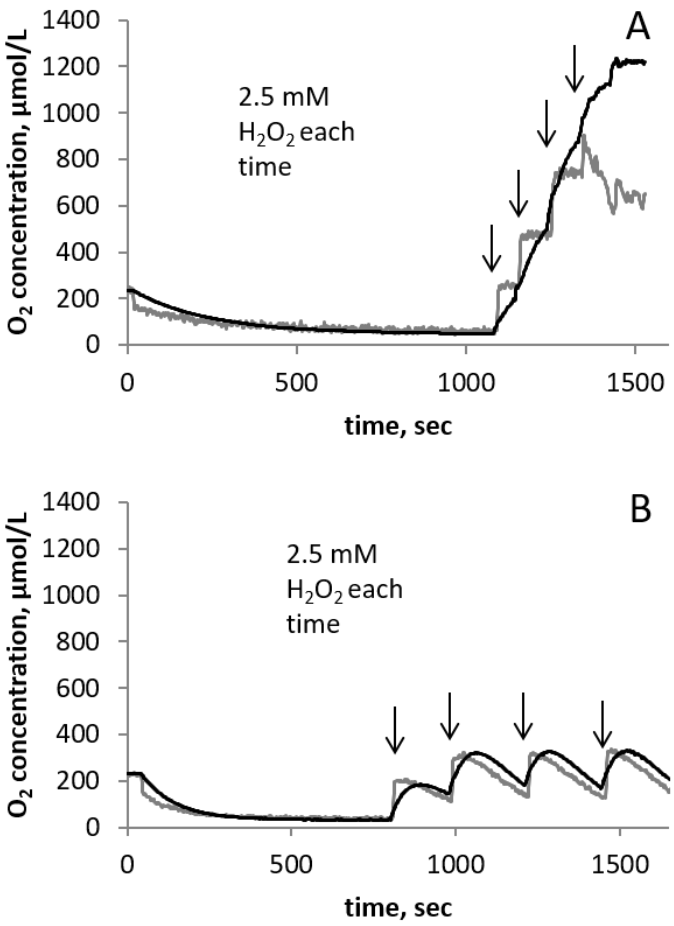


Figure 2. *Measurement of oxygen consumption and production from H_2O_2 addition (2.5 mM each time, indicated by arrows) in bulk and particle for a coimmobilizate (Z_{basic2}_Tv_DAAO: 600 U/ g carrier; Z_{basic2}_BP_KatA: 50 000 U/ g carrier). (A) shows a faster addition rate, leading to unbalanced O_2 production and finally bubble formation in bulk (after 1400 s). (B) shows a slower addition rate and a balanced O_2 production. Bulk oxygen (black line, continuous measurement); Intraparticle oxygen (grey line, continuous measurement).*

In one experiment (Figure 2a) 2.5 mM H_2O_2 were added after dissolved O_2 in bulk and particle was removed, however the addition was done so fast that each addition (every 100 s) lead to an increase of around 250 - 300 µmol O_2/L and a total accumulation in bulk of above 1200 µmol O_2/L in less than 400 s. These findings show an accumulation of O_2 in the system due to the fact that oxygen was still diffusing back from bulk to particle leading to a higher total increase of oxygen in particle than from H_2O_2 addition alone.

In another experiment (Figure 2b) oxygen was reaching around 300 µmol/L after each H_2O_2 addition (every 200 s) and dropped to 150 µmol/L before the next addition occurred, showing that when the same amount of H_2O_2 was added at specific time points that after 800 s the oxygen concentration in bulk and particle at the final addition was ~250 µmol/L and no accumulation occurred. Timing H_2O_2 addition after initial O_2 in bulk and particle was reached again proved to be the key for a controlled O_2 production in the reaction system.

While these results showed that a controlled oxygen production and consumption could be easily achieved for a short time around 10 min, the question was raised how much the activities of both enzymes are affected by H_2O_2, because if production and consumption of oxygen are not balanced anymore an accumulation or a lack of oxygen might occur in the system. Consequently the next step was increasing reaction time and finding a way quantifying possible inactivation of both enzymes.

3.4. Multiple H_2O_2 Addition: Monitoring of Enzyme Inactivation

Previous experiments focused on generating oxygen in particle, while maintain a controlled reaction in the reactor. Neglected so far has been the issue of inactivation of one or both enzymes by H_2O_2, an issue that is even more important when the reaction is continued over a longer time span (1 h). Therefore this section focuses on finding ways to quantify inactivation occurring over time by using data gained from oxygen monitoring.

In Figure 3 two experiments are shown following semi-continuous addition of H_2O_2. Figure 3a is showing bulk and intraparticle oxygen courses of a controlled addition over an hour of the same amount of H_2O_2 (1.25 mM), leading to an oxygen concentration between 100 and 250 µmol/L in bulk and intraparticle.

Figure 3c is showing bulk and intraparticle oxygen courses of another addition of H_2O_2 to a coimmobilizate. However in this experiment initially added H_2O_2 concentration was too high (2.5 mM) and even after reducing the concentration (to 1.25 mM) the preparation was strongly affected by inactivation over time.

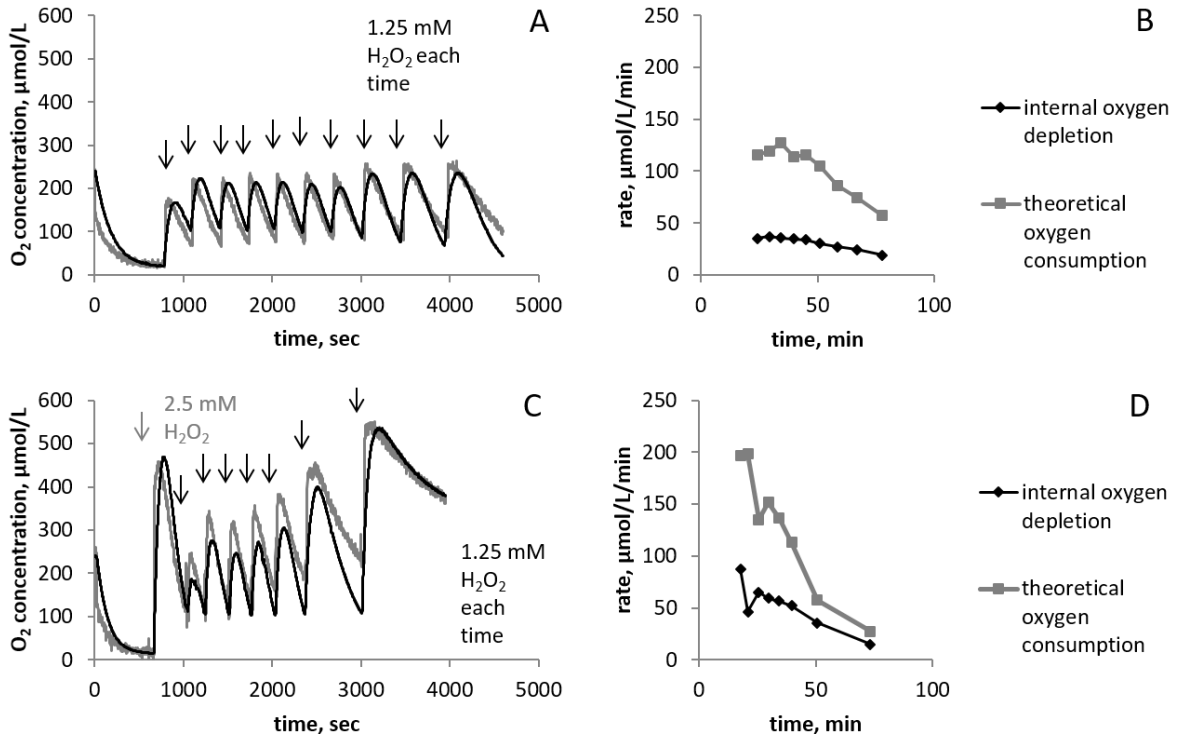


Figure 3. Monitoring of enzyme inactivation. *(A) and (C) Bulk and intraparticle oxygen courses of a semi-continuous H_2O_2 addition; arrows indicate H_2O_2 addition; Bulk oxygen (black line, continuous measurement); Intraparticle oxygen (grey line, continuous measurement). (B) and (D) Theoretical Oxygen consumption (TOC) and Internal Oxygen Depletion (IOD) rates of measurements (A) and (C).*

These experiments were used for 2 rate calculations. These rates should allow quantification and identification of inactivation occurring over time. The first rate was called the theoretical oxygen consumption rate (TOC rate). It is calculated from the amount of O_2 produced in mM (known from H_2O_2 added in mM) and the time span in min from observing an increase in O_2 intraparticle until O_2 is reaching the lowest concentration again. When the same amount of H_2O_2 is added at each addition this rate should be the same for each addition, if no inactivation of either enzyme is happening. The rate will decrease at an inactivation of Z_{basic2}*_Tv_DAAO*, of Z_{basic2}*_BP_KatA* or both. Identifying which enzyme is mostly affected

by inactivation can be done by observing behavior of intraparticle oxygen. If maximum reached intraparticle oxygen is increasing Z_{basic2}*_Tv_DAAO* is most likely affected by inactivation: Slower consumption of O_2 by a lower active immobilized Z_{basic2}*_Tv_DAAO* is leading to a higher O_2 accumulation in particle. A decrease of maximum reached intraparticle oxygen is indicating an inactivation of Z_{basic2}*_BP_KatA*. O_2 production is slowing down and therefore the accumulation inside the particle is lower. The second rate mentioned before can also be used as a tool to determine which of the two enzymes is more affected by inactivation. This rate is the internal oxygen depletion rate (IOD rate). IOD rate is actually the rate of decrease in oxygen concentration after the maximum of oxygen concentration in particle is reached. This rate is reflecting the actual activity of Z_{basic2}*_Tv_DAAO* observed inside the particle. However comparison of this rate for drawing conclusion about inactivation of Z_{basic2}*_Tv_DAAO* is strongly dependent on value of maximum O_2 reached intraparticle, since the observable activity of Z_{basic2}*_Tv_DAAO* is O_2 dependent. It is therefore advisable using IOD rate as another tool for inactivation analysis completion, not independently without taking the TOC rate or intraparticle O_2 concentration into account.

Figure 3b is showing the corresponding calculated TOC and IOD rates for each enzyme. TOC rates are in the first 30 min between 100 and 120 µmol/L/min, then start to decline until finally after an addition time of 60 min TOC rate was only half the initial rate (60 µmol/L/min). Calculated rates indicated that barely any inactivation occurred in the first 30 min of H_2O_2 addition and afterwards ongoing inactivation could be observed. IOD rate decreased in 1 h to half the initial rate (35 µmol/L/min) as well. In Figure 3d TOC rate decreased in 60 min from 200 µmol/L/min to 30 µmol/L/min further indicating inactivation from the start of H_2O_2 addition and therefore also showing that a too high H_2O_2 is inactivating from start. IOD rates mirrored the inactivation by dropping from 90 to 15 µmol/L/min.

Use of these rates combined with monitoring of oxygen in bulk and in particle showed that under controlled conditions of H_2O_2 addition it was possible maintaining an active preparation

for over an hour.

As another way of monitoring inactivation a possible accumulation of H_2O_2 in bulk was explored. H_2O_2 was measured at several time points during semi-continuous addition before the next H_2O_2 was added using CPA without enzyme or substrate present. Results in Figure 4 indicated no increase of H_2O_2 over the course of 1 hour. Every 2-3 min 1250 µM H_2O_2 were added and between 100-200 µM were detected before the next addition.

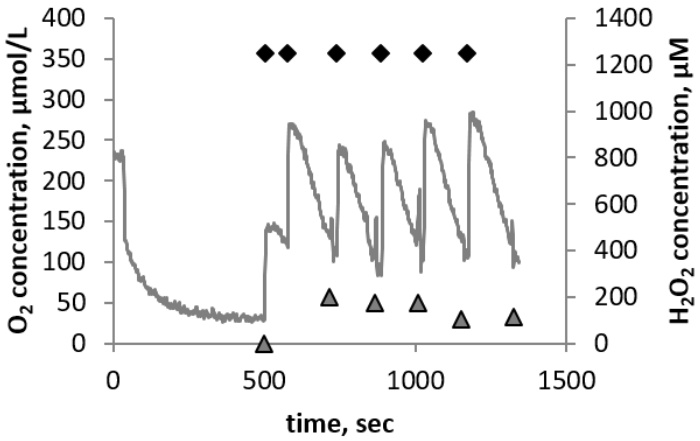


Figure 4. *Intraparticle oxygen course (grey line) of a semi-continuous H_2O_2 addition. Black squares: Amount of H_2O_2 added at time point. Grey triangles: measured H_2O_2 in supernatant at time point.*

All experiments indicating that the approach of semi-continuous H_2O_2 addition is leading to a longer stable production period for the conversion of D - Met with immobilized *Z_{basic2}_Tv_DAAO* than previously established without an additional intraparticle oxygen source for cofactor (FAD) recycling.

3.5 Bubble-free Fed-batch Reactor Design via Continuous H_2O_2 Addition

After establishing a semi-continuous controlled H_2O_2 addition for a coimmobilizate of *Z_{basic2}_Tv_DAAO* and *Z_{basic2}_BP_KatA* a logic next step was developing a continuous application of H_2O_2 for a bubble free fed-batch reactor (Figure 5).

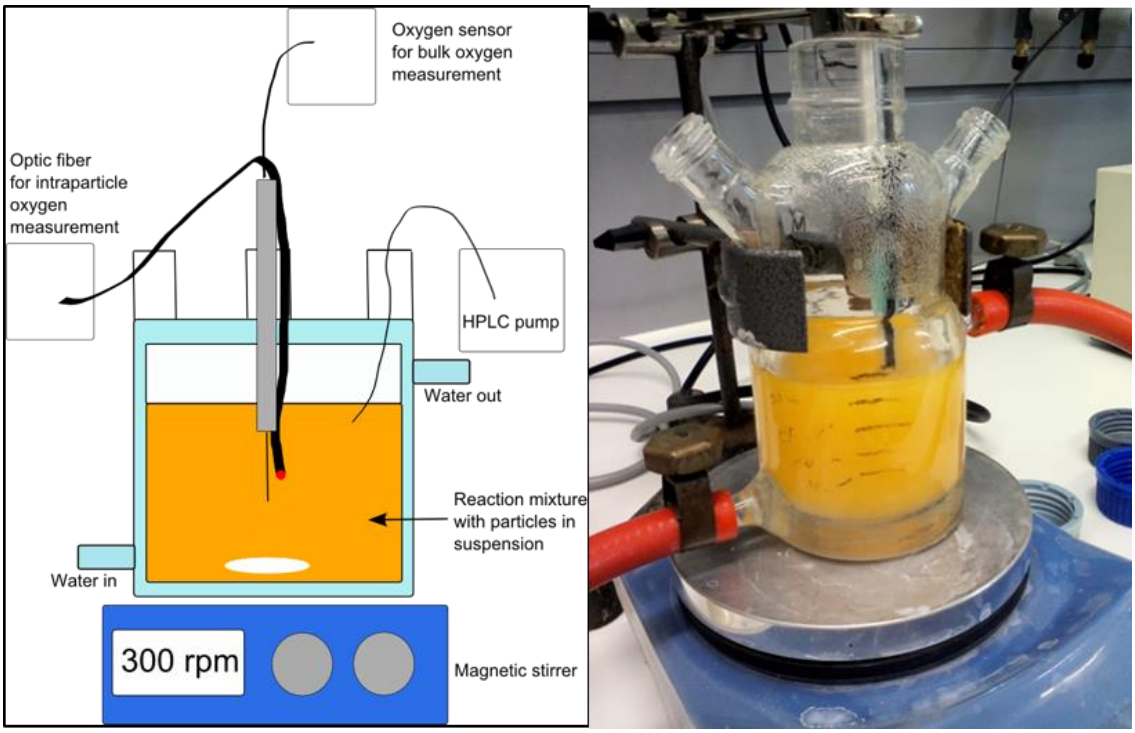
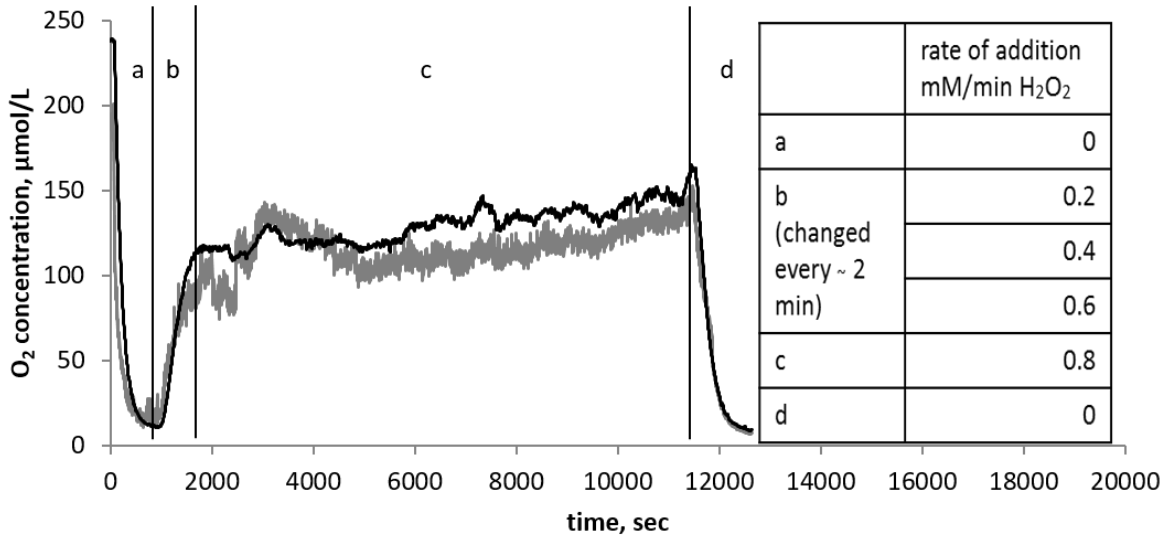


Figure 5. *Set-up for scaled-up reactor for running a conversion of D-Met with a labeled (orange) coimmobilizate of Z_{basic2}_Tv_DAAO and Z_{basic2}_BP_KatA.*

Enabling a controlled continuous addition of H_2O_2 demanded a change from manual addition to a HPLC pump, allowing a smoother controlled addition. This also provided the opportunity to extent reaction time (studying the stability of the coimmobilized preparation over a longer period of time in presence of different H_2O_2 concentrations) and thus volume increase from fed-batch operation was therefore expected to be higher than in previous experiments (Starting volume was 4 mL, with a possible increase to 5 mL in 1.5 h). It seemed therefore a logical consequence trying to scale up the reaction system. System was scaled up to 100 mL a

starting reaction volume in a 200 mL flask. Volume could therefore be doubled while H_2O_2 was fed to the system. In most experiments a final volume between 130 -150 mL was reached when addition rates of H_2O_2 were between 0.5 and 1 µmol/mL/min. Other parameters of the system such as temperature (30°C) and stirring speed (300 rpm) were adopted from smaller scale experiments for this study.

Studies of preparations with controlled continuous H_2O_2 addition showed that preparation remained stable up to 4 hours of total reaction time and was still active after this time (Figure 6). O_2 overproduction in initial phase of H_2O_2 addition could be prevented by starting addition with a gradient, starting with a low flow (0.2 µmol/mL/min) and increasing it every 2-3 min until final flow (0.8 µmol/mL/min) was reached. Addition is stopped at the end to show that *Z_{basic2}_Tv_DAAO* is still active and consuming O_2.



	rate of addition mM/min H_2O_2
a	0
b (changed every ~ 2 min)	0.2
	0.4
	0.6
c	0.8
d	0

Figure 6. *Oxygen courses of a controlled continuous H_2O_2 addition in a scaled-up reactor (Z_{basic2}_Tv_DAAO activity in reactor: 0.38 mM/min measured at 100 % O_2). H_2O_2 addition is started in b and rates of addition are indicated in the table. Bulk oxygen (black line, continuous measurement); Intraparticle oxygen (grey line, continuous measurement).*

Calculation of a suitable flow for activity of coimmobilizate was finally done by calculating the productivity rate (rate of production of α- keto acid by *Z_{basic2}_Tv_DAAO* at 100 % oxygen saturation in solution; in µmol/mL/min) from activity of coimmobilized *Z_{basic2}_Tv_DAAO*. When H_2O_2 addition rate is the same as the productivity rate, conversion of system should be the same as operating at air saturation. Half a mole of O_2 is produced from 1 mole H_2O_2, however for a total balance of H_2O_2 input one has also to consider that H_2O_2 is produced as a byproduct. Therefore taken the recycling of O_2 and H_2O_2 in account in total 1 mole H_2O_2 is given 1 mole of O_2. As final parameters for comparing and analyzing scale-up experiments were the productivity rate, H_2O_2 addition rate and the production rate (all in µmol/mL/min). Production rate was calculated from product detection with DNP assay and are dependent on oxygen produced inside the particle. Production rates of initial 15 min showed a productivity fitting to addition of H_2O_2 added to the system, afterwards however productivity declined. For example in one scale-up experiment H_2O_2 addition rate was 0.9 µmol/mL/min and the initial production rate (first 15 min) was 0.77 µmol/mL/min, the average calculated production rate for 1 h however was 0.34 µmol/mL/min. Most intraparticle oxygen courses indicated no inactivation or a possible limitation of the substrate D - Met, there was no unexplainable increase or decrease in oxygen. Stopping of H_2O_2 addition after 1 h of reaction time showed in most cases a rapid consumption of O_2 intraparticle, proving no strong inactivation of either enzyme and no substrate limitation had occurred. The possible explanation of product degradation was proven by HPLC analysis. Chromatograms showed the formation of another unknown product over time next to detection of alpha-keto acid. This degradation product is probably caused by decarboxylation of α-keto acid, a phenomenon often observed in presence of H_2O_2 [9]. For calculation of a production rate unaffected by product degradation D - Met consumption detected by HPLC analysis was used. This confirmed that the first sample taken of most experiments is little affected by product degradation. Thus the rate calculated from this sample was used for further analysis.

Figure 7 is displaying a correlation between the rate of H_2O_2 addition and the rate of alpha keto acid production. In increase of H_2O_2 addition over a range between 0.1 until 1 µmol/mL/min lead to a corresponding increase in production of alpha-keto acids. Activity of *Z_{basic2}_Tv_DAAO* displayed by coimmobilizate at air-saturation was for all experiments around 0.4 µmol/mL/min (individual deviations were due to usage of crude extract from cell disruption for immobilization). It is important to notice that these experiments displayed that production of alpha-keto acid was directly dependent on addition of H_2O_2 indicating a direct utilization of H_2O_2 as source for oxygen production.

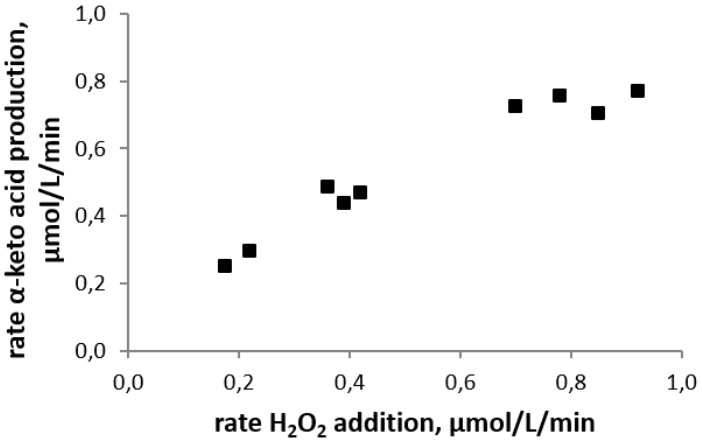


Figure 7. *Correlation between α-keto acid production rate and H_2O_2 addition rate. One black point is indicating one experiment (average α-keto acid production rate calculated from DNP measurements and final H_2O_2 addition rate).*

Application of coimmobilizate in the new set-up for scaled-up fed-batch proved to be not without difficulties, however previous studies in smaller scale allowed gaining experience and understanding of most common issues governing these preparations. Therefore most issues like oxygen overproduction or inactivation could be resolved with simple solutions.

3.6. Modulation of Bubble-free Fed-batch Reactor

The previously described reactor set-up was tested for two kinds of reaction intensification, an increased amount of biocatalyst and an increased H_2O_2 addition rate for supersaturation of oxygen inside the particle.

The increase of biocatalyst used in a single experiment was mainly performed, because the use of labeled particles in a fed-batch reactor lead to increased noise over time due to volume changes and also a shift of phase could be observed (Figure S7). The previously used amount of 5 mg particle per mL of reaction volume had displayed in recent studies a good signal with reasonable noise for measurement of phase [2]. Higher particle amounts were therefore tested (Figure S8), finally settling on a particle concentration of 15 mg/mL. Particle amounts of 15 and 20 mg/mL showed the best results with a similar small phase shift and low noise at a volume increase of up to 20 %.

Production rates increased corresponding to increased particle amount and adjusted H_2O_2 addition rates. When a particle concentration of 5 mg/mL (productivity rate 0.14 µmol/mL/min) was used with a final H_2O_2 addition rate of 0.16 µmol/mL/min, production rate was 0.12 µmol/mL/min. A threefold increase of particle amount to 15 mg/mL (productivity rate 0.405 µmol/mL/min) with the corresponding H_2O_2 addition rate of 0.39 µmol/mL/min showed a production rate of 0.44 µmol/mL/min.

Another goal of usage of higher hydrogen peroxide addition rates for oxygen supersaturation of the carrier. A way to measure this reaction intensification became the calculation of the degree of intensification:

degree of intensification = rate of α-keto acid production/ rate of activity (at air saturation)

The rate of activity at air saturation is obtained by measurement (bulk oxygen monitoring) of activity of the biocatalyst. In Figure 8 this degree of intensification can be observed compared to addition of H_2O_2. In this figure a black line is indicating at 1. A degree of 1 is indicating the productivity of the reaction is for this coimmobilizate the same as it would be at air saturation. A value of more than 1 is therefore indication a productivity above the initial measured one at air saturation. There are several examples where reaction intensification was possible. Production of α-keto-acids could be increased up to 2.5-fold compared to production at air-saturation.

The response of the system to different addition rates further proved that with this approach oxygen generation and the production of α-keto-acids can be tightly regulated.

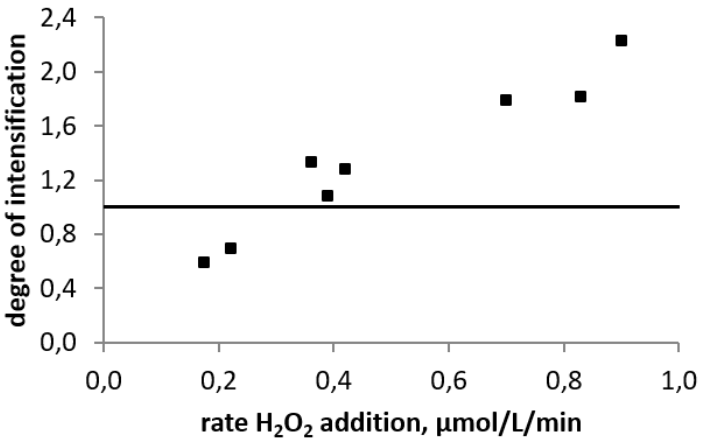


Figure 8. *Influence of H_2O_2 addition on degree of intensification. One black point is indicating one experiment (average degree of intensification and final H_2O_2 addition rate). Degree of intensification is calculated from $Z_{basic2}\_Tv\_DAAO$ activity at 100 % O_2 concentration without additional H_2O_2 and average α-keto acid production rate calculated from DNP measurements.*

We also tested a more conventional approach for increase of oxygen in this set-up. Bubbling in air to feed the bulk with oxygen was successful, however measurement of bulk oxygen was only possible when air was turn off, otherwise the turbulence would have broken the sensor.

It was not possible measuring intraparticle oxygen at a particle concentration of 5 mg/mL, because the noise of measurement was even higher than in the previously described set-up with H_2O_2 addition. After an increase to 15 mg/mL it was possible, however this signal is still disrupted because the stirring had to be turned off for bulk oxygen measurements.

Comparing the conventional approach by using air to the use of H_2O_2 addition showed that using air has several drawbacks: Oxygen measurement is complicated, particles tent to aggregate on the flask surface on top of the liquid due to bubble disturbances and a maximum degree of intensification reached is only 1.5 (compared to 2.4 for H_2O_2).

The proof of reaction intensification highlighted the potential, this design has for not only for overcoming oxygen diffusion limitation in porous particle, but also for an increase of production beyond what is commonly observed at air saturation.

4. Conclusions

Coimmobilization of *Z_{basic2}_Tv_DAAO* and *Z_{basic2}_BP_KatA* and subsequent labeling could be easily performed and analysis showed no issues concerning immobilization yield and measureable activity on the carrier. *Z_{basic2}_Tv_DAAO* proved to be very stable over a longer period of time, when H_2O_2 was added in a controlled protocol. Issues with inactivation often occurred at too high H_2O_2 concentrations. H_2O_2 addition need to be tightly controlled, constant intraparticle O_2 monitoring helped identifying too high H_2O_2 addition or inactivation of one or both enzymes occurring over time, without taking sample to perform an assay or HPLC analysis. Results of oxygen measurements compared to results from assays and HPLC analysis confirmed the affectivity of H_2O_2 addition to increase oxygen availability inside the carrier.

Main drawback of this experimental set-up is that excess H_2O_2 in bulk is degrading the product quite quickly (observable after 15 min of H_2O_2 addition). A possible solution could be product removal or using this approach for a substrate less hydrogen peroxide sensitive. The use of a coimmobilizate of *Z_{basic2}_Tv_DAAO* and *Z_{basic2}_BP_KatA* in combination with H_2O_2 feeding is leading to controlled reaction intensification, compared to the activity of the immobilized enzyme measured with initial reaction rates. The productivity of the reactor is higher and definitely easier achieved than in an air bubbled reactor that is limited and controlled by kL_a.

In the future the limits of the reaction intensification should be explored based on parameters and issues stated in this thesis. Another possibility would be the application on the established system on other enzymes, e.g. GOX. The advantage of GOX would be that this enzyme is not as affected by H_2O_2 as DAAO is. Furthermore GOX and catalase coimmobilizates were already studied in another context [7, 8].

5. References

[1] Trost, E.-M., Fischer, L.

`Minimization of By-product Formation during D-Amino Acid Oxidase Catalyzed Racemate Resolution of D/L- Amino Acids´.

Journal of Molecular Catalysis B: Enzymatic, 2002, 19 -20, 189-195.

[2] Tishkov, V.I., Khoronenkova, S.V.

`D-Amino Acid Oxidase: Structure, Catalytic Mechanism, and Practical Application´.

Biochemistry (Moscow), 2005, 70 (1), 40-54.

[3] Buto, S., Pollegioni, L., D'Angiuro, L., Pilone, M.S.

`Evaluation of D-Amino Acid Oxidase from *Rhodotorula gracilis* for the Production of α-Keto Acids: A Reactor System´.

Biotechnology and Bioengineering, 1994, 44, 1288-1294.

[4] Bolivar, J. M., Schelch, S., Mayr, T., Nidetzky, B.

`Dissecting Physical and Biochemical Factors of Catalytic Effectiveness in Immobilized D-Amino Acid Oxidase by Real-Time Sensing of O_2 Availability Inside Porous Carriers´.

ChemCatChem, 2014, 6 (4), 981-986.

[5] Bolivar, J. M.; Consolati, T.; Mayr, T.; Nidetzky, B.:

`Shine a Light on Immobilized Enzymes: Real-time Sensing in Solid Supported Biocatalysts´.

Trends in biotechnology, 2013, 31, 194 – 203.

[6] Hernandez, K., Berenguer-Murcia, A., Rodrigues, R.C., Fernandez-Lafuente, R.
`Hydrogen Peroxide in Biocatalysis. A Dangerous Liaison´.
Current Organic Chemistry, 2012, 16, 2652-2672.

[7] Bankar, S.B., Bule, M.V., Singhal, R.S., Ananthanarayan, L.A.
`Co-immobilization of Glucose Oxidase - Catalase: Optimization of Immobilization Parameters to Improve the Immobilization Yield´.
International Journal of Food Engineering, 2011, 7 (2), 8.

[8] Reuss, M., Buchholz, K.
`Analysis of the Coupled Transport, Reaction, and Deactivation Phenomena in the Immobilized Glucose Oxidase and Catalase System´.
Biotechnology and Bioengineering, 1979, 21 (11), 2061-2081.

[9] Fernández-Lafuente, R., Rodriguez, V., Guisán, J.M., Guisán, J.M.
`The Coimmobilization of D-Amino Acid Oxidase and Catalase Enables the Quantitative Transformation of D-Amino Acids (D-Phenylalanine) into α-Keto Acids (Phenylpyruvic Acid) ´.
Enzyme and Microbial Technology, 1998, 23, 28-33.

[10] Schneider, K., Dorscheid, S., Witte, K., Giffhorn, F., Heinzle, E.
`Controlled Feeding of Hydrogen Peroxide as Oxygen Source Improves Production of 5-Ketofructose from L-Sorbose using Engineered Pyranose-2-oxidase from *Peniophora gigantea*´.
Biotechnology and Bioengineering, 2012, 109, 2941-2945.

[11] Pollegioni, L., Molla, G.

`New Biotech Applications from Evolved D-Amino Acid Oxidases´.

Trends in Biotechnology, 2011, 29 (6), 276-283.

[12] Pollegioni, L., Caldinelli, L., Molla, G., Sacchi, S., Pilone, M.S.

`Catalytic Properties of D-Amino Acid Oxidase in Cephalosporin C Bioconversion: A Comparison between Proteins from Different Sources´.

Biotechnology Progress, 2004, 20, 467-473.

[13] Wiesbauer, J., Bolivar, J. M., Mueller, M., Schiller, M., Nidetzky, B.

`Oriented Immobilization of Enzymes Made Fit for Applied Biocatalysis: Non-Covalent Attachment to Anionic Supports using Z_{basic2} Module´.

ChemCatChem, 2011, 3, 1299–1303.

[14] Fernández-Lafuente, R., Rodríguez, V., Mateo, C., Fernández-Lorente, G., Arminsen, P., Sabuquillo, P., Guisán, J.M.

`Stabilization of Enzymes (D-Amino Acid Oxidase) against Hydrogen Peroxide via Immobilization and Post-immobilization Techniques´.

Journal of Molecular Catalysis - B Enzymatic, 1999, 7, 173-179.

[15] Betancor, L., Hidalgo, A., Fernández-Lorente, G., Mateo, C., Fernández-Lafuente, R., Guisán, J.M.

`Preparation of a Stable Biocatalyst of Bovine Liver Catalase using Immobilization and Postimmobilization Techniques´.

Biotechnology Progress, 2003, 19, 763-767.

[16] Switala, J., Loewen, P.C.

`Diversity of Properties among Catalases´.

Archives of Biochemistry and Biophysics, 2002, 401, 145-154.

[17] Pollegioni, L., Buto, S., Tischer, W., Ghisla, S., Pilone, M.S.

`Characterization of D-Amino Acid Oxidase from *Trigonopsis variabilis*´.

Biochemistry and Molecular Biology International, 1993, 31 (4), 709-717.

6. Supporting Information

6.1. Supporting Tables

Table S1. *Yield and effectiveness factors of immobilizate and coimmobilizate in all steps of a consecutive coimmobilization (final loading: 600 U/ g carrier* Z_{basic2}*_Tv_DAAO and 50 000 U/ g carrier* Z_{basic2}*_BP_KatA; labeled with 2.5 mg Ru(dpp) dye/ g carrier); all values are given in %. Indices a, b, c for method of measurement (a…Coupled Peroxidase Assay (CPA); b…* H_2O_2 *assay; c… bulk oxygen sensor measurement).*

Properties	first immobilized *Z_{basic2}_Tv_DAAO*	first immobilized *Z_{basic2}_BP_KatA*
Yield *Z_{basic2}_Tv_DAAO*	98[a]	97[a]
Yield *Z_{basic2}_BP_KatA*	97[b]	96[b]
η *Z_{basic2}_Tv_DAAO* immobilizate	8.5[c]	-
η *Z_{basic2}_BP_KatA* immobilizate	-	7[b]
η *Z_{basic2}_Tv_DAAO* Coimmobilizate	5.5[c]	4[c]
η *Z_{basic2}_BP_KatA* Coimmobilizate	3[b]	3.5[b]
labeling yield	100	100
η *Z_{basic2}_Tv_DAAO* Coimmobilizate labeled	3[c]	2.5[c]
η *Z_{basic2}_BP_KatA* Coimmobilizate labeled	4.5[b]	3[b]

6.2. Supporting Figures

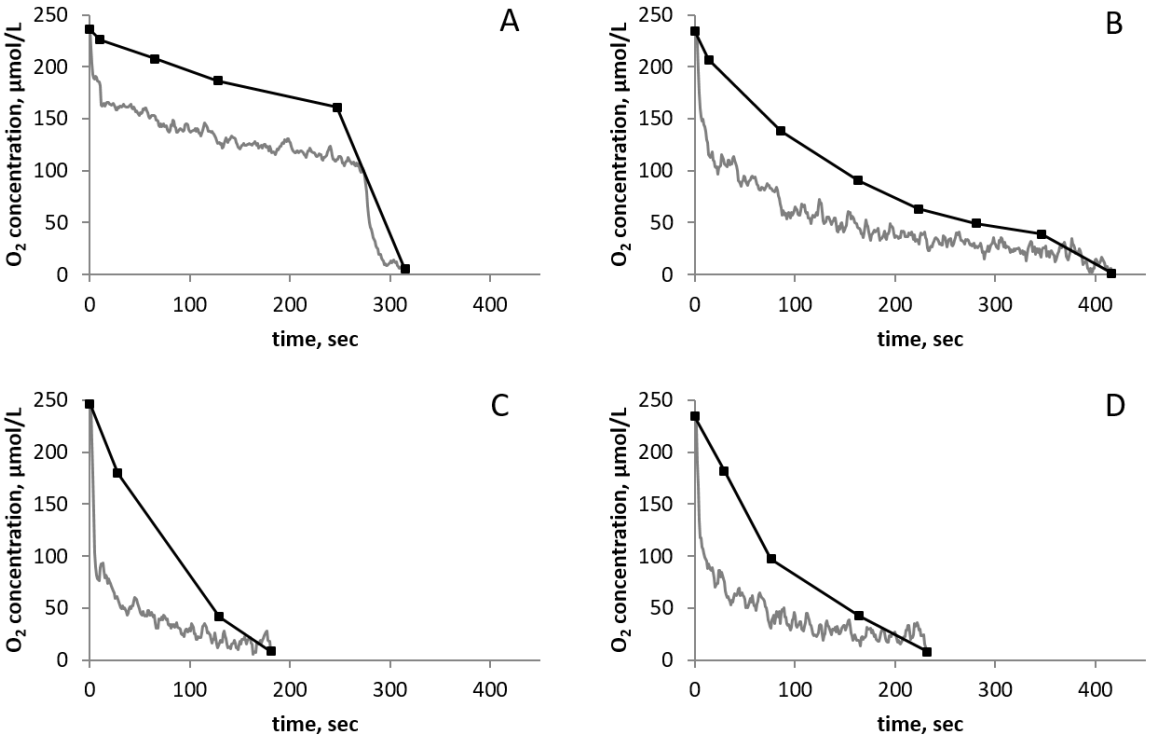


Figure S1. *Measurement of oxygen consumption after addition of 200 mM* D*-Met to (A) immobilized DAAO* Z_{basic2}*_Tv_DAAO (200 U/g carrier), (B) immobilized* Z_{basic2}*_Tv_DAAO (400 U/g carrier), (C) immobilized* Z_{basic2}*_Tv_DAAO (600 U/g carrier) and (D) immobilized* Z_{basic2}*_Tv_DAAO (800 U/g carrier). Bulk oxygen (black line with dots, point measurement at each dot); Intraparticle oxygen (grey line, continuous measurement).*

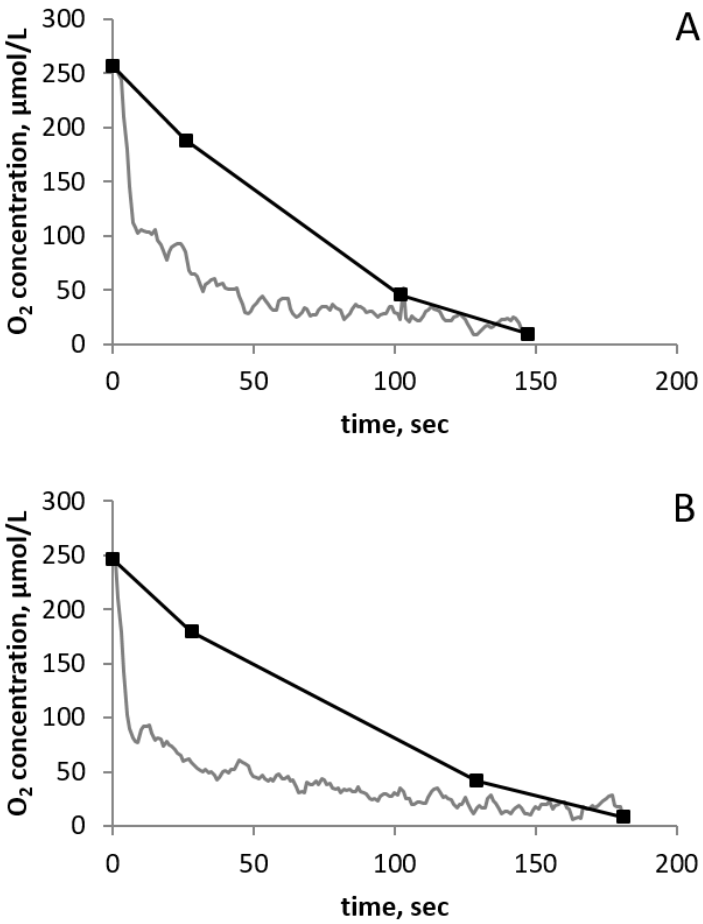


Figure S2. *Measurement of oxygen consumption after addition of 200 mM D-Met to (A) immobilized Z_{basic2}_Tv_DAAO (600 U/g carrier) and (B) coimmobilized Z_{basic2}_Tv_DAAO (600 U/g carrier) and Z_{basic2}_BP_KatA (10 000 U/ g carrier). Bulk oxygen (black line with dots, point measurement at each dot); Intraparticle oxygen (grey line, continuous measurement).*

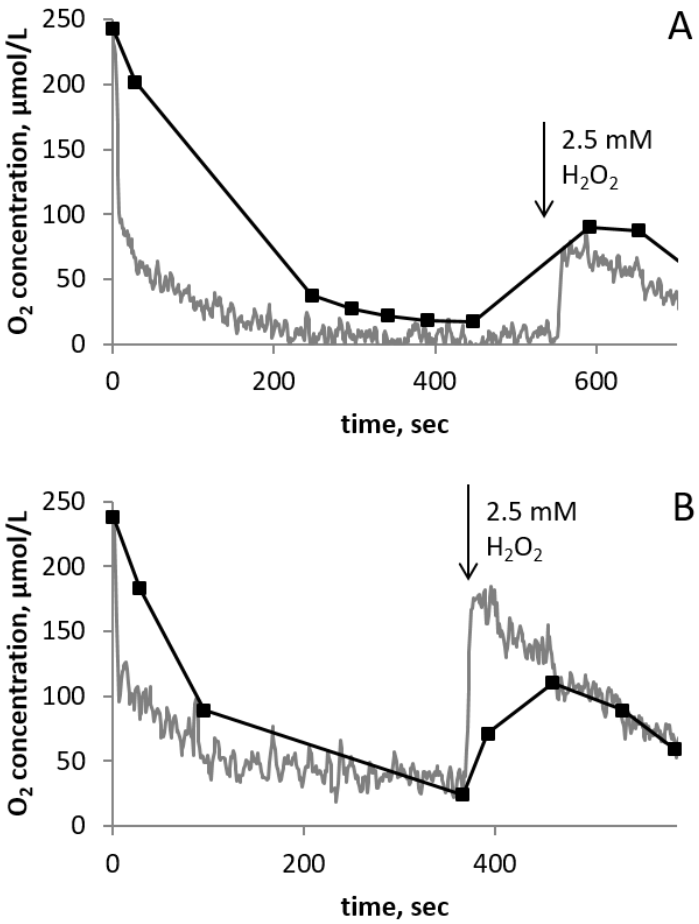


Figure S3. *Measurement of oxygen consumption after addition of 200 mM D-Met to (A) coimmobilized Z_{basic2}_Tv_DAAO (600 U/g carrier) and Z_{basic2}_BP_KatA (10 000 U/ g carrier) and (B) coimmobilized Z_{basic2}_Tv_DAAO (600 U/g carrier) and Z_{basic2}_BP_KatA (50 000 U/ g carrier). Bulk oxygen (black line with dots, point measurement at each dot); Intraparticle oxygen (grey line, continuous measurement); Arrows indicate H_2O_2 addition.*

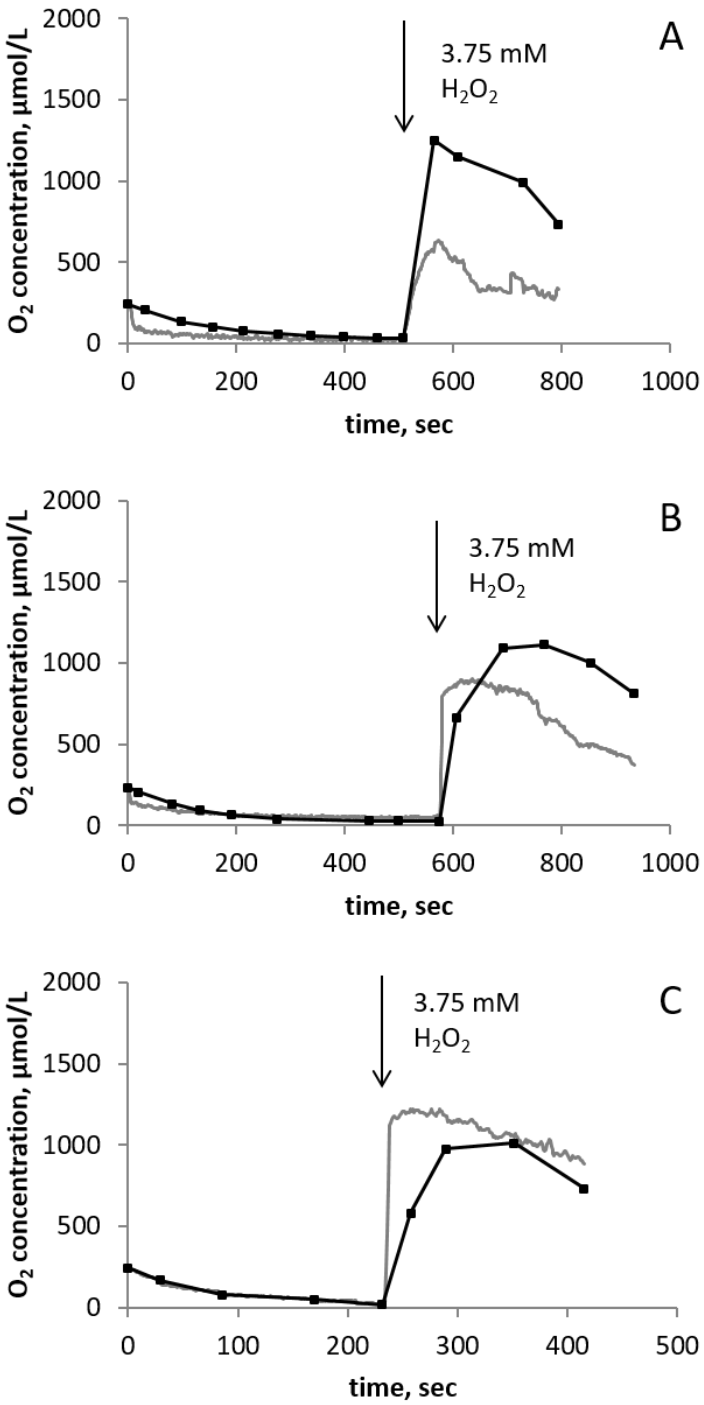


Figure S4*. Comparison of oxygen measurement for an oxygen consumption and production with different combinations of free and immobilized* Z_{basic2}*_Tv_DAAO and* Z_{basic2}*_BP_KatA. (A) immobilized* Z_{basic2}*_Tv_DAAO (600 U/g carrier) and free* Z_{basic2}*_BP_KatA (equivalent to 50 000 U/ g carrier), (B) coimmobilized* Z_{basic2}*_Tv_DAAO (600 U/g carrier) and* Z_{basic2}*_BP_KatA (50 000 U/ g carrier) and (C) free* Z_{basic2}*_Tv_DAAO (equivalent to 600 U/g carrier) and immobilized* Z_{basic2}*_BP_KatA (50 000 U/ g carrier). Bulk oxygen (black line with*

dots, point measurement at each dot); Intraparticle oxygen (grey line, continuous measurement); Arrows indicate H_2O_2 addition.

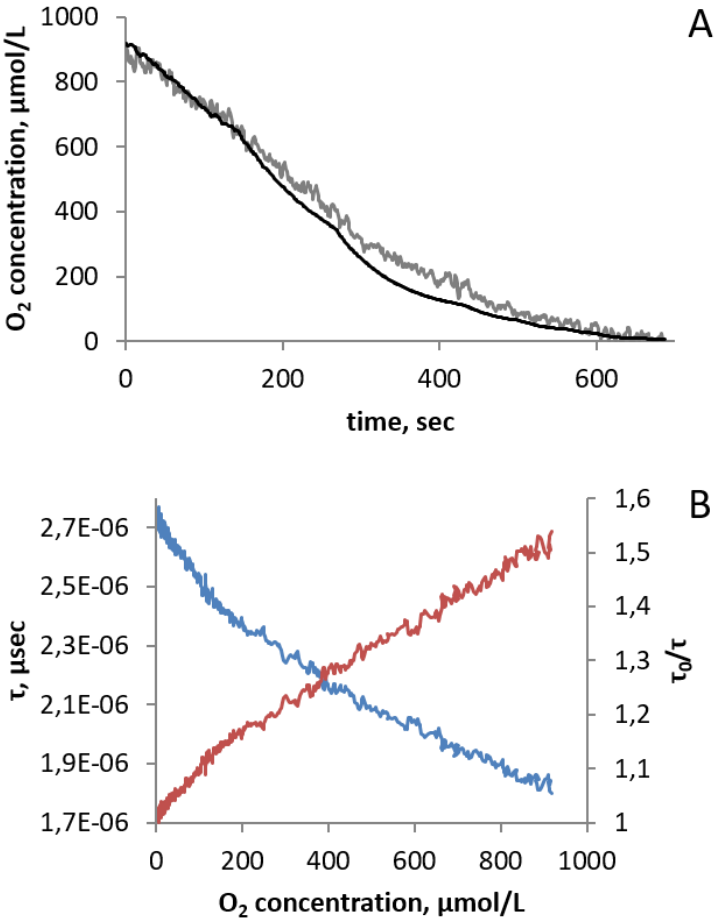


***Figure S5**. Calibration of $Ru(dpp)_3$ labeled on coimmobilizate of DAAO (Z_{basic2}_Tv_DAAO 600 U/g carrier) and Z_{basic2}_BP_KatA (50 000 U/ g carrier). (A) After O_2 increase with pure O_2, O_2 was decreased again with Glucose oxidase and Glucose; graph is showing the controlled decrease of O_2. (B) Calculated life time (τ) for $Ru(dpp)_3$ over a range between 0 and 1000 µmol/L O_2. Bulk oxygen (black line, continuous measurement); Intraparticle oxygen (grey line, continuous measurement).*

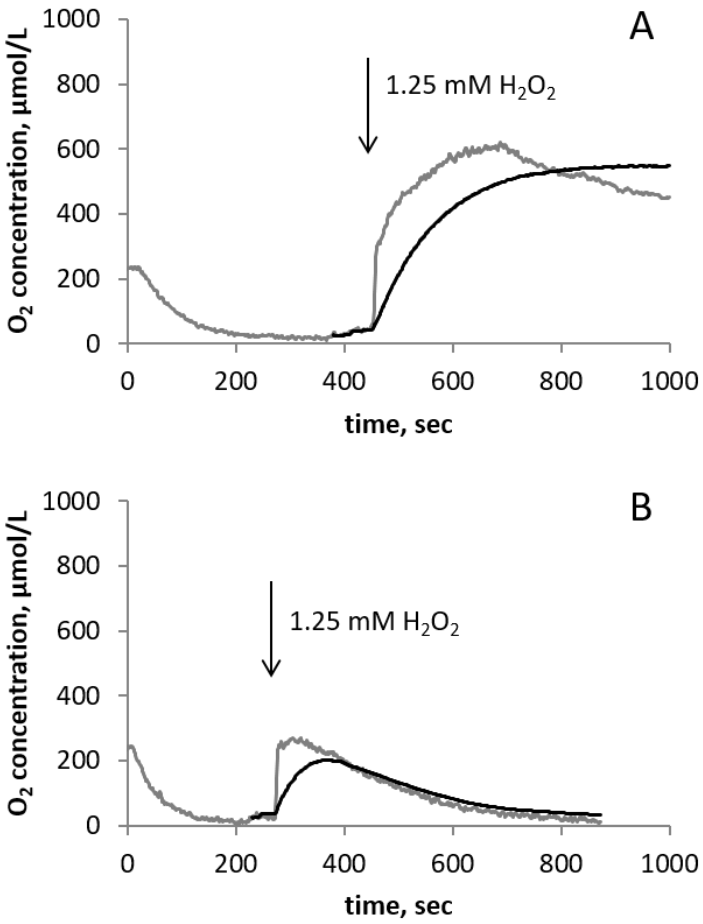


Figure S6. *Bulk and Intraparticle oxygen measurement of (A) immobilized* Z_{basic2}*_BP_KatA (50 000 U/ g carrier) and (B) coimmobilized* Z_{basic2}*_Tv_DAAO (600 U/ g carrier) and* Z_{basic2}*_BP_KatA (50 000 U/ g carrier). Oxygen was depleted in both cases by bubbling in* N_2 *and* H_2O_2 *was added afterwards (indicated by arrow). Bulk oxygen (black line, continuous measurement); Intraparticle oxygen (grey line, continuous measurement).*

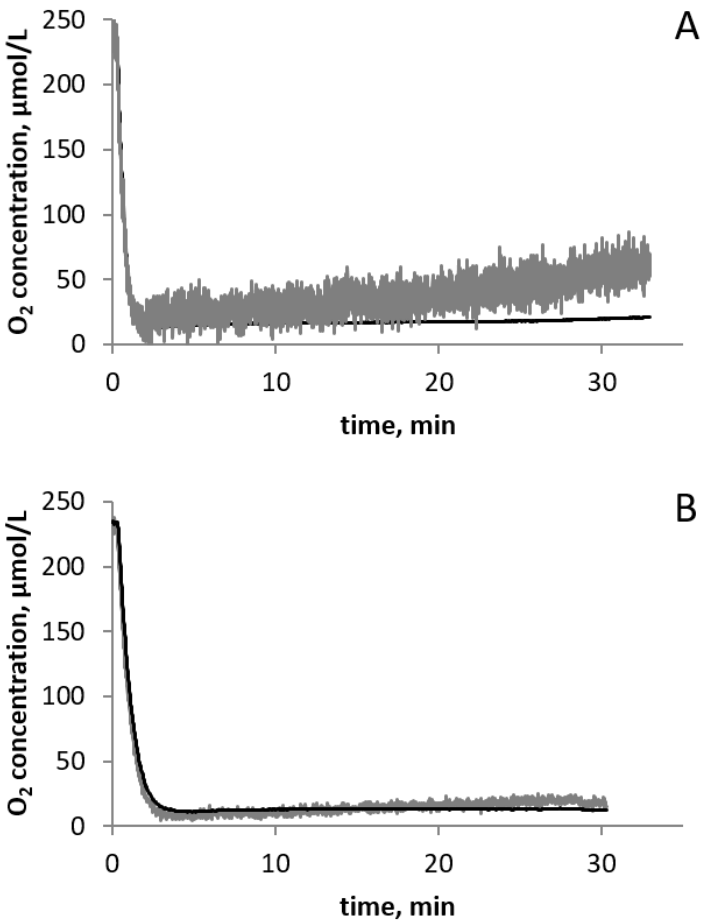


Figure S7*. Signal shift and noise of intraparticle measurement at volume increase of 20 % in reactor, when particle concentration is (A) 5 mg/ mL and (B) 20 mg/ mL in reactor. Bulk oxygen (black line, continuous measurement); Intraparticle oxygen (grey line, continuous measurement).*

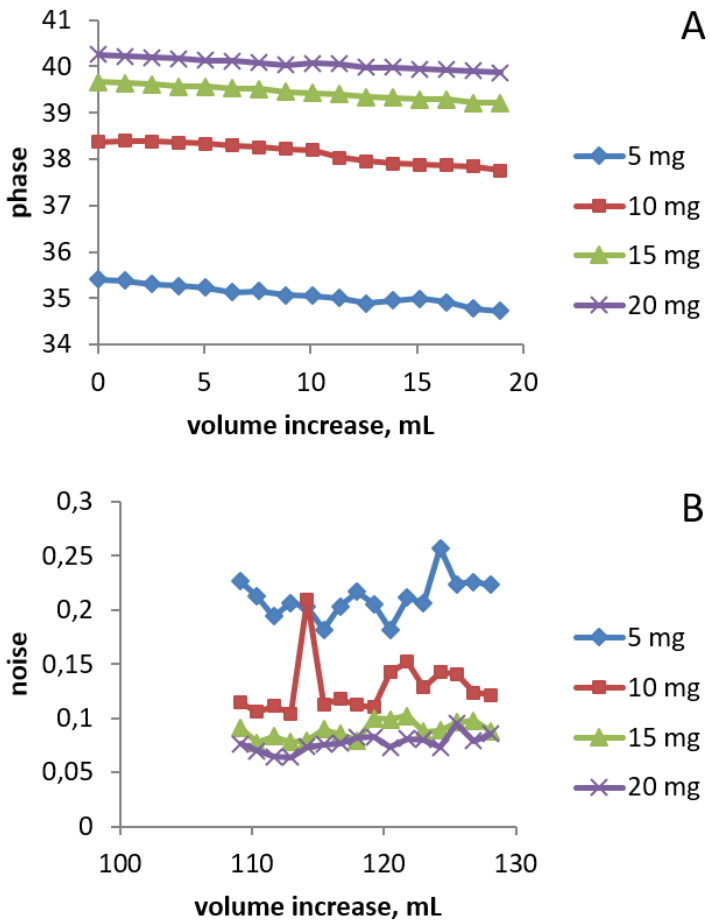


Figure S8. *(A) Phase and (B) noise of* $Ru(dpp)_3$ *labeled on carrier Relisorb 400 in correlation to particle amount (initial concentration: 5-20 mg/mL) and volume change (initial reactor volume 100 mL).*

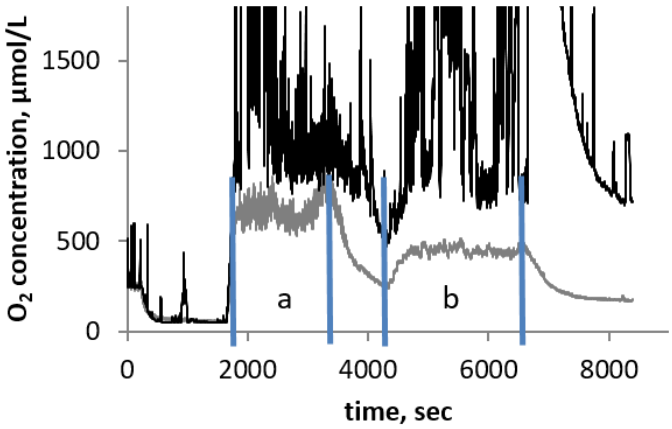


***Figure S9**. Overproduction of O_2 in a reactor containing coimmobilized Z_{basic2}_Tv_DAAO (activity: 0.11 mM/min) and Z_{basic2}_BP_KatA with a continuous H_2O_2 addition. Flow rate at a was 0.45 mM/min and at b 0.1 mM/min. Bulk oxygen (black line, continuous measurement); Intraparticle oxygen (grey line, continuous measurement).*

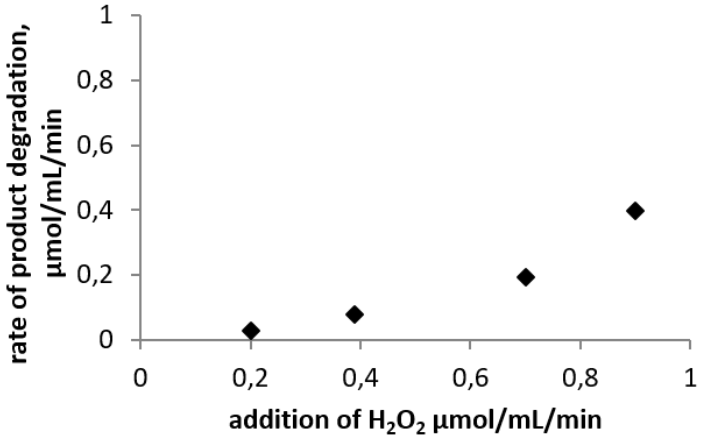


***Figure S10**. Product degradation calculated from D-Met consumption and α-keto acid production detected with HPLC over a reaction time of 60 min in correlation with H_2O_2 addition rate in fed-batch reactor with coimmobilizate.*

6.3. Supporting Methodology

6.3.1 DNP assay

In various papers [1-4] an assay for the quantification of alpha- keto acids was found. Modified for the use of bulk samples from reactions with coimmobilizate the following protocol was developed:

150 µL sample (or for the blank: PBB pH 8) are pipetted into a 1.5 mL epi and then the same amount of DNP is added. The epi is placed in an end-over-end rotator (20 rpm) and left there for 10 minutes incubation. When alpha keto acids are present within the sample a color change to bright yellow occurs. After 10 minutes 600 µL of NaOH are added and put again on the rotator for 10 minutes. The yellow color is changing to reddish-brown when the base is added. After the assay is finished 500 µL are transferred into a cuvette and then diluted with 500 µL of PPB pH8. The measurement is performed at 440 nm. For calibration α-Keto-γ-(methylthio)butyric acid sodium salt was used as a standard.

6.3.2 Calibration of $Ru(dpp)_3$ on Coimmobilizate of Z_{basic2}_*Tv_DAAO* and Z_{basic2}_*BP_KatA*

Coimmobilizate labeled with Ru(dpp)3 was kept under suspension at 30 °C. Stirring was stopped and oxygen concentration in bulk and particle was increased by bubbling pure oxygen into the flask containing the coimmobilizate in suspension. After 5-10 min, oxygen in bulk and particle was measured with oxygen sensors, fiber-optic oxygen microoptode connected to a fiber optic oxygen meter (model microx TX3, PreSens) and a fiber-optic cable connected to a fiber optic oxygen meter (model pH-1 mini, PreSens). When oxygen concentration was around 1000 µmol/L, calibration measurement was started by adding Glucose and Glucose oxidase (GOX) ensuring oxygen decrease down to 0 µmol/L in bulk and particle at a steady rate. Life time (τ) for $Ru(dpp)_3$ was calculated over a range between 0 and 1000 µmol/L O_2.

6.4. Supporting References

[1] Oguri, S., Watanabe, K., Nozu, A.; Kamiya, A.;

`Screening of D-Amino Acid Oxidase Inhibitor by a New Multi-assay Method´.

Food Chemistry, 2005

[2] D'Aniello, A., D'Onofrio, G., Pischetola, M., (...), Petrucelli, L., Fisher, G.H.;

`Biological Role of D-Amino Acid Oxidase and D-Aspartate Oxidase: Effects of D-Amino Acids´.

Journal of Biological Chemistry, 1993

[3] Sikora, L.A., Marzluf, G.A.;

`Regulation of L-Phenylalanine Ammonia-Lyase by L-Phenylalanine and Nitrogen in *Neurospora crassa* ´.

Journal of Bacteriology, 1982

[4] Nuutinen, J.T., Timonen, S.;

`Identification of Nitrogen Mineralization Enzymes, L-Amino Acid Oxidases, from the Ectomycorrhizal Fungi *Hebeloma spp.* and *Laccaria bicolor*´.

Mycological Research, 2008

List of Abbreviations

BSA	Bovine Serum Albumin
CPA	Coupled Peroxidase Assay
D-Met	D-Methionine
DAAO	D-Amino Acid Oxidase
DNP	2,4-Dinitrophenylhydrazine
FAD	Flavin Adenine Dinucleotide
GOX	Glucose Oxidase
IOD	Internal Oxygen Depletion
PPB	Potassium Phosphate Buffer
Relisorb 400	Company name of carrier
$Ru(dpp)_3$	Dichloride(4, 7-diphenyl-1, 10-phenantroline) ruthenium (II)
TOC	Theoretical Oxygen Consumption
*Tv*DAAO	DAAO from *Trigonopsis variablis*
Z_{basic2}	Strongly positively charged tag
Z_{basic2}*_Tv_DAAO*	DAAO from *Trigonopsis variablis* tagged with Z_{basic2}
Z_{basic2}*_BP_KatA*	Catalase from *Bordetella pertussis* tagged with Z_{basic2}
η	Catalytic Effectiveness Factor

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe. Das in TUGRAZonline hochgeladene Textdokument ist mit der vorliegenden Masterarbeit identisch.

Datum Unterschrift