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Development of packed-bed reactors for intensification of oxygen-dependent oxidations using immobilized enzymes

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AFFIDAVIT

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Abstract

Biocatalytic oxidation reactions are very important in schemes for the synthesis of active pharmaceutical ingredients and in fine chemical industry. Generally, employing biocatalysts for oxidation reaction has the potential to overcome some drawbacks of conventional chemical oxidations as enzymes have superior regio- and stereoselectivity, they are known for their low environmental impact as well as for enabling much safer and cost-effective operation procedures.

Implementation of oxidative enzymatic driven reactions into industrial valuable processes is known to be difficult, as there are issues of enzyme stabilization, basic strategies for substrate and oxygen supply as well as reactor design and operation needed to be addressed. A fundamental bottleneck identified, is the low solubility of oxygen in liquid.

Enzyme immobilization on porous spherical supports is shown in this work. Design, implementation and operation of a packed bed reactor for continuous oxidation reaction, operating in single liquid-phase under the premise of high concentration of oxygen dissolved, is presented. Due to combination of microreactor- and flow-technology and due to application of high pressurized conditions, solubility and hereby consistent supply of oxygen was orders of magnitude intensified.

Proof of concept of reactor operation is shown for glucose oxidase in combination with catalase, whereas product yield of up to 90 percent is presented. A process engineering approach accessing basic process operation understanding and knowledge for the designed packed bed reactor is presented including proof of feasibility of catalyst reuse, exploration of maximum operation capability for the plant and approaches aiming process intensification.

As implementation of different bi-enzymatic systems on the example of D-amino acid oxidase/catalase has been proven having high concentration of product formed, this designed packed bed reactor can be seen as platform technology opening a wide range of feasible implementation of different other enzymatic oxidation reactions.

Zusammenfassung

Biokatalytische Oxidationsreaktionen sind sehr wichtige Bestandteile von Syntheseschemata zur Produktion von Aktiven Pharmazeutischen Wirkstoffen (API) und können auch in der Feinchemie eingesetzt werden. Die Nutzung von Biokatalysatoren hat im Allgemeinen das Potential einige Unzulänglichkeiten konventionell geführter Oxidationsreaktionen zu überwinden, da Enzyme über überlegene Regio- und Stereoselektivität verfügen, für ihre geringe umweltbelastende Wirkung bekannt sind und eine viel sicherere und kosteneffektivere Prozessführung ermöglichen.

Erwiesenermaßen wird die Implementierung enzymatisch betriebener Oxidationsreaktionen in industriell wertvolle Prozesse als schwierig deklariert, da Problemstellungen bezüglich Enzymstabilisierung, grundlegende Strategien der Substrat- und Sauerstoffversorgung als auch Fragestellungen bezüglich Reaktorkonfiguration und Reaktoroperation behandelt werden müssen. Als grundlegender Engpass ist die schlechte Löslichkeit von Sauerstoff in Flüssigmedien hervorzuheben.

In dieser Arbeit wird ein Weg der Enzymstabilisierung mittels Immobilisation an porösen sphärischen Trägermaterialien vorgestellt. Konstruktion, Implementierung und Funktionsweise eines Festbettreaktors (packed bed reactor) wird präsentiert, welcher in einphasigem Flüssigmedium unter der Prämisse hoher Sauerstoffkonzentration betrieben wird. Erzielt durch Kombination von Mikroreaktortechnologie und Strömungstechnik (Flow Technologies) und unter Anwendung von Hochdruckbedingungen wurden die Löslichkeit und hierdurch die konstante Sauerstoffversorgung um einige Größenordnungen intensiviert.

Ein konzeptioneller Beweis der Reaktoranwendung mittels Glucose-oxidase in Kombination mit Katalase wird vorgestellt, in welchem Produktausbeuten von bis zu 90 Prozent erreicht wurden. Es wird ein verfahrenstechnischer Ansatz präsentiert, mit dem Zugang zu grundlegendem Betriebsverständnis und Wissen um den konstruierten Festbettreaktor erlangt werden konnten. Weiteres wurde der konzeptionelle Beweis der hypothetischen Wiederverwendbarkeit des Biokatalysators erbracht, welcher von der Erkundung der maximalen Betriebsspanne des Anlagenkonstrukts inklusive Ansätze zur Prozessoptimierung abgerundet wurde.

Die Implementierung weiterer Bi-enzymatischer Systeme wird anhand des Beispiels D-Aminosäureoxidase/Katalase vor Augen geführt, wobei abermals hohe Produktausbeuten erzielt werden konnten. Hierdurch liegt der Schluss nahe, dass der konstruierte Festbettreaktor als Plattform Technologie für ein breites Spektrum möglicher Anwendung weiterer enzymatischer Oxidationsreaktionen gesehen werden kann.

Table of contents

1 Introduction

Chemical oxidations are a powerful synthetic tool, which are generally conducted under high pressure and high temperature conditions.[1] Oxidation reactions contribute about 30 percent of total production volume of bulk chemical industry.[2] Generally, metal catalysts, sulfoxides or amine oxides and organoperoxides are used in fine- and pharmaceutical industry for oxidations, generating considerable amount of waste.[2] The use of solvents with a poor environmental profile, expensive noble metal catalysts and the need of additional and cost-demanding synthetic steps as well as the need to protect and deprotect labile functional groups, are some drawback worth mentioning.[3] Additionally, poor chemical selectivity and harsh reaction conditions initiate a demand for better reagents.[2]

Using biocatalysts for oxidation reactions would have the potential to overcome many of these limitations. Due to their superior regio- and stereoselectivity, combined with their low environmental impact, they could substitute a wide range of chemically catalysed oxidation reactions and can thus be used in fine chemical industry as well as in pharmaceutical industry.[3] Furthermore, using enzymes provide the opportunity to work in aqueous condition, which enables much safer operation procedures.[2] Biocatalytic oxidation reactions are very important in schemes for the synthesis of active pharmaceutical ingredients. Biocatalysts high selectivity can also be used for conversions of compounds having multiple chiral centres or multiple step syntheses.[4]

Enzyme format can basically be used in their isolated form or as whole cells, either of them in suspension or immobilized. Nevertheless, the format of the catalyst mostly depends on the requirements of the process headed for, and further influences the way of operation mode possible for this process.[5] Soluble enzymes are quite unstable under conditions of stirring and bubbling, which is needed to be done to achieve a large interfacial area, aiming to enhance gas transport into the reaction media. Irreversible inactivation of enzymes could possibly be founded by the mechanical shear stress due to different kinds of mixing.[6] Although enzymes are pre-eminently made to perform in living being cells, they become steadily more important as industrial catalyst in food chemistry, for pharmaceutical applications, in bulk chemical industry etc.

Enzymes and techniques how to use them properly may be essential for a future with more cost-effective and more ecologically friendly pharmaceutical-, textile-, and chemical industry. But, generally spoken, enzymes are not very suitable for industrial applications, reasoned by their usual instability and possible inactivation behaviour, due to either substrates- or products interaction. Furthermore, enzymes are naturally soluble, are having low to moderate stability and can be inhibited not only due to products and substrates, also due to many other factors.[7] Isolation of enzymes is still a cost-demanding process and if used once and still active, recovery of enzymes is technically very difficult. For most chemical processes the reuse or continuous use of a catalyst is imperative, considering technical and economic reasons. Immobilization of enzymes could be seen as an appropriate approach to address some of these issues[8], and can also be used to improve the control of reactions and avoid product contamination, which is in particular important for food- and pharmaceutical chemistry.[7]

From the first industrial use of immobilization techniques until today, stabilized and meant by that immobilized catalysts, improved the performance of industrial processes. Generally spoken, immobilization can be explained as the physical confinement of enzymes within a beforehand defined region of space, aiming to preserve their activity and to use them in a continuous manner. The mode of attachment of an enzyme to a matrix, the support carrying this matrix and the enzyme itself, are the main components of an immobilized enzymatic system. Immobilization techniques can be categorized as irreversible and reversible. Immobilization via adsorption and via disulphide bonds are part of the latter. Covalent coupling between functional groups, entrapment within beads or fibres and crosslinking by functional reactants, can be classified as irreversible methods. The choice of support primarily depends on specific enzyme properties and needs, but also on the later use and the final contribution to process costs. Carriers, or supports, can be categorized in inorganic and organic and further in porous or non-porous particles. Bio-compatibility, resistance to microbial attack and low cost availability as well as physical resistance to compression are some fundamental features, supports must have. Additionally, a support also has to be hydrophilic and chemically stable. Further, hydrodynamics and mechanical properties of carriers are tightly bound to the kind of reactor and reaction media to be used.[8] So, in conclusion, immobilization of enzymes implies the decision among different alternatives for binding and material usage, which is dependent on the type of the enzyme itself and also on the indented use. Vice versa, once the decision for a specific immobilization strategy on distinct kind of carriers has been made, this decision will also influence all future contingencies in regard of reactor design and operation.

Oxygen is used as substrate in many different kinds of reactions, like those catalysed by oxygenases or oxidases. Here normally, enzymes are utilized in the form of resting cells or in their isolated form.[9] Molecular oxygen acting as oxidizing agent is clearly exceeding over stoichiometric oxidants, such as permanganate or nitric acid, from both an environmental and economical point of view. Already highly efficient reaction systems have been developed and are becoming increasingly popular in the chemical manufacturing industry.[10]

A fundamental bottleneck for enzymatic oxidations has been found due to the low solubility of oxygen in liquid of about 0.25 mM at atmospheric pressure and 25°C, resulting in an upper limit on volumetric productivity and low biocatalytic turnover rates.[2] As the enzyme clearly underperforms due to poor solubility of oxygen in water[2], one can say that, in respect of catalytic efficiency, the concentration of oxygen in solution is critical.[5] So, main challenge is to establish the required stoichiometry for chemical transformations, having sufficient quantities of oxygen within the reaction media.[11] Oxygen may also become a limiting factor during scale-up and especially for biocatalytic oxidations. But the supply of oxygen through hard mixing or bubbling with gas may considerably increase the potential of deactivation rate of certain enzymes.[3] As conventional air-supply may be insufficient in many cases, pure oxygen could be used in order to support enhanced enzymatic activity.[5]

The concentration of dissolved gases can be increased due to forcing higher pressure into the reaction system. By this means, reaction rates of oxygen dependent enzymes can be boosted. Thus, industrial applications of gas-liquid processes are nowadays commonly operated under high pressure. Due to gas compressibility, elevated system pressure is known to cause considerable influence on mass-transfer and flow behaviour.[12] Furthermore, according Henry's law, increased pressure is imperative to be able to maintain the same concentration of dissolved gas when the reaction-temperature is elevated.[11] In order to enhance reaction efficiency, improve solubility of gaseous reactants, and to attain high transport performance, the majority of gas-liquid processes driven in microreactors are carried out under elevated pressure of 10-100 bars. Namely oxidation-reactions, Fischer-Tropsch synthesis, carbonylation, hydroformylation and hydrogenation, etc.[13]

Batch reactions, for example, are not able to come up with higher heat and mass transfer rates, as known to be provided by channelled flow systems, like microreactors.[11] Microreactor technology has also been becoming increased respected in chemical industry, as there are significant advantages such as the numbering up method, or the fact that investment in both expense and time from lab scale to ready to use industrial applicable scale is reduced due to miniaturization of devices.[14] Compared to conventional batch systems, gases are much easier to use in flow, reasoned due to the possibility to apply the higher pressure of the system to accomplish a higher degree of solubility. An additional benefitting fact for combination of continuous processing and microreactor-systems is the possibility to exactly regulate the gas-flow by just regulating the volume of flow.[11] Due to their multiple advantages as high surface to volume ratios and the possibility of continuous operation[12], microreactors are generally acting as process intensification tool. Multiphase reaction systems, synthesis of pharmaceuticals and cross coupling reactions are often cited examples, where microreactors are in operation.[15] Examples for state-of-the-art continuously operated microreactors are the Falling Film Micro Reactor, Tube in Tube Reactor, cStirred Tank Reactor and the packed bed micro-reactor. Exact explanations for these reactor setups can be found in the literature.[16,17,18]

Process engineering methods and tools can be used to cover issues required for development of new biological processes, interdependently. Decisions about the biocatalyst format, method of oxygen supply and the choice of reactor format as well as the operation mode have to be made, aiming the establishment of better manufacturing processes.[5]

As a matter of principle, a chemical reactor has to be able to fulfil certain requirements. Providing a proper environment in terms of pressure, substrate concentration and temperature, bringing all reactants into contact with each other and finally to the active site of a catalyst. The removal of product in an appropriate timely manner, as well as in some cases the retrieval of the catalyst after reaction, are some necessary tasks, needed to be accomplished. Understanding of thermodynamics, enzyme kinetics and reaction pathways, as well as knowledge about flow dynamics, mixing, heat and mass transfer are imperative for successful reactor- and process engineering. Combining miniaturized technologies with microfluidic systems administers the possibility of significant improvements for biocatalytic reactions over conventional batch processes.[19] The total mass transfer coefficient (k_La) for gas-liquid reactions can noteworthy be improved due to the use of microreactors. Compared to conventional reactors, the total gas-liquid interfacial area is enhanced by 1-2 orders of magnitude.[20] Cost-efficient and thus economic use of biocatalysts, improved process control and reduction of reactor size are some advantages of continuously driven operation in regards of biocatalytic processes compared with established batch methods.[21]

Aiming to establish a continuously operated packed bed reactor, yielding constant substrate conversion rates, case studies regarding two different oxidases, are going to be presented. For this studies glucose oxidase and D-amino acid oxidase will be immobilized in a proper way and implemented within a packed bed reactor setup will be demonstrated.

High activity and a moderate price made glucose oxidase (GOX 1.1.3.4) an ideal enzyme-candidate to be tested in the designed continuous flow reactor. Glucose oxidase catalyses the oxidation of β-D-glucose to D-glucono-δ–lacton, which hydrolyses to gluconic acid spontaneously. The reaction of the flavoprotein, originated in *Aspergillus niger*, can catalyse this oxidation reaction under oxygen consumption, in which flavin adenine dinucleotide (FAD) reacts as prosthetic group to $FADH_2$. Regeneration of FAD is accomplished due to molecular oxygen acting as hydrogen acceptor, but under production of hydrogen peroxide as side-product. Conclusive, the consumption of dissolved oxygen and of the substrate, as well as the production of hydrogen peroxide and gluconic acid is stoichiometrically coupled and thus equimolar.[22] Glucose oxidase is also known for being a fast acting enzyme, so only low enzyme concentration is required to observe oxygen limited reaction rate.[23]

D-Amino acid oxidase (DAAO) from *Trigonopsis variabilis* (1.4.3.3) can oxidize R-configured α- amino acids.[24] Due to the enzymes kinetic resolution of racemic mixtures, DAAO can produce optically pure amino acids. Molecular oxygen is utilized as co-substrate by the flavin cofactor dependent oxidase, so continuous supply of oxygen during the process is required. Enzyme's stability is jeopardized due to interaction between the solubilized DAAO and gas-liquid interface, caused by aeration. Also hydrogen peroxide, a side-product of this reaction, has a deleterious effect on the enzyme stability. Biotechnological synthesis of 7-amino-cephalosporanic acid (7-ACA), a precursor for the synthesis of penicillin antibiotics, is a very well-known example for the industrial usage of DAAO.[4]

In order to guarantee enzymatic function and prevent enzyme inactivation, removal of hydrogen peroxide, which is in both cases formed as side-product, is necessary. Literature reports that the use of catalase is the most effective way to achieve this, as catalase is capable of breaking down one mole of hydrogen peroxide to one mole of H_2O and a half mole of O_2. These reports also indicate the reduction of the stoichiometric oxygen requirements by half per each reaction step of the oxygenation reaction, including cumulative diminution of the oxygen requirements for the entire process.[3] The reaction is forced to proceed in the product-side direction by removal of hydrogen peroxide additionally, and as a consequence GOX and DAAO are protected from oxidation.[25]

Main purpose, laid in this work, was to generate a packed bed reactor-setup for continuous enzymatic conversion. As it was obvious that there were many points which needed to be addressed, preliminary experiments were conducted in the work "Preparation of a packed bed reactor containing immobilized oxidases for gas-liquid-solid bioconversions".[26] The choice for a continuous operated flow reactor, intended for enzymatic conversions in which the catalyst is immobilized on carrier packed within a HPLC-column, was made. During the experimental course of this preliminary work, carrier characterization as well as evaluation of potential HPLC-columns, intended to act as packed bed column, was done. Immobilization strategies were examined, regarding stabilization of glucose oxidase and D-amino acid oxidase, as well as co-immobilization of catalase. Proof of concept of reactor operation as well as experiments in consideration of achieving basic process understanding and knowledge were further proceeded. Hypothesis as especially the proof of basic reactor operation concept, manageable pressure levels regarding continuous operation, catalysts reusability and impressions regarding biocatalytic intensification due to residence time modulation as well as due to high concentration of oxygen dissolved demanded to be verified. In purpose to clarify these and further assumptions regarding possible limitations of the designed plant and exploring means with the intent to overcome them, basis for process engineering considerations were strictly followed within this thesis. Selection of enzymatic supports including determination of immobilization parameters, as immobilization yield and catalytic effectiveness factor was done. Based on the knowledge about enzyme stabilization properties, design, implementation and operation of a pressurized packed bed reactor was pursued. Successful setup of the continuous reactor operation was achieved via systematically approached basic process operation understanding and knowledge by evaluation of either gas- and substrate-flow conditions, determination of flow regimes, exploration of physical pressure limitation and evaluation of possible mass transfer limitation. Further, exploration of enzyme stability and productivity regarding glucose oxidase and D-amino acid oxidase as well as exploration of residence time was performed. In respect to overcome identified bottlenecks of reactor operation, maximum reactor operation window for immobilized oxidative biocatalyst was evaluated and expanded, correlation of product yield and residence time was stated and verified, and oxygen solubility within liquid media was intended to be intensified by application of high pressurized conditions, additionally. In respect to verify issued hypothesis, examples of high pressurized continuous reactor operation are shown and feasibility of implementation of different bi-enzymatic systems on the example of D-amino acid oxidase was proven, in regard to state the designed packed bed reactor as general platform technology for a variety of oxidative biocatalytic applications.

2 Materials and Methods

2.1 Chemicals

Glucose oxidase (β-D-Glucose: oxygen 1-oxidoreductase, EC 1.1.3.4, GOX; type II) was bought from Sigma-Aldrich GmbH (Vienna, Austria).[27] The glucose oxidase (GOX) derives from *Aspergillus niger* and has been solubilized in potassium phosphate buffer (PPB) at pH 7.0. Additionally, catalase (CAT) from *bovine liver*, EC 1.11.1.6, as well as catalase from *Bordetella pertussis*, modified as terminal fusion protein with Z_{basic2}, was used.[28] All other reagents as polyethylenimine etc., were also purchased from Sigma-Aldrich GmbH (Vienna, Austria).[29]

D-Amino acid oxidase (*Tv*DAAO), originated in *Trigonopsis variabilis*, was received from the hosting institute. The enzyme, provided as frozen *E. coli* cells, was previously modified with a Z_{basic2}_tag, enabling orientated ionic adsorption on supports during immobilization.[30,28]

All carrier-materials were open-handed gifts from Resindion (Milano, Italy), whereby the focus for this work was set on medium-sized particles, carrying either sulfonate- or epoxy-functional groups. A full list of carriers including exact chemical and particle characteristics can be found in the appendix (5.1). Technical equipment, especially the micro-reactor setup and corresponding software and spare parts, were kindly provided by Microinnova Engineering GmbH (Allerheiligen bei Wildon, Austria).

2.2 Selection of supports & determination of immobilization yields

2.2.1 Glucose oxidase immobilization on Sepabeads® EC-EP/M

Sepabeads® EC-EP/M are porous hydrophilic enzyme-carriers, manufactured by RESINDION S.r.l., a subsidiary of Mitsubishi Chemical Corporation. This support is generally characterized as highly porous hydrophilic activated absorbent, furthermore attributed as physically and chemically stable by the manufacturer. Promising low swelling tendency in highly molar solution and in common solvents and outstanding mechanic-osmotic resistance, made this carrier line ideal for pressurized packed bed reactor operation experiments. These spherical beads can be further delineated as a matrix of polymethacrylate with a corresponding median pore diameter of 10 to 20 nm. The polymethacrylate support is providing epoxide functional groups with an oxirane content of min. 100.0 µmol/g of wet carrier for enzyme immobilization. The true density is determined at higher than 1.1 g/mL and the average particle size for M-graded supports are in the range of 200.0 to 500.0 µm.[31] The principle of immobilization used was ionic interaction on beforehand amino functionalised supports.

2.2.2 D-Amino acid oxidase immobilization on ReliSorb™ SP405/EB

Carrier ReliSorb™ SP405/EB has a specific gravity of higher than 1.1 grams per millilitre with a corresponding particle size of 200.0 to 500.0 µm. The carrier particles are typified as rigid, low swelling and highly porous polymeric matrix. The manufacturer, RESINDION S.r.l., promises an outstanding mechanic-osmotic resistance including basic physical and chemical stability. Support ReliSorb™ SP405/EB provides furthermore a mean diameter of 300.0 µm. The matrix is composed of crosslinked highly porous polymethacrylate with an average pore radius of 40 to 50 nm. Providing sulphonic functional groups, the beads can be used as strong cation exchange resin for biomolecules capture and purification. The white appearing spherical beads are stable over the entire pH range and temperature stability ranges from 2°C to 60°C.[32] Both kinds of carriers are primarily developed for protein chromatography and enzyme immobilization.[24]

2.3 Enzyme Immobilization

2.3.1 Activation of epoxy-carriers using polyethylenimine (PEI)

Support Sepabeads® EC-EP/M is manufactured to carry functional epoxy-groups on his surface. These oxirane-groups can be aminated to carry amino-groups by incubation with polyethylenimine. To facilitate enzyme binding, the particles were incubated using 10.0 % (w/v) PEI in water solution at pH 10.0 to prepare polyethylenimine modified supports. Particles to PEI solution had to have a ratio of 1:10, so the proper amount of particles had to be diluted with each 10.0 mL of PEI. The chemical modification was performed in an end-over-end rotator at room temperature, overnight. After finishing the transformation into amino-carrying supports, the carriers were washed and filtered two times, either with 100 mM sodium acetate buffer at pH 5.0 and with 100 mM sodium hydrogen carbonate buffer at pH 9.0. Finally a washing step with distilled water and then with 50 mM PPB was performed.[29]

2.3.2 Immobilization of glucose oxidase on PEI-aminated carriers

A GOX-stock solution with a concentration of 10.0 mg/mL GOX has been prepared for immobilization. The afterwards measured enzyme activity of this GOX-stock solution was determined to plan the immobilization as followed.

Generally 100.0 mg of wet carrier particles were offered to 1.0 mL of a GOX solution, to satisfy a 1 to 10 ratio between amount of particles and offered enzyme solution. The GOX-solution contained the amount of enzyme activity desired, diluted in 50 mM potassium phosphate buffer (PPB) at pH of 7.0. Each carrier should be loaded with 5, 50, 200 and 500 Units of GOX-activity per grams of carrier, respectively. Immobilization time was set to three hours, carried out at room temperature under constant stirring. Mixing was performed using an end-over-end rotator device, which guaranteed well mixing of the particles and hereby enhanced possible enzyme attachments. Immobilization procedure can be defined as ionic adsorption of the enzyme on the amino-functionalized carrier. After immobilization, the carriers have been washed two times with a 4-fold amount of 50 mM PPB at pH 7.0. Enzyme activities in the obtained supernatants and on the particles as well, were measured. Determination of GOX-enzyme activity in supernatants has been done using the coupled peroxidase assay. Enzyme activity, actually bound on particles, was measured via oxygen consumption rate using the optical fiber sensor PreSens MICROX TX3 and the corresponding software.[27]

2.3.3 Co-immobilization of catalase (CAT)

Co-immobilization of catalase (CAT) was based on the principle of ionic adsorption and was therefore similarly performed as the immobilization of GOX. A proper amount of catalase was offered to the particles, considering a 100-fold higher catalase immobilization approach as intended for GOX. CAT-stock solution of 0.2 mg/mL was created, which had a specific volumetric enzyme activity of about 47375.0 U/mL. Immobilization was performed in a time period of three hours, using an end-over-end rotator device. After immobilization, three washing steps with 50 mM PPB pH 7.0 were conducted and catalase-activity was determined on both supernatants and particles. Enzyme activity was determined by hydrogen peroxide consumption rate, measured spectrophotometrically, later described as catalase activity-measurement.

2.3.4 Immobilization of D-amino acid oxidase (Z_{basic2} *Tv*DAAO) on sulfonate carriers

Z_{basic2}_tag modified enzymes were prosperously produced in previous years by the hosting institute, which granted access to its protein repository. D-Amino acid oxidase, already modified with a Z_{basic2}_tag, was received as frozen *E. coli* cell extract. The modification of DAAO provides access to have an oriented ionic adsorption on porous carriers.[28] Disruption of *E. coli* cell extract was either obtained using French press cell disrupter device or by sonication. French press is characterized by pressing a beforehand unfrozen cell suspension at high pressure of about 1500 psi thought a small orifice. Cell disruption by sonication was performed for six minutes, a pulse-setting of 0204 and with amplitude of 60%. For both methods of cell disruption, cell extract needed to be carefully unfrozen in 50 mM PPB at pH of 7.0. Additionally, 50 mM PPB pH 7.0 twice the volume of the cell extract was added, before starting cell disruption.

Free Z_{basic2} *Tv*DAAO has been received after centrifugation for 30 minutes at 4°C. The enzyme activity of this cell extract was measured via oxygen consumption rate to plan the following DAAO immobilization on the sulfonate functional carriers. Immobilization was carried out in similar conditions as for GOX. About 100 mg of carrier were incubated with the proper amount of Z_{basic2} *Tv*DAAO solution, diluted in 50 mM PPB at pH of 7.0, and additionally containing 250 mM NaCl, for 3 hours. After immobilization, washing steps followed and finally the DAAO-enzyme activity was determined by DAAO-activity measurement via O_2-decrease in both, the supernatants and directly on the particles.[30]

2.3.5 Co-immobilization of catalase (Z_{basic2}_BP_KAT*A*) on sulfonate carriers

Z_{basic2}_BP_KAT*A* was also beforehand expressed in E. *coli* and received as frozen cell extract from the hosting institute. The Z_{basic2} modification of catalase, as previously reported for Z_{basic2} *Tv*DAAO, facilitates an oriented ionic adsorption on porous carriers. Cell disruption and harvesting of the free Z_{basic2}_catalase was achieved as already descripted in the part Immobilization of D-amino acid oxidase.

To further plan the co-immobilization, catalase enzyme activity was determined by catalase activity-measurement. Co-immobilization was similar achieved as mentioned in Section 2.3.3. A convenient amount of free catalase cell extract was offered to carrier ReliSorb™ SP405/EB, considering a 100-fold higher catalase immobilization approach as intended for DAAO. Immobilization was performed in an end-over-end rotator device for a time period of three hours. Again three washing steps with 50 mM PBB pH 7.0 were conducted and enzyme activity was determined on the collected supernatants and on the particles itself.

2.4 Characterization of suitable supports for reactor operation

As decision was made to design and operate a packed bed reactor, aiming to access continuous enzymatic production, selection of convenient supports had to be done. By using the previously attained knowledge about enzyme immobilization due to the work "Preparation of a packed bed reactor containing immobilized oxidases for gas-liquid-solid bioconversions"[26], most criteria were already stated to continue with immobilization experiments. Medium sized supports carrying either functional epoxy- or sulfonate-groups, were chosen for immobilization of GOX or DAAO respectively, as they were already basically explored and proven as promising. Experimental evaluation of immobilization scope in the range of 5 up to 200 U/g of carriers for GOX and 50 up to 500 U/g for DAAO was therefore performed. Based on this already achieved knowledge considering particle's configuration in terms of shape and pore-size, Sepabeads EC-EP/M were selected for GOX and ReliSorb™ SP405/EB for DAAO.

Objectives, needed to be known for making a reasonable decision and verifying already possessed knowledge regarding the usage of supporting carriers for enzyme immobilization, are the catalytic effectiveness factor of immobilization and the corresponding immobilization yield. The catalytic effectiveness factor (η) for an enzyme immobilization can be calculated by the division of the measured activity actually immobilized on the carrier with the theoretically immobilized activity (Equation 1).

2.4.1 Determination of catalytic effectiveness factor

Equation 1: Formula for calculation of catalytic effectiveness factor η

$$\eta = \frac{activity\ immobilized\ [\frac{U}{g}]}{theoretically\ immobilized\ activity\ [\frac{U}{g}]} * 100$$

2.4.2 Determination of immobilization yield

Immobilization yield can be calculated by the division of the theoretically loaded enzyme activity to the true offered activity, both multiplied with a factor of 100 to get immobilization yield in percent (Equation 2).

Equation 2: Formula for calculation of immobilization yields

$$immobilization\ \ yield\ [\%] = \frac{theoretical\ loading\ \ \left[\frac{U}{g}\right]}{true\ offered\ activity\ \left[\frac{U}{g}\right]} * 100$$

2.5 Design, implementation and operation of the pressurized packed bed reactor

2.5.1 Reactor Set-up

A beforehand well-chosen HPLC column (HPLC Column Blank and End Fittings ½ OD x 10 mm ID x 25 cm of Supelco solutions within™, Bellafonte, PA 16823-0048 USA) was packed with certain amounts of catalyst. This column was selected, based on the attained knowledge during the experiments for the "Preparation of a packed bed reactor containing immobilized oxidases for gas-liquid-solid bioconversions".[26] Pressure drop studies, evaluation of physical limitations regarding operations as packed bed reactor as well as evaluation of stability and reliability of applied flow conditions was hereby done. Results for possible pressure drop were determined to be at low values and only minor deviations of applied flow-rates were recognized. As blocking materials, identified during the mentioned experimental course, were successfully removed and performance of the column was again proven to be suitable, decision for implementation was felt.

Catalyst is defined as support carrying the bi-enzymatic system. Due to packing of this catalyst within the HPLC column, a packed bed reactor was generated. The cross sectional area of the packed bed reactor is $7.9*10^{-5}$ square meters and the volume of the HPLC-column is 19.6 mL. A filter device at the end of the column was installed, aiming to hold the catalyst carriers within the reaction-column confined.

This packed column was implemented into a micro-reactor-setup (Figure 5), capable of substrate- and gas-feeding. Two specific pressure-controlling back pressure valves, Upchurch Scientific®[33], connected in series, were used to increase back pressure within the reactor. These pressure-controlling back pressure valves were used instead of the initially implemented Swagelok® back-pressure-controlling valve, reasoned on one hand due to higher applicable pressure and on the other hand due to guaranteeing more stable conditions. Corresponding software tools (Microinnova Process Development Software; Version 6.0) were used to log operations data of the packed bed reactor operation. Substrate flow, pressure patterns of the two different pressure sensors (Keller Type PA-21Y /30bar/ 81524.11) and time logs as well, were controlled by the software and logged as well. Substrate feeding was performed using Knauer high pressure dosing pump type 2.1S and gas-supply was enabled by operating EL-FLOW® select mass flow meters, a specific controller for gases of Bronkhorst® company. The overall operation window for this reactor setup was in the range of 5.0 up to 35.0 bars, recorded for the first pressure sensor (P1) and 5.0 to 32.59 bars for the second sensor (P2). This pressure-value was mainly built up by the incorporated back pressure regulating valves. It was possible to deliver both, substrate and gas, with flow velocities of 2.5 up to 20.0 mL/min, maintaining stable flow behaviour and avoiding leakages at standardized conditions of 21°C and atmospheric pressure. Pure oxygen was used as oxygen source and compression of pressurized gas and liquid substrate was facilitated in a beforehand defined mixing zone, mainly forced by both back pressure regulating valves. This forced way of compression before entering the catalysts-area, is on one hand enhancing oxygen concentration dissolved within the media, and on the other hand preventing phenomena of bubble coalescence which likely produces gas pockets between the particles. The amount of packed catalyst of about 10 grams combined with already mentioned flow conditions enabled values for residence time of 1.7 to about 6.9 minutes. The deviation of residence time values form the ones already announced in the report of "Preparation of a packed bed reactor containing immobilized oxidases for gas-liquid-solid bioconversions"[26], can be reasoned by the more

accurately conducted determination via bed voidage and in respect of viscosities of the applied media and the increased amount of packed catalyst as well.

2.5.2 General proof of principle of stable maintenance of reactor operation

Substrate flow was implemented and evaluated within the packed bed reactor setup. Standard conditions for substrate delivering in the range of 2.5 up to 20.0 mL per minute were examined. Based on pressure drop determination for media sized carrier-particles[26], empirical experiments were conducted. Also standard gas flow conditions were set in the range of 2.5 up to 20.0 mL per minute, using pure oxygen, and experiments aiming to explore physical pressure limitations of the packed bed reactor-setup were performed.

2.5.3 Approaching basic process operation understanding and knowledge

2.5.3.1 Determination of flow regimes via Reynolds number

A commonly well-known parameter to characterize fluid flow is the Reynolds number. Flow qualities are generally influenced by the density and viscosity of a fluid and also by geometrical reactor properties.[34] For the geometrical properties of the constructed packed bed reactor, Reynolds number was calculated using the spherical equivalent particle diameter (x), the superficial fluid velocity (U_s) and the density of the fluid, which is flowing through the packed bed. These terms are divided by the viscosity of the fluid (pf) and a term respecting the bed voidage (Ɛ). Previous calculation of the cross sectional area of the packed bed was necessary and for the fluid density assumption was made (water at 22°C with 997.0 kg/m^3). Reynolds numbers were calculated for substrate flow conditions in the range of 2.5 up to 20.0 mL per minutes, for both carriers and media (Equation 3).

Equation 3: Calculation of Reynolds number[35]

$$Re = \frac{x\, U_s\ \rho f}{\mu\,(1-\mathcal{E})}$$

2.5.3.2 Evaluation of possible mass transfer limitation

From an engineering point of few, reactor design should enable that the catalyst can operate in a kinetically controlled way. So evaluation of mass transfer phenomena is an imperative task, heading for successful design and development of reactor platform technologies. The continuing calculations are based on the Supporting Information of the work of Bolivar et al.[24]

2.5.3.2.1 Internal mass transfer limitation

For calculation of tortuosity factor ϒ, Beekman Equation was used (Equation 4). The variable Ɛ describes the porosity factor, which was determined as 0.42 previously, to get the Reynolds number.

Equation 4: Beekman Equation

$$\Upsilon^2 = \frac{\mathcal{E}}{(1-(1-\mathcal{E})^{\frac{1}{3}})}$$

Using the Stokes-Einstein equation (Equation 5), calculation of effective diffusion coefficient of oxygen was achieved. The tortuosity factor $\mathcal{T}$ and the porosity factor ε are already known due to the previous calculations. The diffusion coefficient D in water at 30°C (1.6×10^{-9} $m^2 s^{-1}$) was taken for support Sepabeads EC-EP/M and the diffusion coefficient of glutamine ($7.6 * 10^{-10}$ $m^2 s^{-1}$) was taken for support ReliSorb™. Radii of molecules were either taken for glucose ($7.5 * 10^{-10}$ m) or D-methionine ($3.2 * 10^{-9}$m), respectively.

Equation 5: Stokes-Einstein Equation

$$D_{eff} = D \frac{\mathcal{E}}{\mathcal{T}} \left(1 - \frac{r_{molecule}}{r_{pore}}\right)$$

All previous performed equations were intended to finally get the variables needed for determination of the observable Thiele modulus, which can be used to get information about possible existing internal mass transfer limitations (Equation 6).

Equation 6: Observable Thiele modulus

$$\Phi = (\frac{R}{3})^2 \frac{V_{obs}}{D_{eff}[O_2]}$$

2.5.3.2.2 External mass transfer limitation

After calculation of these fundamental parameters, determination of Schmidt numbers for both carrier-substrate combinations were possible (Equation 7). μ_L is the liquid velocity (taken for the lowest possible flow conditions of 2.5 mL/min with $5.3 * 10^{-4}$ ms^{-1}), ρ_L is the liquid viscosity (water 997.0 kgm^{-3}) taken for both supports and substrates, and D_{AL} is the previously determined individual effective diffusion coefficient, calculated using Stokes-Einstein equation.

Equation 7: Calculation of Schmidt number

$$Sc = \frac{\mu_L}{\rho_L D_{AL}}$$

Finally using all these results, determination of Sherwood number for the spherical particles within the packed bed reactor was possible (Equation 8).

Equation 8: Correlation of the Sherwood number in a packed bed reactor[34]

$$Sh = 0.95\, Re_p^{0.5} Sc^{0.33}$$

Aiming to account the effect of external mass-transfer, Damköhler number can be used (Equation 9). Knowledge or alternatively estimation of mass-transfer coefficient is imperative to account external mass-transfer effects. Calculations are based on the given correlations in Pauline M. Doran's Bioprocess Engineering Principles.[34] Damköhler numbers were expressed for both kinds of catalyst-carrier combinations, the reactions rate v_{max} was determined to 300 mM/min and corresponding factors were calculated. All equations and definitions of Sherwood number, Reynolds number and Schmidt number can be seen in the literature.

Equation 9: Determination of Damköhler number (Da)

$$Da = \frac{rate\ of\ reaction}{rate\ of\ mass\ transfer}$$

2.5.3.3 Regarding enzyme stability and productivity

To see if production makes good economic sense, determination of turn over frequencies (TOF) and turn over numbers (TON) is imperative. The turn over frequency is defined by the number of substrate converted to the number of catalyst used per value of residence time, both in molar relations (Equation 10).

Equation 10: Determination of turn over frequency (TOF)

$$TOF = \frac{number\ of\ substrate\ converted\ [mol/min]}{number\ of\ catalyst\ [mol]}$$

The turnover number gives the relation of substrate molecules converted to the number of catalyst used per unit time of entire reactor operation (Equation 11).

Equation 11: Determination of turnover number (TON)

$$TON = \frac{number\ of\ substrate\ converted\ [mol/min]}{number\ of\ catalyst\ [mol]}\ x\ time\ of\ reactor\ operation\ [h]$$

The number of catalyst used is hereby defined as the amount of enzyme actually immobilized on the carrier-supports, which are packed within the packed bed reactor column. This amount of enzyme can be calculated using the immobilization yields [U/g], which needs to be further converted to millimoles of glucose-oxidase per grams of carrier. The molecular weight for glucose-oxidase monomer was taken with a value of 80 kDa, and the specific enzyme activity with a value of 124 [U/mg]. Entire amount of catalyst packed within the reaction column [10 g] as well as conversion to the basic unit [mol] needed to be respected (Equation 12).

Equation 12: Determination of the number of catalyst used

$$Nr.of\ catalyst\ used\ [mol] = \frac{immo.yield\left[\frac{U}{g}\right]}{spec.enzyme\ act.\left[\frac{U}{mg}\right]} * \frac{carrier\ packed\ [g]}{MW\ GOX\ \left[\frac{mg}{mmol}\right]} * \frac{1[mol]}{1000[mmol]}$$

The number of substrate converted within the packed bed column also needed to be determined beforehand. This can be accomplished with the relation of substrate concentration per reactor volume and the corresponding residence time (Equation 13).

Equation 13: Calculation of the number of substrate converted

$$Nr.of\ substrate\ converted\ \left[\frac{mol}{min}\right] = \frac{concentration\ of\ substrate\left[\frac{mol}{L}\right] * reactor\ volume\ [L]}{residence\ time\ \mathrm{T}\ [min]}$$

2.5.3.4 Determination of residence time

As catalyst particles are fixed within the packed bed reactor-setup, residence time is a crucial factor to get an idea of possible reaction time between substrate and enzyme. Substrate is steadily spilled with certain flow rates into the beforehand well defined reaction space, where the enzymes are retained within this packed bed column. Hereby different time intervals, in which enzymatic conversion occurs, are achieved. Using the correlation of reactor volume (V) and flow rate (F), calculation of residence time (τ) for different flow settings can be done (Equation 14).

Equation 14: Correlation of reactor volume and flow with residence time τ [34]

$$\tau = \frac{V}{F}$$

2.5.4 Conversion studies for glucose oxidase

Glucose oxidase was immobilized on support Sepabeads EC-EP/M and a 100-fold amount of catalase-activity was co-immobilized. Basically glucose oxidase activity aimed to be immobilized on the support was planned to be in the range of 10 up to 20 Units per grams. 10 grams of this hereby established catalyst were packed within the HPLC-column to create the packed bed reactor. Substrate and co-substrate, 50 mM glucose and pure oxygen, were delivered constantly in beforehand well-defined feeding conditions. Aiming to enhance solubility of oxygen in the media, the reactor setup was driven under high pressure. Different feeding strategies, flow conditions and certain pressurized conditions were examined, in respect to establish long time steady state conversion. Process parameters as pH of 7.0 and temperature of 21°C were kept constantly and average product conversion has been determined.

Speaking about the general procedure of operating the packed bed reactor, one has to mention the starting procedure. Basically, after plugging the HPLC-column packed with immobilized biocatalyst within the packed bed reactor setup, substrate flow was implemented at certain flow rates. Hereby, the column and the carrier packed inside were spilled with substrate and after reaching stable substrate flow conditions, gas was delivered co-currently. The compression of gas within the liquid media was forced due to application of pressurized conditions, which were generated using the back pressure-controlling valves. Reaching steady state conditions needed to be awaited, before it was possible to start the experiments regarding reactor operation capabilities. Basically, steady state operation conditions were reached, when no air bubbles were recognized within the packed bed reactor setup. So transient starting conditions were overcome, when in principle all gas was dissolved within the media and all experiments were conducted in these steady state conditions. After finishing the experimental course, set-off of the packed bed reactor system was done in the reversed way. Gas flow was in principle the first to shut down, followed by release of pressure due to disconnection of the back pressure holding valves. Finally substrate flow was stopped and the reaction column was filled with PPB, 50 mM buffer solution four times the volume of the HPLC-column, in advance to stabilize the biocatalyst.

2.5.5 Evaluation of maximum reactor operation stability under steady state conversion

As continuous operation for the packed bed reactor was established, experiments regarding maximum reactor operation time and catalyst stability were conducted. Glucose oxidase/catalase bi-enzymatic catalyst was used in previously mentioned conditions for these experiments, aiming to prove catalysts stability under steady state conversion. Searching for possible enzyme washout and material throwbacks, as well as evaluation of general long time functionality of the intended reactor platform technology, was headed for.

2.5.6 Exploration of the feasibility of implementation of different bi-enzymatic systems

Using the already attained knowledge, conversion studies regarding D-amino acid oxidase were done at pH-values of 7.0 and 8.0, respectively. The immobilization approach was planned according to the immobilization of glucose oxidase and catalase, so D-amino acid oxidase enzyme activity in the range of about 20 Units per grams of support and a 100-fold amount of catalase were immobilized onto carrier particles ReliSorb™ SP405/EB. Also determination of achievable impact of residence time on product formation was evaluated. Finally, experiments regarding evaluation of possible substrate limitation for DAAO in combination with evaluation of feasibility of catalyst-reuse at pH of 8.0 were accomplished.

2.6 Analytics

2.6.1 Enzymatic Assays

2.6.1.1 Coupled Peroxidase-Assay

Enzyme activity of soluble GOX in supernatants was measured due to quantification of hydrogen peroxide released, using spectrophotometric assay. 700.0 µl of 50 mM potassium phosphate buffer (PPB) at pH 7.0, 100.0 µl of 0,2% (V/V) N,N,-dimethylaniline, 100.0 µl of 15 mM 4-aminoantipyrin, 100.0 µl of 1.0 M glucose acting as substrate and 20.0 µl of 2.0 mg/mL concentrated horseradish peroxidase (HRP) were mixed within a disposal OD-cuvette. Finally 50.0 µl of properly diluted sample were, already inside the spectrophotometer chamber, added to start the reaction. The reaction product, quinonediimine dye, was measured using a DU 800 UV-vis spectrophotometer (Backman Coulter, Inc., Fullerton, CA, USA), which is absorbing at 565 nm. The specific GOX enzyme activity was determined and in general 1 Unit was defined as the amount of enzyme, which was necessary for the oxidation of 1 µmol of glucose per minute.[27] To measure DAAO-supernatants 100.0 µl of 100 mM D-methionine was used, acting as substrate and PPB at pH of 8.0.

2.6.1.2 Activity measurement via quantification of initial oxygen-consumption rate

GOX enzyme activity was quantified via determination of initial oxygen-consumption rate in beforehand oxygen saturated reaction conditions. An optical fiber sensor with product name PreSens MICROX TX3 and the corresponding software (P500V520) of PreSens GmbH, Regensburg Germany, were used. Measurement was performed in a mini glass-container consisting of 3.8 mL of 50 mM KH_2PO_4 at pH 7.0, under constant magnetic stirring. This mini stirred tank reactor was placed in a water bath, guaranteeing optimal temperature distribution at 30°C. Either 20.0 µl of 10.0 mg/mL GOX-solution or 50.0 µl of general sample were added. To measure enzyme activity on particles, 100.0 µl of properly diluted particle solution of the initial 100.0 mg carrier per mL of immobilized GOX suspension had to be added. After reaching a stable oxygen-concentration plateau, the reaction was started by adding 200.0 µl of 2.5 M glucose, acting as substrate. GOX-activity was measured using the initial O_2-consumption slope and calculated (Equation 15), resulting in Units/mL. In case of measuring enzyme activity on particles, recalculation had to be done to obtain Units/grams as result. To get useable measurement data, the falling-off of the O_2–consumption rate had to be significantly higher than natural physically decreasing due to diffusion. Additional continuous stirring was necessary. The sensor has to be calibrated following PreSens MICROX TX3 manual, additionally.[27]

Equation 15: Calculation of volumetric enzyme activity [U/mL]

$$a\frac{U}{mL} = \frac{slope\ \left[\frac{\mu mol}{s * L}\right] * 60 * total\ Volume\ [mL]}{\frac{1000}{added\ amount\ of\ Enzyme\ [mL]}}$$

2.6.1.3 DAAO-activity measurement via O_2-decrease

Enzyme-activity of DAAO was measured equally as for GOX. 200.0 µl of D-methionine were used, acting as substrate, and the pH-value of the 50 mM KH_2PO_4-buffer was beforehand set to 8.0. 50.0 µl of enzyme solution was added to the properly stirred particle-substrate solution. Enzyme activity could be determined using the same equation (Equation 15).

2.6.1.4 Catalase activity-measurement

Catalase activity can be determined via initial H_2O_2-consumption rate, which is utilised as substrate by the enzyme. Catalase activity measurement for either soluble or immobilized catalase was performed by photometrical measurement at 240 nm wavelength. Spectrophotometer Varian Cary 50 Bio UV-Visible was used under steady temperature conditions of 30°C and continuous high shaking. After blanking a Quartz cuvette with 2000.0 µl of KH_2PO_4 – buffer (PPB) at pH 7.0, 2000.0 µl of hydrogen peroxide and 80.0 µl either of soluble catalase or properly diluted particle solution was added. Each measurement was carried out for six minutes and the catalase activity was calculated finally (Equation 16), resulting in U/mL which is representing the µmol of H_2O_2 consumed per minute and mL enzyme solution.[29]

Equation 16: Determination of catalase enzyme activity [U/mL]

$$k * \frac{20.0}{0.8} * \frac{2000.0\ [\mu L]}{sample\ volume\ [\mu L]} * dilution\ factor$$

2.6.1.5 Hexokinase Assay

For determination of glucose concentration Fluitest® GLU HK enzymatic in vitro test of Analyticon® Biotechnologies AG, Lichtenfels/Germany was used. By quantification of $NADP(H)_2$ as reaction by-product, glucose concentration can be directly determined. After preparing the ready to use solution, following the manufacturer's description, 1.0 mL of this solution was added to 20.0 µl of sample. Measurement of $NADP(H)_2$ was executed for 45 minutes on DU 800 UV-vis spectrophotometer (Backman Coulter, Inc., Fullerton, CA, USA), after waiting 8 minutes for reaching kinetic upward slope. [36]

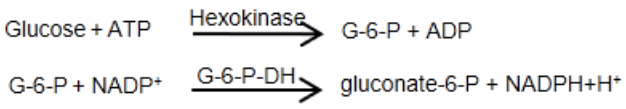

Figure 1: Reaction mechanism to determine glucose concentration using hexokinase and glucose-6-phosphate-dehydrogenase. [36]

Each time of preparing a new Fluitest® GLU HK enzymatic in vitro test solution, creation of a corresponding calibration curve was necessary (Figure 2). The linear range of photometer DU 800 UV-vis spectrophotometer (Backman Coulter, Inc., Fullerton, CA, USA) was determined at a maximum absorption at 340 nm between 1.8 and 2.0. Ideally, measurement results to create a calibration curve should not overtop this physical limit. Certain glucose dilutions in the range of 2 mM up to 20 mM were made and absorption at 340 nm wavelength was measured, to finally produce a calibration curve. An example for calibration can be seen in the appendix (54).

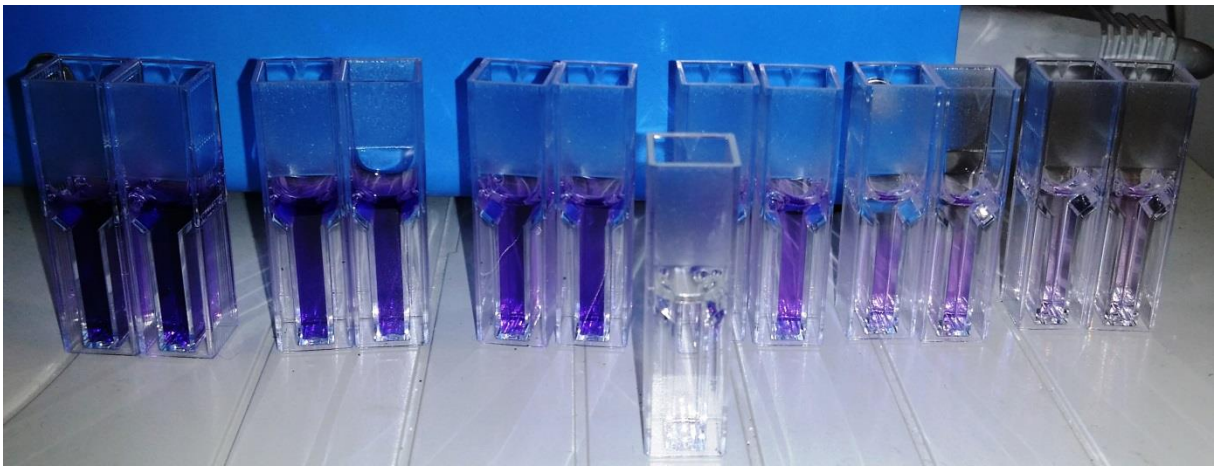

Figure 2: Preparation of calibration curve with several concentrations of glucose, fabricated in duplicates. High to low concentrations of glucose in the sequence from left to right, the blank is located in front of these samples.

For following determinations of glucose concentration using the same measurement approach, similar dilutions of the samples to be measured, had to be produced (Figure 22,Figure 23).

2.6.2 HPLC-methods

2.6.2.1 Determination of glucose-and gluconic acid-concentration

Quantification of product- and substrate concentration was done simultaneously using HPLC Merck Hitachi. Phenomenex column with 5-µm sized amine particles (Luna 5u NH_2 100A, reverse-phase)[37] with 20 mM phosphoric acid (H_3PO_4) solution as eluent was used, aiming to separate glucose and gluconic acid. Gluconic acid was quantified with Merck Hitachi UV Detector L-7400 at 205 nm wavelength, resulting in peaks with a retention time of 1.3 minutes. Glucose concentration was determined by Merck LaChrom RI Detector L-7490, basically with resulting peaks after 2 minutes. HPLC method was operated with up to 100.0 bars and an oven temperature set on 40°C. Mobile phase was delivered with 1.5 mL per minute. Samples to be measured were directly taken out of the micro-reactor and beforehand quenched with 200 mM hydrochloric acid (HCl). Peak integration was accomplished with Chromeleon-software, gluconic acid and glucose concentrations were calculated using previously created calibration.

2.6.2.2 Determination of D-methionine and α-Keto-γ-(methylthio) butyric acid (KMBA)

D-Methionine and α-Keto-γ-(methylthio) butyric acid (KMBA) quantification were performed on HPLC Schimadzu SPD-20A. Both, substrate and product concentrations, were measured with SPD-20A prominence UV/VIS detector at 210 nm wavelength. Kinetex® 5µm C18 100 Å, LC Column 50 x 4.6 mm was used for separation of the analytes, oven temperature was set to 40°C and 5,0% of acetonitrile were delivered, acting as organic solvent. Tetrabutylammonium bromide (TBAB) was used as running buffer with a concentration of 40 mM. The specific retention time at an eluent flow of 1.0 mL/min was 0.6 and 3.6 minutes for D-methionine and KMBA, respectively. Peak integration was accomplished using Chromeleon-software and concentration of D-methionine and KMBA was based on beforehand prepared calibration.

3 Results & Discussion

3.1 Selection of supports & determination of immobilization yields

3.1.1 Glucose oxidase immobilization on Sepabeads EC-EP/M

Certain amounts of glucose oxidase activities in the range of 5 up to 200 Units per grams were offered to a beforehand defined amount of carrier Sepabeads EC-EP/M (Table 1). For all immobilization approaches immobilization yields were in the range of 97.0 to 100.0%. Basically, higher effectiveness factor was found at the lowest offered amount of enzyme activity, with an effectiveness factor of 37.9 and a corresponding immobilization yield of 99.4%. Vice versa, the lowest effectiveness factor for immobilization was found at 200 U/g offered, with 6.1 and a corresponding immobilization yield of 97.9%.

Table 1: Results of immobilization trails for support Sepabeads EC-EP/M with glucose oxidase (GOX)

Sepabeads EC-EP/M	Offered GOX Activity [U/g]	Immobilization Yield [%]	Immobilized GOX-Activity [U/g]	Effectiveness Factor η
	5.0	99.4	1.9	37.9
	10.0	99.3	2.5	24.6
	50.0	98.5	6.6	13.2
	100.0	97.8	10.5	10.5
	200.0	97.9	12.2	6.1

GOX-activities, measured immobilized on carrier Sepabeads EC-EP/M, are in the range of 1.9 up to 12.2 Units per gram of carrier. Worth to mention is the low difference of immobilized enzyme activity between offerings of 100 U/g and 200 U/g, namely 10.5 U/g and 12.2 U/g. (Figure 3). Immobilization results by offering 100 U/g to the support with measureable immobilized glucose oxidase activity of 10.5 U/g seemed to be the most appropriate approach. Herby an effectiveness factor of 10.5 with a corresponding immobilization yield of 97.8% was met.

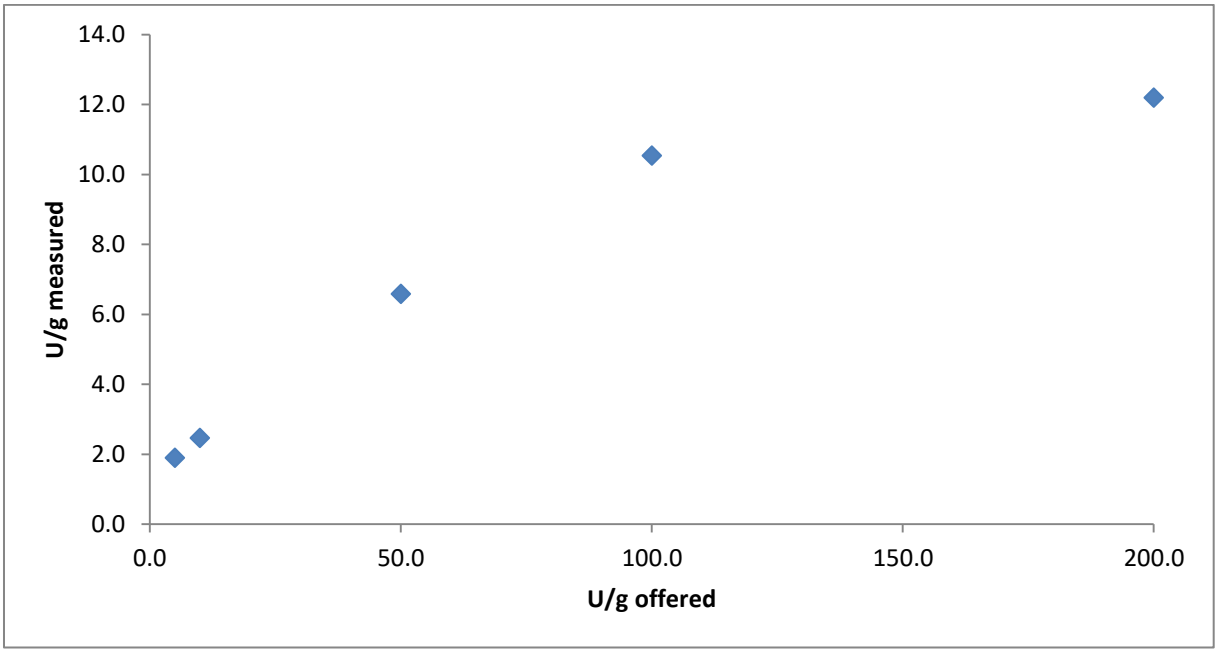

Figure 3: Graphical representation of immobilization experiments for support Sepabeads EC-EP/M with glucose oxidase. Activity of immobilizates [U/g] is shown on the y-axes, offered GOX-activity on the x-axes.

3.1.2 D-Amino acid oxidase immobilization on ReliSorb™ SP405/EB

D-Amino acid oxidase activities have been offered to support ReliSorb™ SP405/EB in a range of 50 up to 500 U/g (Table 2). Compared to the results for GOX-immobilization, values of catalytic effectiveness factor are quite low. But, besides for DAAO-offerings of 500 U/g, immobilization yields are again determined to be near to 100.0 percent. At the highest applied offering, the worst effectiveness factor of 6.1 and a corresponding immobilization yield of 82.3% were achieved. Highest effectiveness factor was met at an offering of only 50 U/g of DAAO-activity, with a factor of 32.5 and an immobilization yield of 95.1%.

Table 2: Results of immobilization trails for support ReliSorb™ SP405/EB with D-amino acid oxidase

SP405/EB	Offered DAAO-Activity [U/g]	Immobilization Yield [%]	Immobilized DAAO-Activity [U/g]	Effectiveness Factor η
	50.0	95.1	15.2	32.5
	200.0	97.5	23.6	12.7
	500.0	82.3	24.9	6.1

Immobilized DAAO-activities measured on support ReliSorb™ SP405/EB reached form 15.2 up to 24.9 U/g (Figure 4). Differences of actually immobilized activity for offerings of either 200 U/g or 500 U/g are in the narrow range of 2 U/g. So, for further immobilization approaches 200 U/g of D-amino acid oxidase activity will be offered. This decision can clearly be reasoned due to the high enzyme losses by offering far more enzyme activity as can be bound on the supports.

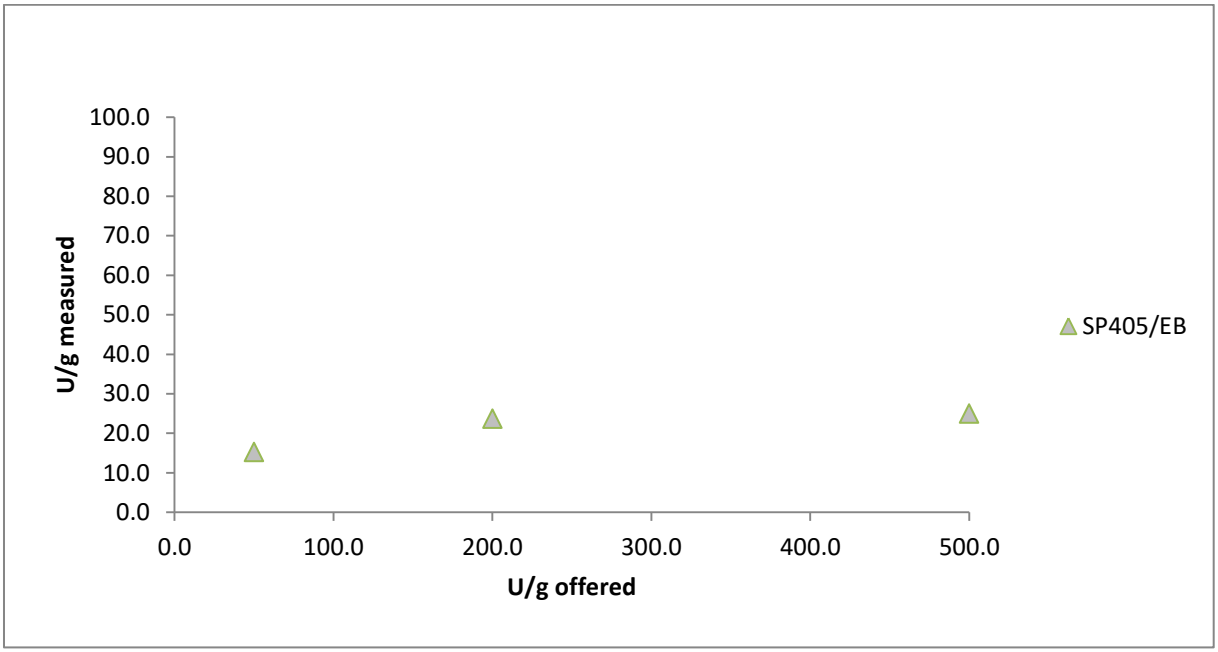

Figure 4: Graphical representation of immobilization trails for ReliSorb™ SP405/EB with D-amino acid oxidase. Activity of immobilizates [U/g] is represented on the y-axes, offered enzyme activity on the x-axes.

3.2 Design, implementation and operation of the pressurized packed bed reactor

3.2.1 Reactor-Setup

Main purpose, laid in this work, was to generate a packed bed reactor-setup for continuous enzymatic conversion, leading to valuable products. Regarding catalysts stability, the decision to head for immobilized enzyme form packed within a column to create a packed bed reactor, has been felt. Gas and substrate were co-currently delivered, using either EL-FLOW® select mass flow meters or Knauer high pressure dosing pump type 2.1S, respectively. Mixing of substrate and gas was forced in a beforehand defined mixing zone, whereas pressure was generated due to implementation of two specific back-pressure valves, which were connected in series. As can be seen in the construction plan, one pressure sensor was implemented on each side of the packed bed reactor (P1, P2). Product was collected in a collection vessel located at the end of the reactor-setup, after being processed by the biocatalyst (Figure 5).

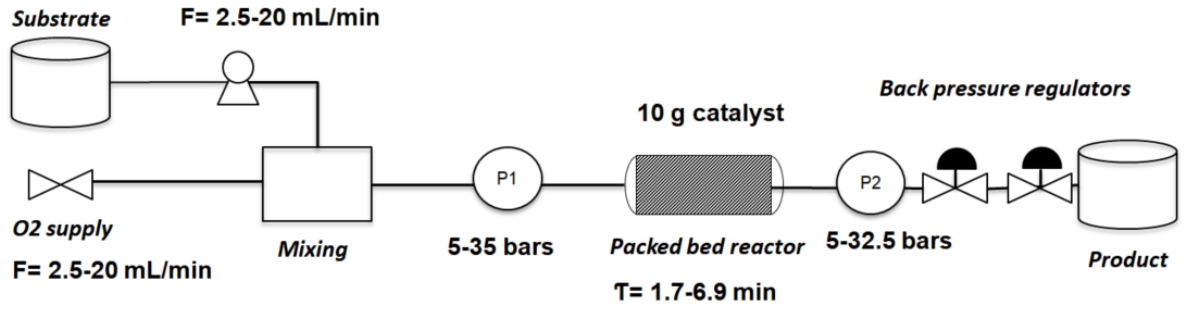

Figure 5: Plant-design for implementation of the packed bed reactor.

3.2.2 Study of assembly of different variables

3.2.2.1 Standard substrate flow

Either glucose or D-methionine was delivered with "Knauer high pressure dosing pump" (type 2.1S), both in a concentration of 50 mM. All experiments were conducted with a substrate flow from 2.5 up to 20.0 mL/min at standard conditions of 21°C and atmospheric pressure. The substrate was fully dissolved in liquid, guaranteeing accurate concentrations delivered to the catalyst. The catalyst was hereby spilled with substrate and afterwards product formation was evaluated using analytical methods. The range of possible substrate flow velocity was initially evaluated (Figure 6), both without and with packed catalyst. Supply was possible within the mentioned range in a continuous manner without leakages, permitting long time stable operation.

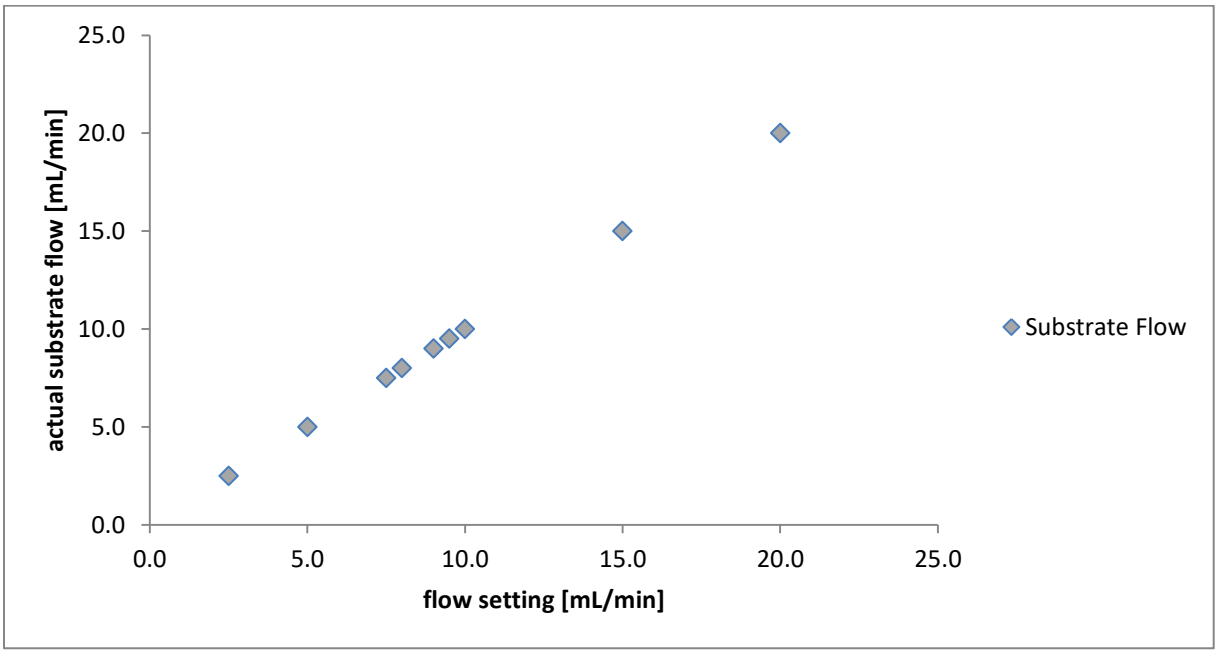

Figure 6: Graphical representation of substrate flow [mL/min] for packed bed reactor operation. Pump flow-settings are represented on the x-axes, actual substrate-flow is shown on the y-axes.

3.2.2.2 Standard gas flow

Acting as terminal oxidant, pure oxygen was delivered and controlled with EL-FLOW® mass flow meter (Bronkhorst®) and its corresponding software. Gas flow rates in the range of 2.5 up to 20.0 mL/ min were intended to be achieved at standard conditions of 21°C and atmospheric pressure. As the literature indicates, oversaturation with oxygen is imperative to enhance oxidative enzymatic activity[12]. It was possible to create stable flow conditions for oxygen, aiming to provide the packed bed reactor with gas in the mentioned range. Regarding oxygen supply, stable reactor operation was possible as no leakages were identified by continuously done examinations.

3.2.2.3 Physical pressure limitation of the packed bed reactor

According to Henry's law, higher pressure can improve concentration of oxygen dissolved in the liquid phase. Thus evaluation of possible pressure limitation was performed. The implementation of the HPLC-column into the packed bed reactor set-up using the two backpressure regulators leaded to stable pressure values, tested for the range of liquid and gas flow defined in 3.2.2.1 and 3.2.2.2.

3.2.2.4 Pressure impact on solubility of oxygen

Reason for applying high pressure within the packed bed reactor system is the intention for intensification of the concentration of oxygen dissolved within liquid. It is reported that the systems pressure can considerably influence the flow and mass-transfer behaviour due to gas compressibility, resulting in enhanced oxidative reactions.[14] As already mentioned before, pressure-controlling back pressure valves were used to intensify pressure, all discussed data are generated at isothermal conditions within the packed bed reaction system at 21°C.

Doing so, oxygen was compressed within the substrate solution in a beforehand designed mixing zone, by increasing the entire systems pressure. At atmospheric conditions of 1.013 bars, it is possible to dissolve about 42.4 mg of oxygen within one litre of medium. The dissolved concentration of oxygen within a liquid can be manifold according Henry's law. Following the rule of ideal gases, intensification of dissolved oxygen can be calculated. At 5.0 bars the concentration of oxygen dissolved can be enhanced to a value of 213.4 mg/L. Intensification of pressure, and hereby resulting intensification of concentration of oxygen dissolved was conducted in the range of 5.0 bars up to 35.0 bars. Maximum pressurized operational conditions were found at 35.0 bars, giving a concentration of oxygen dissolved in media of about 1500.5 mg/L (Figure 7).

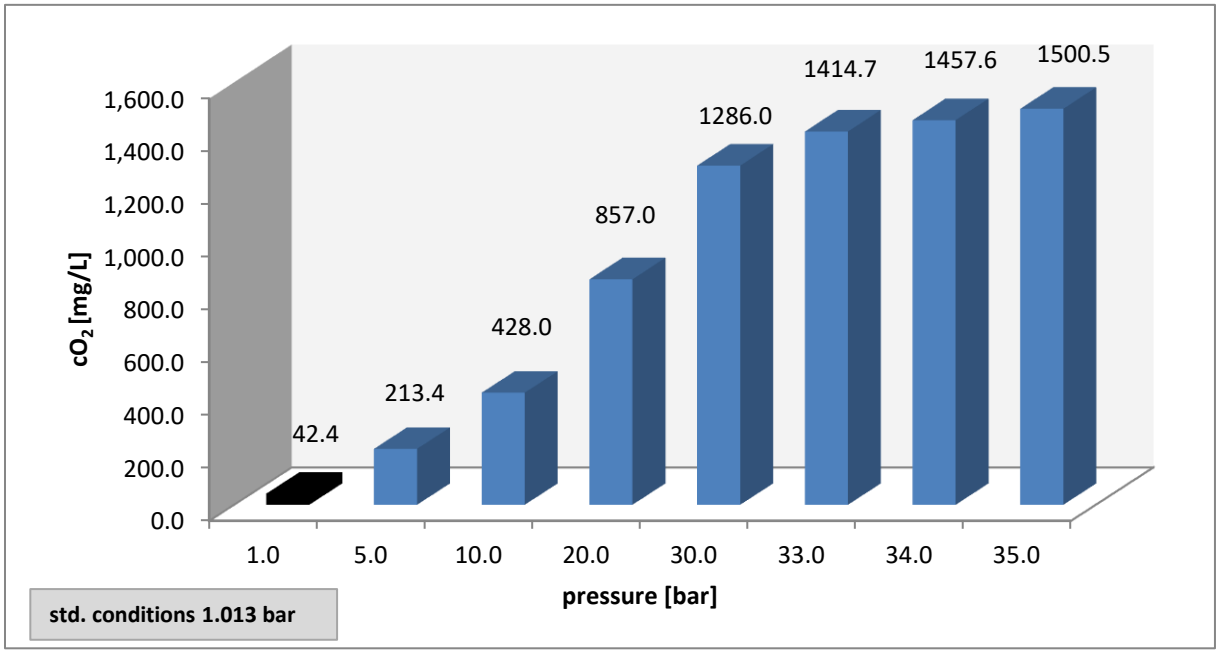

Figure 7: Graphical representation of the correlation of pressure and concentration of oxygen dissolved in media at designated pressure-values. Standard conditions are indicated black. All values are calculated at isothermal conditions of 21°C, on which the packed bed reactor was kept during the experiments.

Regarding compressibility of gases, calculations of the contribution of enhanced oxygen concentration within the liquid media were performed, for flow settings of 5.0, 10.0 and 20.0 mL/min, respectively. Enhanced oxygen concentration was translated to total volume of gas at standard conditions of 1.013 bars, what is named fictive gas-volume. As can be seen, oxygen volume is significantly contributing to total volume, without being compressed by pressure (Table 3). Results are calculated using the rule of ideal gases; standard temperature was set to 21°C.

At standard conditions of 1.013 bars, 42.4 mg/L of oxygen can be dissolved within the liquid. The value of dissolved concentration of oxygen can be intensified due to pressure. For pressurized

conditions of 25.0 bars, this concentration is enhanced to a value of 1071.5 mg/L. At 30.0 bars there are 1286.0 mg/L of oxygen dissolved and at 35.0 bars there are 1500.5 mg/L.

Assuming flow-rates of 5.0 mL/min, the fictive volume at standardized conditions of 1.013 bars is 5.2 mL, for the corresponding concentration of oxygen dissolved of 42.4 mg/L. At pressurized conditions of 25.0 bars, the concentration of oxygen dissolved is enhanced to a value of 1071.5 mg/L, whereas the fictive volume is determined as 9.1 mL of oxygen volume for flow-rates of 5.0 mL/min. Having concentration of oxygen dissolved of 1500.5 mg/L, this fictive volume reaches a value of 10.7 mL, for the same assumed flow–rate of 5.0 mL/min. So conclusive, the shown fictive volume is the volume needed, to satisfy the demand of oxygen solvable due to application of pressurized conditions. These results were used as basis to select appropriate flow-rate at the EL-FLOW® mass flow meter (Bronkhorst®). For example at pressurized conditions of 35.0 bars and a desired flow-rate of 20.0 mL/min, 42.9 mL of oxygen had to be delivered, to guarantee an oxygen volume satisfying the solubility of oxygen of 1500.5 mg/L. (Table 3)

Table 3: Total oxygen volume of enhanced oxygen concentration dissolved under standard conditions of 1.013 bars and 21°C without compression (fictive volume). In the left column concentration of oxygen dissolved at corresponding pressure values is shown, on the right three columns fictive oxygen volume for these oxygen concentration at standard pressurized conditions (1 bar) are demonstrated.

Pressure [bar]	**cO_2 [mg/L]**	**5.0 [mL/min]** ***(at 1.0 bar) Vol. [mL]***	**10.0 [mL/min]** ***(at 1.0 bar) Vol. [mL]***	**20.0 [mL/min]** ***(at 1.0 bar) Vol. [mL]***
1.013	42.4	5.2	10.3	20.7
1.0	41.8	5.2	10.3	20.6
5.0	213.4	5.8	11.6	23.3
10.0	428.0	6.6	13.8	26.5
15.0	642.5	7.5	14.9	29.8
20.0	857.0	8.3	16.6	33.1
25.0	1071.5	9.1	18.2	36.4
30.0	1286.0	9.9	19.8	39.7
31.0	1328.9	10.1	20.2	40.3
32.0	1371.8	10.2	20.5	41.0
33.0	1414.7	10.4	20.8	41.6
34.0	1457.6	10.6	21.1	42.3
35.0	1500.5	10.7	21.5	42.9

3.2.3 Approaching basic process operation understanding and knowledge

3.2.3.1 Determination of flow regimes via Reynolds number

3.2.3.1.1 Calculation of Reynolds number for support Sepabeads EC-EP/M

To identify the flow regime within the packed bed reactor, Reynolds numbers were expressed for different volumetric flow rates in the range of 2.5 up to 20.0 mL/min (Table 4). Superficial fluid velocity through the packed bed reactor, filled with 10 grams of catalytic active support Sepabeads EC-EP/M was expressed, to further calculate the Reynolds number. In means of simplicity, density and viscosity of water was assumed for 50 mM concentrated glucose media. Reynolds numbers are reaching from 0.3 up to 2.7, in order from the lowest up to the highest applied superficial velocity. The numbers are clearly indicating that the packed bed reactor is operated in laminar flow conditions whereas in principle low axial dispersion can be assumed within the packed bed microreactor.[34]

Table 4: Reynolds numbers for certain values of superficial fluid velocity of 50 mM glucose on support Sepabeads EC-EP/M. Pump ratios (Q) are given in [mL/min], superficial fluid velocity (U_s) in [m/s]. Reynolds number is displayed in the last column (Re N°).

Q [mL/min]	U_s [m/s]	Re N°
2.5	$5.3*10^{-04}$	0.3
5.0	$1.1*10^{-03}$	0.7
7.5	$1.6*10^{-03}$	1.0
8.0	$1.7*10^{-03}$	1.1
9.0	$1.9*10^{-03}$	1.2
9.5	$2.0*10^{-03}$	1.3
10.0	$2.1*10^{-03}$	1.3
15.0	$3.2*10^{-03}$	2.0
20.0	$4.2*10^{-03}$	2.7

3.2.3.1.2 Calculation of Reynolds number for support ReliSorb™ SP405/EB

Also for support ReliSorb™ SPSP405/EB, determination of flow regime was performed (Table 5). Same superficial fluid velocity values were used to express the ratio of inertial to viscous forces for substrate flow of 50 mM D-methionine through the packed bed reactor. Viscosity and density of water was taken, for 50 mM D-methionine, aiming to simplify the determination approach. In this case, Reynolds numbers are reaching from 0.3 up to 2.3, in order from the lowest fluid velocity up to the highest. Based on the values, flow regime of reactor operation with the mentioned carrier and substrate combination can be identified as laminar.[38]

Table 5: Reynolds numbers for certain values of superficial fluid velocity. Pump ratios (Q) are given in [mL/min], superficial fluid velocity (U_s) in [m/s]. Reynolds number is displayed in the last column (Re N°).

Q [mL/min]	U_s [m/s]	Re N°
2.5	$5.3*10^{-04}$	0.3
5.0	$1.1*10^{-03}$	0.6
7.5	$1.6*10^{-03}$	0.9
8.0	$1.7*10^{-03}$	0.9
9.0	$1.9*10^{-03}$	1.0
9.5	$2.0*10^{-03}$	1.1
10.0	$2.1*10^{-03}$	1.2
15.0	$3.2*10^{-03}$	1.7
20.0	$4.2*10^{-03}$	2.3

3.2.3.2 Determination of residence time

3.2.3.2.1 Glucose oxidase

As already mentioned in the material and methods part, residence time is one of the very most crucial factors regarding quantification of enzymatic transformations in biocatalysis. The residence time is defined by the relation of volume to substrate flow. Respecting the calculated density, taken by the packed particles within the column of 0.58 g/cm^3 and the particles density of 1.1 grams per millilitre, volume of Sepabeads® EC-EP/M packed within the reactor-column can be expressed. With a volume of 17.2 mL for 10 grams of packed particles, residence time of substrate within this column can be calculated in respect to certain applied substrate flow rates (Figure 8). As substrate can only flow through the not occupied regions of space of either the reaction column and the pores of the support, residence time of 1.7 minutes can be reached at maximum substrate spilling with a flow-rate of 10.0 mL per minute. At substrate flow conditions of 8.1 mL/min the corresponding residence time is 2.1 minutes, at 7.0 mL/min 2.5 minutes and at 5.0 mL/min 3.5 minutes. The maximum achievable contact time between substrate and the immobilized catalyst, so where substrate is delivered inter-particularly and intra-particularly can be expanded to 6.9 minutes at minimal flow conditions of 2.5 mL/min.

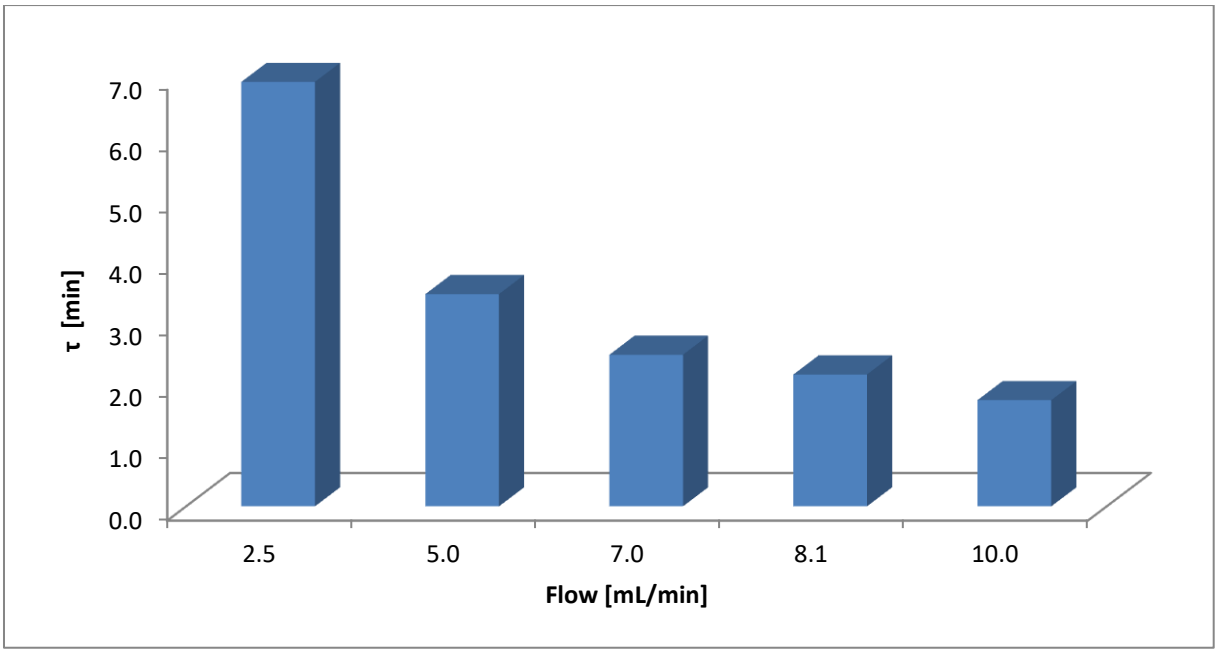

Figure 8: Graphical representation of residence time (τ) at different substrate flow conditions for carrier Sepabeads® EC-EP/M.

3.2.3.2.2 D-Amino acid oxidase

As values of the true density of the particles, given by the producer, and the density of packed material inside the reactor-column are exactly the same, determination of residence time for carrier ReliSorb™ SP405/EB is resulting in similar values. Once more, data are shown from the lowest to the highest substrate flow rates, giving residence times of 6.9 minutes and 1.7 minutes for distinct substrate flow conditions, respectively (Figure 9). Exact numbers can be taken from the upper part (Glucose oxidase).

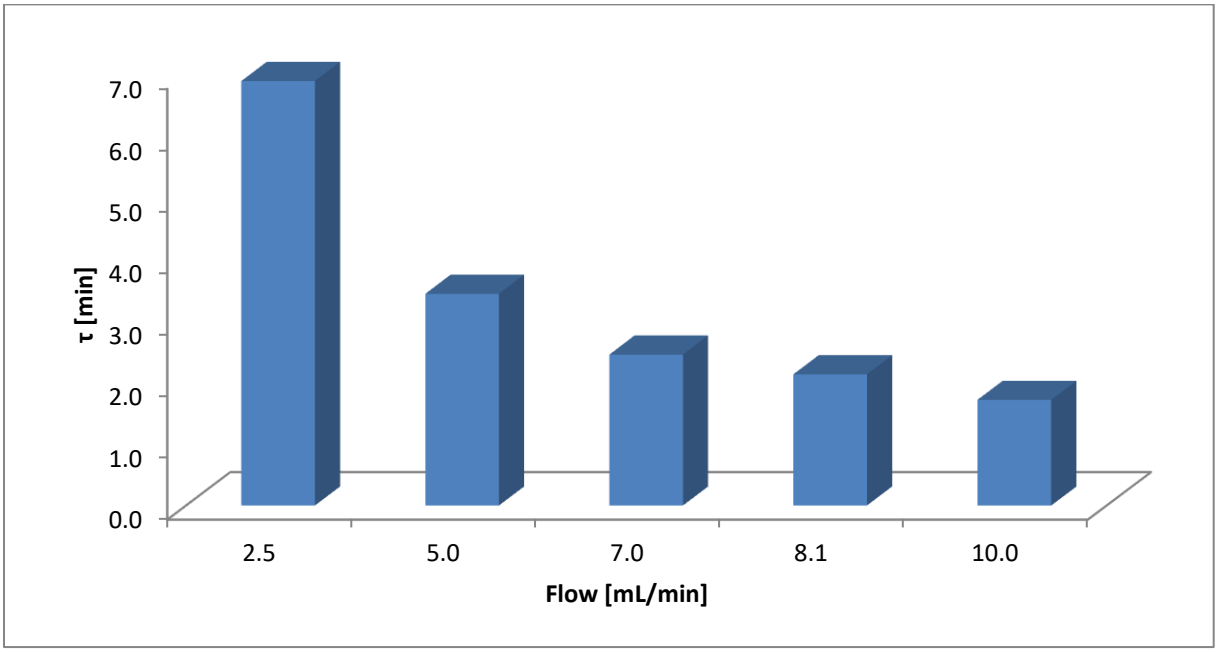

Figure 9: Graphical representation of residence time distribution (τ) at different substrate flow conditions for support ReliSorb™ SP405/EB.

3.2.3.3 Conversion studies for glucose oxidase

Bringing attained knowledge to the field, an example of continuously driven glucose conversion can be shown (Figure 10). Conversion of glucose into gluconic acid by the bi-enzymatic system, consisting of GOX and catalase immobilized on the mesoporous support, was evaluated.

Fundamental knowledge about the immobilized biocatalyst in combination with the designed packed bed reactor was achieved by conducting the experimental course of "Preparation of a packed bed reactor containing immobilized oxidases for gas-liquid-solid bioconversions".[26] In these experiments, basic operation capabilities were evaluated, aiming the development of a platform-technology considering continuous biocatalysis. Having results concerning establishment of the packed bed reactor setup, flow-rate stability and possibility to manage pressure values as well as gas-supply in mind, it was expected to achieve further enhancement of substrate conversion. As most oxidative enzymatic reactions are limited by the availability of the electron acceptor oxygen, especially optimization on the thermodynamic level according Henry's law, aiming to improve oxygen transfer rate, was headed for. Continuous substrate conversion was already proven at certain conversion rates, which was anticipated to be farther intensified and stabilized due to interdependent enhancement regarding steadiness of pressure and flow-regime. In terms of substrate flow, further intensification of enzymatic conversion rate was awaited, due to prolongation of residence time and better control of high pressure level.

For the presented example, 10 grams of catalyst were packed within the reaction-column; substrate and pure oxygen as co-substrate were delivered co-currently. The HPLC-column, packed with catalyst, was implemented within the packed bed reactor setup and operation was started by spilling 50 mM glucose-substrate through the catalysts column. After reaching stable substrate flow conditions, delivery of oxygen was initialized. Mixing of substrate and gas was forced due to pressurized conditions in the beforehand defined mixing area. During the transient condition at the beginning of the operation, compression of oxygen within the liquid substrate was expected, according to Henry's law. After reaching bubble-free flow condition, evaluation of biocatalytic performance was started and samples were taken periodically to quantify product formation, using HPLC-measurements.

Measurable enzyme activities of about 10 Units per grams and 310 Units per grams, for glucose oxidase and catalase respectively, were brought onto support Sepabeads® EC-EP/M. Substrate flow was kept stable at 10.0 mL/min of delivery rate, resulting in a residence time of 1.7 minutes. Gas flow-rate was kept in the range of 9.0 to 10.0 mL/min constantly, too. Regarding substrate- as well as gas-flow-rates, it can be reported that both of them were successfully kept constant during the entire reactor operation time, according to the previous stated expectations.

Homogeneous liquid phase with high concentration of oxygen dissolved was expected after reaching steady state conditions, aiming to provide optimal conditions for the glucose oxidase performed conversion. These steady state conditions were recognized due to disappearance of gas-bubbles within the reactor setup, and conclusively verified by product quantification using analytical methods, ex-situ.

It was possible to achieve gluconic acid formation of about 35 mM, which means in other words, a product conversion of about 70 percent. Continuous reactor operation was performed for about 3.8 hours, maintaining consistent formation-profile, without measureable catalysts loss or inactivation. Comparing to the results previously presented[26], further intensification of values of substrate

conversion rate can be recognized and anticipation about continuous steadiness of gluconic acid yield can be verified.

Concerning expectation for final stabilization of pressurized conditions during reactor operation, it was possible to hold pressure constant in the range of 30.0 to 33.0 bars for pressure sensor 1, and for pressure sensor 2 about 28.0 up to 32.59 bars were hold (Figure 11).

In terms of dissolved concentration of oxygen at high pressurized conditions of 28.0 to 32.59 bars, there are between 1200.2 mg/L and 1397.1 mg/L dissolved within the liquid. For the pressure sensor in front of the reaction column, values of the concentration of dissolved oxygen are for 30.0 bars 1286.0 mg/L and for 33.0 bars even 1414.7 mg/L.

Reactor operation is displayed over a period of time of about 4 hours, having stable conversion profile at the described pressurized steady-state conditions. 50 mM glucose was delivered with a flow-rate of 10.0 mL/min consistently, and hereby a residence time of 1.7 minutes was met. Regarding the achieved contact time between substrate and catalyst, turnover frequencies of $4.0*10^3$ per minute were possible to attain. Speaking of total operability of the packed bed reactor operation, it is immanent to bring in the turnover number. Having 3.8 hours of reactor operation, the general productivity of the biocatalyst can be characterized by a turnover number of $2.1*10^5$, assuming stable conversion profile of 35 mM gluconic acid during the entire reactor operation experiment.

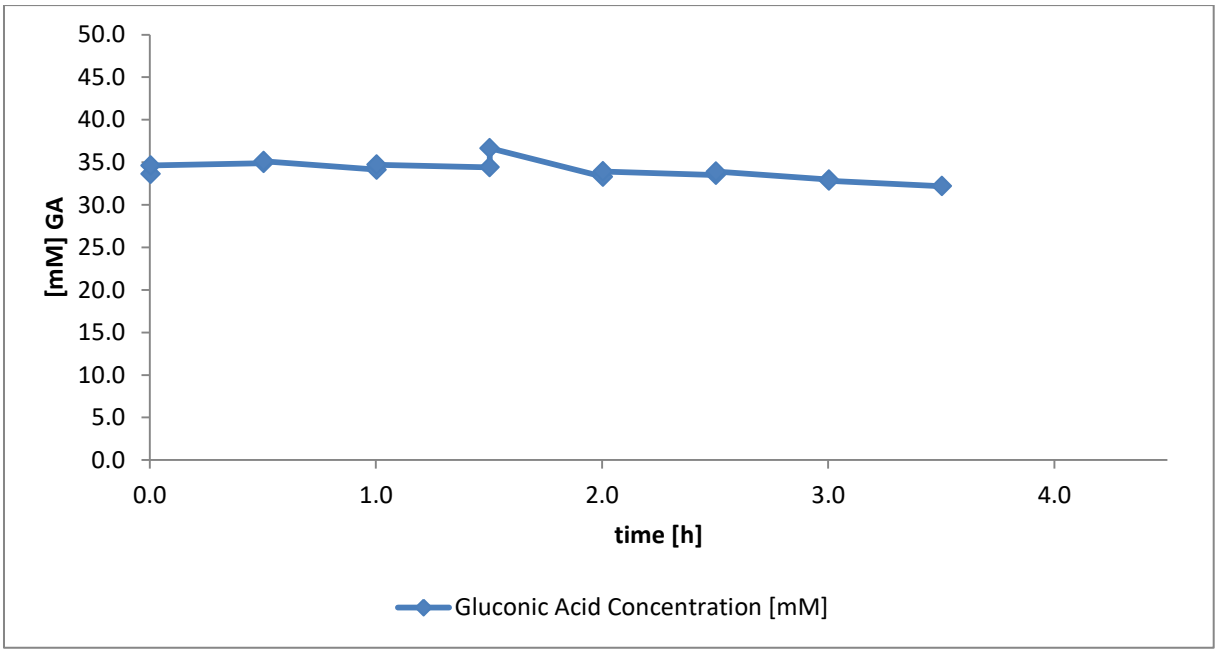

Figure 10: Graphical representation of gluconic acid conversion [GA] over time. 10 g of catalyst (10 U/g GOX and 310 U/g CAT immobilized) were packed and reactor operation was conducted at isothermal conditions of 21°C. Substrate flow-rate of 10.0 mL/min and gas-flow rate of 9.0 to 10.0 mL/min were kept constantly. Pressurized conditions measured for pressure senor one were 30.0 up to 33.0 bars and for pressure sensor two 28.0 up to 32.59.

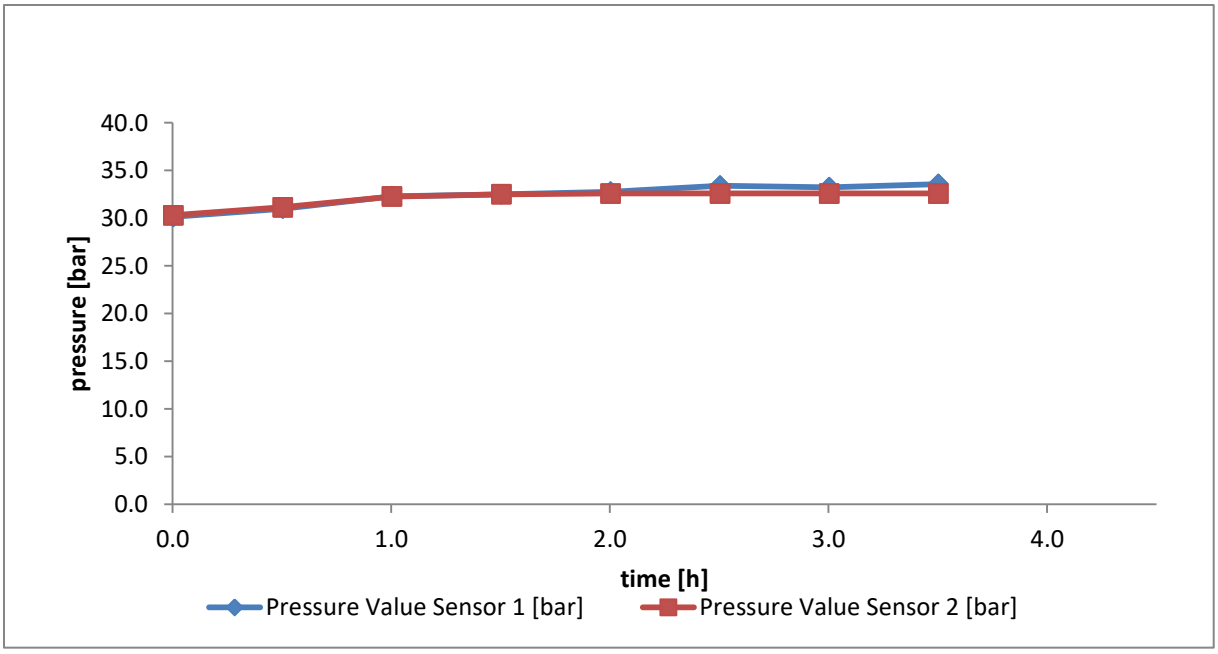

Figure 11: Graphical representation of the corresponding pressure profiles for gluconic acid production at isothermal conditions of 21°C.

3.2.3.4 Evaluation of maximum reactor operation stability under steady state conversion

Most reactor operation experiments were performed in a range of four to six hours, six to eight hours or even up to ten hours. To get an idea of possible catalyst stability, while having stable operation mode, reactor performance was evaluated for about 12.5 hours (Figure 12).

Basically, 10 grams of catalytically active support Sepabeads® EC-EP/M were packed within the HPLC-column to create the packed bed reactor setup. Glucose oxidase activity of 16 U/g and catalase activity of 370 U/g were measured immobilized on the support. During the evaluated time, substrate conversion in the range of 15 to 20 mM was achieved, consistently.

Different substrate flow conditions were applied to get further information of reactor capabilities in terms of possible impact of residence time on conversion-rate, additionally. This can especially be recognized by the different conversion behaviour displayed in the corresponding graphical representation (Figure 12). Starting at the beginning of the experimental course, substrate flow rates were consecutively reduced from 20.0 mL/min down to 2.5 mL/min. Gas-flow rates, which were started at 0.8 hours of reactor operation, were accordingly reduced from 10.0 mL/min down to 2.5 mL/min. A constant variation of the corresponding gluconic acid concentration going hand in hand with the manipulation of the rate of substrate supply can be recognized. Higher substrate flow-rates were applied at the beginning, followed by consecutive reduction, where higher conversion rates for the product of more than 20 mM can be highlighted in the timespan of 5.5 to 6.5 hours. After this initial 6 hours, flow rate was enhanced which can also be seen in an ongoing decrease of substrate conversion. Another peak at about 11 hours of reactor operation with increase in gluconic acid concentration is worth mentioning, whereas flow rate was decreased to 2.5 mL/min. In the experimental course indication for direct correlation of substrate delivery rate and product conversion can be found, which can be reasoned by the impact of residence time during continuous catalytic processes.

As a standard strategy for starting reactor operations was already described in the former example in words, the pressure profile of this reactor operation is shown additionally (Figure 13). As can be seen comparing both graphs, a certain period of time is required to start the reactor operation at the beginning and some time is also required to terminate the operation at the end. This transient state can be identified on one hand by construction of pressure at the beginning and on the other hand by the destruction of pressure at the end of the entire experimental course. Looking at the graphical representation of gluconic acid conversion over time (Figure 12), increase of product concentration at the beginning of the experimental course can be recognized according to the corresponding built up of pressurized conditions, and the contrary can be recognized for a timespan necessary to terminate the experimental course.

Recapitulating, high operational stability of the designed packed bed reactor is clearly proven, effectiveness of immobilization approach for glucose oxidase and catalase onto this kind of carrier as well, and indications for feasibility of intensification due to manipulation of the residence time were found. Generally, no catalyst inactivation, matters of material damages or general reactor setup drawbacks have been identified during this long time conversion study. The obvious lower product concentration in comparison to the previously shown experiment can be explained due to difficulties of continuous pressure hold up, as can be recognized by the measured values of pressure sensor one. As this experiment was primarily intended to prove long term operational stability of the designed

plant and to state a relation of product formation rate and substrate-delivery rate additionally, these lower concentrations can be seen as insignificant for the outcome of this experiment.

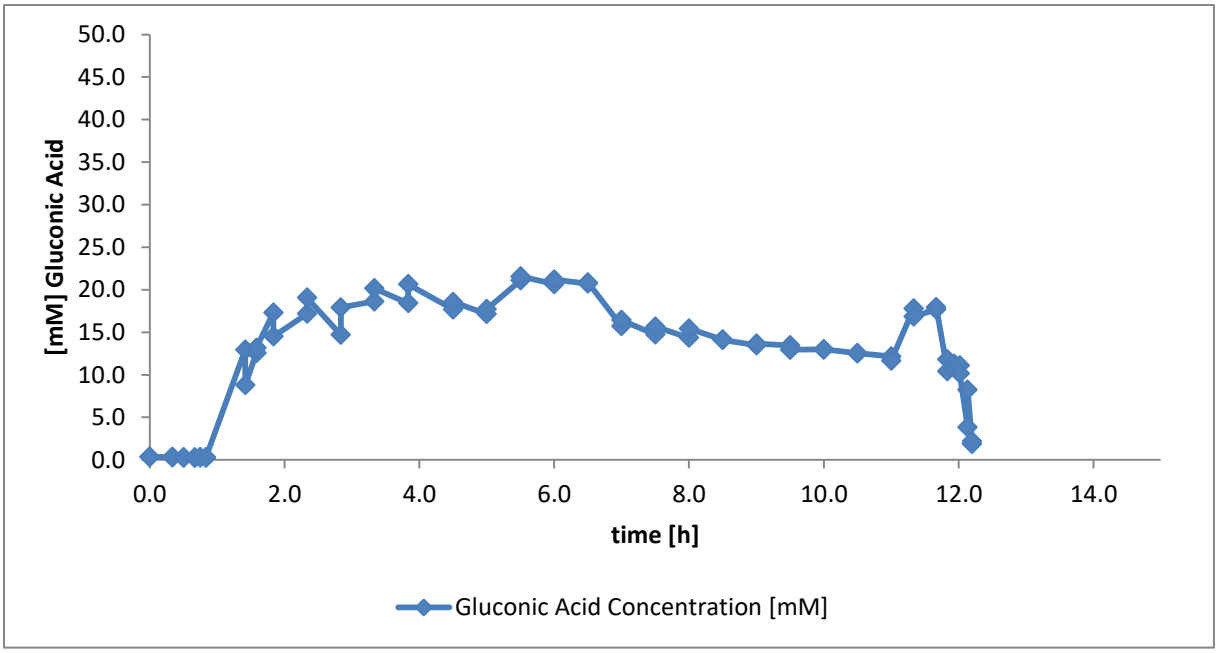

Figure 12: Graphical representation of gluconic acid conversion over time. 10 grams of immobilized catalyst (16 U/g GOX and 370 U/g CAT) were packed within the reactor-column; operation was performed at 21°C. Different substrate-flow rates were applied in the range of 2.5 up to 20.0 mL/min and gas flow-rates between 2.5 up to 10.0 mL/min. Pressurized conditions were in the range of 32.6 up to 35.9 bars for pressure sensor one and 32.59 bars for pressure sensor two, respectively.

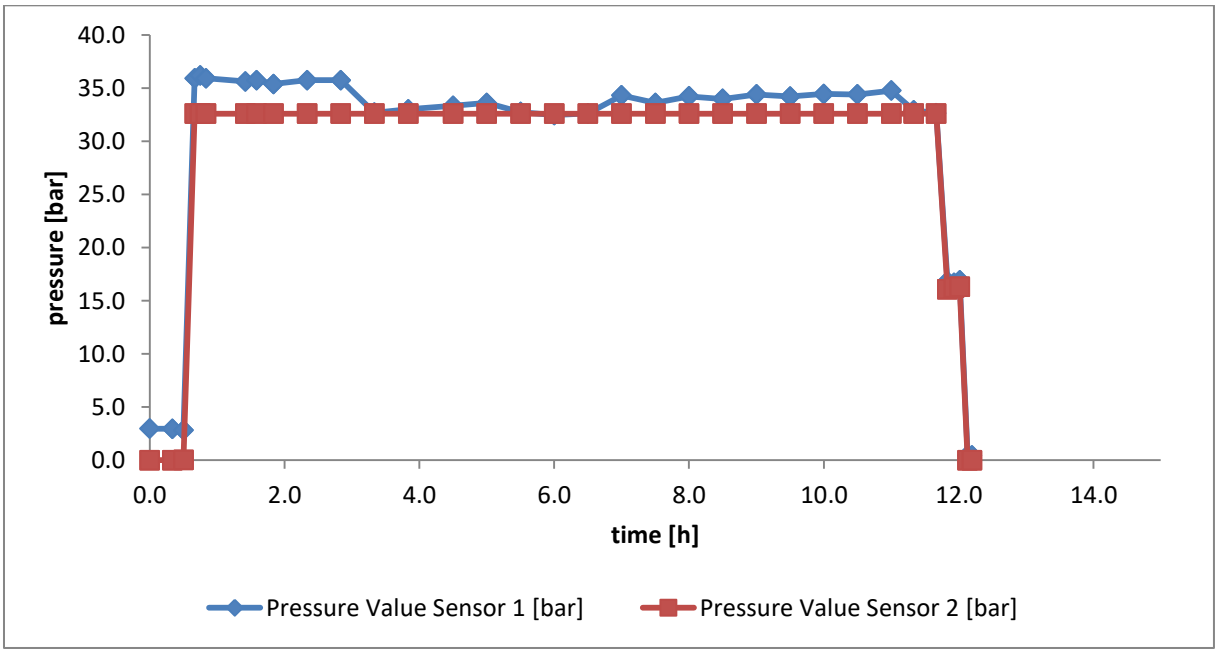

Figure 13: Graphical representation of the corresponding pressure profiles for gluconic acid production.

3.2.4 Feasibility for implementation of different bi-enzymatic systems

3.2.4.1 Conversion studies regarding D-amino acid oxidase at pH-value of 7.0

Heading for more valuable products and more challenging kinds of enzymes in terms of immobilization and operation, D-amino acid oxidase was implemented as oxidative active biocatalyst. On one hand, D-amino acid oxidase can be used for production of pure amino acids by kinetic resolution of racemic mixtures, but on the other hand it is known that D-amino acid oxidases stability is highly affected by aeration and suffering from the gas-liquid interphase in its soluble form.[4] Basically stabilization and implementation of this enzyme within the packed bed reactor framework could be seen as further success of our studies.

As already mentioned, D-methionine can be converted to α-Keto-γ-(methylthio)butyric acid (KMBA) by enzymatic catalysis using D-amino acid oxidase. Continuing the experimental course done for glucose oxidase/catalase bi-enzymatic system, DAAO and catalase were equitably immobilized on support ReliSorb™ SP405/EB. 10 grams of this catalytically active support were packed to create the packed bed reactor setup, and reactor operation was started according to the already delineated standard operation procedures. Pure oxygen and D-methionine were delivered in beforehand determined values, samples were taken periodically and quantified afterwards with HPLC-measurements.

About 10 U/g of DAAO and 270 U/g of catalase enzyme activity were measured after immobilization on the carrier ReliSorb™ SP405/EB, for the demonstrated experiment. 50 mM D-methionine at pH of 7.0 was delivered consistently, and pure oxygen as acting co-substrate under high pressurized conditions was additionally pumped in. As can be seen in the graphical representation (Figure 14), conversion of D-methionine to α-Keto-γ-(methylthio) butyric acid was achieved in the range of about 18 mM, which indicates a value of substrate-conversion of about 36 percent. Stable substrate flow of 10.0 mL/min was kept during this experimental approach constantly, resulting in a residence time of 1.7 minutes. Consistent conversion was achieved for more than 2.5 hours. Pressure values were kept in the range of about 35.0 bars and 32.59 bars, for pressure sensor 1 and pressure sensor 2, respectively (Figure 15). Hereby, the concentration of dissolved amount of oxygen was in the range of either 1500.5 mg/L or 1397.1 mg/L, in order for both determined pressurized conditions.

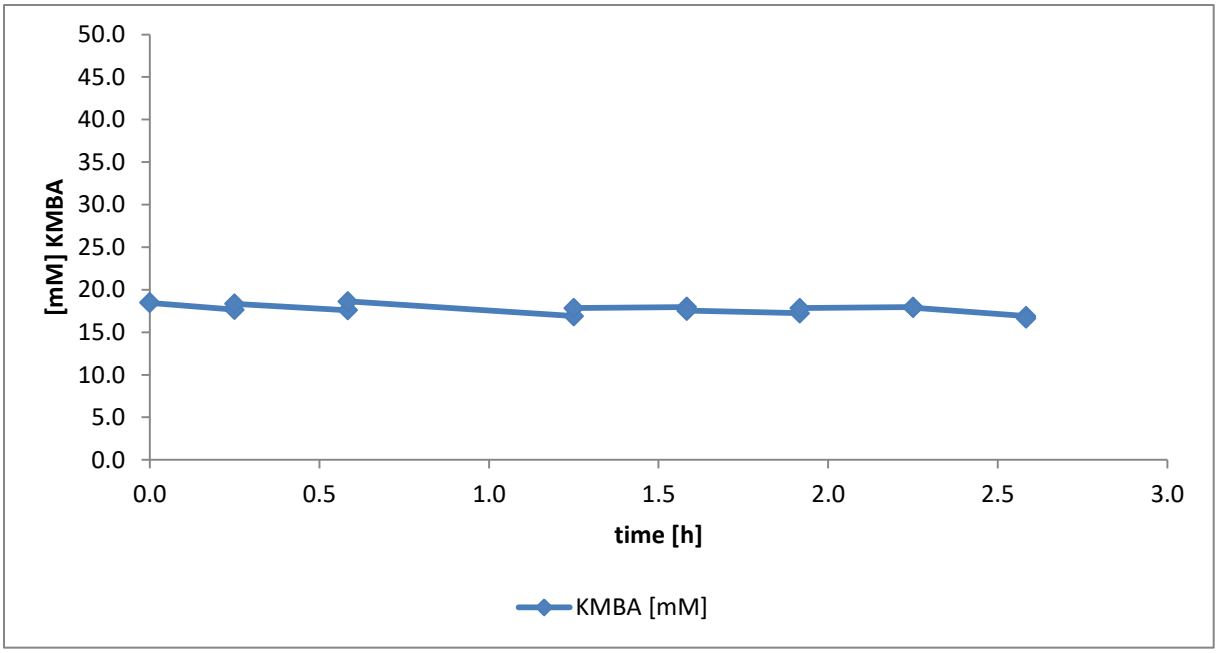

Figure 14: DAAO catalysed formation of KMBA under steady state conditions with a pH-value of 7.0 and 21°C. 10 grams of catalyst (10 U/g DAAO and 270 U/g CAT immobilized) were packed within the reaction column. Substrate flow was kept constant at 10.0 mL/min and pressure profiles measured were 35.0 bars and 32.59 bars for pressure sensor one and two, respectively.

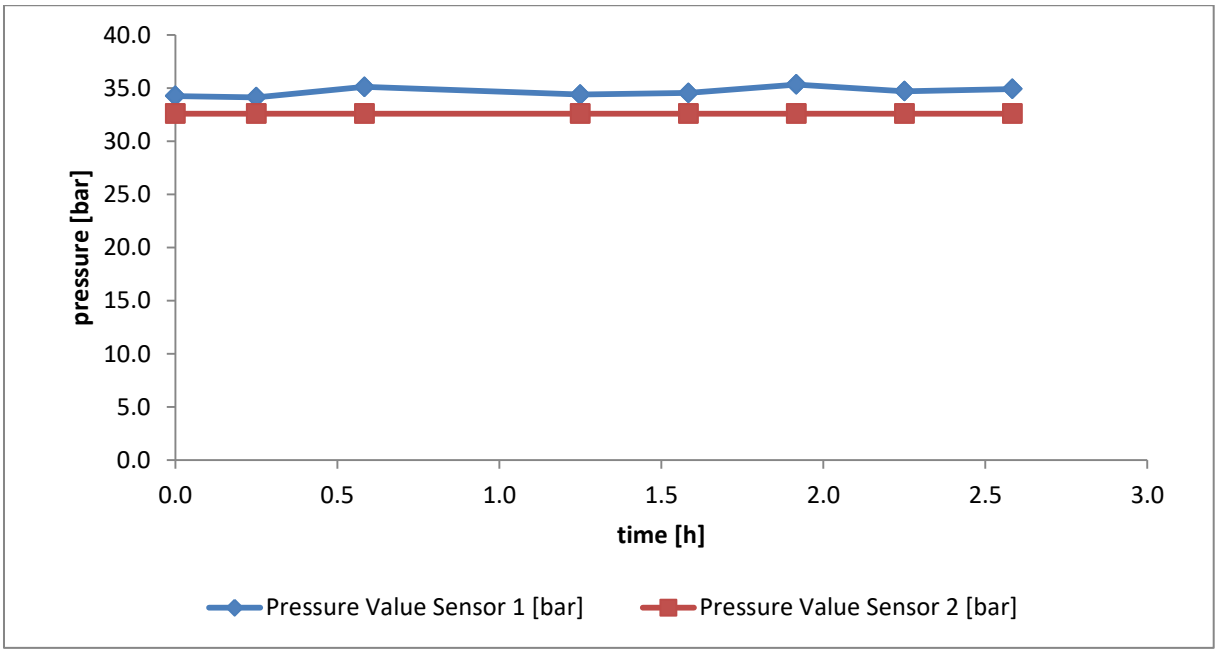

Figure 15: Corresponding pressure profile for steady state DAAO-catalysed conversion.

3.2.4.2 Conversion studies regarding D-amino acid oxidase at pH-value of 8.0

Heading for process intensification, substrate modification regarding optimization of pH-value was evaluated. Hereby 50 mM concentrated D-methionine at pH-conditions of 8.0 was implemented, acting as substrate. 10 grams of catalyst with immobilized DAAO and catalase were packed, whereby about 24 U/g of DAAO and about 240 U/g of catalase were determined after immobilization procedures on support ReliSorb™ SP405/EB. As can be taken from the graph (Figure 16), α-Keto-γ-(methylthio) butyric acid (KMBA) formation was in the range of 35 mM, which means 70% in terms of substrate conversion-rate, having constant substrate-flow rate of 5.0 mL/min. That fertile amount of product formation has been kept stably and data for about 6 hours are presented. Speaking about high pressurized conditions built up within the packed bed reactor setup, pressure values of about 33.0 to 34.0 and again 32.59, for pressure sensor 1 and 2, respectively were hold, permanently over the entire time of operation (Figure 17). Following from these values, determination of available dissolved concentration of oxygen within the D-methionine media resulted in 1457.6 mg/l and 1397.1 mg/L, calculated for both pressure sensors, in front and after the catalytically active reaction column.

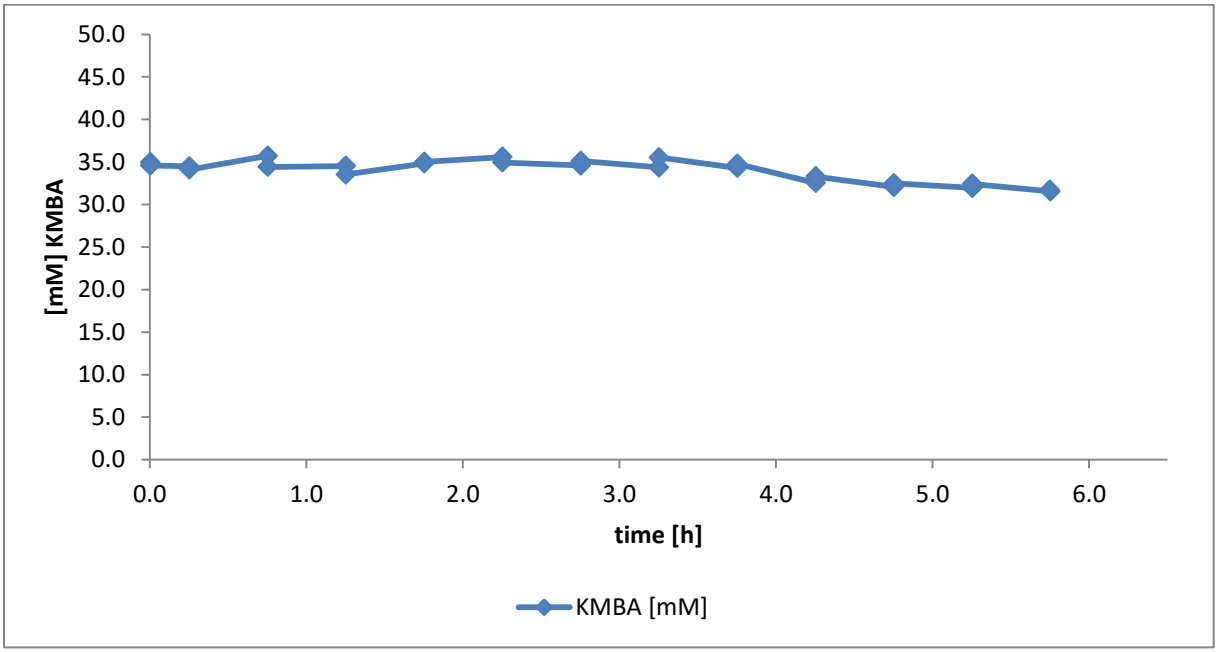

Figure 16: Graphical representation of KMBA formation for DAAO/catalase application within the packed bed reactor at pH-conditions of 8.0. 10 grams of catalyst (24 U/g DAAO and 240 U/g CAT measured immobilized) were packed and substrate was constantly delivered with a flow-rate of 5.0 mL/min. Pressure determined was 33.0 up to 34.0 bars and 32.59 bars for pressure sensor one and two, respectively.

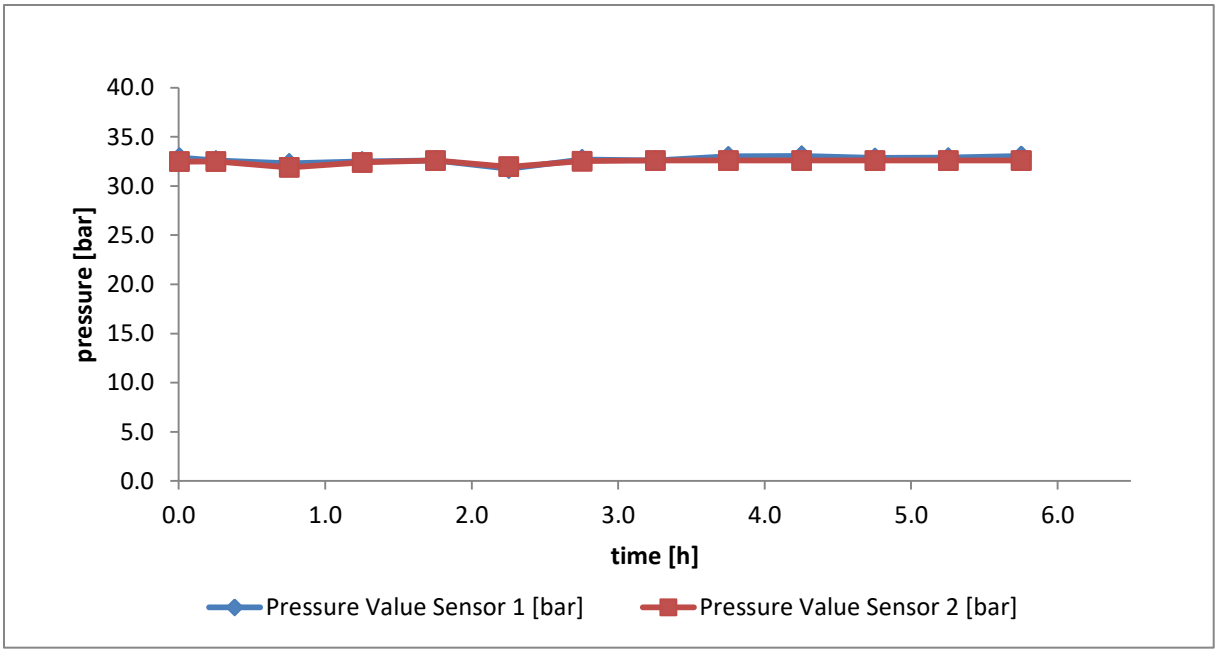

Figure 17: Graphical representation of pressure profiles for implementation of DAAO/CAT-bi enzymatic system within the packed bed reactor at pH conditions of 8.0.

3.2.4.2.1 Determination of achievable impact of residence time on product formation

As promising product values were achieved during the conducted experiments, further issues about possible achievable impact of residence time demanded to be answered. General controllability of the amount of product formed due to application of different residence times for the interaction of the catalyst with the substrate was the fundamental thesis, which needed to be verified.

Modifications in terms of substrate-delivery rate were conducted, due to application of different flow conditions, aiming to influence catalytic productivity of the bi-enzymatic DAAO/CAT-system. Certain assigned substrate flow conditions were tested, resulting in residence times from starting with 1.7 minutes for highest flow rate, 3.5 minutes for a substrate delivering of 5.0 mL/min and even 6.9 minutes of residence time for the lowest flow condition of 2.5 mL/min (Table 6).

Table 6: Residence time distribution at different substrate flow conditions for 10 grams of catalytic support packed

Flow [mL/min]	Residence time τ [min]
10.0	1.7
5.0	3.5
2.5	6.9

A clear correlation of product formation and the mean residence time, where the catalyst is in contact with the substrate, has been found (Figure 18). At the lowest contact time of 1.7 minutes, α-Keto-γ-(methylthio) butyric acid formation yielded in 29 mM. Already 35 mM product formation was possible to reach with a residence time of 3.5 minutes and with a residence time of 6.9 minutes even 45 mM of KMBA-formation was determined. As can be seen by these correlations, a clear relation of residence time to amount of product formed in the packed bed reactor application was established and controllability of product formation due to substrate flow rate has been proven. Summarizing on a percentage basis, 45 mM of KMBA can be translated into 95 percent of converted substrate into product.

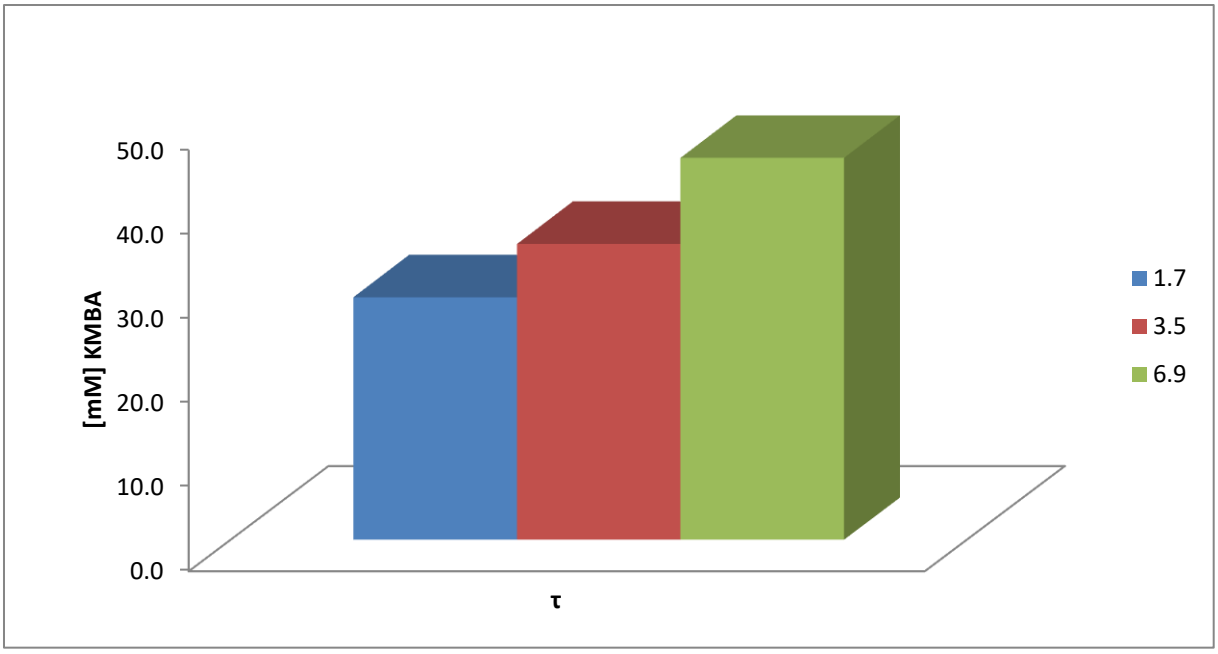

Figure 18: Correlation of residence time (τ) with product formation ([mM] KMBA) by DAAO-application within the packed bed reactor.

Furthermore, having a mean residence time of 6.9 minutes and corresponding conversion of D-methionine into a product yield of about 45 mM, results for turnover frequency and turnover number are $3.8*10^2$ min^{-1} and $2.3*10^4$, respectively. By applying a substrate flow condition of 5.0 mL/min, resulting in a corresponding residence time of 3.5 minutes, product formation of 35 mM was measured. Stable plateau of KMBA formation under these conditions was evaluated for about 1.0 hours, resulting in a turnover frequency of $5.9*10^2$ min^{-1} and a corresponding catalytic turnover number of $3.5*10^4$. A mean residence time distribution of 1.7 minutes was created by supplying substrate flow of 10.0 mL/min. Product formation of 28 mM was steadily determined, resulting in a turnover frequency of $9.4*10^2$/min and a turnover number of $5.7*10^4$. All turnover numbers are expressed for a period of one hour, in respect of general commensurability.

3.2.4.3 Experiments regarding evaluation of possible substrate limitation for D-amino acid oxidase in combination with evaluation of the feasibility of catalyst reuse at pH-condition of 8.0

Curiosity has arisen, if substrate concentration may also present a limiting factor, as enzyme activity and oxygen concentration dissolved within the media are actually proven as to do so. In purpose to evaluate possible limitations due to the substrate properties, 100 mM D-methionine was applied. Basically catalyst particles, which were used in the previous packed bed reactor experiment, were reused. Assuming no significant washout of the enzyme, the activities in this experiment were 24 U/g for DAAO and 240 U/g for catalase and 10 grams of these catalyst particles were repacked within the packed bed reactor. 100 mM D-methionine at pH of 8.0 and pure oxygen were co-currently delivered, product formation was determined by HPLC-measurement.

For a substrate flow rate of 9.0 mL/min, residence time of 1.9 minutes was achievable, resulting in a value of product formation of 27 mM. Using 5.0 mL/min, the residence time was nearly doubled up and it was possible to measure product formation of 45 mM. At the lowest substrate flow rate of 2.5 mL/min and a corresponding residence time of 6.9 minutes, it was possible to determine even 76 mM of α-Keto-γ-(methylthio) butyric acid, converted by the applied catalyst (Table 7 & Figure 19).

Table 7: Residence time distribution at different substrate flow conditions for 10 grams of catalytic support packed

Flow [mL/min]	Residence time τ [min]
9.0	1.9
5.0	3.5
2.5	6.9

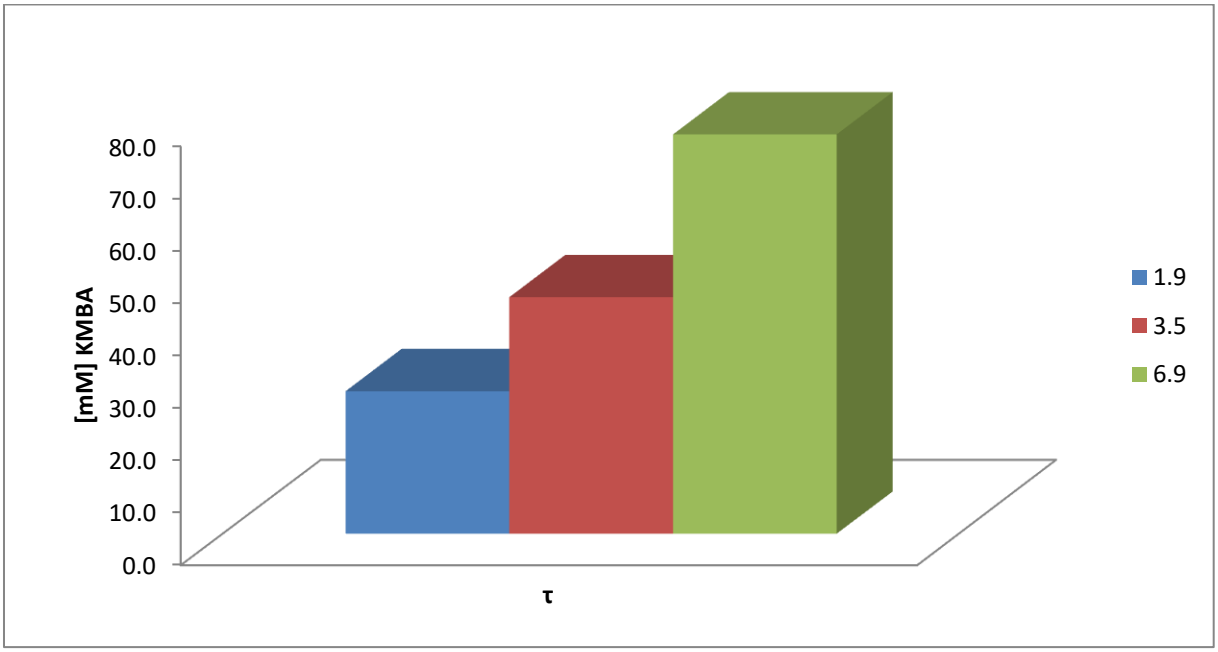

Figure 19: Graphical representation of the correlation of residence time (τ) with product formation ([mM] KMBA).

High pressurized conditions were kept constant during the entire reactor operation experiment (data not shown), with values of about 35.0 bars and 32.59 bars for pressure sensor one and two, respectively. The amount of dissolved oxygen can be stated as 1500.5 mg/L for 35.0 bars and 1397.1 mg/L for 32.59 bars.

Conclusive to previously attained results, three different plateaus for formation of KMBA, in respect to each designated residence time, were established (Figure 19). In order from the shortest interaction time of the catalyst with media to the longest, product formations of 27 mM, 45 mM and 76 mM were measured. As can be seen in the stable continuous manner of product formation, no detectable issues of enzyme washout or inactivation were detected during an entire reactor operation time of 7 hours.

Furthermore, experiments regarding catalyst stability, and in this term proving reusability of already applied catalyst, were implemented within the experimental course additionally.

Referring to terms comparing catalytic issues as stability and productivity, turnover frequencies and turnover numbers are presented. For KMBA production of 27 mM with a corresponding residence time of 1.7 minutes catalysts turnover frequency is $9.1*10^2$ per minute. This certain conversion rate was measured for a period of 1.3 hours, giving a corresponding turnover number of $7.3*10^4$. The plateau for product formation of 45 mM was recognized over a period of operation time of 2.0 hours, resulting in a turnover frequency for the catalyst of $7.6*10^2$ per minute and a corresponding turnover number of $9.1*10^4$. Turnover frequency and turnover number for the highest measured conversion rate of 76 mM are $6.4*10^2 min^{-1}$ and $6.7*10^4$, respectively. KMBA-formation plateau was determined for 1.8 hours, which was used to express the corresponding turnover number.

In terms of reusability, it is worth to mention that the catalyst was used for about 7 hours in this approach, and in the former approach data for 6 hours are presented. Conclusive stability of the bi-enzymatic DAAO/CAT-system can be described with a turnover number of $3.0*10^5$, assuming an operation time of 13 hours with a residence time of 6.9 min and a mean productivity of 45 mM. In terms of amount of product formed, or substrate converted, it is safe to say that there are clear indications for substrate-concentration acting as limiting factor.

3.2.4.4 Mathematical evaluation of possible mass transfer limitations

Clear indication for limitations due to substrate concentration can be recognized by the comparison of the graphical representation of product formation plateaus for experiments handling with 50 mM and 100 mM substrate concentration (Figure 18 & Figure 19). Mathematical proof was searched for by determination of possible external and internal mass transfer limitations.

3.2.4.4.1 Internal mass transfer limitation

Internal mass transfer is defined as transfer of substrates from the outer surface of solid supports through the pores of the porous carriers to the enzyme, immobilized on and inside the mesoporous structure of the support. As can be understood in this definition, internal mass transfer phenomena mainly depend on the features of the solid support and the rheological properties of the substrate carrying media.

Tortuosity factor, which is commonly used to describe diffusion in porous media, gives a value of 0.5. Using the result for the effective diffusion coefficient of $8.4*10^{-10}$ m^2s^{-1} for oxygen and both, tortuosity and porosity factors determined previously for the packed bed reactor setup, determination of observable Thiele modulus resulting in a value of 0.1 is possible (Table 8).

Table 8: Calculation of possible internal mass transfer limitations for support Sepabeads EC-EP/M

Factor	Abbreviation	Results
Tortuosity factor	τ^2	0.5
Effective diffusion coefficient of O_2	D_{eff}	$8.4*10^{-10}$
Observable Thiele modulus	ϕ	**0.1**

As packing is very similar for support ReliSorb™ SP405/EB, tortuosity factor was also determined to 0.5. The effective diffusion coefficient of oxygen for the combination of the support-carrier and D-methionine results in a value of $3.9*10^{-10}$ m^2s^{-1} and a corresponding observable Thiele modulus of 0.2 has been calculated (Table 9).

Table 9: Calculation of possible internal mass transfer limitations for support ReliSorb™ SP405/EB

Factor	Abbreviation	Results
Tortuosity factor	τ^2	0.5
Effective diffusion coefficient of O_2	D_{eff}	$3.9*10^{-10}$
Observable Thiele modulus	ϕ	**0.2**

Values of observable Thiele modulus are in the range of 0.1 up to 0.2 in order for both carrier-substrate combinations. Using established criteria token from Bioprocess Engineering Principles (Chapter 13)[34], evaluation of the internal effectiveness factor can be made. Both values are basically extracted due to extrapolation of the presented graph, originated by *R. Aris, (Mathematical Theory of Diffusion and Reaction in Permeable Catalysts)*[34]. Both observable Thiele moduli are smaller than 0.3, but close to the transition to be limited. Qualification taken for this evaluation is the criteria of first order kinetics for both enzymatic transformations regarding observable Thiele modulus, determination and categorization of internal mass-transfer limitations was done according Weisz's criteria, which can be used for all geometries and reaction kinetics.[34]

Before expressing each term for the equations mathematically, some assumptions had to be made. First of all, isothermal conditions are imperative, as enzyme activity can be influenced by temperature. Mass transfer should only occur by diffusion, so no or only negligible convection through the carrier pores are assumed to be happening. It has to be possible to describe the diffusion using Fick's law, having a constant effective diffusivity, additionally. Also substrate concentration is important, as the concentration should only change in the radial direction of the supports-configuration. The particles should be seen to be in steady state, so no enzyme inactivation is allowed to occur and generally immobilization of the enzyme on the carrier should be achieved in a uniform way, all over the entire carriers-structure. Finally no discontinuity of concentration at solid-liquid interface has to be assumed.[34]

3.2.4.4.2 External mass transfer limitation

The diffusion of the reactants across the surface layer of fluid around the catalyst is a part of mass transport phenomena, which is generally described by external mass transfer. Basically, the dimensionless Damköhler number is defined as the relation of the maximum possible reaction rate to the maximum achievable rate of mass-transfer.

Possible external mass transfer limitations are evaluated by the expression and correlation of Schmidt number, Sherwood number and Reynolds number (Table 10), which can be assumed for spherical particles packed as packed bed reactor. For carrier Sepabeads EC-EP/M, with corresponding glucose medium, Schmidt number has a value of 633.7 and Sherwood number of 4.7. Finally, the Damköhler number results in a value of 0.5.

Table 10: Parameters to evaluate external mass transfer limitations for support Sepabeads EC-EP/M. Schmidt number (Sc), Sherwood number (Sh) and Damköhler number (Da) are expressed.

Term	Value
Sc	633.7
Sh	4.7
Da	0.5

For support ReliSorb™ SP405/EB also external mass transfer behaviour was evaluated (Table 11). Schmidt- and Sherwood numbers give results of 1365.1 and 5.5, respectively. The value of the Damköhler number has been calculated, resulting in the dimensionless term of 0.7.

Table 11: Parameters to evaluate external mass transfer limitations for support ReliSorb™ SP405/EB. Schmidt number (Sc), Sherwood number (Sh) and Damköhler number (Da) are expressed.

Term	Value
Sc	1365.1
Sh	5.5
Da	0.7

Regarding possible external mass transfer limitations, the diffusion of reactants from the layer of fluid around the catalyst-support was evaluated, by calculation of Damköhler number. Damköhler numbers for both, the transport of glucose to carrier Sepabeads EC-EP/M and the transport of D-methionine to support ReliSorb™ SP405/EB were expressed, yielding in values of 0.5 and 0.7, respectively. As these values are near to the criteria of 1.0, external mass transport limitations could be happening in both examined applications of the packed bed reactor system.

Concluding accessed results for determination of internal- and external mass transfer limitations one can say that both values are closer to the transition of mass transfer limitations. Furthermore, empirical evaluation and here included applied enhanced substrate concentration made clear evidence that the concentration of substrate is acting as limiting factor.

3.2.4.5 Regarding enzyme stability & productivity

3.2.4.5.1 Glucose oxidase

3.2.4.5.1.1 Turnover frequency

A convenient way to describe the catalytic effectiveness of an enzyme is the turnover frequency (TOF). Since the turnover frequency compares the number of substrates converted by the number of catalyst used in a certain period of time, this term can be seen as mass-independent. Thus, using turnover frequency as value for enzyme effectiveness allows the comparison of different catalytic systems.[39]

Turnover frequencies for different residence times are shown (Table 12), assigned to certain amounts of converted substrate molecules. For the highest achievable residence time with the corresponding lowest substrate flow setting, turnover frequencies are in the range of $2.9*10^2 min^{-1}$ up to $1.4*10^3 min^{-1}$. For the highest applied substrate flow with a residence time of 1.7 minutes, turnover numbers are in the range of $1.2*10^3 min^{-1}$ up to $5.8*10^3 min^{-1}$. Data are presented for substrate conversion values of 10 up to 50 mM, as can be seen in the table.

Table 12: Turnover frequencies for designated residence time values. Immobilization yield for glucose oxidase (98.6%) was used to calculate the number of catalyst molecules. The dimension of TOF is time [min^{-1}].

Number of substrate converted [mM]	TOF	TOF	TOF
τ [min]	**6.9**	**3.5**	**1.7**
10	286.4	572.8	1148.9
15	429.6	859.2	1723.3
20	572.8	1145.5	2297.7
25	716.0	1431.9	2872.2
30	859.2	1718.3	3446.6
40	1145.5	2291.1	4595.5
45	1288.7	2577.5	5169.9
50	1431.9	2863.8	5744.3

As literature indicates, values for the most catalysts used in biotransformation are in the range of 10 up 1000 per second, which would be 60 up to 6000 per minute.[39]

3.2.4.5.1.2 Turnover number

General productivity of a biocatalyst can be characterized by the turnover number (TON). This dimensionless number gives the relation of the number of substrates converted, by the number of catalyst used. In the approach of this work, the value was expanded by the time of operation of the packed bed reactor, in hours. Basically this way of calculation would better refer to the total turnover number, by definition. But in the case of our experiments, no significant loss of enzyme activity has been determined. Reasoned by that, we are not able to accurately speak about total turnover number. In the limited time available for all experiments, it was not possible to determine the end of the lifetime of the used catalyst, which can be explained due to the very high results regarding productivity and stability over several hours.

Turnover numbers are expressed for distinct stable substrate conversion rates over assigned hours of reactor operation, under the premise of 6.9 minutes of mean residence time (Figure 20). For a stable substrate conversion rate of 10 mM turnover numbers in the range of $1.7*10^4$ up to $1.7*10^5$ are able to be achieved, for durations of reactor operations from one hour up to ten hours. With an assigned stable conversion of 30 mM turnover numbers are ranging from $5.2*10^4$ up to $5.2*10^5$, for one hour of reactor operation and ten hours of reactor operation, respectively. At hypothetical full substrate conversion of 50 mM, turnover numbers would even exceed from values of $8.6*10^4$ up to $8.6*10^5$, for reactor operation times of one hour up to ten hours.

Referring to the literature, TON's for enzymatic applications are in the range of 10^3 up to 10^6.[39]

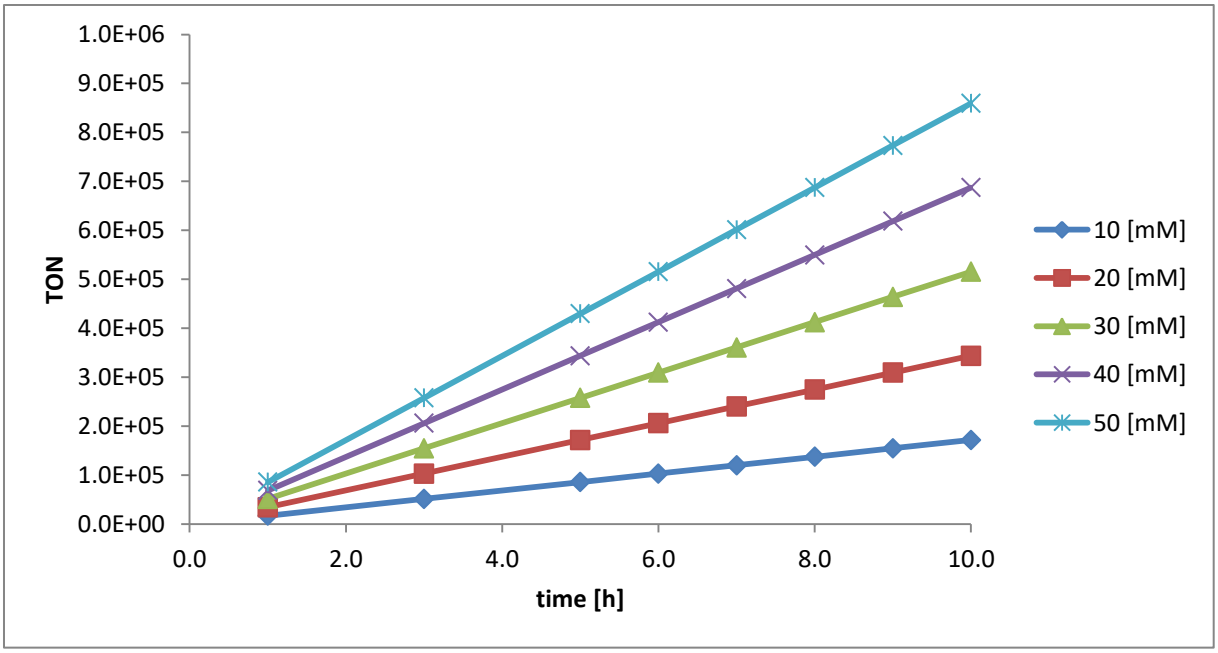

Figure 20: TON's determined for certain amounts of substrate converted and different durations of reactor operation. Turnover numbers are determined for certain assigned substrate conversion rates (10-50 mM). TON's are represented on the y-axes, reactor operation time [h] on the x-axes.

3.2.4.5.2 D-Amino acid oxidase

3.2.4.5.2.1 Turnover frequency

Determination of turnover frequencies and turnover numbers were similarly performed for the D-amino acid oxidase enzymatic system, applied in the packed bed reactor. Turnover frequencies for the longest possible residence time of 6.9 minutes are in the range of $8.4*10^{1}min^{-1}$ up to $4.2*10^{2}min^{-1}$. For the fastest substrate delivering rate, with a corresponding residence time of 1.7 minutes, turnover frequencies are in the range of $3.4*10^{2}min^{-1}$ up to $1.7*10^{3}min^{-1}$, assigned for substrate conversion-rates from 10 mM up to 50 mM (Table 13).

Table 13: Turnover frequencies for designated residence time values. Immobilization yield for DAAO (96.3 %) was used to calculate the number of catalyst. The dimension of TOF is time [min^{-1}].

Number of substrate converted [mM]	TOF	TOF	TOF
τ [min]	**6.9**	**3.5**	**1.7**
10	83.9	167.8	336.7
15	125.9	251.8	505.0
20	167.8	335.7	673.3
25	209.8	419.6	841.7
30	251.8	503.5	1010.0
40	335.7	671.4	1346.7
45	377.7	755.3	1515.0
50	419.6	839.2	1683.3

3.2.4.5.2.2 Turnover number

Determination of turnover number respecting total operability of the packed bed reactor, so the total time of rector operation in hours, was continued.

Turnover numbers are presented for assigned values of substrate converted, respecting different reactor operation windows and mean residence time of 6.9 minutes (Figure 21). For the lowest assigned substrate conversion of 10 mM turnover numbers are in the range of $5.0*10^3$ up to $5.0*10^4$ in order of one hour of stable reactor operation up to ten hours. Speaking of 30 mM of substrate-conversion, turnover numbers are determined to values of $1.5*10^4$ up to $1.5*10^5$, again regarding reactor operation windows of one up to ten hours. For the highest assigned value of substrate conversion of 50 mM, so hypothetical full conversion, turnover numbers are in the range of $2.5*10^4$ up to the $2.5*10^5$, for one hour of stable operation or the 10-fold operational time, respectively.

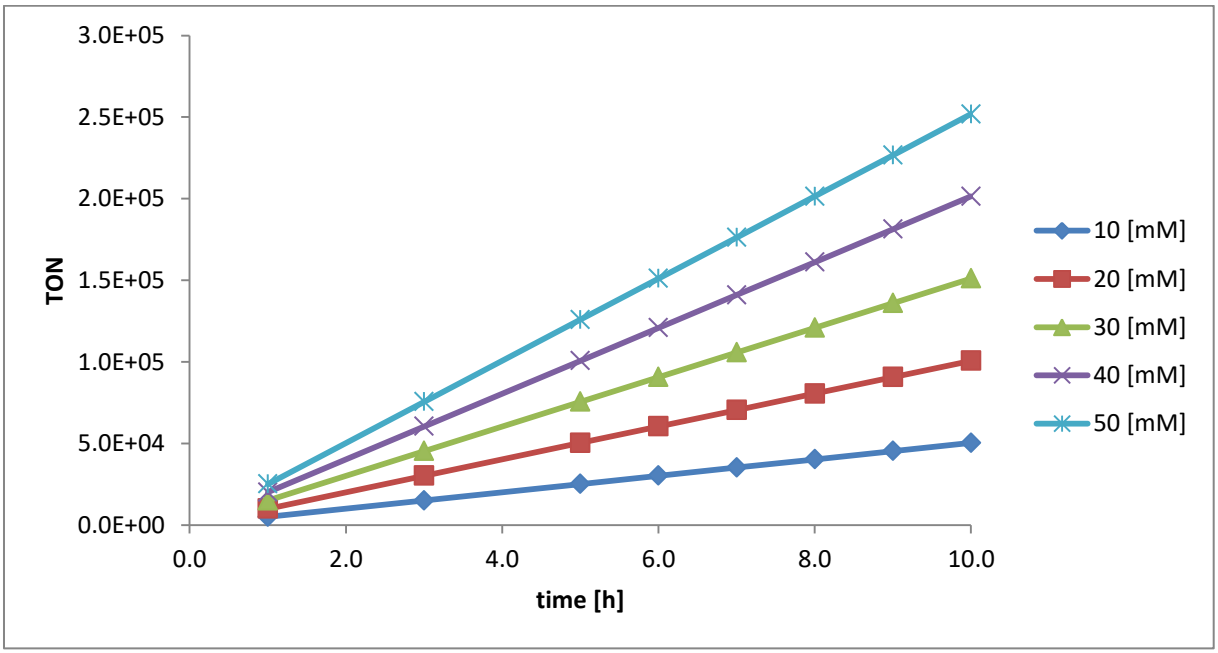

Figure 21: TON's determined for certain amounts of substrate converted and different durations of reactor operation. Turnover numbers are determined for certain assigned substrate conversion rates (10-50 mM). TON's are represented on the y-axes, reactor operation time [h] on the x-axes.

4 Conclusions

4.1 Selection of supports & determination of immobilization yields

Using biocatalytic catalysed reactions, is one way to overcome some drawbacks, as limitations in productivity, selectivity and the general environmental questionable outcome, of pure chemical oxidations. The main issue needed to be addressed in terms of biotransformation, is the poor stability regarding harsh bioprocessing techniques, as are required in conventional large scale production. As immobilization of enzymes could overcome some of these issues, immobilization experiments for glucose oxidase, catalase and D-amino acid oxidase were done.

All immobilization yields for glucose oxidase on carrier Sepabeads® EC-EP/M evaluated, were in the range of 97.0 to 100.0% and values of catalytic effectiveness factor in the range of 6.1 up to 37.9 were met. Actual immobilized GOX-activities on carrier-particles, resulted in quite low values compared to the very effective immobilization, displayed on immobilization yields. On one hand, this fact could be explained in losses of enzyme activity due to conformational changes and inactivation during the process of immobilization, or on the other hand by the impossibility of appropriate enzyme activity determination with the applied measurement method, as it may not be possible to solve enough oxygen within the mini-glass container-vial used in the activity measurement via determination of O_2-consumption rate. As about 10 to 20 U/g of immobilized activity were required for further implementation in the packed bed reactor-experiments, GOX immobilization was standardized to an immobilization approach offering 100 Units per grams of support. Immobilization experiments with D-amino acid oxidase on carrier ReliSorb™ SP405/EB were also conducted. With immobilization yields between 80.0 and 100.0% and also low catalytic effectiveness factor values, the standardized immobilization approach was set, offering 200 Units per grams of carrier. It was possible to meet a catalytic effectiveness factor of 12.7 with a corresponding immobilization yield of 97.5%. So about 20 U/g should be observed on the particles for reactor operation studies.

For both enzyme carrier combinations, higher catalytic effectiveness factors were generally found by lower offerings of enzyme activity. Suitability of ionic adsorption either on supports carrying modified amino functional groups or oriented immobilization via usage of Z_{basic2} tag was proven, as activities measured immobilized on supports were found sufficient for further possible implementation within a packed bed reactor setup. Catalase co-immobilization was performed according the properties of the main enzymes and the support, all along resulting in an activity of about 10-fold higher for this acting co-enzyme.

4.2 Design, implementation and operation of the pressurized packed bed reactor

Handling multiphase reaction processes can be seen as state of the art challenge, playing a significant role in pharmaceutical and chemical production processes. Multiphase flow, in particular handling gas liquid mixtures to provide oxygen and substrate to an oxidative biocatalyst at the same time in an appropriate manner, have influenced developments in micro-reaction technology and flow chemistry.[40] Main advantage of continuous driven processes is that the reagents are injected consistently within the defined reaction space of a reactor setup, aiming that a certain amount of reagents is able to react at a given time in a controllable manner. One can further define an ideal catalytic continuous flow process by using a heterogeneous catalyst as stationary phase, which is supplied continuously with all starting reagents by injection. The ideal outcome is defined by receiving the desired product without the need of any separation- and purification steps.[41] Having these broad requirements in mind, evaluation of the designed packed bed reactor can be done.

Delivery of substrate and oxygen in a continuous manner was achieved aiming to consistently supply the catalyst, packed within the reaction-column. Both, substrate and gas-flow were manageable and interaction time of the catalyst with substrate and co-substrate were mainly depended on the flow-settings and the amount of catalyst packed itself. As continuous flow conditions were set and the catalyst was retained within the defined region of the HPLC-column, product recovery was no specific issue needed to be addressed. In this work, operation of standard gas- and substrate- flow was done in a range of 2.5 up to 20.0 mL/min defined at standard conditions of 1.013 bars and 21°C, without issues of discontinuity or leakages. Product was collected at the outflow of the packed bed reactor column and determination of productivity was achieved by analytical methods as HPLC and enzymatic essays.

Having Henry's law of ideal gases in mind, the reactor setup was pressurized at isothermal conditions, aiming to enhance concentration of oxygen dissolved within the media. In the designed reactor-system, intensification of pressure was carried out of up to 35.0 bars. Standard oxygen concentration dissolved of 42.4 mg/L at 1.013 bars was orders of magnitude intensified. At pressurized conditions of 35.0 bars, it was possible to attain a concentration of oxygen dissolved within the liquid media of 1500.5 mg per litre. As oxygen can be broader defined as gaseous substance and basically gases can be seen in technical terms as compressible liquids, no or even minor volume changes were be expected by this kind of intensification. Without pressurized force, the volume of the concentration of oxygen dissolved within the liquid would be orders of magnitude higher and thus likely influencing reactor operation conditions in an undesired manner.

Regarding implementation of the pressurized packed bed reactor, determination of average residence time and Reynolds number was accomplished. According the definition by Osborne Reynolds, laminar flow regime was identified for both carrier-substrate combinations. The volume of the fixed packed bed was calculated and after determination of flow conditions, residence time was expressed. Dependent on the applied substrate flow, mean contact time between the fixed catalyst and the substrate was in the range of 1.7 up to 6.9 minutes. A standardized method to start reactor operation was established. The time required for the reactor start and shut-down, were identified as transient state and experiments were generally conducted under steady-state conditions.

Conversion studies for glucose oxidase/catalase bi-enzymatic system yielded in 70 percent of substrate conversion, where 35 mM gluconic acid was continuously produced under pressurized

conditions of up to 33.0 bars. Turnover frequencies of $4.0*10^3$ per minute were possible to attain and the overall productivity of the presented reactor operation can be expressed with a turnover number of $2.1*10^5$, for stable conversion of substrate into gluconic acid during an entire reactor operation over 3.8 hours. Evaluation of maximum achievable reactor operational stability for GOX/CAT immobilized on Sepabeads EC-EP/M under steady-state product conversion was presented, whereas proof of high operational stability for the catalyst was attained. Entire operational stability was evaluated over a timeframe of 12.5 hours, resulting in a turnover number for the catalyst of $4.3*10^5$.

The feasibility for the implementation of different bi-enzymatic systems within the packed bed reactor setup was evaluated likewise. Hereby conversion studies regarding D-amino acid oxidase as oxidative active catalyst in combination with catalase as co-enzyme were performed at certain pH-values. Bio-oxidative conversion of D-methionine into α-Keto-γ-(methylthio) butyric acid (KMBA), 50 mM concentrated, was demonstrated for 2.5 hours having stable conversion profile of 18 mM at pH of 7.0. Pressure values were kept in the range of 35.0 bars and 32.6 bars, having hereby oxygen concentration dissolved available of 1500.5 mg/L and 1397.1 mg/L, respectively. After optimization in regards of the pH-range, even up to 35 mM KMBA formation was reached with the applied DAAO/CAT bi-enzymatic system. Determination of available dissolved oxygen within the D-methionine media resulted in 1457.6 mg/L and 1397.1 mg/L. 70% product formation was achieved in a period of time of 6 hours. Continuing research, determination of achievable impact of different flow conditions on product formation was evaluated. Three different substrate-delivery rates of 2.5, 5.0 and 10.0 mL/min were applied on the packed bed reactor operation, resulting in residence times of 1.7, 3.5 and 6.9 minutes. A clear correlation of the mean contact time of the catalyst with D-methionine media was proven, resulting in conversion rates of 29 mM, 35 mM and 45 mM for α-Keto-γ-(methylthio) butyric acid formation, in the order for the fastest to the lowest substrate feeding method. Worth to mention, 45 mM KMBA yield can be translated in 90% of substrate conversion. In terms of common comparability of stability and productivity of the enzyme, turnover frequencies and turnover numbers are expressed. Turnover frequencies are of up to $9.1*10^2$ and turnover numbers are in the range of $6.7*10^4$ to $9.1*10^4$.

Finally experiments regarding evaluation of possible substrate limitations for D-amino acid oxidase in combination with evaluation of the feasibility of catalyst reuse at pH-condition of 8.0 were carried out. An obvious indication for limitations due to substrate concentration was found, as conversion into α-Keto-γ-(methylthio)butyric acid of up to 76 mM were attained by doubling up the substrate concentration. Due to determination of possible internal- and external mass transfer limitations an explanation was searched for. As can be taken from the results, indications for existing internal mass transfer limitations were found. Values of Damköhler numbers indicate existing external mass transfer limitations, which coupled with the laminar flow conditions determined within the system, seemed to be the main bottleneck of the packed bed reactor. Verification of impact of residence time on product yield was accomplished additionally, as again stable plateaus of substrate conversion in correlation with certain values of residence time were established.

4.3 Outlook

Continuous processing can also be connected to future trends towards green and sustainable manufacturing. Demands as maximizing efficiency, minimizing waste and rise of profitability are included in this statement, not only the simple avoidance of toxic waste and materials. In terms of cost, equipment size, energy consumption and waste generation, batch processing has clearly been outdistanced by continuous flow technology. Further needs to be met, considering the establishment of a green process, are high product quality, safety and efficiency, which should be met from the very beginning on lab scale. Applying ideas from both, green engineering and green chemistry on designing continuous processes, may fulfil some of the requirements of the 21th century. Also being known for their precise residence time control and shorter process times with good reproducibility, continuous flow technology already gained high popularity in the academic field.[42]

Finally closing the thesis from an ambitious engineering point of view, I will present one further calculation. The highest amount of product formed was in the range of about 75 mM, with a corresponding residence time of 6.9 minutes. Which amount of time of stable reactor operation would be needed to produce one kilogram of product?

Aiming to solve this issue, basic standard conditions as substrate and gas flow, concentration of both of them, isothermal conditions and stable catalyst behaviour infinitively, need to be assumed. The example taken for this calculation is extracted from the mentioned DAAO-catalysed conversion of D-methionine into KMBA. As can be seen in the formula defining calculation of residence time, three interdependent topics could be addressed heading forward to solve the question. Simplifying this approach, one could set the mean residence time and therewith the substrate delivering rate constant. Now basically reactor specification in respect of the reaction volume needs to be adjusted. The volume of the column, or the volume packed with catalytically active supports, was 17.2 mL. 76 mM of KBMA (MW 170.2 g/mol) were already proven to be producible and 5.9 mol of KMBA would be necessary to attain a product amount of one kilogram. Basically intensification of catalytically active reactor volume from 17.2 mL up to 77.4 litres would be required. Another approach, heading to solve that issue, could be done by speaking about reaction time. 76 mM of KMBA are formed per 6.9 minutes of residence time, so aiming to produce 5.9 mol of this valuable product would need an entire reactor operation time of 517.3 hours at continuous operation conditions.

Conclusively, using microreactor technology enables high conversion efficiency while having reduced reaction time. The possibility to work in novel process windows, as under high temperature or high pressurized conditions and also high productivity per unit of reaction volume, are feasible to achieve[43], what was actually proven by this thesis. As biotransformation are expected to open many new operation windows for food-and pharmaceutical industries, such reactor setup can in principle be used as platform-technology for implementation of a variety of different other enzymatic oxidative reactions.

5 Appendix

5.1 List of carriers and their characteristics for enzyme immobilization

Table 14: List of carriers and their characteristics for enzyme immobilization, created by Leopold Heydorn

Product name	Functional group	Particle size [µm]	Mean diameter [µm]	Pore size [nm]	Pore volume [mm^3/g]	Surface area [m^2/g]	Density [g/cm^3]
Sepabeads® FP-SP 400 [9]	Sulfonate	75-200	120	60-80			
Sepabeads® EC-EP [9]	Epoxy	100-300	206	10-20			
ReliZyme® 113 (EP113) (S grade) [1]	Epoxy	100-300		20-50			
ReliZyme® 403 (EP403) (S grade) [1]	Epoxy	100-300		40-60			
ReliZyme® 113 (EP113) (M grade) [1]	Epoxy	200-500		20-50			
ReliZyme® 403 (EP403) (M grade) [1],[8]	Epoxy	200-500		40-60	900-1000	50-60	
ReliSorb™ 400/SS (SP400/SS) [2]	Sulfonate	50-150		80-100			~ 0,8 $^+$
ReliSorb™ 400 (SP400) [2]	Sulfonate	75-200		80-100			~ 0,8 $^+$
ReliSorb™ SP405/EB (SPSP405/EB) [2]	Sulfonate	200-500		80-100			~ 0,8 $^+$
ECR8204F [3]	Epoxy	150-300		30-60			
ECR8209F[3]	Epoxy	150-300		60-120			
ECR8215F[3]	Epoxy	150-300		120-180			
ECR8204M [3]	Epoxy	300-710		30-60			
ECR8209M [3]	Epoxy	300-710		60-120			
ECR8215M [3]	Epoxy	300-710		120-180			
ECR8285 M [3]	Epoxy + Butyl	300-710		40-50			
ECR8285 [3] **(2015)**	Epoxy + Butyl	300-710		40-50			
C 100	Sulfonate	300-1200					
SP Sepharose Fast Flow [4]	Sulpho-propyl	45-165	90*				
SP Sepharose	Sulpho-	24-44	34*				

High Performance [5]	propyl					
6% BCL Agarose beads	Hydroxyl	20-50				
6% BCL Agarose beads [6]	Hydroxyl	50-150				
CPG (Trisoperl®) (VitraBio) [11]	Silanol	50-100	161	1521	43	
CPG, Native; 300 Å [7]	Silanol	70-130	32,18	1487	176,03	0,28
CPG, Native; 1000 Å [7]	Silanol	70-140	103,78	1326	54,06	0,3
CPG, Native; 2000 Å [7]	Silanol	70-140	208,8	1150	25	0,36
PSSP 42 (Porous sphere. silicate p.) [?]	Silanol		7	700	203	
PPS [10]	Silanol		7,5	600	267	
SBA-15 [10]	Silanol		7,5	1300	682	

+ indicates bulk density * indicates the median particle diameter of the Q_3-distribution

5.1.1 Sources for list of carriers and their characteristics

[1] http://www.resindion.com/images/stories/resindion/download/RESINDION_brochure_Reliy me_SepabeadsEC_set2015_web.pdf

[2] http://www.resindion.com/images/stories/resindion/download/RESINDION_brochure_Relio rb_set2015_web.pdf

[3] http://www.purolite.com/RelId/610608/ISvars/default/Enzyme_Carriers.html

[4] http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences/ products/AlternativeProductStructure_17466/17072901

[5] http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-at/products/AlternativeProductStructure_17466/17108701

[6] http://www.abtbeads.com/size-exclusion-chromatography/crosslinked-agarose-beads/6-bcl-agarose-bead-standard-50-150-m.html

[7] Certificate of analysis of each respective CPG from Biosearch Technologies

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5.2 Fluitest® GLU HK enzymatic in vitro test solution – Calibration

5.2.1 Calibration for the 1st to the 3rd reactor run

Table 15: Glucose calibration values for run 1 to 3 of the reactor

Glucose concentration [mM]	Absorption [340nm]
20.0	1.8
15.0	1.5
10.0	1.0
7.5	0.8
5.0	0.5

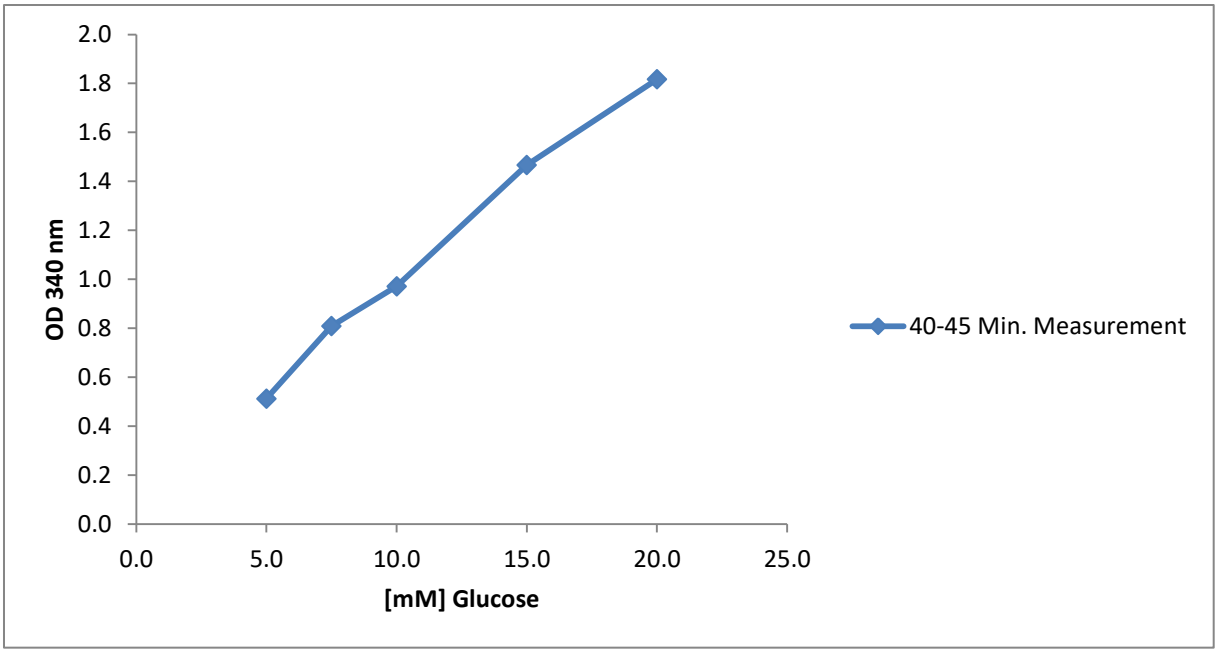

Figure 22: Glucose calibration values for run 1 to 3 of the reactor.

5.2.2 Calibration for the 4th and 5th reactor run

Table 16: Glucose calibration values for the 4th and 5th reactor run

Glucose concentration [mM]	Absorption [340nm]
20.0	1.7
15.0	1.3
10.0	0.9
7.5	0.7
5.0	0.5
2.0	0.2

Corresponding absorption values of glucose concentrations in the range of 2 mM up to 20 mM were measured on DU 800 UV-vis spectrophotometer (Backman Coulter. Inc. Fullerton. CA. USA) at 340 nm wavelength. The absorption for 20 mM glucose seemed to be not in a linear range to the others and has thus been omitted by creating the desired calibration curve.

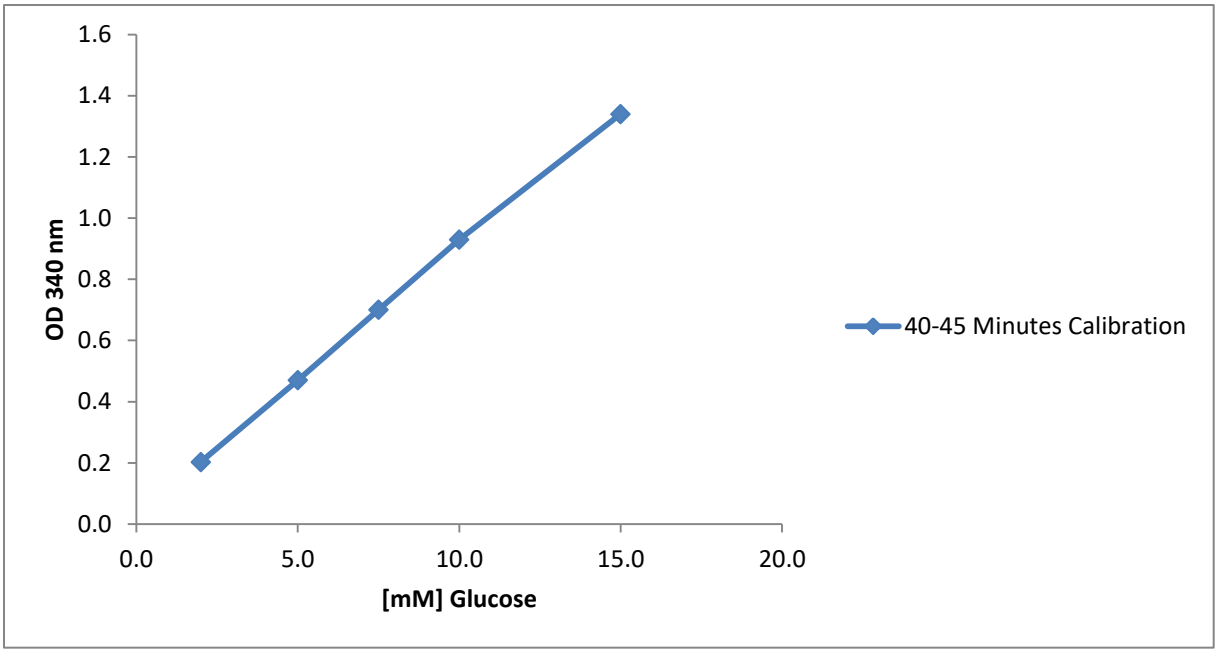

Figure 23: Glucose-calibration for the 4th and 5th reactor run.

Determination of glucose concentration remained during reactor run was done using these calibration results.

5.3 HPLC-Methods

5.3.1 Determination of glucose-and gluconic acid-concentration

Table 17: Calibration of glucose and gluconic acid

Chemical Solution			**Detector Measured**
Glucose	Concentration [mM]	Intensity mV	**RI**
	50.0	811545	
	40.0	650885	
	30.0	482570	
	25.0	402885	
	12.5	204286	
	5.0	78442	
	2.5	40942	
Gluconic Acid	Concentration [mM]	Intensity mV	**UV 205 nm**
	50.0	2222728	
	40.0	1711239	
	30.0	1316432	
	25.0	1119320	
	12.5	544644	
	5.0	221757	
	2.5	112511	

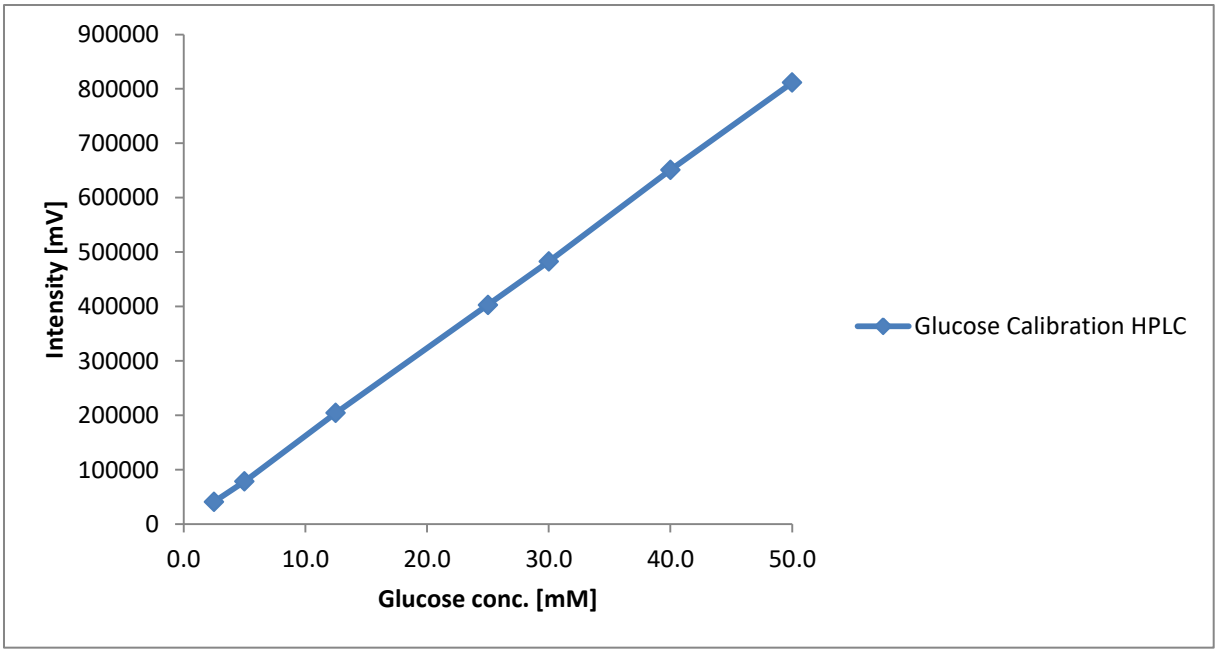

Figure 24: Calibration-curve for HPLC of glucose.

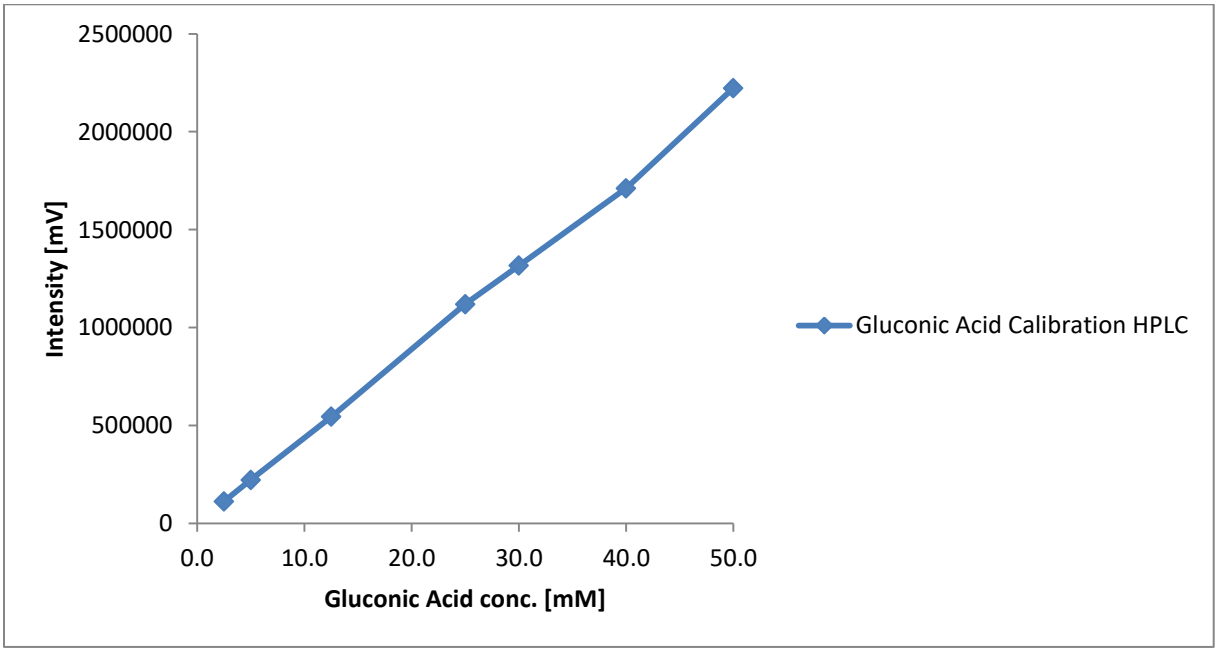

Figure 25: Calibration-curve for HPLC of gluconic acid.

5.3.2 Determination of D-methionine and α-Keto-γ-(methylthio) butyric acid (KMBA)

Table 18: Calibration of D-methionine and KMBA

Analytes/Conc.[mM]	Area	Analytes/Conc.[mM]	Area
D-Methionine/50	0.0634	KMBA/50	0.8093
D-Methionine/40	0.0561	KMBA/40	0.6753
D-Methionine/25	0.0427	KMBA/25	0.4507
D-Methionine/20	0.0368	KMBA/20	0.3769
D-Methionine/12.5	0.0263	KMBA/12.5	0.2422
D-Methionine/10	0.0219	KMBA/10	0.1942
D-Methionine/5	0.0116	KMBA 5	0.1023
D-Methionine/2.5	0.0061	KMBA 2.5	0.0505

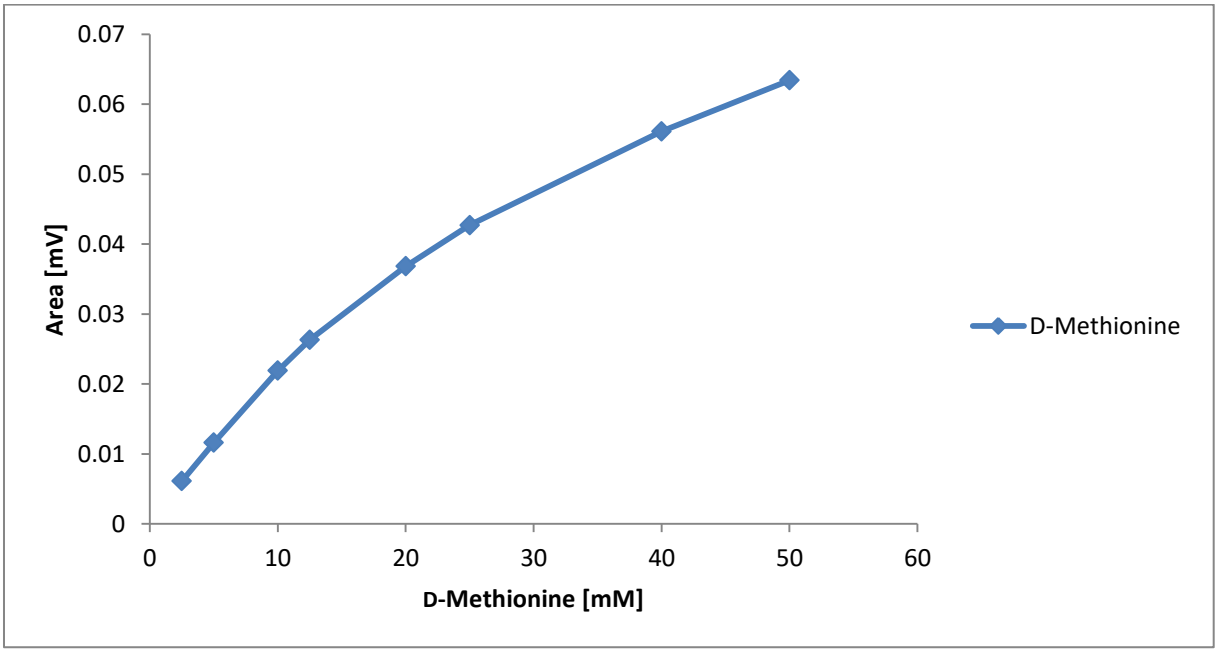

Figure 26: Calibration-curve for HPLC of D-methionine.

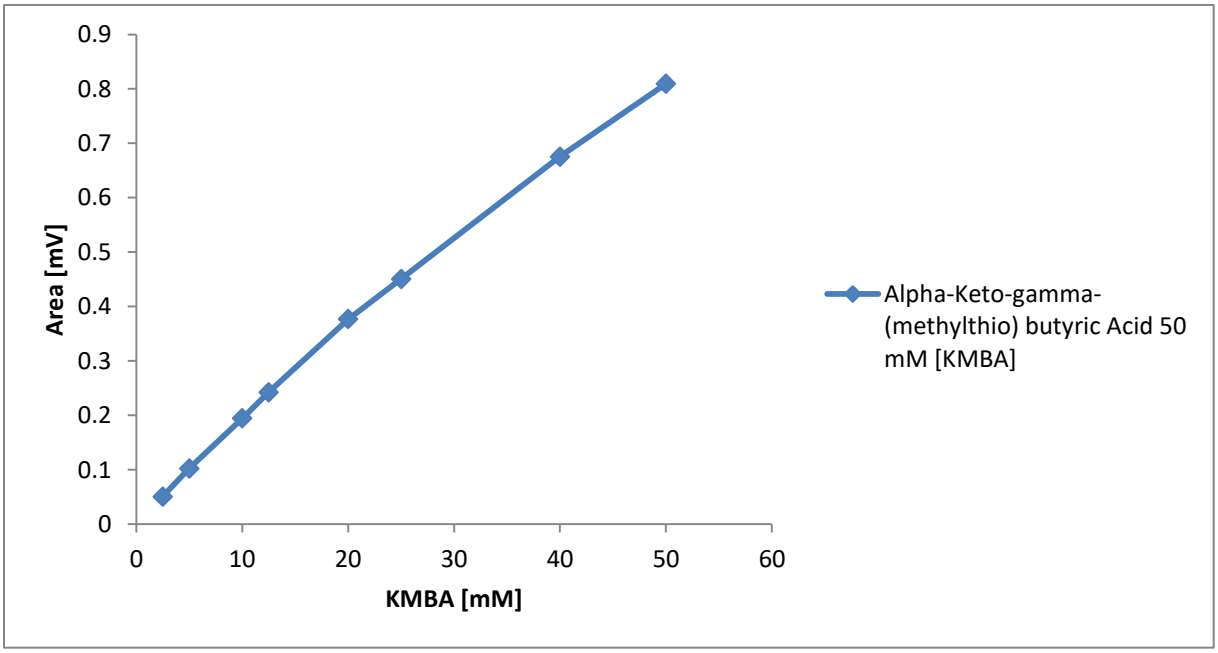

Figure 27: Calibration-curve for HPLC of KMBA.

5.4 List of abbreviations

mM	millimole
mL/min	millilitre per minute
U/g	units per grams
GOX	glucose oxidase
CAT	catalase
DAAO	D-amino acid oxidase
mmol/L	millimole per Litre
PEI	polyethylenimine
% (w/v)	percentage weight per volume
Rpm	revolutions per minute
PPB	potassium phosphate buffer
pH	potentia hydrogenii (latin term), power of hydrogen (Carlsberg Foundation; Eng.)
mg	milligrams
mL	millilitre
U/mL	Units per millilitre; volumetric enzyme activity
psi	pound-force per square inch, unit of pressure
NaCl	sodium chloride
U/g	Units per grams, mass based specific enzyme activity
kg/m^3; g/m^3	kilogram per cubic meter; gams per cubic metre, SI derived unit of density
°C	degree Celsius, temperature scale according International System of Units (SI)
m^2/s	metre squared per second; SI unit of kinematic viscosity, specific relative angular momentum and thermal diffusivity.
m	metre
mM/min	millimole per minute; term to describe rate of enzymatic reactions
m/s	metre per second, unit of both velocity and speed
mol	mole, unit of measurement for amount of substance

min/h/s	abbreviation of minute/hour/second
%(V/V)	percentage concentration of volume per volume
M	molar; number of moles per litre
L, mL, µL	volume of litre, millilitre, microliter
nm	nanometre, unit for wavelength of light
$NADP(H)_2$	Nicotinamide adenine dinucleotide phosphate
TOF	turnover frequency
TON	turnover number
hPA	hectopascal, SI unit of pressure and stress equals to 10^2 pascals
kDa	kilo Dalton

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