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High throughput carrier based fluorescent enzyme assays

Masterarbeit

zur Erlangung des akademischen Grades eines
Diplom-Ingenieurs
der Studienrichtung Biotechnologie
erreicht an der
Technischen Universität Graz

Institut für Analytische Chemie und Lebensmittelchemie
Technische Universität Graz

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Graz, January 2012

„Ich werd nie mehr so rein und so dumm sein wie weißes Papier.“
(**Element of Crime**)

Abstract

The use of immobilized enzymes is a common method in industrial applications. Therefore, it is also important to characterize these enzymes immobilized on particles. Most of the enzyme assays are based on absorption measurements and are hence not appropriate for immobilized enzymes. The aim of this thesis was the development of enzyme assays for esterases immobilized on beads for industrial applications. Therefore, two different assays based on fluorescing substances were developed. In one of the assays esters of fluorophores were used as substrate. In the second one, a more universal one, a fluorescent pH-indicator was used for the determination of the activities of immobilized esterases. Applications based on fluorescence have several advantages. The most important ones are a low detection limit and a high reproducibility. In a further part of the thesis enzyme assays were also investigated in organic solvents because a lot of enzyme reactions in industrial scale are performed in such solvents.

Kurzfassung

Die Verwendung von immobilisierten Enzymen ist eine übliche Methode bei industriellen Anwendungen und deswegen ist es wichtig, diese auf Partikeln immobilisierten Enzyme zu charakterisieren. Das Ziel dieser Diplomarbeit war die Entwicklung eines Enzymassays für auf Partikeln immobilisierten Esterasen. Zwei verschiedene Enzymassays, basierend auf Fluoreszenz, wurden im Zuge dieser Diplomarbeit entwickelt. Bei einem Assay werden veresterte Fluorophore als Substrate verwendet. Der zweite, universellere Assay verwendet einen fluoreszierenden pH-Indikator zur Bestimmung von Esterase Aktivitäten. Die Verwendung von auf Fluoreszenz basierenden Assahts mys hat viele Vorteile. Eine niedrige Nachweisgrenze und eine hohe Repruduzierbarkeit sind nur zwei der vielen Vorteile. Im weiteren Verlauf der Arbeit wurden Enzymassays auch in organischen Lösungsmitteln untersucht, da im industriellen Maßstab Enzymreaktionen oftmals in solchen Lösungsmitteln durchgeführt werden.

Statutory Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

Place	Date	Signature

Danksagung

It is time to say thank you.....

Zu Begin möchte ich mich bei meinen Betreuern Univ.-Prof. Dipl.-Chem. Dr.rer.nat. Ingo Klimant, Ass.-Prof. Dipl.-Chem. Dr.rer.nat. Torsten Mayr und Dr. Peter Remler bedanken, die es mir überhaupt erst ermöglicht haben meine Masterarbeit zu verfassen. Besonderer Dank gilt meinem Betreuer Torsten Mayr, der mir während meiner ganzen Zeit mit Rat und Tat zur Seite gestanden ist. Ebenfalls möchte ich mich bei Dr. Peter Remler bedanken, der mir vor allem bei der Chemikalienanschaffung sehr behilflich war. Außerdem danke ich Dr. Ferdinand Zepeck, der mein Ansprechpartner von der Firma Sandoz war und mich mit zahlreichen Enzymproben während meiner Arbeit versorgt hat.

Selbstverständlich möchte ich an dieser Stelle auch die zahlreichen Institutsmitglieder erwähnen, welchen ich ebenfalls viel zu verdanken habe. Zahlreiche Diskussionen zu den verschiedensten Uhrzeiten brachten mich sowohl fachlich als auch persönlich sehr viel weiter.

Starring: Klaus, Lisi, Daniel, Birgit, Tobi, Sergey, Philipp, Christoph, Spela, Babsi, Yvonne, Luki, Günter, Gunter, Fabian, Eveline, Herbert, Chrissi, Iris,....

Außerdem möchte ich noch all meinen anderen Freunden danken, die mich auf meinem Weg begleitet haben, mich motiviert haben und mit denen ich in den letzten Jahren zahlreiche schöne Momente verbracht habe. Des weiteren möchte ich meinen Eltern und meiner Familie großen Dank aussprechen, die mich immer unterstützt haben und die zu jeder Zeit hinter mir gestanden sind. Zu guter Letzt möchte ich mich noch besonders bei meiner Freundin Hanna bedanken. Vielen Dank für die sagenhafte Unterstützung während der letzten Jahre!

In diesem Sinn: „Finger weg von meiner Paranoia“ (Element of Crime)

.....and good bye

Graz, im Jänner 2012 Clemens Gamerith

Abbreviations

BCECF	2',7'-bis(carboxyethyl)-5(or 6)-carboxyfluorescein
EC	enzyme commission
EDA	eosin diacetate
EtOH	ethanol
FA	fluorescein amid
FDA	fluorescein diacetate
FODE	fluorescein octadescylester
g	gram
HOMO	highest occupied molecular orbital
HPTS	8-hydroxypyren-1,2,6- trisulfonic acid
LUMO	lowest unoccupied molecular orbital
mg	milligram
min	minute
mL	milliliter
mM	millimolar
MTBE	methyl-tertiary-butylether
MTP	microtiter plate
mU	milliunit
nm	nanometer
nM	nanomolar
NZL	novozyme lipase
IC	internal conversion
ISC	intersystem crossing
PE	polyethylene
PET	photoinduced electron transfer
PMT	photomultiplier tube
pNPA	para-nitrophenyl acetate
PP	polypropylene
QY	quantum yield
R^2	correlation coefficient
RA	resorufin acetate
s	second
SOP	standard operating procedure
SPB	sodium phosphate buffer
TOA	tetraoctylammonium
U	unit

UV	ultraviolet
VR	vibrational relaxation
WL	wavelength
μM	micromolar

Contents

Part I.

Introduction

1. Introduction

The aim of this thesis was to design an enzyme assay for the pharmaceutical company Sandoz which supported this thesis by supplying resources. In more detail, a high-throughput fluorescence based assay for the determination of activities of esterases immobilized on particles was investigated. Different approaches were examined. For example, different esters of fluorophores were used as substrates or various fluorescent pH-indicators were tested.

Fluorescence based enzyme assays are used in a wide range of applications. Starting with intracellular pH-measurements up to varying assays for enzyme discovery and engineering many applications are used nowadays. [1] [2] [3] Especially the high-throughput assays for industrial applications get more and more important due to the increasing quantity of known and mutated enzymes. It is essential to find an appropriate way to differentiate between a huge variety of enzymes. The detailed structure of an enzyme is often less useful for the improvement of an enzyme than the information delivered by a high-throughput enzyme assay. [1]

At the moment Sandoz uses an absorption measurement to determine the activities of immobilized enzymes. A certain amount of beads is added to a buffered solution. The substrate, p-nitrophenyl acetate (p-NPA) , is added and the stirred solution is incubated at 25°C. Every minute a certain volume has to be transfered to a cuvette. After the settlement of the beads and the measurement of the absorption at 405nm, the sample has to be transfered back to the reaction vessel. A schematic representation of the system is shown in figure 1.1.

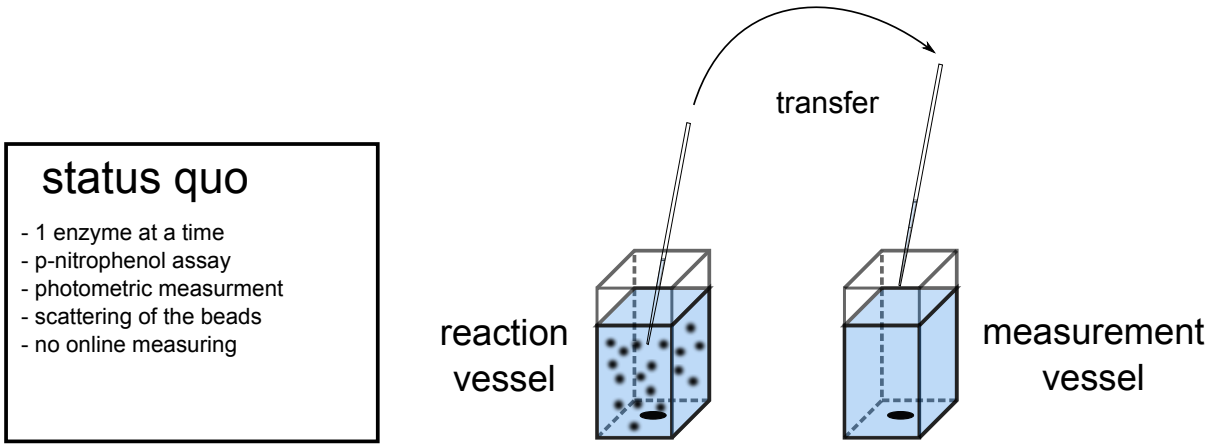

Figure 1.1.: Schematic representation of the enzyme activity measuring at the moment

At the end a standard operating procedure (SOP) should describe the new assay, which has preferably the following advantages:

- rapid
- online measurements
- easy handling
- inexpensive
- universal
- high reproducibility

Part II.

Theoretical background

2. Luminescence

In the year 1888 the German physicist and science historian Eilhard Wiedemann was the first scientist to introduce the term luminescence. Many different types of luminescences are known nowadays (table 2.1) and they have all one thing in common - the emission of light. According to table 2.1 the different modes of excitations define the various kinds of luminescence. A lot of different kinds of compounds, such as organic, inorganic and organometallic compounds are able to emit light. The following chapter focuses on the phenomenon of photoluminescence, especially fluorescence, and is based on information taken from [4] and [5]. Further information about other excitation modes is available in relevant literature, for example [6].

Table 2.1.: Types of luminescence

Phenomenon	Mode of excitation
Photoluminescence	Absorption of light
Radioluminescence	Ionizing radiation
Cathodoluminescence	Cathode rays
Electroluminescence	Electric field
Thermoluminscence	Heating after prior storage of energy
Chemiluminescence	Chemical process
Bioluminescence	Biochemical process
Triboluminescence	Frictional and electrostatic forces
Sonoluminescence	Ultrasounds

2.1. Photoluminescence

Photoluminescence is based on the absorption of a photon which can lead to the promotion of an electron to an electronic excited state. An excited molecule can return to the ground state using a lot of different de-excitation pathways. Figure 2.1 gives an overview of the various possible de-excitation pathways.

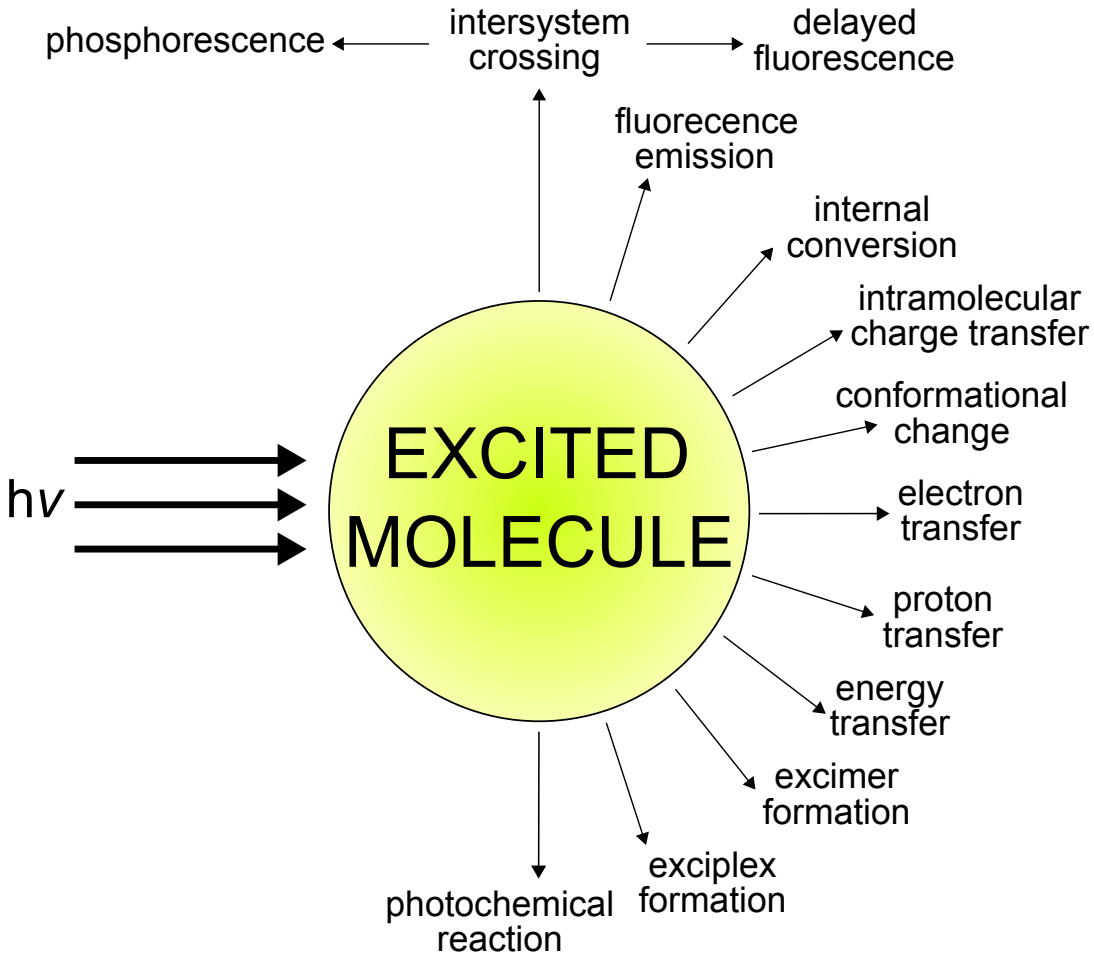

Figure 2.1.: Possible de-excitation pathways

Out of this diversity only a few pathways cause the emission of light, e.g. fluorescence. All of these de-excitation pathways are in competition with fluorescence emission if they occur during a defined time window when the molecule is in the excited state. A lot of different factors can influence molecular fluorescence. The following schedule (table 2.2) shows a general view of possible parameters that can influence molecular fluorescence.

2.1.1. Electronic State Transitions

Aleksander Jablonski conceptualized a diagram (figure 2.2) which is very useful for visualizing the possible transitions between the electronic states of a molecule. First of all, the absorption of a photon takes place which is a very fast process (about 10-15 s). The

Table 2.2.: Different parameters influencing the emission of fluorescence

Ions	Polarity
pH	Pressure
Temperature	Quenchers
Hydrogen bonds	Viscosity

molecule in the ground state (non excited state, S_0) gets excited by lifting an electron commonly from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO). The selection rules define the possible transitions between electronic states. As transitions between states of different multiplicities are forbidden, the electrons in the S_0 are excited to the S_1 state. That implies that singlet-singlet and triplet-triplet transitions are allowed. In contrast, singlet-triplet transitions are forbidden. The excitation to higher order singlet states is also allowed but not very common. If visible light is used, the electrons of π -bonds are involved. To excite electrons of σ -bonds, higher energy is required (e.g. UV). Absorption normally occurs at higher energy levels than the emission. Therefore, the wavelengths of fluorescence are longer than the ones of absorption. This shift between the absorption maximum and the emission maximum is better known as "Stokes' Shift" and occurs because of the energy loss due to vibrational relaxations in the excited state.

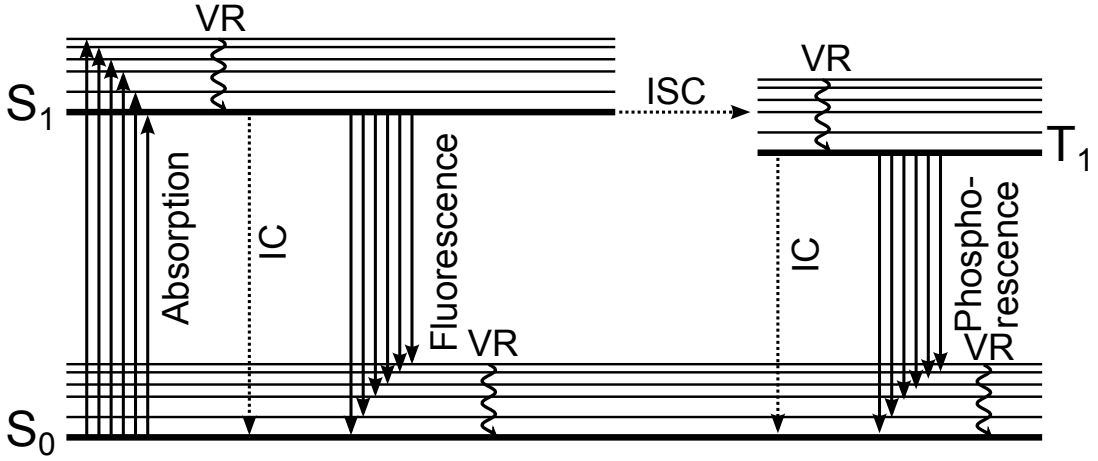

Figure 2.2.: Perrin-Jablonski-diagram, IC: internal conversion; ISC: inter system crossing; RV: vibrational relaxation

Every electronic state has different vibrational levels and after absorption the molecule is in a vibrational excited state. Absorption is immediately followed by the vibrational

relaxation to the lowest vibrational state and is commonly faster than the other de-excitation-processes.

2.1.2. Internal Conversion (IC)

The internal conversion is a transition between two electronic states which have the same spin multiplicity. It does not lead to the emission of light. In case the molecule is not in the lowest vibrational level after this non-radiative transition, a vibrational relaxation to the lowest level takes place again. A much larger energy gap between S_1 and S_0 leads to a less efficient conversion compared to the transition between S_2 and S_1.

2.1.3. Fluorescence

The transition (between S_1 and S_0 states) emitting photons is called fluorescence. According to Kasha's rule, fluorescence always occurs from the lowest vibrational level of the excited state. As fluorescence emission normally takes place from the S_1 to the S_0 state, the spectral characteristics do not depend on the excitation wavelength. Although the emission of a photon is as fast as absorption there is a certain delay before fluorescence occurs. This short delay (10^{-10}-10^{-7} s) , where the molecules stay in the excited state, is called 'life time' and depends on the type of molecule as well as the surrounding.

2.1.4. Intersystem Crossing (ISC)

According to the selection rules, this process is forbidden. However, inter system crossing is possible due to spin-orbit interaction. Intersystem crossing is the transition between a singlet electronic state and a triplet electronic state. Therefore, it is a non-radiative transition between two states of different multiplicities. As this transition has almost the same rate, it can compete with processes like fluorescence.

2.1.5. Phosphorescence

Once a molecule is in a triplet state T_1 (after vibrational relaxation in the lowest vibrational state) a transition to S_0 can take place even though it is again a forbidden transition. In solution at room temperature the most probable way for a molecule to reach the S_0 is non-radiative de-excitation. A lot of collisions with solvent molecules facilitate ISC and vibrational relaxation to the S_0 state. On the other hand, radiative de-excitation, called

'phosphorescence', can take place especially at low temperatures or in a rigid medium. As the T_1 to S_0 transition is forbidden, the rate constant of phosphorescence is hence very low. Since the T_1 state is energetically lower located than the S_1 state, the emission maximum of phosphorescence is even more shifted to higher wavelengths than the one of fluorescence.

2.1.6. Delayed Fluorescence

If the energy gap between the T_1 and the S_0 state is small and the life time of T_1 is long enough, a reverse ISC can take place. This process is better known as delayed fluorescence ($T_1 \rightarrow S_1$) because it has the same spectral characteristics as fluorescence but a much longer decay time constant. This longer decay time constant is achieved through the longer delay in the T_1 state. As delayed fluorescence is thermally activated, higher temperatures increase the efficiency.

2.1.7. Life Time

A molecule only stays in an excited state for a certain time which is called life time τ_S. As there are various possibilities for an excited molecule to reach the ground state, we have to define different rate constants for all of these processes. For example k_r^S is the rate constant for the radiative deactivation $S_1 \rightarrow S_0$ with the emission of fluorescence. k_{nr}^S, on the other hand, is the rate constant for the non-radiative deactivation processes (internal conversion and intersystem crossing). The following equation (2.1) shows the lifetime of a S_1 state and the rate constants k_r^S and k_{nr}^S

$$\tau_S = \frac{1}{k_r^S + k_{nr}^S} \tag{2.1}$$

2.1.8. Quantum Yields

The ratio between absorbed and emitted photons is called Φ_F (fluorescence quantum yield) and is defined by equation 2.2.

$$\Phi_F = \frac{k_r^S}{k_r^S + k_{nr}^S} = k_r^S \tau_S \tag{2.2}$$

2.2. Fluorescent *pH*-Indicators

Currently a large variety of applications are available for the use of fluorescent *pH* indicators. [7] [8] [9] In general, fluorescent *pH* indicators offer a much better sensitivity than common dyes which are based on a color change (phenolphthalein). Therefore, such indicators are widespread in fields of chemical application as in analytical chemistry, bioanalytical chemistry or cellular biology as well as medicine. [10] In fluorescent *pH* determinations the concentrations of the acidic and basic forms are used according to the Henderson-Hasselbalch equation. [11]

$$pH = pK_a + log\frac{[B]}{[A]} \tag{2.3}$$

Equation 2.3 can be adapted for fluorometric titrations as follows:

$$pH = pK_a + log\frac{I - I_A}{I_B - I} \tag{2.4}$$

In this equation I is the measured fluorescence intensity, I_A and I_B are the intensities of the acidic respectively basic form of the indicator at the same wavelength. As a matter of fact, the Henderson-Hasselbalch equation is a simplification since the *pH* is in reality defined by the activity of protons and not by their concentrations. Hence, the activity coefficient is not only depending on temperature, but also on other facts like ionic strength, structural changes of the medium and specific interactions depending on the chemical nature of the indicator.

2.2.1. Common Fluorescent *pH*-Indicators

Depending on their basis of elementary processes, it is possible to divide these indicators into three classes:

1. Photoinduced proton transfer, no electron transfer (hydroxycoumarins)
2. Photoinduced electron transfer, no proton transfer (PET)
3. Neither photoinduced proton nor electron transfer (fluorescein, eosin Y)

2.2.2. Fluorescein and its Derivatives

Fluorescein is probably the most common fluorescent dye and has been used in various applications. [12] [13] Due to the existence of two consecutive transitions (figure 2.3) this indicator dye has a broad range of pH response. Derivatives like the BCECF (2',7'-bis(carboxyethyl)-5(or 6)-carboxyfluorescein) have been designed for intracellular pH measurements by increasing the apparent pK_a. [10] [14] Several applications of fluorescein as pH-indicator can be found in the literature, for example [15]. One advantage of fluorescein is a high quantum yield. The photo-stability of fluorescein is acceptable. A spectrum of fluorescein is shown in figure 2.4. The excitation wavelength is 490nm, the emission wavelength is 514nm. The nonfluorescent, colorless lactone is only formed in aqueous solutions with a pH under 5. The use of solvents like acetone facilitates the forming of the lactone.

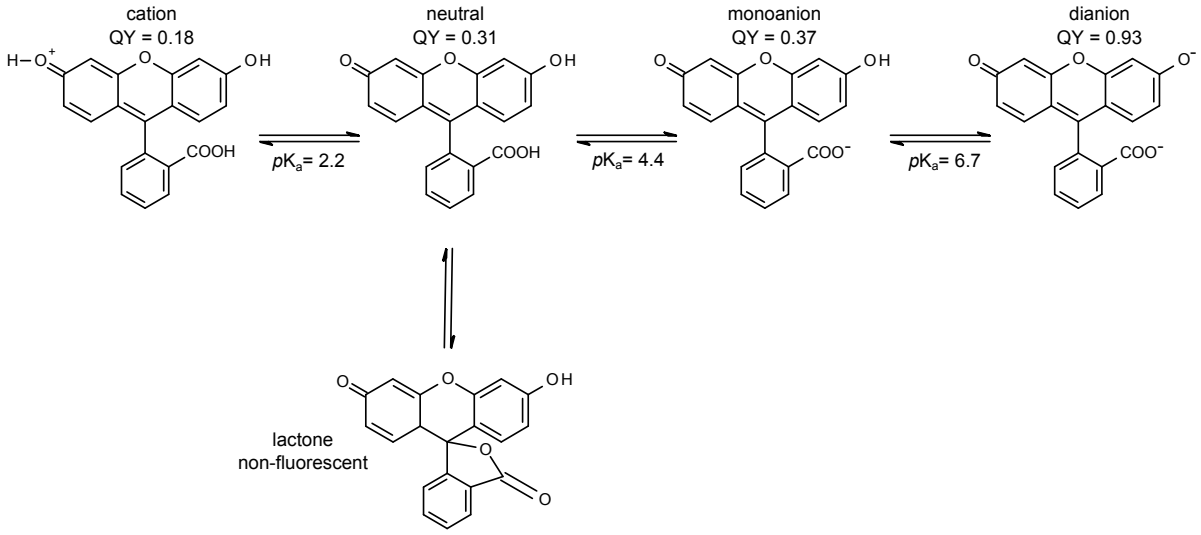

Figure 2.3.: Fluorescein in aqueous solution

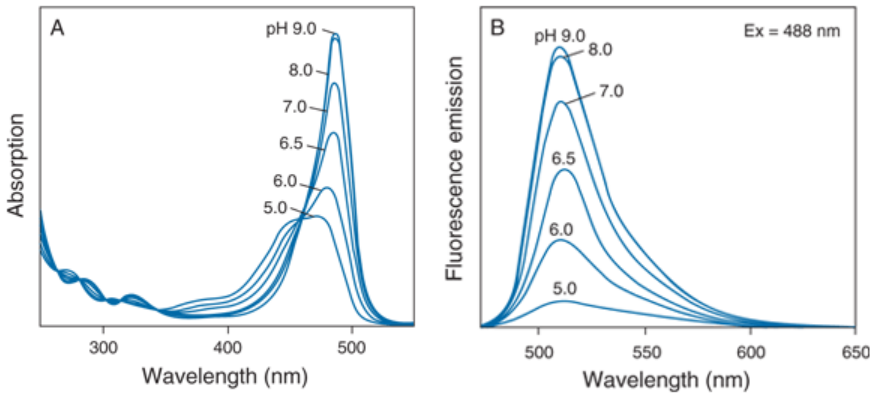

Figure 2.4.: *pH*-dependent spectra of fluorescein, A) absorption spectra, B) emission spectra [16]

2.2.3. Pyranine (8-Hydroxypyren-1,2,6- Trisulfonic Acid, HPTS)

The functionality as a *pH*-indicator is based on an absorption shift. Figure 2.6 shows two spectra of HPTS at different *pH* values. The structure of HPTS is illustrated in figure 2.5. Pyranine has an apparent pK_a of 7.2. Therefore, it is used for intracellular measurements in a *pH* range around 7. [16] HPTS can also be used as CO_2 sensor. [17] [18] [19] This highly water soluble dye is also very photo-stable.

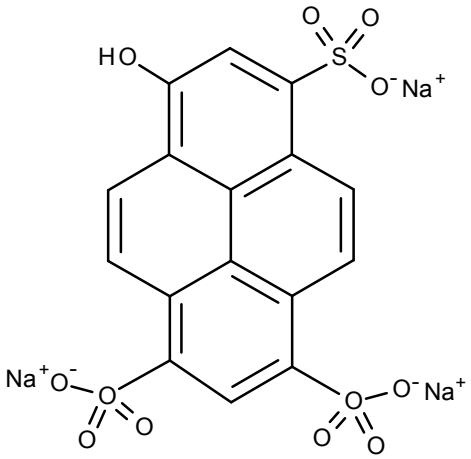

Figure 2.5.: Structure of HPTS

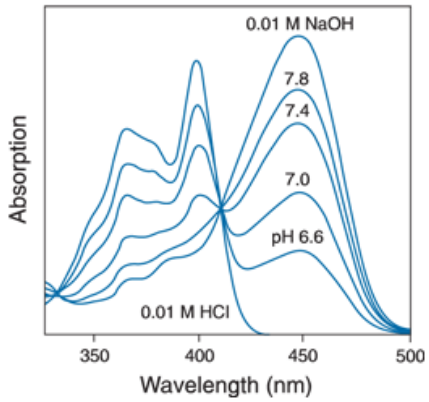

Figure 2.6.: *pH*-dependent spectra of HPTS [16]

3. Enzymes

The following chapter is based on information taken from [1] [20] [21] [11].

Enzymes are biocatalysts and therefore accelerate chemical reactions. Most of the known Enzymes are proteins, but there also exist catalytically active ribonucleic acids, called ribozymes. Like all catalysts, enzymes lower the activation energy of a reaction but are not consumed by the chemical reaction.

3.1. Enzyme Classification

Enzymes catalyze different chemical reactions. Therefore, it is possible to differentiate them according to the various reactions types. The Enzyme Commission number (EC number) classifies due to this chemical reactions. The following table (table 3.1) shows the six main classes of enzymes.

Table 3.1.: Enzyme classification

EC 1	Oxidoreductases
EC 2	Transferases
EC 3	Hydrolases
EC 4	Lyases
EC 5	Isomerases
EC 6	Ligases

There also exist sub- and sub-subclasses. For example EC 3.1 is the subclass for esterases. Other hydrolases can act, for example, on ether bonds (EC 3.3) or peptide bonds (EC 3.4; peptidase). A lot of different sub-subclasses of esterases exist. Depending on the ester which is cleaved, it is possible to differentiate between various esterases (carboxylic ester hydrolases, EC 3.1.1; thioester hydrolases, EC 3.1.2; sulforicester hydrolases, EC 3.1.6).

3.2. Enzyme Kinetics

The most common model used to describe enzyme kinetics is the Michaelis-Menten kinetic. The following simplification of an enzymatic reaction is the basis of the Michaelis Menten Equation [11]:

$$S + E \overset{k_m}{\leftrightarrow} [ES] \overset{k_{cat}}{\rightarrow} E + P$$

(S: substrate; E: enzyme; [ES]: enzyme-substrate complex; P: product; k_m: Michaelis constant; k_{cat}: catylytic constant)

Two main simplifications are necessary to set up the Michaelis-Menten-Equation. One assumption is that the first reaction step is in equilibrium, the other one is that the second reaction step is an irreversible process because the product does not bind to the enzyme anymore.

$$v_0 = \frac{v_{max}[S]}{k_m + [S]} \tag{3.1}$$

(v_0: initial velocity; v_{max}: maximum velocity; [S]: substrate concentration; k_m: Michaelis constant, provides information about the enzymes affinity to the substrate)

3.3. Enzyme Acitivity

The SI unit of the enzyme activity is the katal, defined by $1\,\mathrm{katal} = 1\ \mathrm{mol\,s^{-1}}$. The more frequently used one is the enzyme unit U (equation 3.2),

$$1U = 1\ \frac{\mu molsubstrate}{min} \tag{3.2}$$

(1 U = 16.67 nanokatal). In some cases, it is useful to apply specific activities, e.g. U/g.

3.4. Enzyme Assays

The continuously rising number of different enzymes increases the importance of enzyme activity measuring methods. Especially high-throughput screening methods get more and more relevant. An enzyme assay facilitates the measurement of an enzyme-catalyzed reaction in several ways. [22] A lot of these methods are based on a color change or a light signal. Also fluorescence-based assays can be found in relevant literature [23].

The following chapter focuses on two different assay types which are both based on fluorescence.

3.4.1. Fluorogenic Substrates

As previously described (3.1) enzymes catalyze different chemical reactions. For the enzyme class EC 3.1 (esterases) different esters can be used as substrates. In this case the cleavage of an ester bond results in a modification of the spectral properties of a molecule. Figure 3.1 shows fluorescein diacetate (FDA) as one possible substrate for this method. The esterified fluorescein exists as lacton, which does not fluoresce as described in section 2.2.2. On the contrary, fluorescein is a very common fluorescent dye with high quantum yields at proper *pH* values. In a buffered system it is possible to quantify the conversion. An increasing signal indicates an increasing fluorescein concentration. Other possible esteres are, for example, eosin diacetate (EDA), resorufin acetate (RA) or 3-(2-benzoxazolyl)umbelliferyl acetate.

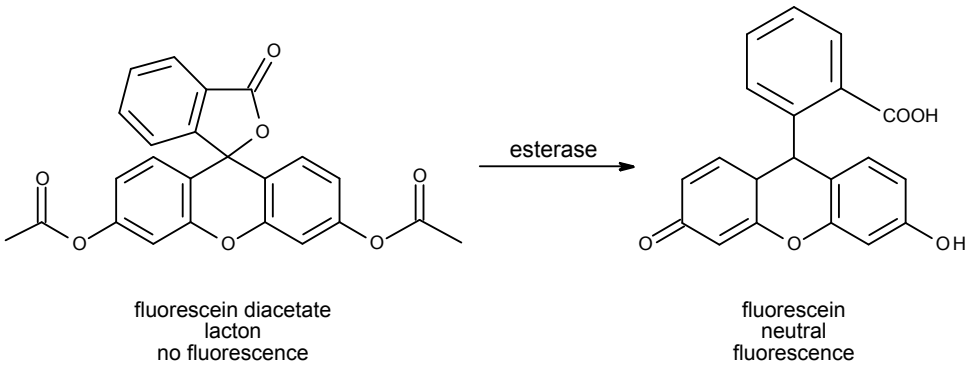

Figure 3.1.: Fluorescein diacetate as substrate for esterases

The main disadvantage of this method obviously is that the activity of the enzymes depends on the substrate. Therefore, it is possible to measure the enzyme activity for one special molecule, but it is not allowed to generalize this activity for other compounds.

3.4.2. *pH*-Sensitive Dyes as Indicators for Enzyme Activity

Using *pH*-indicators is another method to quantify the activity of esterases. On the one hand, it has a more universal applicability because it is possible to characterize the enzyme using the substrate of interest. On the other hand, it is only useful for certain enzyme classes. The essential requirement for enzymes is that the catalyzed reaction has to change the *pH* value. The buffer system is important for this kind of assay. A low buffer capacity has to be used because otherwise too much acid has to be produced by the enzymes to change the *pH* value. The amount of substrate used has to be calculated using equation 2.3.

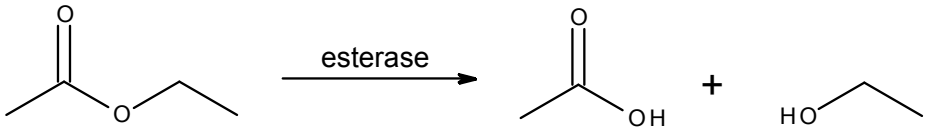

Figure 3.2.: Ethyl acetate as a substrate for esterases.

The conversion of ethyl acetate into acetic acid and ethanol can be visualized using fluorescent *pH*-indicators such as HPTS or fluorescein (section 2.2).

Part III.

Experimental part

4. Experimental Part

4.1. Measurement Set-Up

Unless specified otherwise, the used buffer was a sodium-phosphate-buffer (SPB). Figure 4.1 illustrates the measurement setup inside the F-7000 Fluorimeter (Hitachi) that was used. The main differences to a conventional F-7000 Fluorimeter are an integrated magnetic stirrer and a flow-through-cell. This flow-through-cell coats the cuvette on two sides. Therefore, it is possible to control the temperature inside the cuvette. A small magnetic stir bar enables a continuously mixing of the solution.

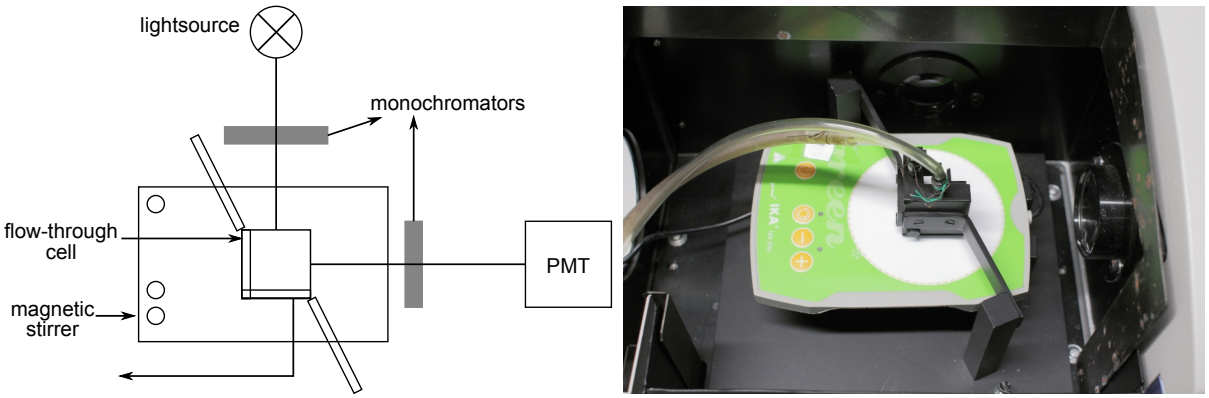

Figure 4.1.: Measurement setup fluorimeter

A high-throughput screening has several advantages. Therefore, a FLUOstar optima microplate-reader (BMG LABTECH, Germany) was used. In figure 4.2 one can see the microplate-reader used for this thesis. The utilization of different microtiter plates (MTP) enables additional changes in the settings. The number of wells defines the number of samples, but the material of the MTP's is also very important. The use of glass MTP's facilitates the use of organic solvents. Figure 4.3 shows the different set-ups for top and bottom measurements.

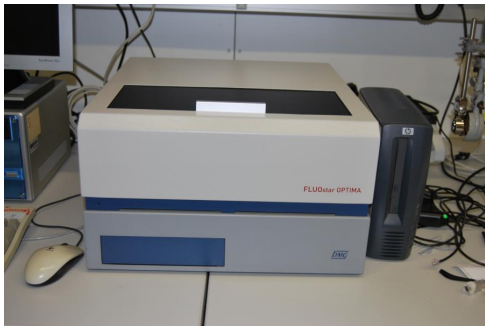

Figure 4.2.: BMG platereader

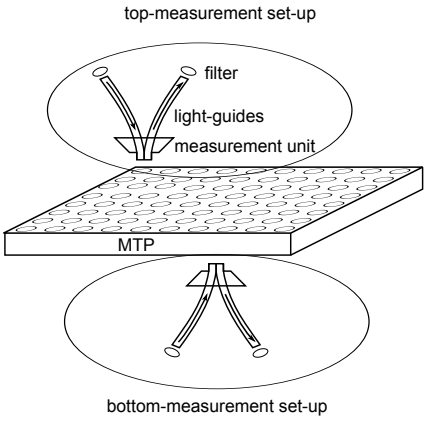

Figure 4.3.: Schematic representation of the top and bottom measurement system

Another possibility to adjust the setup is orbital averaging (figure 4.4). In order to obtain less variations, optimized settings should be used. The radius of the inner circle has an effect on the maximal number of measurements points. A smaller radius results in less possible measurement points. In contrast, too many measurement points can lead to photobleaching. The best results were gained using a scan diameter between 4 and 6 mm. Also the use of appropriate filters is very important.

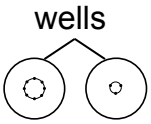

Figure 4.4.: Orbital averaging: the outer circles represent the wells, whereas the inner circles represent the scan diameter

4.2. Chemicals used

Table 4.1.: List of chemicals used

Chemical	Abbr.	Supplier	Comments
CHEMICALS AND SOLVENTS			
5(6)-*N*-Octadecyl-Carboxamidofluorescein	FA		
8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt	HPTS		
8-Hydroxypyrene-1,3,6-trisulfonic acid tritetraoctylammonium	HPTS-TOA		
disodium phosphate		Sigma-Aldrich	
eosin Y		Sigma-Aldrich	
eosin diacetate	EDA	Sigma-Aldrich	
ethanoic acid	acetic acid	Sigma-Aldrich	
ethanol	EtOH	Roth	
fluorescein diacetate	FDA	Sigma-Aldrich	
fluorescein octadecylester	FODE		
fluorescein sodium salt		Sigma-Aldrich	
lipase from *Candida rugosa* - Type VII		Sigma-Aldrich	L1754
lipase immobilized from *Candida antarctica*	CalB	Sígma-Aldrich	73940
methyl-tert.-butylether	MTBE	Sigma-Aldrich	
monosodium phosphate		Sigma-Aldrich	
novozyme lipase 101-109	NZL 101-109	Sandoz	immobilized enzyme kit
sepa-beads EC-EP		Resindion S.l.r.	
propanon	acetone	Roth	
resorufin acetate	RA	Sigma-Aldrich	
resorufin sodium salt		Sigma-Aldrich	
tetraoctylammonium hydroxid	TOA		

4.3. List of Devices used

Table 4.2.: List of devices used

Device	Supplier
FLUOstar optima microplate reader	BMG LABTECH, Germany
BMG 0119 485-P filter	BMG LABTECH, Germany
BMG 0135 520-P filter	BMG LABTECH, Germany
BMG 0142 590-10 filter	BMG LABTECH, Germany
BMG 0207A ABS560 filter	BMG LABTECH, Germany
BMG 0222A ABS492 filter	BMG LABTECH, Germany
MTP 96-well	Greiner Bio-One, Austria
F-7000 fluorimeter	Hitachi, Germany
Cary50 UV-VIS spectrophotometer	Varian, US

4.4. Different Kinds of Beads and Enzymes

4.4.1. Immobilization of a Lipase from *Candida rugosa* on Sepabeads EC-EP

For the immobilization a method was adopted and slightly modified. [24] 75.2 mg enzyme (lipase from *candida rugosa*) were suspended in 15 mL of a 0.75 M sodium phosphate buffer (SPB) (*pH* 7.2). 1.16 g of moist Sepabeads EC-EP were added to this enzyme suspension and incubated at room temperature for 24 hours. During the incubation the reaction vessel was mixed using an end-over-end rotator. The beads with the immobilized enzyme were washed twice with about 50 mL SPB (0.1 M, *pH* 7.3) and resuspended in 20 mL of a 0.1 M SPB, *pH* 7.3. The final concentration of beads was 50 mg/mL.

4.5. Fluorogenic Substrates

Previously described (3.4.1), esters of fluorophores can be used as substrates for esterases activity measurements.

4.5.1. Fluorescein Diacetate

The activities of two different enzymes were investigated within the following conditions:

Enzyme 1: lipase from *Candida antarctica* (CalB) which was already immobilized on beads

Enzyme 2: lipase from *Candida rugosa* which was immobilized according to section 4.4.1

The quantities of SPB (*pH* 7.3, 0.1 M), fluorescein diacetate (dissolved in acetone, stock-solution 1 mM, figure 2.3) and the amounts of beads used are listed in table 4.3. First, the beads were weighed and added. Respectively the Sepabeads suspension (50 mg/mL) was pipeted to a cuvette and covered with buffer. Before starting the reaction by adding the substrate, the solution was preheated to a temperature of 40 °C. During the reaction the temperature was hold constant (40 °C). The reaction solution was stirred with a small magnetic stir bar. The substrate concentration in the solution was 10 μM. A fluorescence spectrometer (Hitachi F-7000, 4.1) was used for the measuring of the fluorescence intensity. The excitation wavelength was 490 nm. The emission at 514 nm was recorded every 7 minutes.

Table 4.3.: Quantities of chemicals used

CalB (mg)	**Buffer, *pH* 7.3, 0.1 M** (μL)	**FDA, 1 mM** (μL)
49.5	1980	20
49.7	1980	20
51.3	1980	20
Sepabeads-suspension 50 mg/mL (*mL*)		
1	980	20
1	980	20
1	980	20

High-Throughput Assay

The following assay consists of two different parts. A very fast 'pre-screening' is followed by a more precise 'main-screening'. The first step is the selection of suitable enzymes (pre-screening). A lot of different enzymes are analyzed at once to visualize whether they have a high activity or not. The enzymes with higher activities are characterized more precisely in the main-screening.

Nine different hydrolases NZL 101-109 were analyzed with this method. The enzymes NZL 101-109 are immobilized on different beads. Information about the beads or the enzymes is not available. The preparation of the samples and the implementation of the assay followed the attached Standard Operating Procedure 1 (SOP1). The method was slightly modified:

Pre-screening: instead of three repetitions per enzyme, only two were done.

About 3 mg of each immobilized enzyme were added into a 96-well-plate (two replications). 195 μL sodium-phosphate-buffer (pH 8.0, 0.1 M) were added to each well. The background-fluorescence was measured using a microplate-reader (BMG FLUOstar OPTIMA, excitation-filter: BMG 0119 485-P; emission-filter: BMG 0135 520-P). 5 μL of a 10 μM FDA-stock solution were added to each well as fast as possible to start the reaction (final concentration of FDA: 250 nM). For general settings of the plate-reader see attached SOP1. The temperature was 37.0 °C $\pm$ 0.2.

Main-screening: Enzyme NZL 104 was investigated

The quantities of applied enzyme for the five point calibration and the enzyme reaction are listed in table 4.4 and 4.5. The concentrations of fluorescein for the calibration are also listed in table 4.4. A 0.1 M sodium-phosphate-buffer, pH 8.0 was added to each well (195 μL each well). After the background fluorescence measurement FDA was added as fast as possible to the reaction wells. The FDA concentration was 150 nM. The temperature during the whole process was 37.0 °C $\pm$ 0.2. The fluorescence intensity was measured with a BMG FLUOstar OPTIMA platereader.

Table 4.4.: Quantities of NZL 104 beads, (mg); different fluorescein concentrations for calibration; 3 repetitions each concentration

	fluorescein concentration (nM)				
	25	50	100	150	200
1	2.3	3,4	2.6	2.7	2.9
2	3.1	2.5	2.4	2.5	2.2
3	2.3	2.6	2.2	2.7	2.4

Table 4.5.: Quantities of NZL 104 beads for the reaction, (mg); 10 replications

	Reaction				
1	2.3	3.1	3.0	2.9	2.9
2	2.6	2.9	3.1	3.6	3.4

4.5.2. Eosin Diacetate (EDA)

Almost the same assay as described in section 4.5.1 can be used for eosin diacetate as a substrate (figure 4.5) The same 9 immobilized enzymes as previously were investigated with this method:

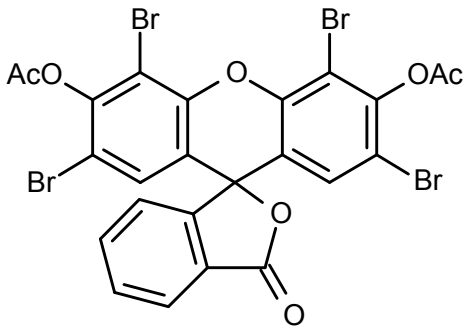

Figure 4.5.: Structure of eosin diacetate

Pre-screening:

Approximately 3 mg of each enzyme were added to a 96 well microtiter plate (2 replications). 185 μL sodium-phosphate-buffer (pH 8.0, 0.1 M) were added to each well. After the background-fluorescence measurement 15 μL of a 250 μM eosin diacetate stock solution (dissolved in acetone) were added to each well.

Main-screening: Enzyme NZL 107 was investigated

Quantities of applied enzyme for the four point calibration and the enzyme reaction are listed in the tables 4.6 and 4.7. The concentrations of eosin for the calibration are also listed in table (4.6). A 0.1,M sodium-phosphate-buffer, pH 8.0 was added to each well (195 μL each well). After the background fluorescence measurement EDA was added as fast as possible to the reaction wells. The EDA concentration was 18.75 μM. The temperature during the whole process was 37.0 °C $\pm$ 0.2. The fluorescence intensity was measured with a BMG FLUOstar OPTIMA plate-reader (filters: excitation: BMG 0135 520-P; emission: BMG 0142 590-10).

Table 4.6.: Quantities of NZL 107 beads, (mg); different eosin concentrations for calibration; 3 repetitions each concentration

	eosin concentration (μM)			
	6.25	12.5	18.75	25
1	2.6	3.1	2.8	2.2
2	2.3	2.4	3.3	2.8
3	2.2	2.6	2.7	2.8

Table 4.7.: Quantities of NZL 107 beads for the reaction, (mg); 10 replications

	Reaction				
1	4.1	4.9	2.5	2.7	2.7
2	3.4	2.2	2.8	2.8	2.6

4.5.3. Resorufin Acetate

Figure 4.6 shows the structure of resorufin acetate.

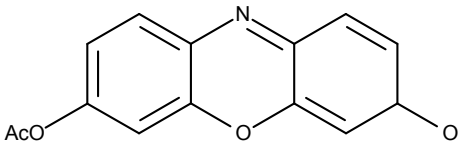

Figure 4.6.: Structure of resorufin acetate

Pre-screening:

About 3 mg of the different enzymes (NZL 101-109) were added into the wells of a 96-well-plate (2 replications). 190 μL sodium-phosphate-buffer (pH 8.0, 0.1 M) were added to each well. The background-fluorescence was measured using a microplate-reader (BMG FLUOstar OPTIMA, excitation-filter: BMG 0207A ABS560; emission-filter: BMG 0142 590-10). 10 μL of a 10 μM resorufin acetate stock solution (dissolved in ethanol) were added to each well as fast as possible to start the reaction (final concentration of resorufin acetate: 500 nM). For general settings of the plate-reader see attached SOP1. The temperature was 37.0,°C $\pm$ 0.2.

4.6. *pH*-Sensitive Dyes as Indicators for Enzyme Activity

In this section a BMG FLUOstar OPTIMA plate-reader was used for the fluorescence measurements. The filters used for fluorescein and HPTS were a BMG 0119 485-P for the excitation and a BMG 0135 520-P filter for the emission.

4.6.1. Fluorescein

Calibration without beads

A 5 mM sodium-phosphate-buffer (*pH* 8.0) was used for the calibration of fluorescein as a *pH*-sensor. Different acetic acid concentrations (table 4.8) were used according to the Henderson-Hasselbalch-equation (2.3). A total volume of 200 μL was applied for this calibration. For the first experiment no immobilized enzymes were added. A 25 mM acetic acid stock-solution was used to achieve the different acid concentrations. The fluorescein concentration was 250 nM.

Table 4.8.: Acetic acid concentrations (mM) for the calibration of fluorescein as a *pH*-sensor

1	2	3	4	5	6	7
0.625	1.25	1.875	2.5	4.37	5	6.25

Calibration with immobilized enzymes

A 10 mM sodium-phosphate buffer, *pH* 8.14, was used for the calibration (total volume 200 μL). For the preparation of the different acid concentrations a 50 mM acetic acid stock solution was used (4-point-calibration: 4.0, 4.5, 5.0, 5.5 mM). The utilized enzyme was the NZL 107 (about 3 mg each well) and was added first to each well of the plate. Different buffer concentrations (5 and 10 mM), fluorescein concentrations (250, 500 and 750 nM) and different temperatures (37°C and room temperature) were investigated (table 4.9).

Table 4.9.: Series of experiments with different parameters

		Temp. (°C)	Acetic acid conc. (μM)	Fluorescein conc. (nM)	SPB pH 8; 5mM (μL)	SPB pH 8; 100mM (μL)	SPB pH 8.14; 10mM (μL)	NZL 101 (mg)	Sepabeads (sec.4.4.1) (mg)
a	a	37	625	250	200	0	0	3	0
	b	37	625	375	200	0	0	3	0
b	a	37	250	250	200	0	0	3	0
	b	37	250	375	200	0	0	3	0
c	a	37	625	250	200	0	0	3	0
	b	37	250	250	200	0	0	3	0
d	a	37	625	250	0	200	0	3	0
	b	37	250	250	0	200	0	3	0
e	a	28.4	3750	250	0	0	200	3	0
	b	37	3750	250	0	0	200	3	0
	c	37	3750	250	0	0	200	0	0
f	a	37	3750	250	0	0	200	0	3
	b	37	3750	250	0	0	200	3	0

4.6.2. HPTS

The first experiment included 200 μL SPB (pH 8.14, 10 mM) and approximately 3 mg NZL 101 beads. The HPTS concentration was 250 nM and the acetic acid concentration was 3.75 mM.

In the further experiments two different enzymes were investigated: NZL 105 and 106. The acetic acid concentrations for the calibration are listed in the following table (4.10). The buffer used was a 10 mM sodium-phosphate buffer at pH 8.14. The HPTS concentration was 2.5 μM. Each well contained about 3 mg of beads (NZL 105 or 106). Ethyl acetate was used as substrate. The concentration of ethyl acetate was 3.75 mM. More details about the precise settings can be found in the appendix SOP2.

Table 4.10.: Acetic acid concentrations (mM) for the calibration of HPTS as a pH-sensor

1	2	3	4
0	1.25	2.50	3.75

4.7. Enzymatic Reactions in Organic Solvents

Methyl *tert*-butyl ether (MTBE) was investigated as an organic solvent. In some cases only MTBE was used, in others sodium phosphate buffer was added.

4.7.1. Fluorescein Octadecylester (FODE)

Fluorescein octadecylester (supplied by the Institute of Analytical Chemistry and Food Chemistry which was prepared according to the literature procedure [25]) was dissolved in MTBE (stock solution 30 mM) and used as a substrate. The enzyme investigated was the NZL 108. The filters used were the same as for the fluorescein diacetate assay (excitation-filter: BMG 0119 485-P; emission-filter: BMG 0135 520-P). The bottom reading mode of the BMG FLUOstar OPTIMA was used for the measurement. Table 4.11 shows the amounts of beads, the concentrations and volumes of the used compounds.

Table 4.11.: FODE as *pH*-sensor

	NZL 108 (mg)	MTBE (μL)	SPB 10 mM *pH* 8.14 (μL)	FODE (mM)
1	0	360	40	0.375
2	≈3	360	40	0.375
3	≈3	360	40	0.750
4	≈3	360	40	1.125

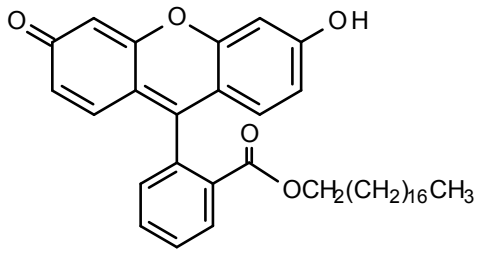

Figure 4.7.: Structure of FODE

4.7.2. 5(6)-*N*-Octadecyl-Carboxamidofluorescein (Fluorescein Amid, FA)

The 5(6)-*N*-octadecyl-carboxamidofluorescein was dissolved in MTBE (stock solution 138 μM) and used as *pH*-indicator. The fluorescein amid was supplied by the Institute of Analytical Chemistry and Food Chemistry which was synthesized according to the literature procedure [25]. Enzyme NZL 108 was analyzed. More details of the used compounds are listed in table 4.12. The following filters were used: excitation filter BMG 0222A ABS492; emission filter BMG 0135 520-P. The top reading mode was used as measuring mode . The acetic acid was added after 74 minutes.

Table 4.12.: FA as *pH*-sensor

	NZL 108 (mg)	**MTBE** (μL)	**SPB 10 mM *pH* 8.14** (μL)	**acetic acid concentration** (mM)	**FA** (nM)
1	≈3	360	40	2.18	3.45
2	≈3	360	40	4.375	3.45
3	≈3	360	40	6.55	3.45
4	≈3	400	0	2.18	3.45
5	≈3	400	0	4.375	3.45
6	≈3	400	0	6.55	3.45

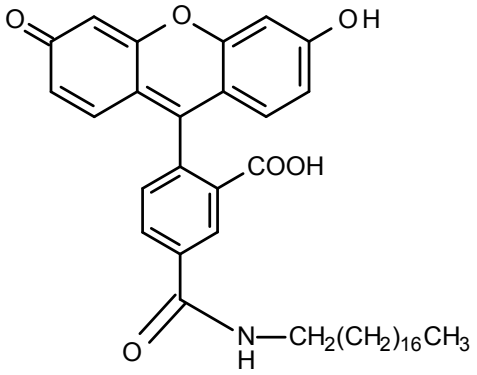

Figure 4.8.: Structure of FA

4.7.3. Fluorescein Diacetate

Two different experiments were done using FDA as substrate. The following tables show the concentrations and volumes of these assays. On the one hand, different enzymes were compared. On the other hand, the enzyme NZL 108 was investigated in more detail. To dissolve the FDA in MTBE, an ultrasonic bath was used for about 20 minutes. The fluorescein was dissolved in acetone.

Table 4.13.: Different enzymes in MTBE and FDA as substrate

Enzyme NZL ≈3 mg	**MTBE** (μL)	**FDA** (μM)
101	400	25
104	400	25
106	400	25
107	400	25
108 (a)	400	25
108 (b)	400	0
no beads	400	25

Table 4.14.: Different enzymes in MTBE and FDA as substrate

Enzyme NZL 108 (mg)	**MTBE** (μL)	**FDA** (mM)	**fluorescein** (μM)
≈3	400	0	0.25
≈3	400	0.25	0

Part IV.

Results and discussion

5. Results

5.1. Introduction

A number of various methods are qualified for enzyme activity measurements, such as radioactive, titrimetric or spectrophotometric methods. These are the most common ones because the instrumentation needed is part of the standard equipment of most biochemical laboratories. Nevertheless, there also occur some problems using these methods, for example the production of radioactive waste or a lack of sensitivity.

p-Nitrophenyl esters can be used as chromogenic substrates for esterases. The conversion can be measured continuously due to the absorption of the reaction product *p*-nitrophenol. Figure 5.1 shows the chemical equation. Such a method is currently used at Sandoz. p-Nitrophenol has an absorption at 410 nm. [1] Different enzyme assays can be found in literature. [26]

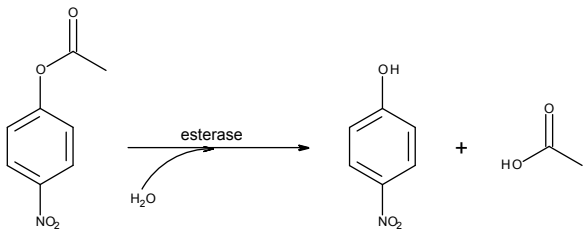

Figure 5.1.: Enzymatic cleavage of p-NPA

Current assay: 30 mg immobilized enzyme is covered with 4.75 mL 50 mM potassium phosphate buffer (pH 8.0). 250 μL 4 mM *p*-NPA are added with stirring and incubated at 25 °C. The substrate concentration in the reaction solution is 0.2 mM. 1 mL of the reaction solution is transfered into a cuvette each minute (until a total reaction time of 6 minutes). Before the absorption is measured, the beads have to settle. After the measurement the whole volume is transfered back to the reaction vessel to minimize the affectation. The use of a calibration curve facilitates the determination of the enzyme activity.

This assay has several disadvantages. The *p*-nitrophenyl acetate only mimic the true substrate of interest. Additionally, parallelization is difficult to achieve.

The use of enzymes immobilized on beads does not only have advantages such as higher stability, easier separation of the reaction broth or reusability, but also severe disadvantages. Handling these beads can be difficult as illustrated in figure 5.2. These beads scatter the light and it is therefore not possible to measure the absorption continuously. It is necessary to separate the beads before a photometric measurement is possible. This could be achieved by filtration or by the removal of a small amount out of the reaction solution and the measurement outside the system. Therefore, a continuous measurement is not possible, the risk of mistakes increases and it is not a quick method after all.

On the contrary the use of fluorimetric assays is advantageous. The two main benefits are the possibility of monitoring directly in the sample and their very high sensitivity. In case of enzymes immobilized on beads the scattering of the beads has a less negative effect. Therefore, these kinds of measuring systems are suitable methods for the aim of this thesis. High-throughput screenings are also possible by using, for example, microplate-readers.

The aim of this master thesis is to develop an assay for the characterization of esterases immobilized on beads. Two different principles are the basis of the experimental part. On the one hand chromogenic substrates, on the other hand fluorescent *pH*-indicators are used for the determination of the activities of esterases.

At the beginning two different enzymes were analyzed. One was bought already immobilized on beads, the other one was immobilized on Sepabeads according to the procedure in section 4.4.1.

The behavior of these two kinds of beads is very different in aqueous solutions (figure 5.2). The behavior of the Novozymes (CalB) varies a lot between the theoretically identical particles. Some of them stick to the surface of the cuvettes, some of them stay on the surface of the liquid and others again sink to the bottom. As there is always a different amount of beads in solution, the light scattering of these beads varies a lot. The attempt to stop stirring before measuring could not improve the results because the beads need a long time to settle. In contrast, the Sepabeads have a more suitable behavior. The dispersion of these beads is homogeneous and if the stirring is turned off, the beads settle within a few seconds. Therefore, it is possible to improve the results by stopping the stirring before the measurement takes place.

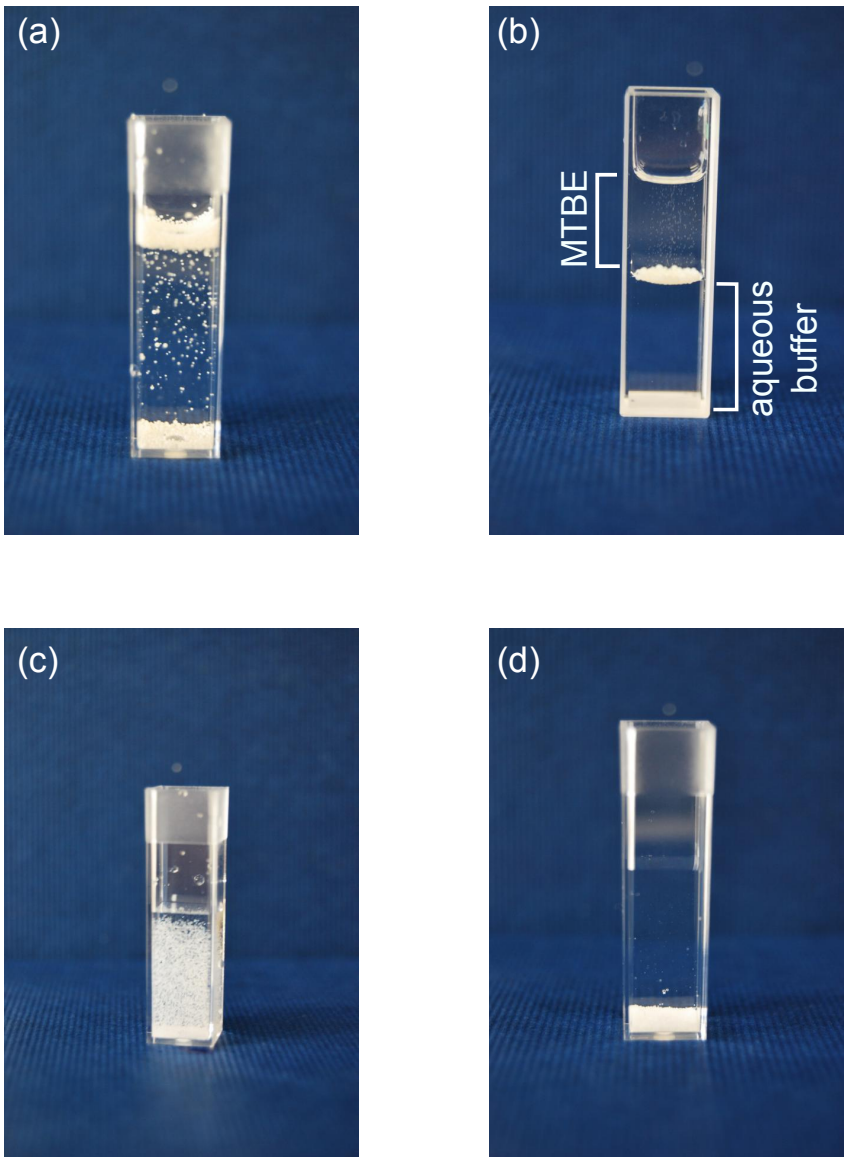

Figure 5.2.: (a) Behavior of Novozymes in aqueous buffered solutions; (b) 3 phase system, MTBE, Novozymes and aqueous buffered phase; (c) Sepabeads stirred in aqueous buffered solution, (d) settled Sepabeads in aqueous buffered solution (after a few seconds)

Three different substrates were investigated: fluorescein diacetate, eosin diacetate and resorufin acetate (figure 5.3). These three substrates have different pk_a-values, prices and excitation/emission wavelengths (table 5.1).

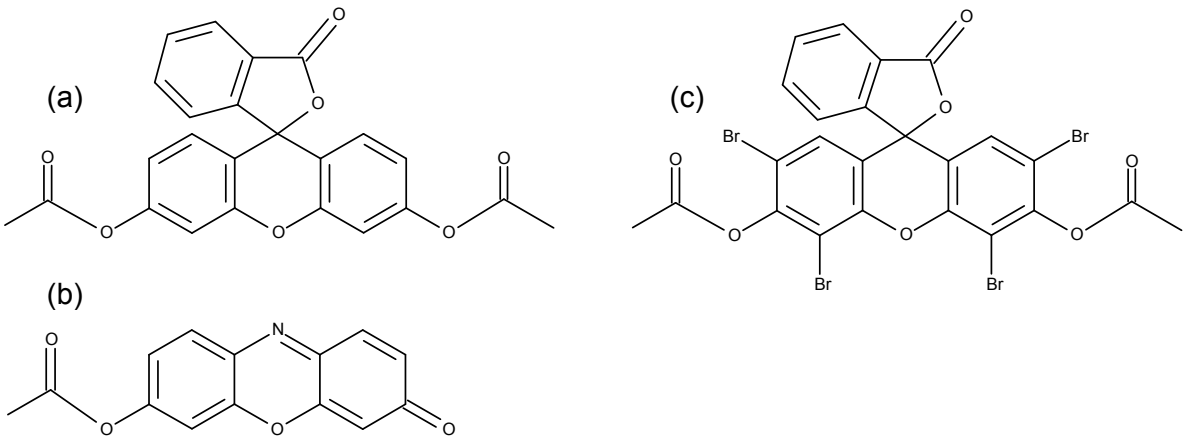

Figure 5.3.: Structural formulas of the three substrates: a) fluorescein diacetate; b) resorufin acetate; c) eosin diacetate

Table 5.1.: Different parameters of the substrates used respectively of the fluorophores after the enzymatic ester cleavage

	pk_a	price (€/g)	excitation WL (nm)	emission WL (nm)
fluorescein diacetate	6.4	≈6	490	514
eosin diacetate	4.2	≈82	524	546
resorufin acetate	5.5	≈2800	572	583

The first substrate selected for this thesis was fluorescein diacetate. FDA has several advantages. Fluorescein has a very high quantum yield and is a widely used fluorescent dye. Therefore, it is well investigated, the price is low and it is established in routine analyses. Another advantage is, for example, the high pH-range.

Both enzymes mentioned above were analyzed according to the procedure in section 4.5.1. Figure 5.4 shows the conversion of FDA into fluorescein. The mixtures were both stirred with a magnetic stirrer and the temperature was kept constant during the whole reaction. The same amount of FDA without enzymes in solution resulted in a low signal. The difference in intensity between the two enzymes is caused by the different behavior of the

beads in aqueous solutions. Whereas the Sepabeads form a homogeneous dispersion with almost all particles in the region of the light beam, a lot of particles of the CalB act as discussed above.

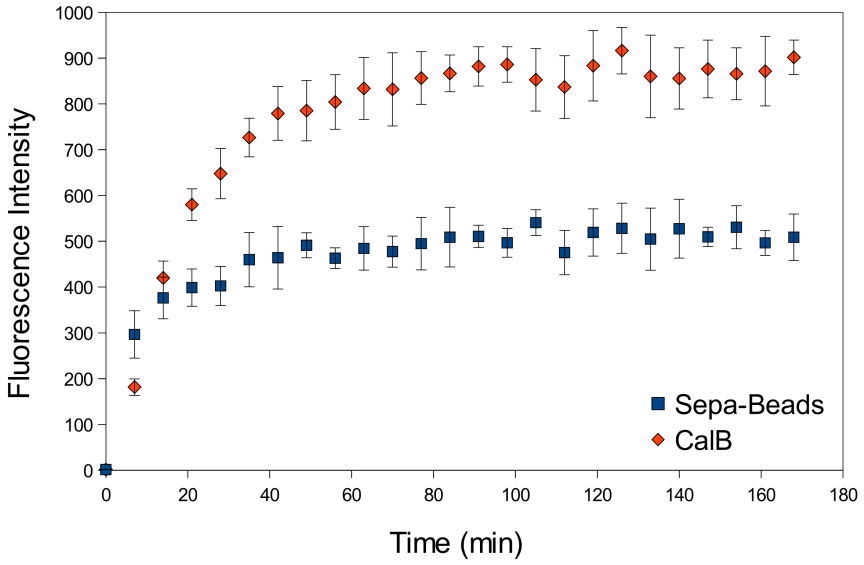

Figure 5.4.: Comparison of different enzymes and different beads. As substrate FDA (10 μM) was used. The excitation wavelength was set to 490 nm. The emission at 514 nm was recorded every 7 minutes.

This experiment showed that it is possible to visualize the conversion of FDA into fluorescein, but also pointed out some disadvantages. The quantification of fluorescein in the presence of beads is difficult. However, the use of beads, for example Sepabeads, that settle within a few seconds would enable the quantification of the conversion.

These different kinds of beads result in various problems. Consequently, 9 different enzymes directly obtained from Sandoz (Kundl, Austria) were used for the rest of this thesis to simulate the situation in their laboratory. These 9 enzymes were already immobilized on beads. The behavior of these different beads were similar to the beads on which the *CalB* was immobilized. Therefore, it was not possible to use the first method for the quantification of the conversion. Furthermore, using the previous method is time-consuming as only one sample can be analyzed at the same time. Since Sandoz purchased these particles themselves, no detailed information about these beads, e.g. the surface, were available.

5.2. High-Throughput Assay

5.2.1. Use of Esters of Fluorophors as Substrate

The first experiment showed that it is possible to visualize the conversion of FDA using a fluorescence spectrometer. For the implementation of a high-throughput assay the use of a microplate-reader is advantageous. The main benefit is the high number of wells that can be measured simultaneously. Another positive effect is the smaller volume which has to be used. This results in smaller amounts of chemicals and therefore a lower price. The possibility to measure from the top is very important for this kind of measuring system. The measurement with a bottom fiber optic system would arise problems because the beads settle on the bottom.

The number of available enzymes is permanently increasing. Hence, a fast method to screen this huge variety is needed. This section describes a high-throughput assay based on the enzymatic ester cleavage of an esterified fluorophore. The assay is divided into two parts: the pre-screening and the main-screening.

Pre-screening assay - selection of the best enzymes

The purpose of this screening is to eliminate the less effective enzymes in a short time. About 30 different enzymes can be screened at once using a microtiter plate with 96 wells. 9 different enzymes, all immobilized on different beads, were investigated (NZL 101-109). Figure 5.5 shows the different conversions of FDA into fluorescein. As shown in the figure 5.5, different enzymes gain different fluorescence intensities. In principle, a higher signal indicates more converted substrate. The absolute intensity is not significant, as different beads show diverse self-fluorescences. Therefore, the most important value is the slope at the beginning of the graphs. The slope indicates the activity of the enzyme. Nevertheless, an enzyme with no signal should be either investigated again or should be excluded for the main-screening.

Another important parameter is the validation by the standard deviation. In this case only two replications of each enzyme have been done. Therefore, the standard deviation could not be calculated. Instead, error bars were used to evaluate the variance. The following figures show the error bars of 8 enzymes. Figure 5.6 shows the 4 enzymes with low deviation, figure 5.7 the 4 enzymes with high deviation. The two replications of the enzyme NZL 102 differed widely from each other. Therefore, this enzyme was not evaluated. One reason for this problem could be a higher concentration of FDA in one of the wells.

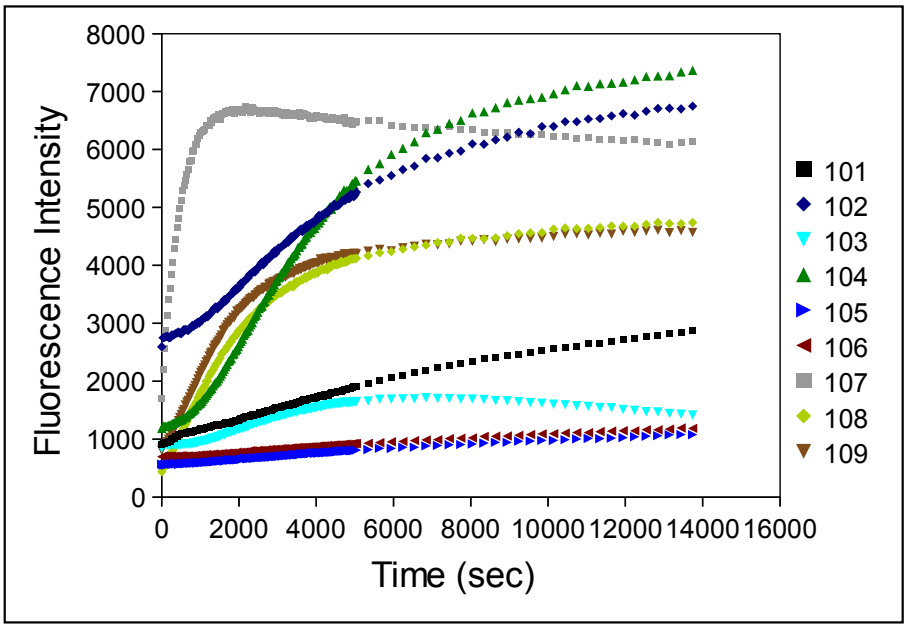

Figure 5.5.: Course of enzymatic conversion of FDA into fluorescein by the enzymes NZL 101-109

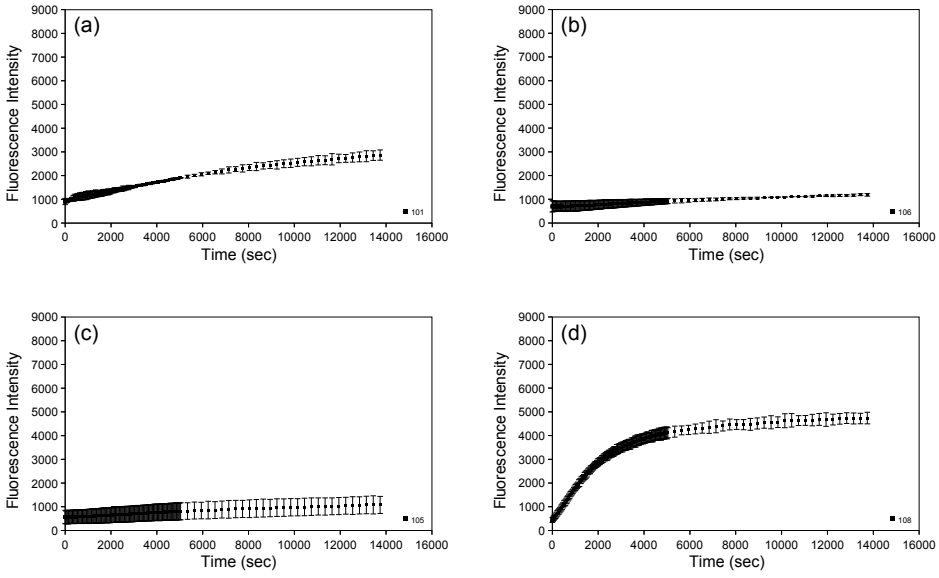

Figure 5.6.: Course of enzymatic conversion of FDA into fluorescein by the enzymes (a) NZL 101, (b) NZL 106, (c) NZL 105 and (d) NZL 108

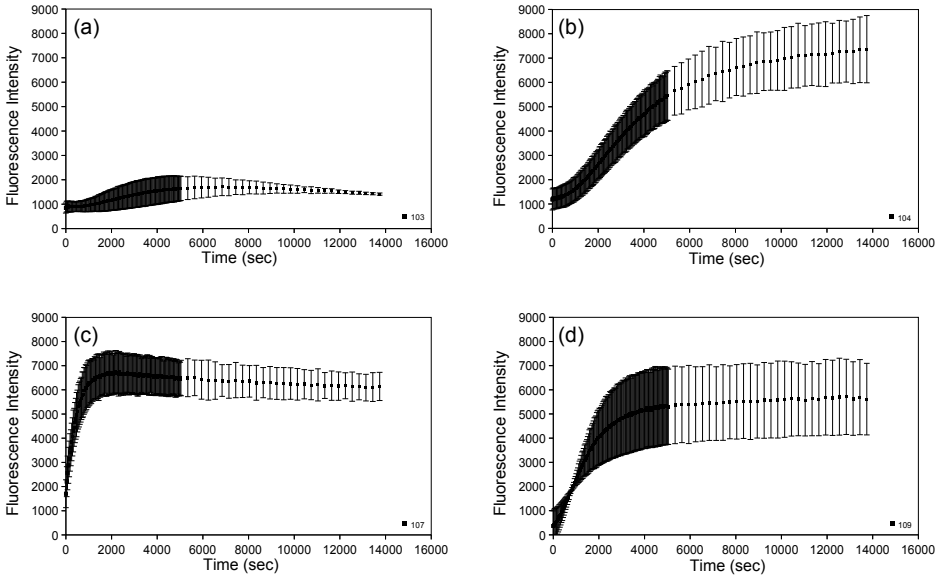

Figure 5.7.: Course of enzymatic conversion of FDA into fluorescein by the enzymes (a) NZL 103, (b) NZL 104, (c) NZL 107 and (d) NZL 109

Although some enzymes have a high deviation, in most of the cases the shapes of the curves are similar between the two replications. The curve of the NZL 109 enzyme is the only exception. The results of this enzyme are not convincing. To improve the results more replications are necessary. All things considered, this method is useful to differentiate between various enzymes according to their enzyme activities. The most promising ones are investigated in more detail using the main-screening.

As previously mentioned, two other substrates were investigated. The figures 5.8 and 5.9 show the results of the pre-screening with EDA and RA as substrates. Other enzyme assays using these substrates can be found in literature (e.g. [27]).

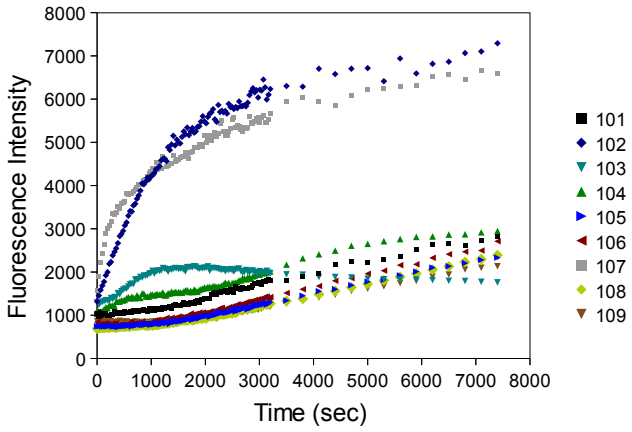

Figure 5.8.: Course of enzymatic conversion of EDA into eosin by the enzymes NZL 101-109

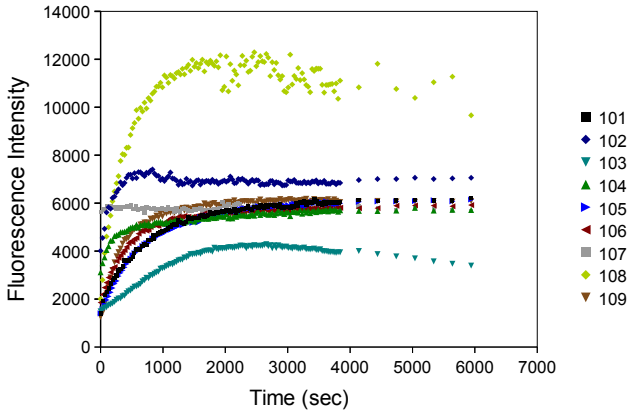

Figure 5.9.: Course of enzymatic conversion of RA into resorufin by the enzymes NZL 101-109

Main-screening assay - characterization of the best enzymes

According to the pre-screening, where FDA was used as the substrate, the enzyme NZL 107 has the highest activity. Other enzymes, for example NZL 104 and 108, also gained reasonable activities. In contrast, the enzymes NZL 101, 103, 105 and 106 seemed to be impractical for this conversion. For this thesis the enzyme NZL 104 was chosen to be further investigated.

All enzymes show different levels of auto-fluorescence. Therefore, a universal calibration is not possible. Every kind of bead needs a separate calibration. This is another reason why the selection of enzymes in the pre-screening is very important. Making a calibration with each kind of beads is rather time-consuming. In the case of fluorescein diacetate a 5 point calibration was used for the quantification of the conversion. The following 5 fluorescein concentrations were used for this calibration: 25, 50, 100, 150 and 200 nM (3 replications each concentration). In order to get a good correlation coefficient (R^2) the value at 100 nM was declared an outlier. In figure 5.10 one can see the calibration with beads (NZL 104). The correlation coefficient is very good (R^2=0.999).

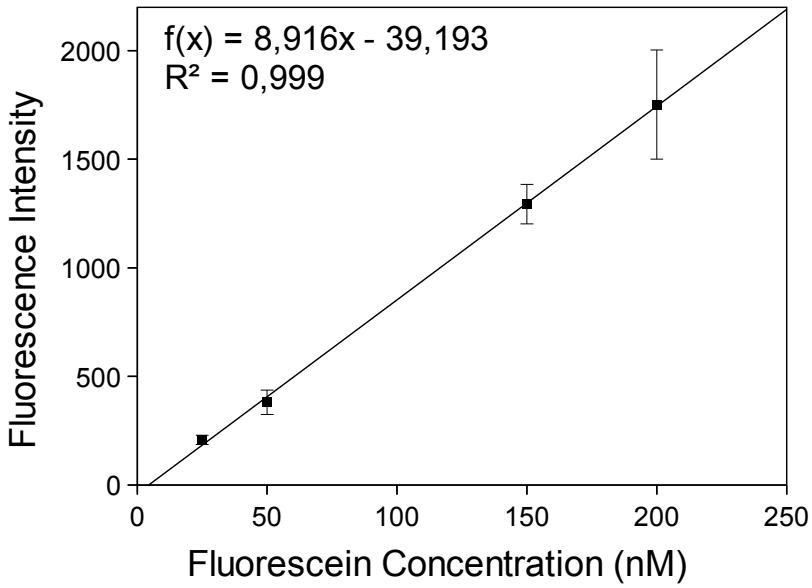

Figure 5.10.: Calibration curve for the enzymatic conversion of FDA into fluorescein by the enzyme NZL 104

For the determination of the activity of the enzyme NZL 104 10 replications were done. As the beginning of the conversion is more important, the concentration of FDA used was located at the head of the calibration curve (150 nM). The figure 5.11 shows the conversion of FDA using the calibration mentioned above. According to figure 5.11, the enzyme NZL 104 converts the total amount of the FDA used.

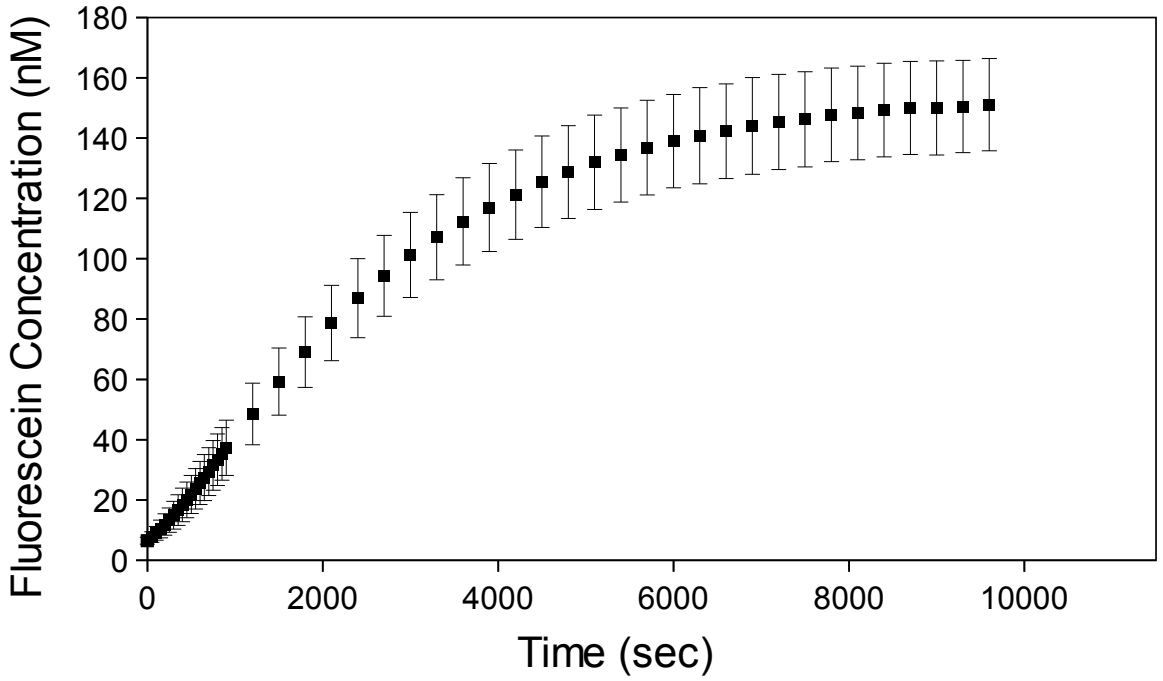

Figure 5.11.: Enzymatic conversion of FDA into fluorescein by the enzyme NZL 104

The following equation (5.1) illustrates how the amount of beads used and the self-fluorescences of the beads were considered (x... measured intensity; x_0... intensity of beads in buffer; m... amount beads (mg)).

$$y = \frac{x - x_0}{m} \tag{5.1}$$

Therefore, the graph in figure 5.11 is normalized to 1 mg of beads. The activity of the enzyme NZL 104 was calculated using the slope of the linear area at the beginning of the graph in figure 5.11. The slope is $0.04\,nM/s$ and as the substrate contains two esters the resulting activity is **0.96 mU/g**. The rather low activity can be a result of the low FDA concentration used in this assay. A higher fluorescein concentration can solve this problem but other problems possibly occur. A high concentration of fluorescein can lead

to a non linear behavior of the curve due to adsorption of fluorescein on the surface of the beads. Figure 5.12 shows different FDA concentrations and the resulting conversions. As one can see from the graph, too low concentrations of FDA affect the slope at the beginning. According to figure 5.12, the slope at the beginning stays constant when using 15 μM or more per 50 mg applied beads. Hence, for the second substrate (eosin diacetate) the concentration was adjusted to higher values.

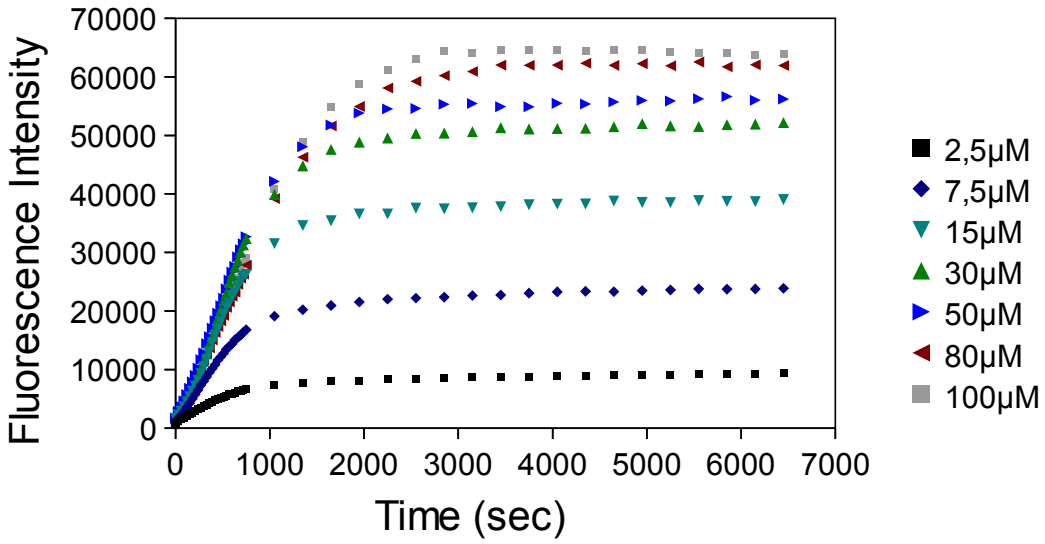

Figure 5.12.: Use of different FDA concentrations (2.5 - 100 μM); 50 mg Sepa-beads (section 4.4.1); 2 mL SPB (pH 7.3; 0.1 M); temperature: 37 °C; excitation-filter: 0119 485p; emission-filter: 0135 520

The enzyme NZL 107 was also investigated using this main screening but, instead of FDA, eosin diacetate was used as the substrate. The calibration curve is shown in figure 5.13. Figure 5.14 displays the conversion of eosin diacetate into eosin. The slope of the linear area at the beginning of the graph was used for the activity determination of the enzyme NZL 107. The applied amount of beads and the background fluorescence were again considered for the calculation of the activity (according to equation 5.1). The slope amounted to 0.068 μM/s. Hence, the activity was **1.6 mU/mg**. The assay used at Sandoz is designed to have 0.5-1.5 mU/assay, thus the calculated activities are in the same range.

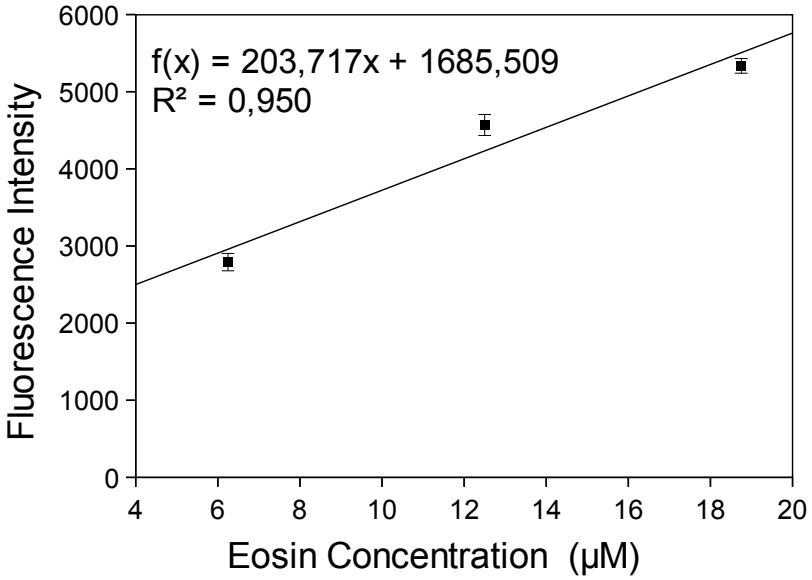

Figure 5.13.: Calibration curve for the enzymatic conversion of EDA into eosin by the enzyme NZL 107

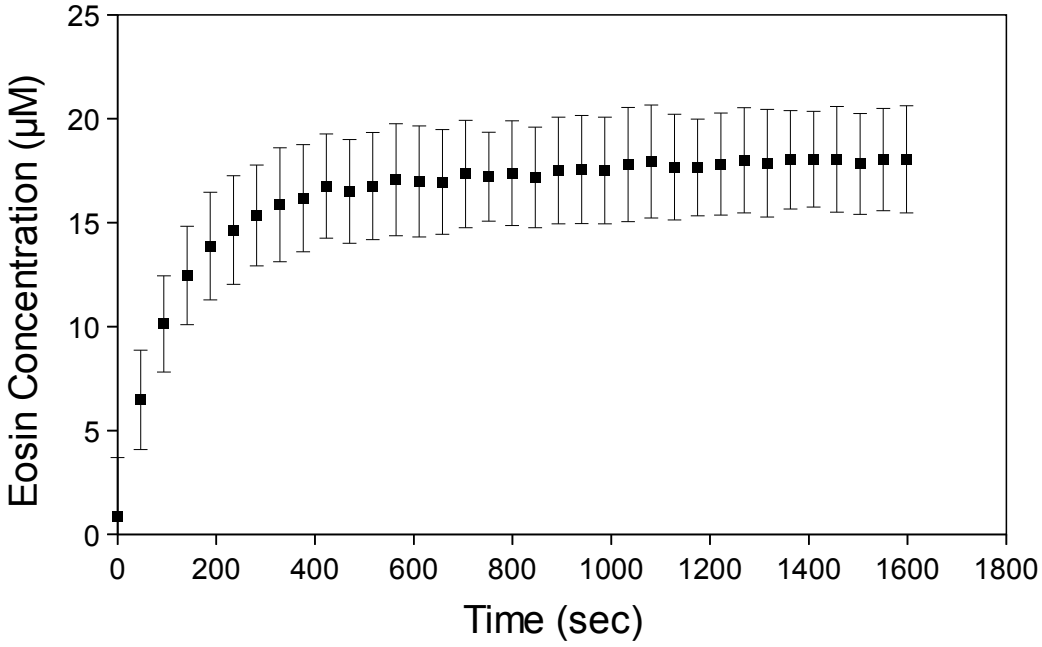

Figure 5.14.: Enzymatic conversion of EDA into eosin by the enzyme NZL 107

5.3. Use of *pH*-Indicators in Aqueous Buffered Solutions for Tracing Activities of Esterases

5.3.1. Fluorescein as *pH*-Indicator

The first *pH*-indicator examined in this thesis was fluorescein (section 2.2.2). The area from minus one to plus one around the pK_a-value of a buffer and indicator is linear. [28] Therefore, only a certain area is defined, were a linear dependence is provided (figure 5.15). In the case of fluorescein ($pK_a = 6.4$) and SPB ($pK_a = 7.2$) this linear area reaches from *pH* 6.2 to 7.4 (figure 5.16). The concentrations of the buffer and the *pH*-indicator are also very important. The sensitivity of the assay increases if the buffer has a low concentration and/or the concentration of *pH*-indicator is high. As high concentrations of indicator can lead to adsorption at the surface of the beads, a 5 mM SPB was used at the beginning. [1]

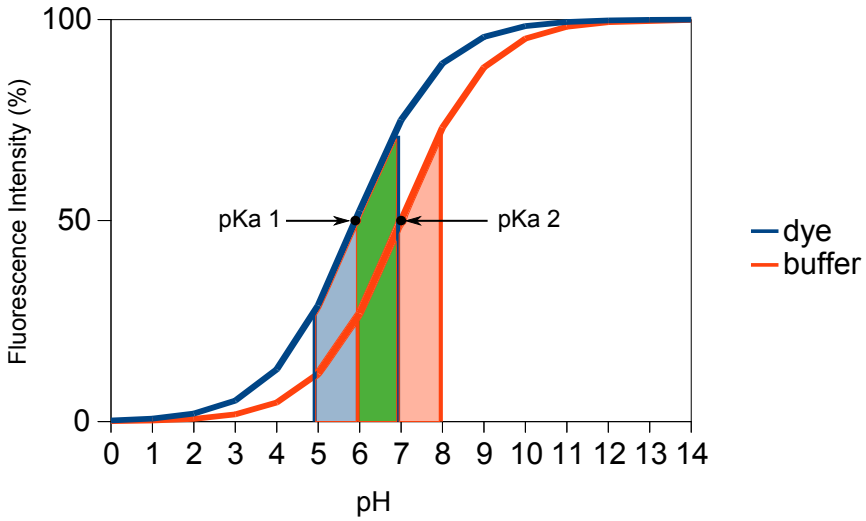

Figure 5.15.: Schematic representation of the *pH*-indicator system. The green area represents the theoretical overlapping linear area of two compounds with different pK_a-values.

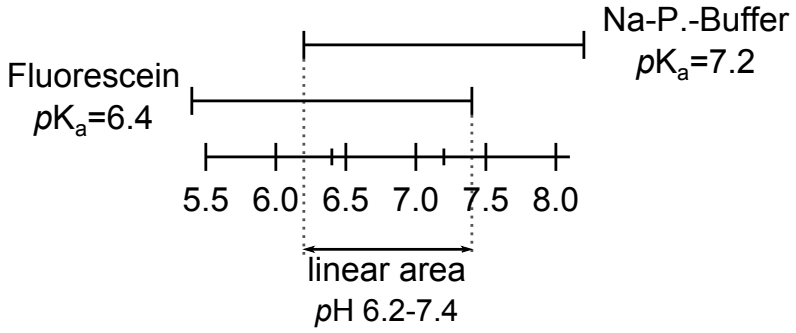

Figure 5.16.: Linear range of fluorescein and SPB

The acid concentrations for the calibration were calculated according to the Henderson-Hasselbalch equation (equation 2.3). At first a calibration without beads was made. In the graph shown in figure 5.17 one can see the expected sigmoidal behavior of the curve. For the calibration only a part of the linear area in the middle of the curve was used. The linear regression of this area is visualized in figure 5.18. A very good correlation coefficient was achieved by this linear regression.

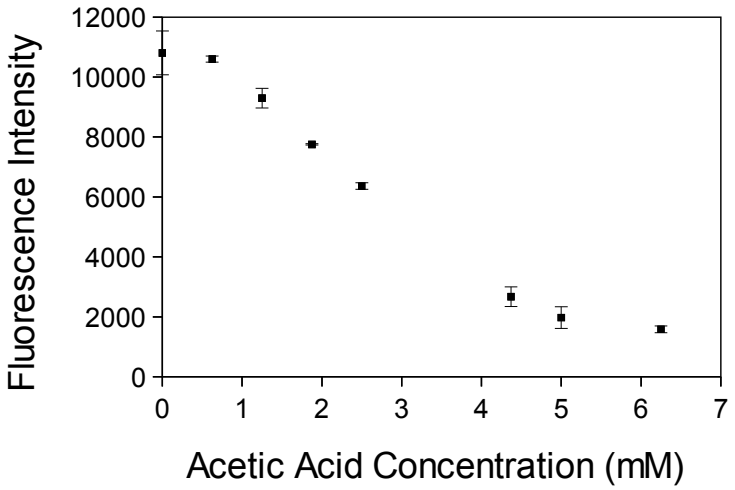

Figure 5.17.: Fluorescein as *p*H-indicator; calibration without beads; fluorescein-concentration: 250 nM; SPB: 200 μL *p*H 8.14, 10 mM;

The calibration without beads worked properly. However, the same experiment with beads failed. As figure 5.19 shows, the fluorescence intensities decrease over time. Different experiments were done to examine this phenomenon.

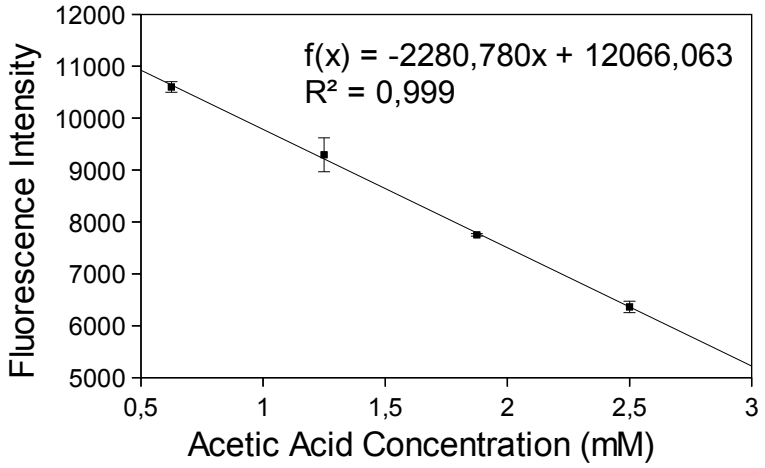

Figure 5.18.: Linear range of the curve in graph 5.17

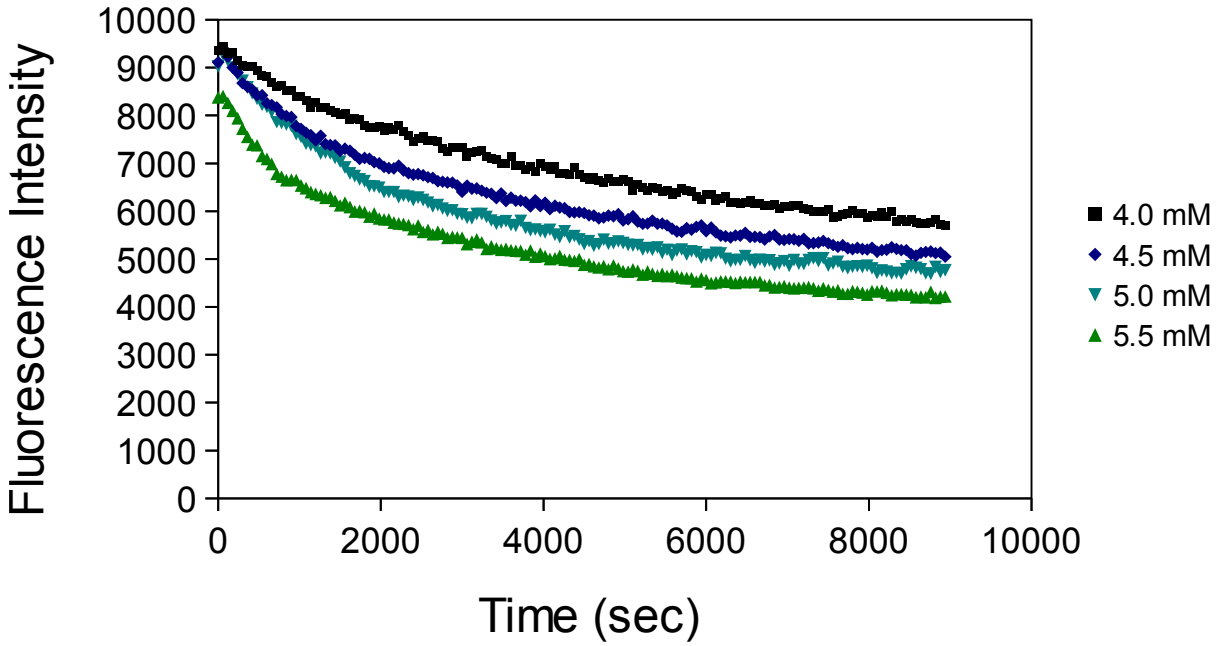

Figure 5.19.: Fluorescein as *p*H-Indicator; enzyme: NZL 107; fluorescein-concentration: 250 nM; SPB: 200 μL *p*H 8.14, 10 mM; different acetic acid concentrations (4.0, 4.5, 5.0, 5.5 mM)

In figure 5.20 6 diagrams (a-f) of different experiments are shown. The conditions for the experiments are listed in table 4.9. Unless otherwise stated the used enzyme was the NZL 101.

The first two graphs ((a) and (b)) show the difference between varying fluorescein and acetic acid concentrations, with 0.625 mM used for the diagram (a) and 0.25 mM acetic acid used for the diagram (b). Whether a lower or a higher acid concentration was used, it always showed the same result. A higher fluorescein concentration increased the signal but the behavior of the curve did not change. The fluorescence intensity still decreased over time.

The diagrams (c) and (d) compare two different buffers. In diagram (c) a 5 mM SPB was used, whereby in diagram (d) a 100 mM SPB was used. Respectively two different acid concentrations (0.625 mM and 0.25mM) were examined. Expectedly, in both diagrams the higher acid concentrations gained the lower intensities. The use of a buffer with a higher capacity changed the behavior of the curve. The decrease of the signal was less marked but still too sharp for an application.

Temperature can influence the fluorescence intensities. Diagram (e) shows the different behavior at two temperatures. Graph a (in diagram (e)) was recorded at room temperature (28.4 °C), graph b at 37 °C. Both intensities decreased again over time. Only if no beads were added (graph c), the signal stayed constant over time.

The last diagram (f) compares two different kinds of beads. The beads used in graph a were Sepa-beads EC-EP with immobilized lipase according to section 4.4.1, whereas in graph b the so far used NZL 101 beads were applied. The beginning of the curve shows the signal without acid in the solution where the intensity stayed constant for both kinds of beads. After 480 seconds the acid was added and immediately both intensities decreased to a lower value. After the addition of acetic acid the signal of the Sepa-beads stayed nearly constant. In contrast, the signal of the NZL 101 decreased by half over time.

The experiments show that temperature, buffer-, acid- and indicator-concentrations do not influence the decrease of the signal. The use of other kinds of beads can solve the problem. Hence, it is possible that the surface of the beads is responsible for this phenomenon. The acid can maybe change the properties of the surface of the beads and therefore, the fluorescein can be adsorbed.

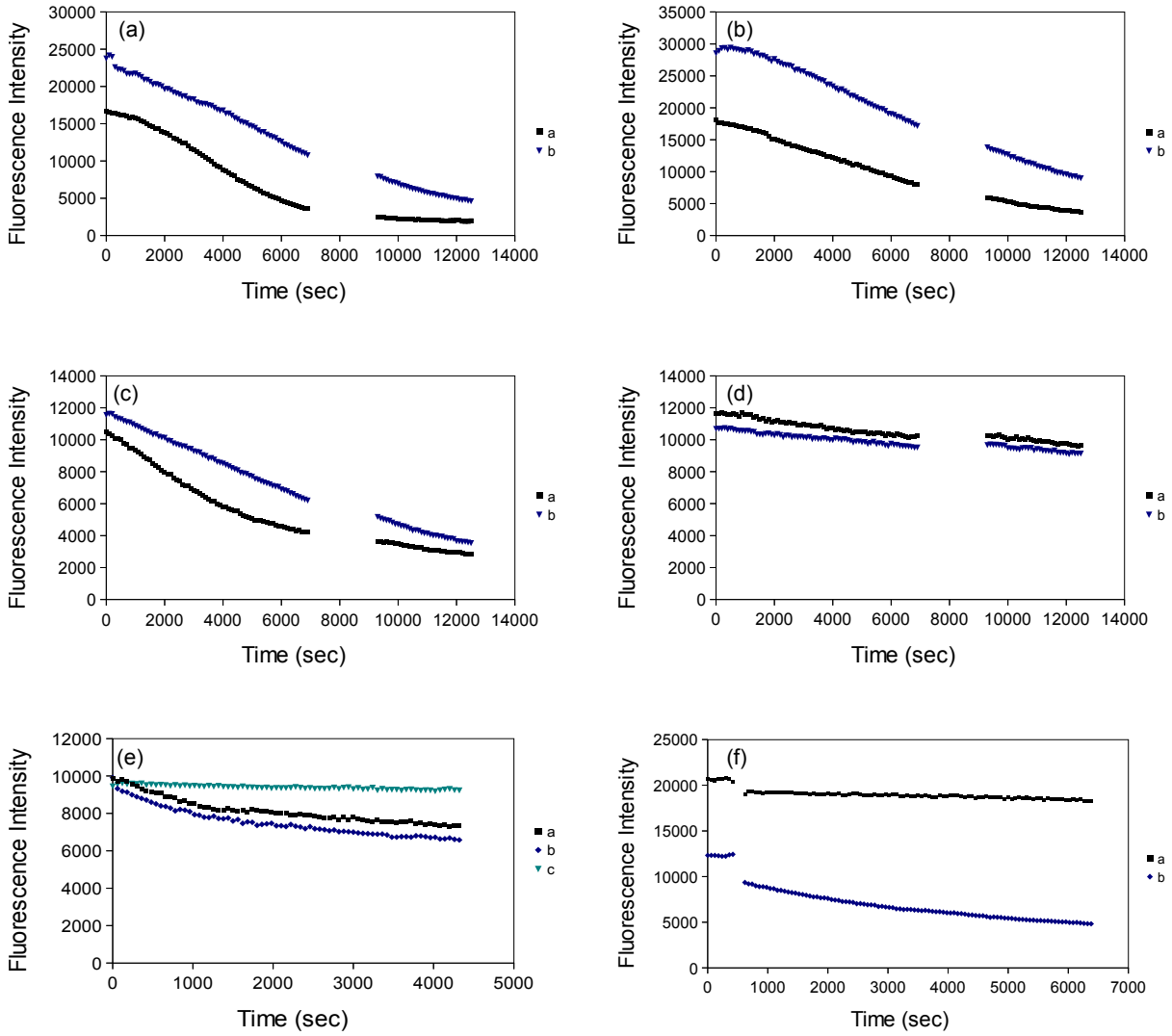

Figure 5.20.: Graphs (a) and (b) show different fluorescein and acetic acid concentrations; graphs (c) and (d) show the influence of different buffer concentrations; graph (e) shows the temperature influence; graph (f) compares different kinds of beads

5.3.2. HPTS as *pH*-Indicator

It was not possible to gain a constant signal for the beads applied by Sandoz. Therefore, another *pH*-indicator, HPTS, was investigated.

8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS) has an pK_a value of 7.3. The difference between the pK_a values of the indicator and the buffer is very important. The closer the pK_a values are, the bigger is the linear area (figure 5.21). [28] Therefore, HPTS is even more appropriate than fluorescein. HPTS has 3 negative charges which lower the adsorption tendency on the beads. It has also a good solubility in water and is inexpensive.

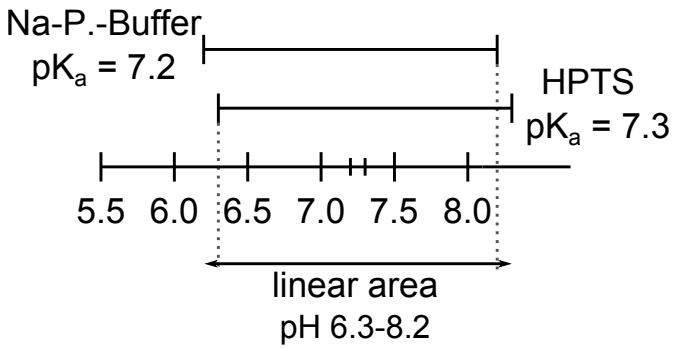

Figure 5.21.: Linear range of HPTS and SPB

Graph (a) in figure 5.22 shows the *pH*-change calculated according to the Henderson-Hasselbalch-equation (equation 2.3). The second graph (b) visualizes the change of fluorescence intensity using the same acetic acid concentrations as for the theoretical curve. The measured behavior of the curve does not correlate completely with the theoretical curve. The beginning of the graph is still linear. Therefore, the concentrations for the calibrations for the further experiments are located at lower values (1-4 mM) to ensure a linear behavior.

The experiment with fluorescein as *pH*-indicator displayed problems with the combination of buffer, beads, indicator and acid. No constant signal could be achieved. Hence, HPTS was used to avoid the previous problem. Figure 5.23 shows that the signal only decreases slightly at the beginning of the graph and stays constant for the rest of the time. Therefore, this indicator was further investigated.

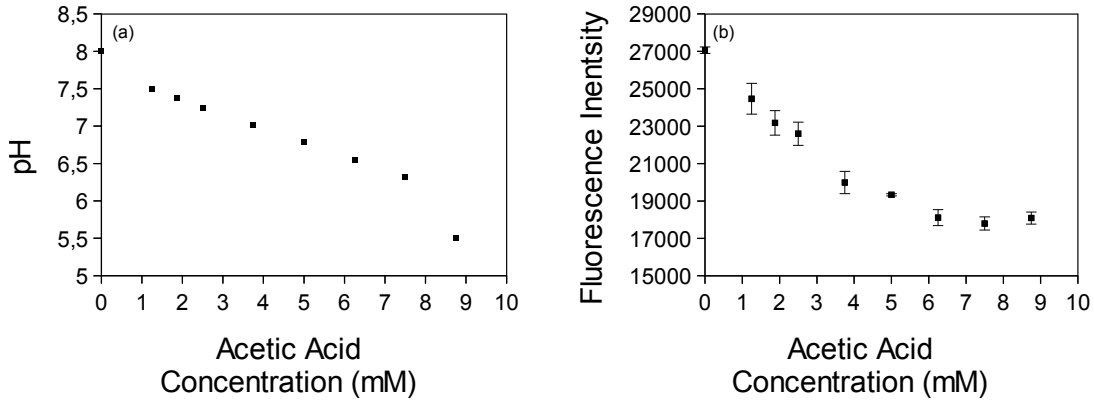

Figure 5.22.: (a) Theoretical dependence of the *pH* as a function of different acid concentrations; (b) Decrease of fluorescence intensities as a function of different acid concentrations

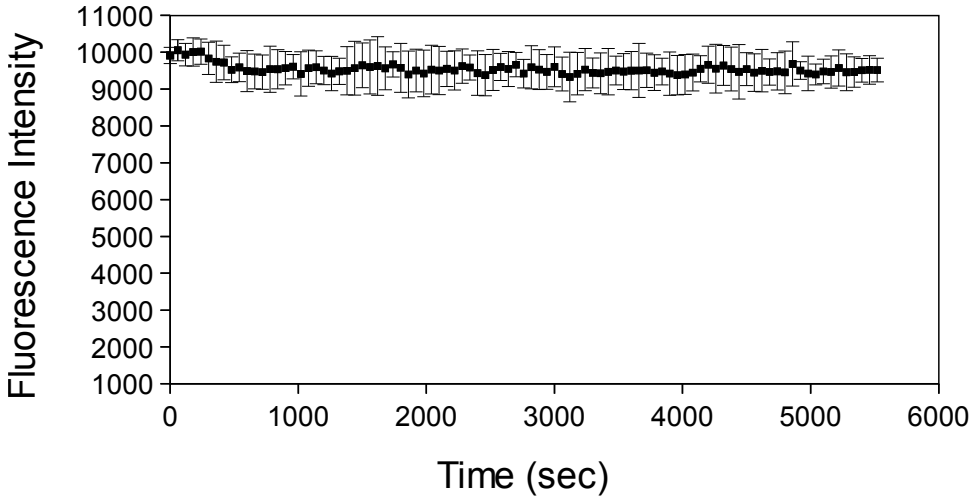

Figure 5.23.: Fluorescence intensity signal of HPTS over time

Two enzymes, the NZL 105 and 106 were analyzed in more detail. For both of the enzymes a calibration curve was recorded (Figure 5.24 (a) and (c)). The used substrate was ethyl acetate (3.75 mM). The conversions are shown in figure 5.24 (b) and (d). Using the slopes at the beginning of the curves it is possible to calculate the activities of the enzymes. As the exact weights of the beads were not determined, the activities are not accurate. The amount of beads used was approximately 3 mg each well and the consequential activities are as follows: **NZL 105 $\approx$ 2.2 mU/mg; NZL 106 $\approx$ 2.8 mU/mg**. It was not possible to verify the complete conversion of the applied substrate. Different enzymes have different *pH* and temperature optima. One possible reason could be an inactivation of the enzymes due to the higher acid concentration. Another explanation could be the released ethanol. As ethanol can act as a weak acid, the *pH*-value could decrease more than expected.

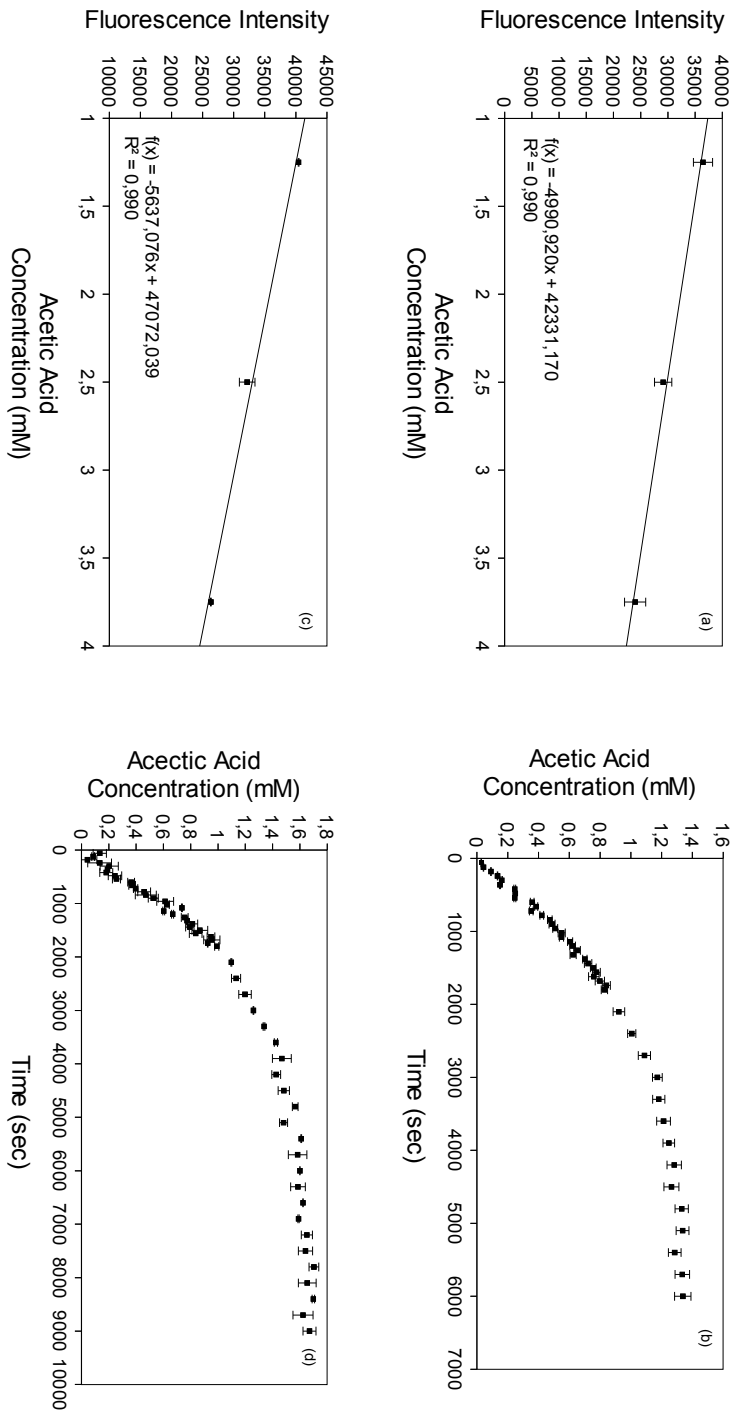

Figure 5.24.: HPTS as *pH*-indicator; (a) calibration curve using acetic acid and the enzyme NZL 105; (b) enzymatic conversion of ethyl acetate into acetic acid by the enzyme enzyme NZL 105; (c) calibration curve using acetic acid and the enzyme NZL 106; (d) enzymatic conversion of ethyl acetate into acetic acid by the enzyme enzyme NZL 106

5.4. Organic Solvents

Enzymatic reactions are not only carried out in aqueous phases, but also in organic solvents. Consequently, this section deals with the use of methyl *tert*-butyl ether (MTBE) as a solvent. The use of organic solvents disables the possibility of using MTPs made of polyethylene (PE) because PE is not resistant to organic solvents. Polypropylene is more resistant but problems still arise when the MTPs are used several times. Therefore, a MTP made of glass was used. MTBE is an ethereal compound and hence, it is important to find an appropriate way to close the MTP. Object slides were fixed with adhesive tape to reduce the evaporation of the MTBE.

The enzymatic cleavage of an ester needs small amounts of water. Hence, a three phase system using buffer/water, MTBE and beads was used. Depending on the quantity of water used in the experiments, different phenomenons can occur. Small amounts of water can form drops and in further consequence stick to the walls of the wells or settle to the bottom. To cover the whole bottom of a well (96-well MTP) about 10-15 percent (about 40 μL) of the whole volume has to be water. Otherwise, it is not guaranteed that all of the bottom is covered. It was desired that the beads stay between the organic and the aqueous phase. Therefore, the choice of indicator and the position of the fiber optics (top or bottom measurement) is very important. Different solution approaches can be used for these assays. A *pH*-indicator can be dissolved whether in the organic or in the aqueous phase. Another approach is the use of an esterified fluorophore which is dissolved in the organic phase. After the enzymatic cleavage of the esters the fluorophore is less lipophilic and is transfered into the aqueous phase. A fluorescence intensity increase in the aqueous phase can be visualized.

As it was possible to measure the conversion of ethyl acetate using HPTS as a *pH*-sensor in aqueous buffered solutions, the first attempt was to use a derivative which is solvable in organic solvents. Therefore, a lipophilic HPTS-*tetraoctylammonium* (TOA) ion pair was investigated (Figure 5.25). The supplied ion pair was synthesized according to the procedure described in [29].

Different HPTS-TOA ratios as well as different water-MTBE ratios were investigated but always resulted in the same problem. The fluorescence signal decreased over time. HPTS-TOA can act as a CO_2-'sensor' [29]. Therefore, one possible reason for the decrease could be the atmospheric carbon dioxide. Only the decrease in fluorescent intensity caused by a pH change is triggered by a shift in the absorption spectra. Therefore, it can be differentiated between a decreasing fluorescence intensity due to *pH*-changes and other phenomenons, e.g. adsorption. In the latter case the maximum of the absorption spectra does not shift from 455 nm to about 405 nm. In fact, only the absorption at 455 nm decreases. Hence, the decrease of the emission signal in this experiment was not the result

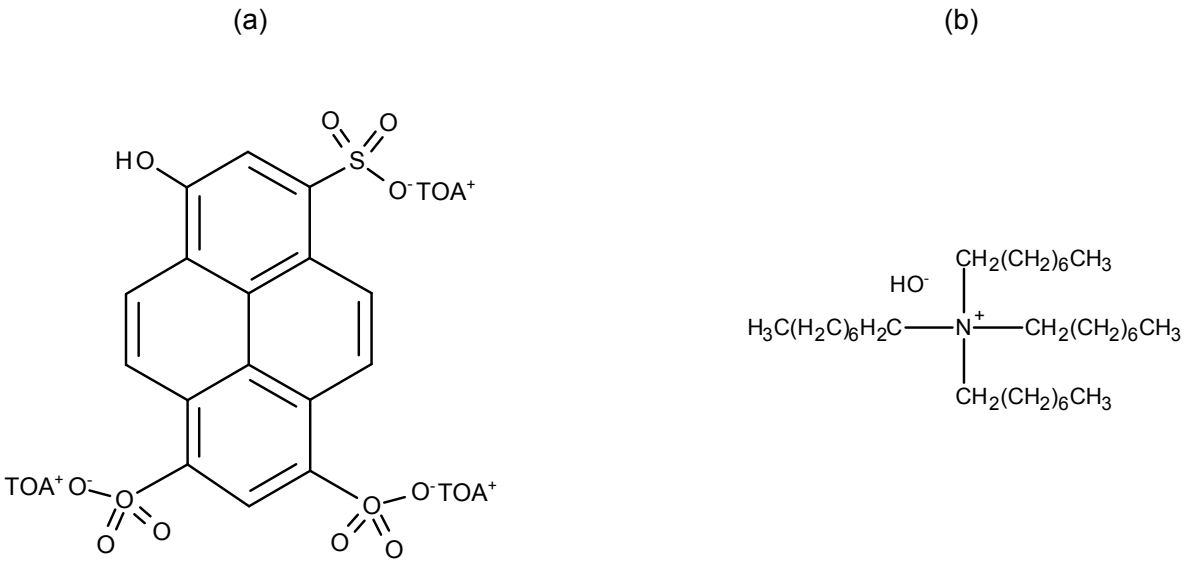

Figure 5.25.: (a) HPTS-TOA ion pair; (b) TOA *hydroxid*

of a *pH* change but of the adsorption of the HPTS-TOA ion pair to the beads. Therefore, this method is impractical.

5.4.1. 5(6)-*N*-Octadecyl-Carboxamidofluorescein (Fluorescein Amid, FA)

This lipophilic fluorescein amid (figure 5.26) is solvable in MTBE and serves as a *pH*-indicator. As one product of an ester cleavage is an acid, FA could indicate this conversion. Two different approaches were investigated. In the first approach 10 percent buffer was used. In the second approach no buffer was added. The enzyme used was NZL 108, the indicator concentration was 3.45 nM. Different acetic acid concentrations were used. Figure 5.27 shows the different behaviors of the curves. The accurate amounts and volumes are listed in table 4.12. The acid was added after 74 minutes.

Figure 5.27 visualizes that the fluorescence intensity does not stay constant. The various acetic acid concentrations resulted, as expected, in higher intensities if less acid was added. Especially if no buffer was added the increase of the signal in the beginning is enormous, whereas the addition of acid resulted in a slow decreasing signal. As this behavior is expected when acetic acid is formed as a reason of an ester cleavage, this method is not practicable. The buffer-acid equilibrium normally adjusts much faster. Hence, this can not be the reason for this decrease.

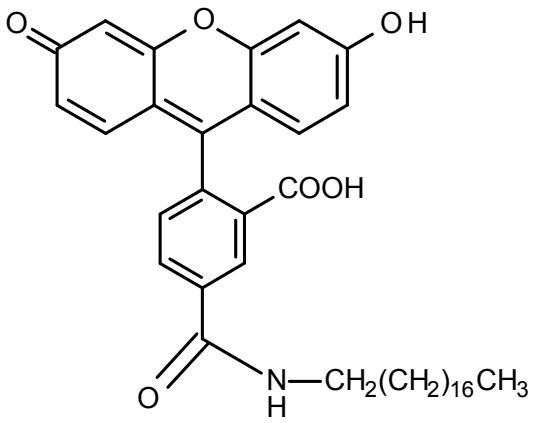

Figure 5.26.: Structure of 5(6)-*N*-octadecyl-carboxamidofluorescein

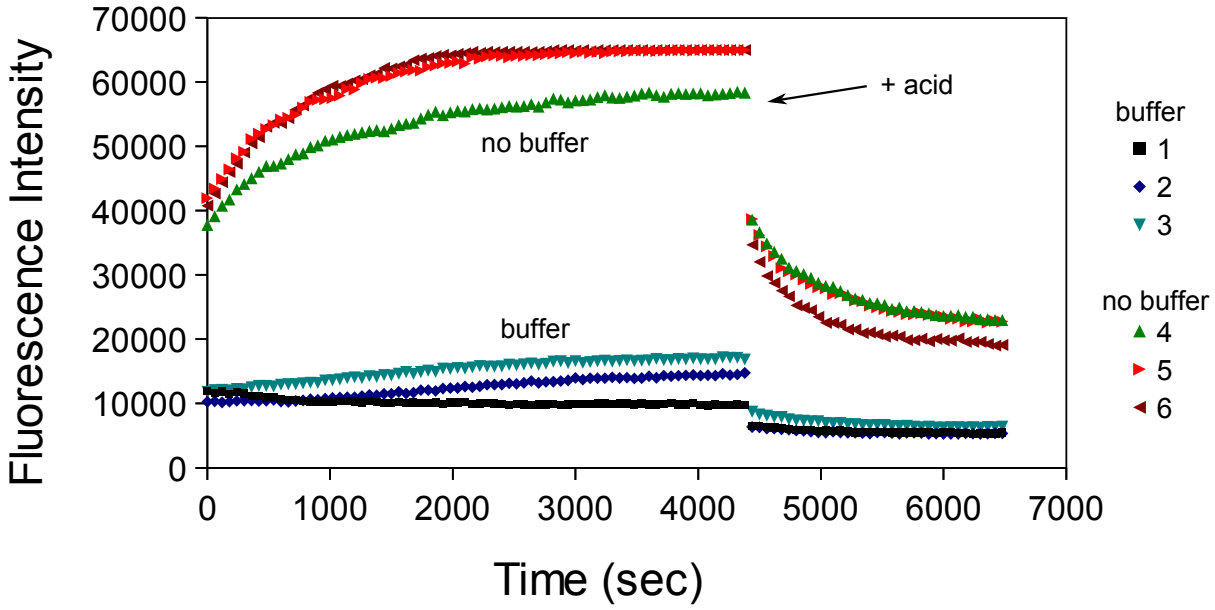

Figure 5.27.: Fluorescein amide as *pH*-indicator 3.45 nM; enzyme NZL 108; 1, 2 and 3 360 μL MTBE, 40 μL 10 mM SPB (*pH* 8.14); 4, 5 and 6 400 μL MTBE, no buffer; acetic acid concentrations: 1 and 4 2.18 mM, 2 and 5 4.36 mM, 3 and 6 6.55 mM

5.4.2. Fluorescein Octadecylester (FODE)

Another approach was the use of a lipophilc fluorescein ester as a substrate. Fluorescein octadeclester (figure 5.28, FODE) is soluble in MTBE. The concept behind this approach was based on a three phase system (buffer, beads and MTBE). At the beginning of the measurement the FODE is dissolved in the upper organic phase (MTBE). After the enzymatic cleavage of the ester, the released fluorescein is transferred to the aqueous phase at the bottom of the well. Hence, an increase of the fluorescence intensity is measurable if the bottom reading mode is used (figure 5.29). The increasing signal of graph 1 in figure 5.29 (no enzyme was added) can be explained by a small solubility of FODE in water or by autohydrolysis. In both cases the fluorescence intensity would increase due to higher fluorescein/FODE concentrations in the aqueous phase at the bottom of the well.

The main problem using this method is that quantifying the conversion of FODE into fluorescein is not possible because both substances emit at around 515 nm. Therefore, the ratio between the product (fluorescein) and the substrate (FODE) can not be determined. In further consequence, it is impossible to determine the activities of the enzymes. However, it is possible to show an approximate overview over the different activities of the enzymes.

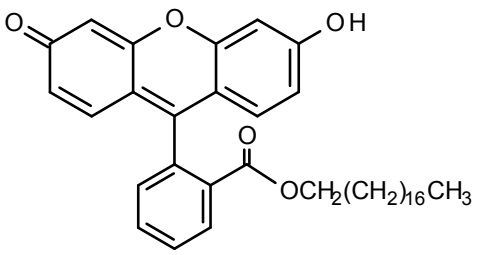

Figure 5.28.: Structure of FODE

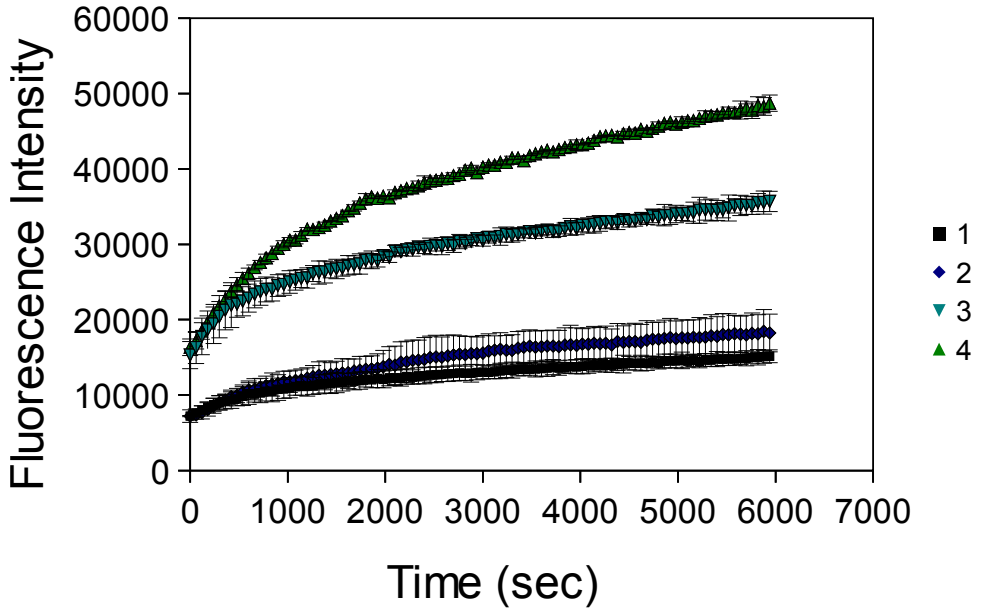

Figure 5.29.: Substrate: FODE; enzyme: NZL 108; (1) no enzyme, 0.375 mM FODE; (2) NZL 108, 0.375 mM FODE; (3) NZL 108, 0.750 mM FODE; (4) NZL 108, 1.125 mM FODE

5.4.3. Fluorescein Diacetate

Fluorescein diacetate was already used as substrate in aqueous solutions. The use of a three-phase-system (buffer/beads/MTBE) is not considered an appropriate approach as the ratio of fluorescein in the aqueous phase respectively the organic phase is not known. As the beads stay between the two phases, the reaction takes place on the interface between both liquid phases. Hence, the amount of converted fluorescein dissolved in MTBE is located behind the particles and it is not possible to measure the fluorescence intensity correctly, neither with the bottom nor with the top reading mode.

Another attempt was the omission of buffer to avoid the previously described problem. As figure 5.30 shows, it is possible to visualize the conversion in MTBE. It is possible to differentiate between various immobilized enzymes. The quantification turned out to be more complicated than expected. The solubility of fluorescein in MTBE is low and higher concentrations of fluorescein in MTBE can lead to precipitation. In figure 5.31 one can see the conversion of FDA into fluorescein. The horizontal graph represents a constant fluorescein concentration (250 nM), whereas the other graph shows the conversion of FDA. To yield the same fluorescence intensity a ten times higher concentration of FDA (250 μM) had to be used. One reason could be, that not all of the FDA was dissolved in

the stock solution. An ultrasonic bath was used to dissolve the FDA in MTBE. Maybe afterwards the FDA precipitated again. Even more problematic is the fact that also acetic acid is released and as fluorescein act as pH-sensor, the released acid can decrease the fluorescence signal.

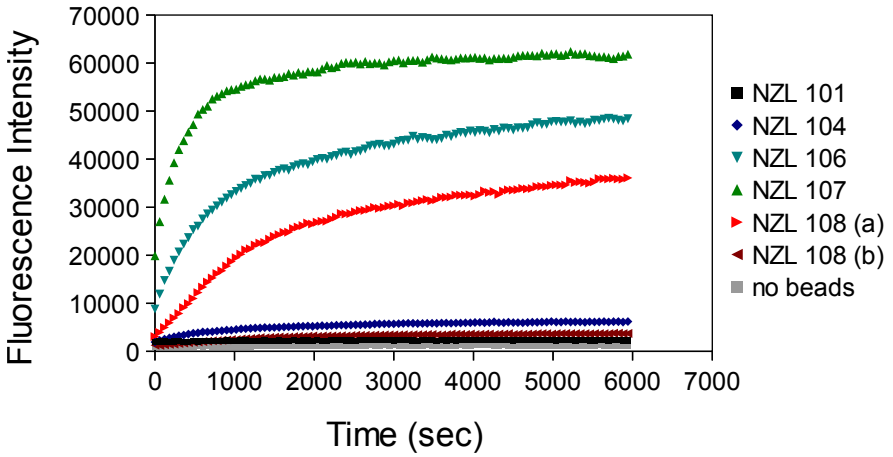

Figure 5.30.: Substrate: FDA; enzymes: NZL 101, 104, 106, 107 and 108; 108 (a) with substrate and (b) without substrate, respectively

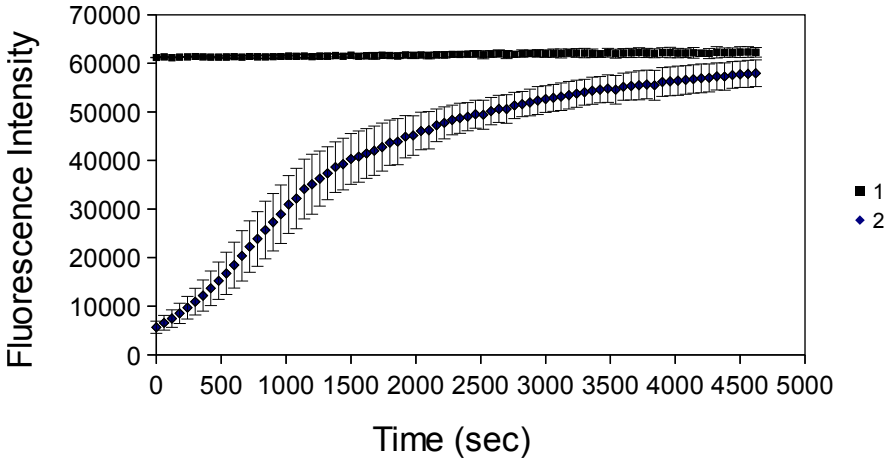

Figure 5.31.: Substrate: FDA; enzyme: NZL 108; (1) constant fluorescein concentration 250 nM, (2) substrate concentration 250 μM

Part V.

Conclusions and outlook

6. Conclusion

In relation to the procedure described in figure 1.1 a lot of improvements were achieved. The following schematic representation (figure 6.1) exemplifies the new designed assays.

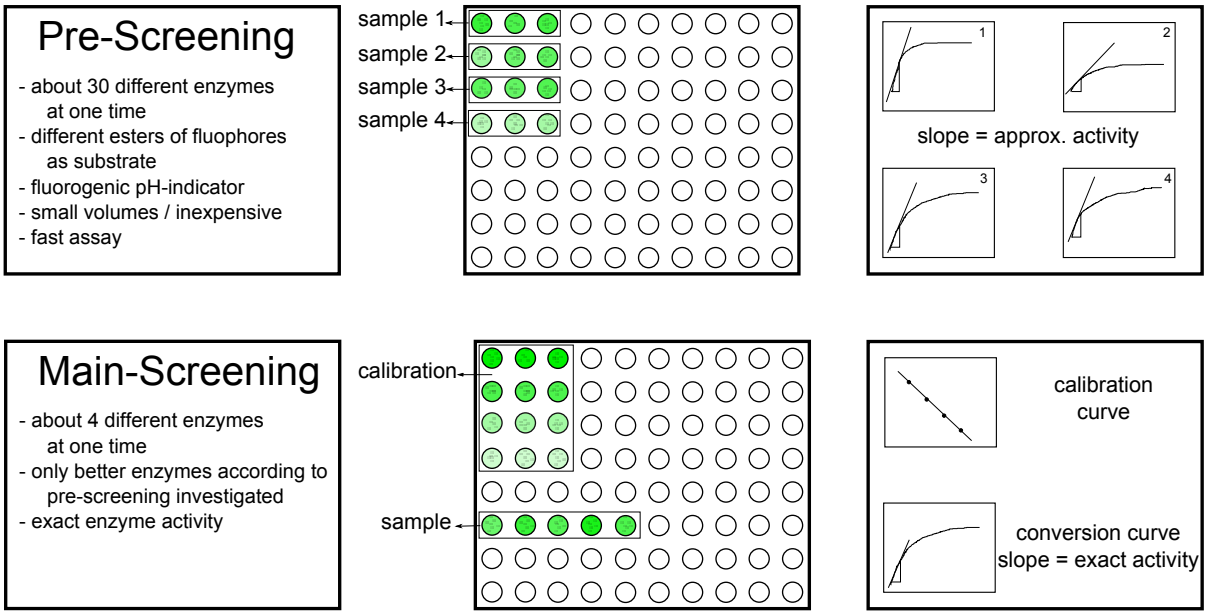

Figure 6.1.: Schematic representation of the new developed assay

The implementation of a pre- and a main-screening makes these assays very fast tools for the determination of enzymatic activities of immobilized enzymes. The use of an application based on matters of fluorescence minimizes the problems occurring due to the light scattering of the beads. In addition the downscaled volumes of a 96 well microtiter plate makes the assay inexpensive. Another advantage is the possibility of the online monitoring of an enzymatic reaction.

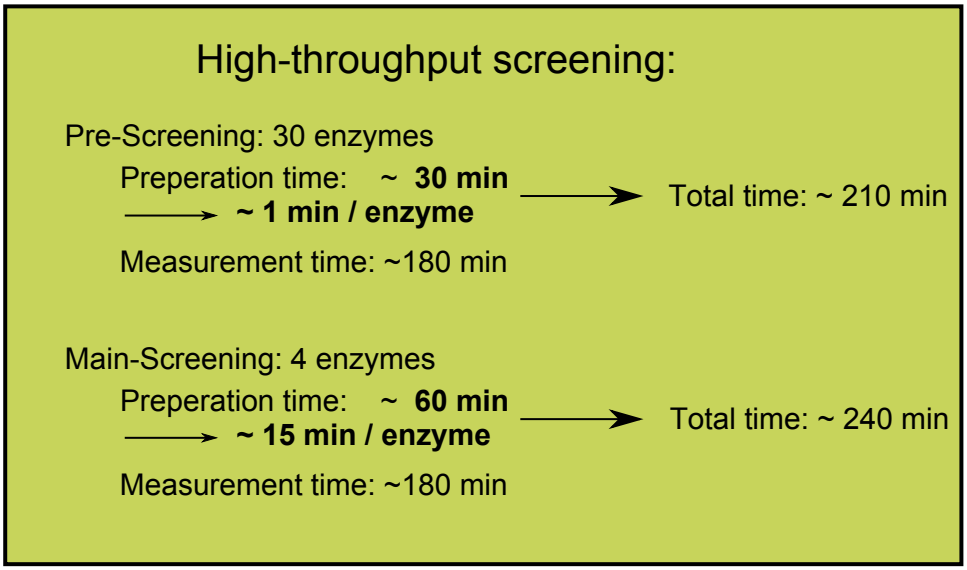

Figure 6.2.: Time required for pre- and main-screening respectively

Figure 6.2 shows the duration of the pre- and main-screening. In the time listed above the corresponding repetitions are already included, 3 for each enzyme in the pre-screening, 10 for each enzyme in the main-screening. The only time needed is the preparation time because the measurement operates automatically. The assay used at the moment needs at least 6 minutes for the measurement of one repetition of one enzyme, preparation time excluded. Hence, the new developed assay is much faster.

One of the main challenges during this thesis was the handling of the particles. As figure 5.2 already showed, the behavior of this beads is very unfavorable. Not only the various behaviors of the beads but also the fact that no information is available concerning the properties, for example the surface, made this thesis a challenge. It was not possible to predict any behavior of the beads regarding the combination with fluorescent dyes nor with organic solvents.

6.1. Fluorogenic Substrates

Three different esters were investigated: fluorescein diacetate, eosin diacetate and resorufin acetate. A standard operating procedure for a high-throughput assay was accomplished. This SOP can be found in the appendix. In comparison to the original procedure many improvements were realized. A lot of different enzymes can be investigated at the same time now. A fast assay enables a first differentiation between the immobilized

enzymes. A second step allows the quantification of the enzymatic reaction and hence a precise determination of the enzymatic activity. All three substrates can be used for the quantification of enzymatic activities, whereby fluorescein diacetate and eosin diacetate are preferable due to their price.

Since the activity of an enzyme is depending on the substrate, an exact prediction of the activity is not permissible if a model-substrate is used. Another possible way for the determination of the activity would be to esterify the real substrate of interest with a chromogenic compound like resorufin or p-nitrophenol. The difference between the real substrate and the model-substrate would be much smaller, but it would still just mimic the real substrate. It is not possible to be sure whether the real substrate would act in the same way as the model-substrate.

6.2. *pH*-Sensitive Dyes as Indicators for Enzyme Activity

Fluorophores as *pH*-indicators are a more universal way to determine the activities of esterases. The release of acid due to an enzymatic ester cleavage can be utilized for the visualization of the conversion. The *pH*-change can be visualized online using *pH*-sensors like fluorescein or HPTS.

The first attempt was to use the well-known *pH*-indicator fluorescein. However, it was not possible to use it as a *pH*-sensor because a steadily decreasing signal caused by the adsorption of dye on the the particles made this method impractical. On the contrary the use of HPTS as *pH*-sensor worked very well. It was possible to design a universal assay for the determination of esterase activity. The utilization of a *pH*-sensors enables the use of the real substrate of interest instead of model-substrates. Hence, this assay is more universally applicable. The standard operating procedure for this assay can be found in appendix SOP2.

6.3. Monitoring of Immobilized Esterase Activity in Organic Solvents

pH measurements in organic solvents are not commonly used and therefore not very well explored. As the *pH*-value is only defined for aqueous solutions it is not sure if the same assay as for aqueous solutions can be used. All things considered, it was possible

to differentiate between various enzymes but it was not possible to determine the exact enzymatic activity. Different approaches were investigated but it proved to be difficult to quantify an enzymatic conversion. Neither different esters of substrates nor different lipophilic pH-indicators could solve the problems concerning the quantification. Hence, it would be possible to use an assay to select the enzymes which have higher activities but for the quantification of the conversion, however, another assay would have to be used. A combination of the fast pre-screening based on the conversion of fluorescein diacetate and the precise assay used at Sandoz at the moment could yield an advantage. It would only be necessary to investigate the better enzymes using the time consuming assay. Another possible approach could be the fluorescence based sensing of p-nitrophenol. 'Coumarin1 in the presence of p-nitrophenol results in a quenching of fluorescence, providing a direct measure of the concentration of p-nitrophenol present in the sample.' These assays were done in 1 % ethanol due to their limited solubility in aqueous solutions. [30] Other organic solvents would have to be investigated.

List of Figures

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Part VI.

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Part VII.

Appendix

7. Appendix

7.1. SOP1

Form: work instruction - process description

1 Objective

The aim of this Standard Operating Procedure (SOP) is to establish a standardized procedure for the selection of immobilized esterases concerning their enzyme activity. There is a great variety of immobilized enzymes available and therefore, it is necessary to identify the most promising ones in an, according to possibilities, easy and fast assay. Furthermore, it is necessary to characterize the selected enzymes due to their enzyme activity.

Therefore, this high-throughput assay consists of two main parts:

1. Selection of the best enzymes (pre-screening)
2. Characterization of the best enzymes (main-screening)

The method for enzyme activity measurement described in this SOP is based on fluorometric principles. An esterfied fluorophore is used as a substrate for different esterases. The substrate does not fluoresce until the ester is hydrolyzed. Hence, it is possible to visualize the conversion online.

2 Field of application

3 Search terms, definitions and abbreviations

Enzyme activity assay, Hydrolase, Esterase, FDA, EDA

Table 1: abbreviations

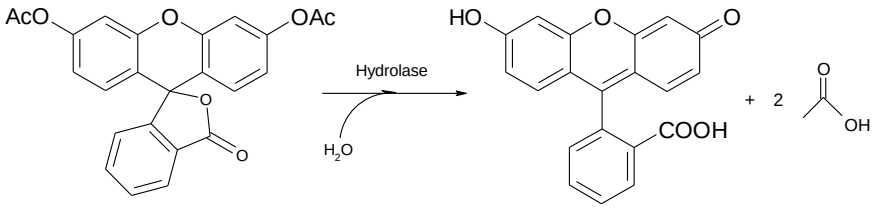
Activity	1 U = 1 µmol/min
Specific activity	U/g
Hydrolase	An enzyme that catalyzes the hydrolysis of chemical bounds (e.g. esterases)
FDA	Fluorescein diacetate
EDA	Eosin diacetate
SOP	Standard Operating Procedure
MW	Molecular weight
IE	Immobilized enzyme
SPB	Sodium phosphate buffer

4 Responsibility

5 Experimental

5.1 Principle

This method is based on the enzymatic cleavage of the non-fluorescent fluorescein diacetate (FDA) into the green fluorescent fluorescein and acetic acid. It is possible to quantify the conversion using a BMG Labtech Microplate reader and the suitable filters for fluorescein. An increasing signal indicates an increasing fluorescein concentration in the reaction well.



Fluorescein diacetate

Fluorescein

Figure 1: Fluorescein diacetate as substrate for esterases

Alternatively it is possible to use eosin diacetate instead of fluorescein diacetate.

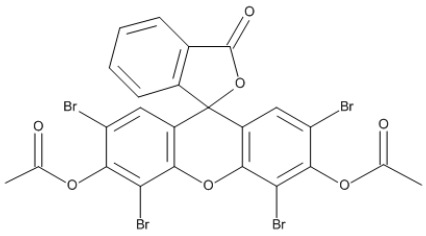


Figure 2: Eosin diacetate

5.2 Chronology

5.1.1 Materials**:**

Table 2: Used chemicals and devices

Fluorescein diacetate	MW: 416.38 g/mol	Sigma-Aldrich F7378
Fluorescein sodium salt	MW: 376.27 g/mol	Sigma-Aldrich 46960
Eosin diacetate	MW: 731.96 g/mol	Sigma-Aldrich 45244
Eosin Y	MW: 647.89 g/mol	Sigma-Aldrich E4009
Acetone		
Sodium phosphate dibasic	MW:141.96 g/mol	Roth P030.2
Sodium phosphate monobasic	MW: 119.98 g/mol	Sigma-Aldrich 71496
H_2O, deionized		
Micro pipettes	2-20 µl, 20-200 µl, 100-1000 µl	e.g. Eppendorf research plus
96 Well microplates	Polystyrene, F bottom	e.g. Greiner bio one 650101
Platereader	FLUOstar OPTIMA	BMG
Filters	BMG 0119 485-P BMG 0135 520-P BMG 0142 590-10	BMG

The chemicals and devices listed in table 2 are only examples and can be replaced by equal products.

Settings

Base settings:

- Pre-screening:

Table 3: general settings for the pre-screening

Test conditions	**Enzyme reaction**
Total volume [µl]	200.0
Immobilized enzymes [mg]	~ 3
FDA [µM]	20
Buffer [mM]	100
pH	8.0
Temperature [°C]	37.0 ± 0.2
Shaking [rpm]	300

- Main-screening:

Table 4: general settings for the main-screening

Test conditions	**Enzyme reaction**
Total volume [µl]	200.0
Immobilized enzymes [mg]	3
FDA [µM]	20
Buffer [mM]	100
pH	8.0
Temperature [°C]	37.0 ± 0.2
Shaking [rpm]	300

Plate reader settings:

- Pre-screening:

Table 5: pre-screening platereader settings for the elimination of the background fluorescence

Test protocol name	Prescr_FDA_0 Prescr_EDA_0	
Microplate	Greiner 96 F-bottom	
General settings	Positioning delay [s]	0.1
	Flying mode	off
	No. of kinetic windows	1
Filter settings	No. of multichromatics	1
	Excitation filter	FDA: BMG 0119 485-P EDA: BMG 0135 520-P
	Emission filter	FDA: BMG 0135 520-P EDA: BMG 0142 590-10
	Gain	Adjust required value [%] 90
Orbital averaging	On	
	Diameter [mm]	4
Pause before cycle	0	
Kinetic window 1	No. of cycles	5
	Measurement start time [s]	0.0
	No. of flashes per well and cycle	15
	Cycle time [s]	
	• Up to 24 wells	50
	• Up to 48 wells	75
	• Up to 64 wells	90
	• Up to 96 wells	125
Temperature	37°C	
Shaking options	Mode	Orbital
	Shaking width [mm]	2 (300rpm)
	Additional shaking	After each cycle
	Shaking time [s]	20

Table 6: pre-screening settings for the enzyme reaction

Test protocol name	Prescr_FDA_1 Prescr_EDA_1	
Microplate	Greiner 96 F-bottom	
General settings	Positioning delay [s]	0.1
	Flying mode	off
	No. of kinetic windows	2
Filter settings	No. of multichromatics	1
	Excitation filter	FDA: BMG 0119 485-P EDA: BMG 0135 520-P
	Emission filter	FDA: BMG 0135 520-P EDA: BMG 0142 590-10
	Gain	Adjust required value [%] 90
Orbital averaging	On	
	Diameter [mm]	4
Pause before cycle	0	
Kinetic window 1	No. of cycles	120
	Measurement start time [s]	0.0
	No. of flashes per well and cycle	15
	Cycle time [s]	
	• Up to 24 wells	60
	• Up to 48 wells	75
	• Up to 64 wells	90
	• Up to 96 wells	125
Kinetic window 2	No. of cycles	15
	Measurement start time [s]	0.0
	No. of flashes per well and cycle	15
	Cycle time [s]	300
Temperature	37°C	
Shaking options	Mode	Orbital
	Shaking width [mm]	2 (300rpm)
	Additional shaking	After each cycle
	Shaking time [s]	20

- Main-screening:

Table 7: main-screening platereader settings for the elimination of the background fluorescence

Test protocol name	Main_FDA_0 Main_EDA_0	
Microplate	Greiner 96 F-bottom	
General settings	Positioning delay [s]	0.1
	Flying mode	off
	No. of kinetic windows	1
Filter settings	No. of multichromatics	1
	Excitation filter	FDA: BMG 0119 485-P EDA: BMG 0135 520-P
	Emission filter	FDA: BMG 0135 520-P EDA: BMG 0142 590-10
	Gain	Adjust required value [%] 90
Orbital averaging	On	
	Diameter [mm]	4
Pause before cycle	0	
Kinetic window 1	No. of cycles	5
	Measurement start time [s]	0.0
	No. of flashes per well and cycle	15
	Cycle time [s]	
	• Up to 24 wells	50
	• Up to 48 wells	75
	• Up to 64 wells	90
	• Up to 96 wells	125
Temperature	37°C	
Shaking options	Mode	Orbital
	Shaking width [mm]	2 (300rpm)
	Additional shaking	After each cycle
	Shaking time [s]	20

Table 8: main-screening settings for the enzyme reaction

Test protocol name	Main_FDA_1 Main_EDA_1	
Microplate	Greiner 96 F-bottom	
General settings	Positioning delay [s]	0.1
	Flying mode	off
	No. of kinetic windows	2
Filter settings	No. of multichromatics	1
	Excitation filter	FDA: BMG 0119 485-P EDA: BMG 0135 520-P
	Emission filter	FDA: BMG 0135 520-P EDA: BMG 0142 590-10
	Gain	Adjust required value [%] 90
Orbital averaging	On	
	Diameter [mm]	4
Pause before cycle	0	
Kinetic window 1	No. of cycles	120
	Measurement start time [s]	0.0
	No. of flashes per well and cycle	15
	Cycle time [s]	
	• Up to 24 wells	60
	• Up to 48 wells	75
	• Up to 64 wells	90
	• Up to 96 wells	125
Kinetic window 2	No. of cycles	15
	Measurement start time [s]	0.0
	No. of flashes per well and cycle	15
	Cycle time [s]	300
Temperature	37°C	
Shaking options	Mode	Orbital
	Shaking width [mm]	2 (300rpm)
	Additional shaking	After each cycle
	Shaking time [s]	20

5.2.3 Preparations

Sodium phosphate buffer (SPB):

Buffer pH 8.0, 0.1 M, 500 ml

Preparation temperature 20 °C, temperature of usage: 37 °C

Dissolve 0.0024 mol of acid component (NaH_2PO_4 , 288mg)
Dissolve 0.0475 mol of basic component (Na_2HPO_4 , 6.74g)
Make up to 500 ml with pure water

Buffer can be stored at room temperature for one week.

FDA stock solutions:

1mM:

Dissolve 0.01 mmol of FDA in 10 ml acetone (4.2 mg FDA, 10 ml acetone)

All of the FDA has to be dissolved before the procedure is continued. If problems occur solving the FDA, use an ultrasonic bath for about 10 minutes.

400µM:

1ml 1mM FDA solution in 1.5ml acetone. Make sure that the solution is well mixed and at room temperature before use.

EDA stock solutions:

1mM:

Dissolve 0.01 mmol of EDA in 10 ml acetone (7.3 mg FDA, 10 ml acetone)

All of the EDA has to be dissolved before the procedure is continued. If problems occur solving the EDA, use an ultrasonic bath for about 10 minutes.

400µM:

1ml 1mM EDA solution in 1.5ml acetone. Make sure that the solution is well mixed and at room temperature before use.

Fluorescein stock solutions:

1 mM:

Dissolve 0.01 mmol of fluorescein sodium salt in 10 ml deionized water (3.8 mg eosin sodium salt, 10 ml deionized water) to produce a 1 mM fluorescein solution.

Used concentrations for calibration:

500µM: 1ml 1mM fluorescein solution in 1ml pure H_2O

400µM: 1ml 1mM fluorescein solution in 1.5ml pure H_2O

300µM: 0.3ml 1mM fluorescein solution in 1ml pure H_2O

200µM: 0.2ml 1mM fluorescein solution in 1ml pure H_2O

Eosin stock solutions:

1 mM:

Dissolve 0.01 mmol of eosin sodium salt in 10 ml deionized water (6.5 mg eosin Y, 10 ml deionized water) to produce a 1 mM fluorescein solution.

Used concentrations for calibration:

500µM: 1ml 1mM eosin solution in 1ml pure H_2O

400µM: 1ml 1mM eosin solution in 1.5ml pure H_2O

300µM: 0.3ml 1mM eosin solution in 1ml pure H_2O

200µM: 0.2ml 1mM eosin solution in 1ml pure H_2O

All stock solutions have to be stored at 2-4°C and under exclusion of light.

Consider the right concentrations according to the real weights of FDA, EDA, fluorescein and eosin!!

5.2.4 Pre-screening:

At the beginning it is important to figure out the right gain for the screenings. Therefore, use any beads of interest for the investigation. Add about 3 mg in a well of a 96 well microtiter plate and cover it with 180µl of SPB. After the addition of 20µl of a 500µM fluorescein/eosin solution adjust the gain to a suitable value (about 90% of the highest measurable signal)

Backgroundfluorescence:

Platereader setup:

- Preheat the platereader to a temperature of 37°C
- Load test protocol Prescr_FDA_0 or Prescr_EDA_0
- Adjust the layout depending on the number of enzymes
- Adjust cycle time according to the number of used wells
- Check if the light guides are in the correct position (top measurement!)

Sample preparation:

- Heat the sodium phosphate buffer and the 400µM FDA/EDA solution to 37°C
- Add about 3mg of immobilized enzymes in each well, 3 repetitions each enzyme
- Add 180µl sodium phosphate buffer to each well
- Put the plate into the platereader

Measurement:

- Select test protocol Prescr_FDA_0 or Prescr_EDA_0
- Enter file name
- Start measurement

Initiation of the reaction:

Platereader setup:

- Load test protocol Prescr_FDA_1 or Prescr_EDA_1
- Adjust the layout depending on the number of enzymes
- Adjust cycle time according to the number of used wells

Sample preparation:

- Add 20µl 400µM FDA/EDA solution (37°C) to each well (as fast as possible, multichannel pipette)
- Put the plate into the platereader

Measurement:

- Select test protocol Prescr_FDA_1 or Prescr_EDA_1
- Enter file name
- Start measurement

DocumentType	gültig ab:	Dok. Nr.	Version	ACIB
Formular	**25.05.2010**	**FO.01**	**1**	**12**

Document Name

Formular: working instruction-process description

Table 9: example layout for 3 enzymes; X1-3 enzyme one; X4-6 enzyme two; X7-9 enzyme three

96	1	2	3	4	5	6	7	8	9	10	11	12
A	X1	X2	X3									
B	X4	X5	X6									
C	X7	X8	X9									
D												
E												
F												
G												
H												

5.2.5 Main-screening

Backgroundfluorescence:

Platereader setup:

- Preheat the platereader to a temperature of 37°C
- Load test protocol Main_FDA_0 or Main_EDA_0
- Adjust the layout (table 2, example for 1 enzyme)
- Adjust cycle time according to the number of used wells
- Check if the light guides are in the correct position (top measurement!)

Sample preparation:

- Heat the sodium phosphate buffer, the 400µM FDA/EDA solution and the fluorescein/eosin stock solutions (for the calibration) to 37°C
- Calibration: Weigh 3mg of immobilized enzyme 15 times and add the right volume of buffer according to table 11 (calibration is only valid if the same beads are used for calibration as well as for the reaction)
- Sample: Weigh 3mg of immobilized enzyme 10 times and add the right volume of buffer according to table 11.
- Blank test: add the right volume of buffer according to table 11; no beads are added
- Put the plate into the platereader

Measurement:

- Select test protocol Main_FDA_0 or Main_EDA_0
- Enter file name
- Start measurement

Initiation of the reaction:

Platereader setup:

- Load test protocol Main_FDA_1 or Main_EDA_1
- Adjust the layout
- Adjust cycle time according to the number of used wells

Sample preparation:

- Calibration: Add the right volumes of fluorescein/eosin stock solutions to the wells according to table 11.
- Blank test: add 20µl 400µM FDA/EDA solution (37°C) to each “blank test well”
- Sample: add 20µl 400µM FDA/EDA solution (37°C) to each “sample well” (as fast as possible, multichannel pipette)
- Put the plate into the platereader

Measurement:

- Select test protocol Main_FDA_1 or Main_EDA_1
- Enter file name
- Start measurement

Table 10: 96 well plate layout for a typically main screening for one IE

96	1	2	3	4	5	6	7	8	9	10	11	12
A	X1	X2	X3									
B	X4	X5	X6		X16	X17	X18	X19	X20			
C	X7	X8	X9		X21	X22	X23	X24	X25			
D	X10	X11	X12									
E	X13	X14	X15									
F												
G											26	27
H												

Table 11: Volumes of solutions added to the wells

X	Buffer [µl]	200µM fluorescein/ eosin solution[µl]	300µM fluorescein/ eosin solution [µl]	400µM fluorescein/ eosin solution [µl]	500µM fluorescein/ eosin solution [µl]	400µM FDA/EDA [µl]
1-3	200	0	0	0	0	0
4-6	180	20	0	0	0	0
7-9	180	0	20	0	0	0
10-12	180	0	0	20	0	0
13-15	180	0	0	0	20	0
16-25	180	0	0	0	0	20
26+27	180	0	0	0	0	20

Evaluation:

Pre-screening:

- subtraction of the averaged background fluorescences
- plot the result as shown in figure 3
- control the standard deviations
- select the enzymes with larger slopes at the beginning for the main-screening

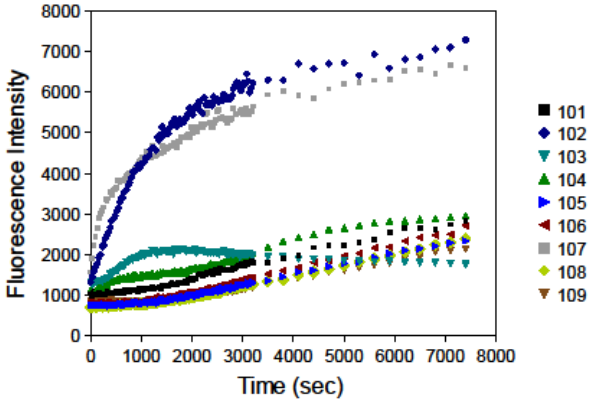


Figure 3: Pre-screening: Substrate: EDA (18.5µM); Enzymes: NZL 101-109

Main-screaning:

- subtraction of the averaged background fluorescences
- calibration curve, linear fit (figure 4), declare outliers, use real concentrations of fluorescein or eosin
- use the linear equation for the visualizing of the conversion (consider the amount of applied beads per well according to equation 1) (figure 5)
- control the standard deviations
- use the slope of the conversion curve for the determination of the enzyme activity

Equation 1:

$$I = \frac{I_1 - I_2}{m_1}$$

I… calculated intensity

I_1… measured intensity

I_2… background intensity

m_1… amount applied beads [mg]

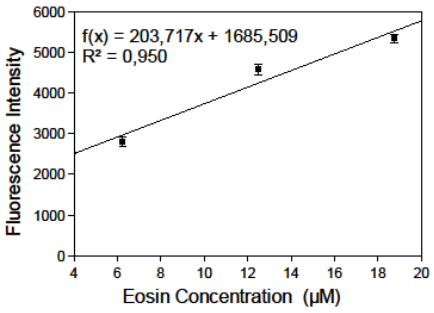


Figure 4: Eosin calibration and linear equation (NZL 107)

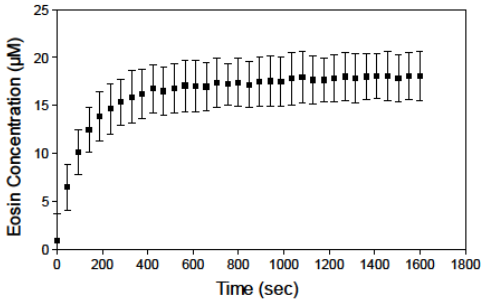


Figure 5: Conversion of EDA into eosin per mg enzyme NZL 107

Enzyme activity:

The slope at the beginning of the conversion curve is used for the determination of the enzyme activity. In due consideration of the real concentrations of FDA and EDA the exact specific activity can be calculated. According to equation 1 the determined activity is per mg applied beads. Important for the calculation of the activity is the fact that both substrates consist of two esters!

Slope = 0.068µM/(s*mg) (according to figure 5)

Reaction volume = $200*10^{-6}$ l

Esters to cleave = 2

1 min = 60 s

1 Unit = 1000 mU

$$\frac{1.6mU}{mg} = \frac{0.068\,\mu mol * 200 * 10^{-6} l * 2 * 60 * 1000}{l * \min * mg}$$

In the case of figure 5 the specific activity of the enzyme NZL 107 was **1.6 mU/mg**.

The amount of applied beads was already considered in equation 1.

5.3 Annotation

In order to get comparable results it is important to ensure a good mixing of all solutions.

A blank test is also important to visualize if a reaction takes place without enzymes. Whereas a second “blank test” (beads without substrate) is useful to check if the beads cause a reaction with the pH-indicator.

As fluorophores often depend on the ionic strength of a solution, it is important to use the same buffer and stock solutions for a whole experiment.

5.4 Troubleshooting

The cap of the microtiter plate can fog, especially when the plate has already been preheated and stays outside the temperate plate reader for a while. The result would be a lower intensity in the first few minutes until the cap is not steamed up any more. To avoid this problem minimize the time where the plate is outside the reader and do not put the plate nor the cap on a cold surface.

6 References

6.1 Corresponding documents

7 Notations

8 Status of the standard operation procedure

Author	**5.1.1**
Checked by	**5.1.1**
Approved by	**5.1.1**
Replaces document No.	**5.1.1**
Date printed	**5.1.1** **21.09.2011 12:52:22**
Filename and path	**5.1.1** **Dokument1**

5.1.1

9 Modification

5.1.1

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7.2. SOP2

Form: work instruction - process description

1 Objective

The aim of this Standard Operating Procedure (SOP) is to establish a standardized procedure for the selection of immobilized esterases concerning their enzyme activity. There is a huge variety of immobilized enzymes available and therefore, it is necessary to identify the most promising ones in an, according to possibilities, easy and fast assay. Furthermore it is necessary to characterize the selected enzymes due to their enzyme activity.

Therefore, this high-throughput assay consists of two main parts:

1. Selection of the best enzymes (pre-screening)
2. Characterization of the best enzymes (main-screening)

The method for enzyme activity measurement described in this SOP is based on fluorometric principles. A fluorophore is used as a pH-indicator for the determination of esterases activities.

The main advantage of this method is that it is possible to investigate the real substrate of interest.

2 Field of application

3 Search terms, definitions and abbreviations

Enzyme activity assay, Hydrolase, Esterase, HPTS

Table 1: abbreviations

Activity	1 U = 1 µmol/min
Specific activity	U/g
Hydrolase	An enzyme that catalyzes the hydrolysis of chemical bounds (e.g. esterases)
HPTS	8-hydroxypyrene-1,3,6-trisulfonic acid
SPB	Sodium phosphate buffer
SOP	Standard Operating Procedure
MW	Molecular weight
IE	Immobilized enzyme

4 Responsibility

5 Experimental

5.1 Principle

The enzymatic cleavage of en ester results in the release of an acid. The pH change due to this acidification can be visualized using 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) as pH-sensor. It is possible to quantify the conversion using a BMG Labtech Microplate reader and the suitable filters for HPTS.

As HPTS has a pH-dependent absorption shift it is possible to visualize the conversion online. A decreasing signal indicates an increasing acetic acid concentration.

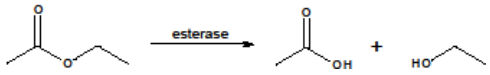

Figure 1: Ethyl acetate as substrate for esterases

Depending on what substrate is used the assay has to be adjusted. In this SOP ethyl acetate is used as model substrate. Therefore, the released acid is acetic acid, which was also used for the calibration. The use of other substrates can result in other released acids and hence, this SOP has to be adjusted.

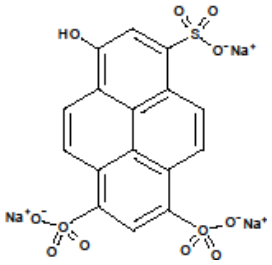

Figure 2: Chemical structure of 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt

5.2 Chronology

5.1.1 Materials**:**

Table 2: Used chemicals and devices

8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt	MW: 524.39 g/mol	Sigma-Aldrich H1529
Acetic acid	MW: 60.05 g/mol	Sigma-Aldrich
Ethyl acetate	MW: 88.105 g/mol	Sigma-Aldrich
Sodium phosphate dibasic	MW:141.96 g/mol	Roth P030.2
Sodium phosphate monobasic	MW: 119.98 g/mol	Sigma-Aldrich 71496
H_2O, deionized		
Micro pipettes	2-20 µl, 20-200 µl, 100-1000 µl	e.g. Eppendorf research plus
96 Well microplates	Polystyrene, F bottom	e.g. Greiner bio one 650101
Platereader	FLUOstar OPTIMA	BMG
Filters	BMG 0119 485-P BMG 0135 520-P	BMG

The chemicals and devices listed in table 2 are only examples and can be replaced by equal products.

Settings

Base settings:

- Pre-screening:

Table 3: general settings for the pre-screening

Test conditions	**Enzyme reaction**
Total volume [µl]	200.0
Immobilized enzymes [mg]	~ 3
HPTS [µM]	2.5
Buffer [mM]	10
pH	8.0
Temperature [°C]	37.0 ± 0.2
Shaking [rpm]	300

- Main-screening:

Table 4: general settings for the main-screening

Test conditions	**Enzyme reaction**
Total volume [µl]	200.0
Immobilized enzymes [mg]	3
HPTS [µM]	2.5
Buffer [mM]	10
pH	8.0
Temperature [°C]	37.0 ± 0.2
Shaking [rpm]	300

Plate reader settings:

- Pre-screening:

Table 5: pre-screening platereader settings for the elimination of the background fluorescence

Test protocol name	Prescr_HPTS_0	
Microplate	Greiner 96 F-bottom	
General settings	Positioning delay [s]	0.1
	Flying mode	off
	No. of kinetic windows	1
Filter settings	No. of multichromatics	1
	Excitation filter	BMG 0119 485-P
	Emission filter	BMG 0135 520-P
	Gain	Adjust required value [%] 90
Orbital averaging	On	
	Diameter [mm]	4
Pause before cycle	0	
Kinetic window 1	No. of cycles	5
	Measurement start time [s]	0.0
	No. of flashes per well and cycle	15
	Cycle time [s]	
	• Up to 24 wells	50
	• Up to 48 wells	75
	• Up to 64 wells	90
	• Up to 96 wells	125
Temperature	37°C	
Shaking options	Mode	Orbital
	Shaking width [mm]	2 (300rpm)
	Additional shaking	After each cycle
	Shaking time [s]	20

Table 6: pre-screening settings for the enzyme reaction

Test protocol name	Prescr_HPTS_1	
Microplate	Greiner 96 F-bottom	
General settings	Positioning delay [s]	0.1
	Flying mode	off
	No. of kinetic windows	2
Filter settings	No. of multichromatics	1
	Excitation filter	BMG 0119 485-P
	Emission filter	BMG 0135 520-P
	Gain	Adjust required value [%] 90
Orbital averaging	On	
	Diameter [mm]	4
Pause before cycle	0	
Kinetic window 1	No. of cycles	120
	Measurement start time [s]	0.0
	No. of flashes per well and cycle	15
	Cycle time [s]	
	• Up to 24 wells	60
	• Up to 48 wells	75
	• Up to 64 wells	90
	• Up to 96 wells	125
Kinetic window 2	No. of cycles	15
	Measurement start time [s]	0.0
	No. of flashes per well and cycle	15
	Cycle time [s]	300
Temperature	37°C	
Shaking options	Mode	Orbital
	Shaking width [mm]	2 (300rpm)
	Additional shaking	After each cycle
	Shaking time [s]	20

- Main-screening:

Table 7: main-screening platereader settings for the elimination of the background fluorescence

Test protocol name	Main_HPTS_0	
Microplate	Greiner 96 F-bottom	
General settings	Positioning delay [s]	0.1
	Flying mode	off
	No. of kinetic windows	1
Filter settings	No. of multichromatics	1
	Excitation filter	BMG 0119 485-P
	Emission filter	BMG 0135 520-P
	Gain	Adjust required value [%] 90
Orbital averaging	On	
	Diameter [mm]	4
Pause before cycle	0	
Kinetic window 1	No. of cycles	5
	Measurement start time [s]	0.0
	No. of flashes per well and cycle	15
	Cycle time [s]	
	• Up to 24 wells	50
	• Up to 48 wells	75
	• Up to 64 wells	90
	• Up to 96 wells	125
Temperature	37°C	
Shaking options	Mode	Orbital
	Shaking width [mm]	2 (300rpm)
	Additional shaking	After each cycle
	Shaking time [s]	20

Table 8: main-screening settings for the enzyme reaction

Test protocol name	Main_HPTS_1	
Microplate	Greiner 96 F-bottom	
General settings	Positioning delay [s]	0.1
	Flying mode	off
	No. of kinetic windows	2
Filter settings	No. of multichromatics	1
	Excitation filter	BMG 0119 485-P
	Emission filter	BMG 0135 520-P
	Gain	Adjust required value [%] 90
Orbital averaging	On	
	Diameter [mm]	4
Pause before cycle	0	
Kinetic window 1	No. of cycles	120
	Measurement start time [s]	0.0
	No. of flashes per well and cycle	15
	Cycle time [s]	
	• Up to 24 wells	60
	• Up to 48 wells	75
	• Up to 64 wells	90
	• Up to 96 wells	125
Kinetic window 2	No. of cycles	15
	Measurement start time [s]	0.0
	No. of flashes per well and cycle	15
	Cycle time [s]	300
Temperature	37°C	
Shaking options	Mode	Orbital
	Shaking width [mm]	2 (300rpm)
	Additional shaking	After each cycle
	Shaking time [s]	20

5.2.3 Preparations

Sodium phosphate buffer (SPB):

Buffer pH 8.0, 0.01 M, 500 ml

Preparation temperature 20 °C, temperature of usage: 37 °C

Dissolve 0.0004 mol of acid component (NaH_2PO_4, 48mg)
Dissolve 0.0045 mol of basic component (Na_2HPO_4, 639mg)
Make up to 500 ml with pure water

Buffer can be stored at room temperature for one week.

HPTS stock solution:

1mM:

Dissolve 0.01 mmol of HPTS in 10 ml pure H_2O (5.2 mg HPTS, 10 ml pure H_2O)

All of the HPTS has to be dissolved before the procedure is continued. If problems occur solving the HPTS, use an ultrasonic bath for about 10 minutes.

50µM:

100µl 1mM HPTS solution in 1.9ml pure H_2O. Make sure that the solution is well mixed and at room temperature before use.

Ethyl acetate stock solution:

30 mM:

Dissolve 30µl of ethyl acetate in 988µl pure water to produce a 30mM ethyl acetate stock solution.

Acetic acid stock solutions:

1 M:

Dissolve 57µl of acetic acid in 995µl pure water to produce a 1M acetic acid stock solution.

Used concentrations for calibration:

40mM: 40µl 1M acetic acid solution in 960µl pure H_2O

30mM: 30µl 1M acetic acid solution in 970µl pure H_2O

20mM: 20µl 1M acetic acid solution in 980µl pure H_2O

10mM: 10µl 1M acetic acid solution in 990µl pure H_2O

All stock solutions have to be stored at 2-4°C and under exclusion of light.

5.2.4 Screenings

At the beginning it is important to figure out the right gain for the screenings. Therefore, use any beads of interest for the investigation. Add about 3 mg in a well of a 96 well microtiter plate and cover it with 190µl of SPB. After the addition of 10µl of a 50µM HPTS solution adjust the gain to a suitable value (about 90% of the highest measurable signal)

5.2.4.1 Pre-screening:

Backgroundfluorescence:

Platereader setup:

- Preheat the platereader to a temperature of 37°C
- Load test protocol Prescr_HPTS_0
- Adjust the layout depending on the number of enzymes
- Adjust cycle time according to the number of used wells
- Check if the light guides are in the correct position (top measurement!)

Sample preparation:

- Heat the sodium phosphate buffer and the stock solutions to 37°C
- Add about 3mg of immobilized enzymes in each well, 3 repetitions each enzyme
- Add 170µl sodium phosphate buffer to each well
- Put the plate into the platereader

Measurement:

- Select test protocol Prescr_HPTS_0
- Enter file name
- Start measurement

Initiation of the reaction:

Platereader setup:

- Load test protocol Prescr_HPTS_1
- Adjust the layout depending on the number of enzymes
- Adjust cycle time according to the number of used wells

Sample preparation:

- Add 10µl 50µM HPTS solution (37°C) to each well
- Add 20µl 30mM ethyl acetate solution (37°C) to each well (as fast as possible, multichannel pipette)
- Put the plate into the platereader

Measurement:

- Select test protocol Prescr_HPTS_1
- Enter file name
- Start measurement

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Formular	**25.05.2010**	**FO.01**	**1**	**11**

Document Name

Formular: working instruction-process description

Table 9: example layout for 3 enzymes; X1-3 enzyme one; X4-6 enzyme two; X7-9 enzyme three

96	1	2	3	4	5	6	7	8	9	10	11	12
A	X1	X2	X3									
B	X4	X5	X6									
C	X7	X8	X9									
D												
E												
F												
G												
H												

5.2.4.2 Main-screening

Backgroundfluorescence:

Platereader setup:

- Preheat the platereader to a temperature of 37°C
- Load test protocol Main_HPTS_0
- Adjust the layout (table 2, example for 1 enzyme)
- Adjust cycle time according to the number of used wells
- Check if the light guides are in the correct position (top measurement!)

Sample preparation:

- Heat the sodium phosphate buffer, the 30mM ethyl acetate, the 50µM HPTS and the acetic acid stock solutions (for the calibration) to 37°C
- Calibration: Weigh 3mg of immobilized enzyme 15 times and add the right volume of buffer and HPTS stock solution according to table 11 (calibration is only valid if the same beads are used for calibration as well as for the reaction)
- Sample: Weigh 3mg of immobilized enzyme 10 times and add the right volume of buffer and HPTS stock solution according to table 11.
- Blank test: add the right volume of buffer and HPTS stock solution according to table 11; no beads are added
- Put the plate into the platereader

Measurement:

- Select test protocol Main_HPTS_0
- Enter file name
- Start measurement

Initiation of the reaction:

Platereader setup:

- Load test protocol Main_HPTS_1
- Adjust the layout
- Adjust cycle time according to the number of used wells

Sample preparation:

- Calibration: Add the right volumes of acetic acid stock solutions to the wells according to table 11.
- Blank test: add 20µl 30mM ethyl acetate solution (37°C) to each “blank test well”
- Sample: add 20µl 30mM ethyl acetate solution (37°C) to each “sample well” (as fast as possible, multichannel pipette)
- Put the plate into the platereader

Measurement:

- Select test protocol Main_HPTS_1
- Enter file name
- Start measurement

Table 10: 96 well plate layout for a typically main screening for one IE

96	1	2	3	4	5	6	7	8	9	10	11	12
A	X1	X2	X3									
B	X4	X5	X6		X16	X17	X18	X19	X20			
C	X7	X8	X9		X21	X22	X23	X24	X25			
D	X10	X11	X12									
E	X13	X14	X15									
F												
G											X26	X27
H												

Table 11: Volumes of solutions added to the wells

X	SPB [µl]	50µM HPTS Solution [µl]	10mM acetic acid solution [µl]	20mM acetic acid solution [µl]	30mM acetic acid solution [µl]	40mM acetic acid solution [µl]	30mM ethyl acetate solution [µl]
1-3	190	10	0	0	0	0	0
4-6	170	10	20	0	0	0	0
7-9	170	10	0	20	0	0	0
10-12	170	10	0	0	20	0	0
13-15	170	10	0	0	0	20	0
16-25	170	10	0	0	0	0	20
26+27	170	10	0	0	0	0	20

Evaluation:

Pre-screening:

- subtraction of the averaged background fluorescences
- plot the result as shown in figure 3
- control the standard deviations
- select the enzymes with larger slopes at the beginning for the main-screening

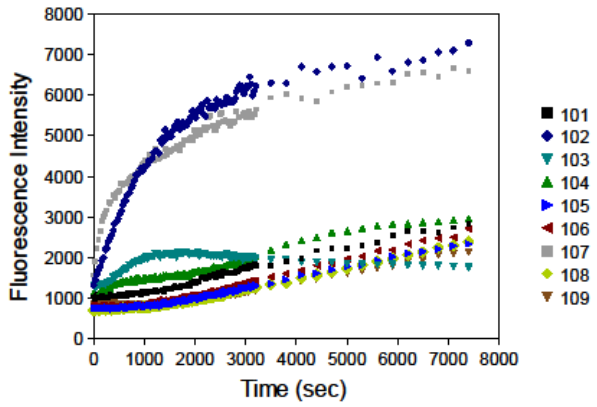

Figure 3: Pre-screening: example picture

Main-screaning:

- subtraction of the averaged background fluorescences
- calibration curve, linear fit (figure 4), declare outliers
- use the linear equation for the visualizing of the conversion (figure 5)
- control the standard deviations
- use the slope of the conversion curve for the determination of the enzyme activity

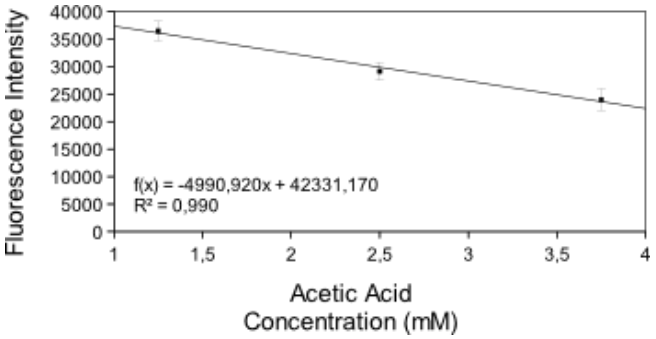

Figure 4: Acetic acid calibration and linear equation (NZL 105)

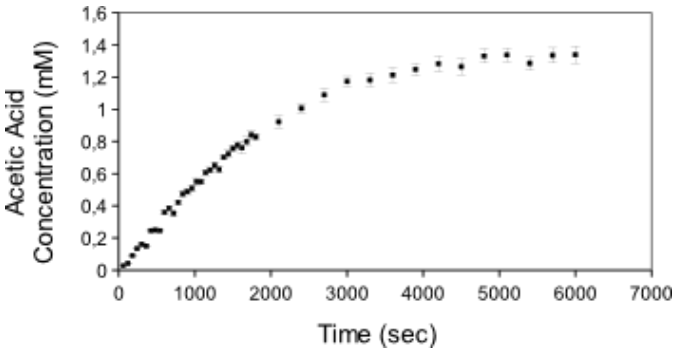

Figure 5: Conversion of ethyl acetate into acetic acid per 3 mg enzyme NZL 105

Enzyme activity:

The slope at the beginning of the conversion curve is used for the determination of the enzyme activity.

Slope = 0.56µM/(s) (according to figure 5)

Reaction volume = $200*10^{-6}$ l

Esters to cleave = 1

1 min = 60 s

1 Unit = 1000 mU

Amount applied beads = 3 mg

$$\frac{2.24 mU}{mg} = \frac{0.56 \mu mol * 200 * 10^{-6} l * 1 * 60 * 1000}{l * min * 3\ mg}$$

In the case of figure 5 the specific activity of the enzyme NZL 105 was **2.24 mU/mg**.

5.3 Annotation

According to which substrate is used this assay has to be adjusted. The calibration has to be performed with the acid which is released by the enzymatic cleavage of the substrate.

The solubility of ethyl acetate in water has to be considered. Therefore, high concentrated ethyl acetate stock solutions are problematic.

In order to get comparable results it is important to ensure a good mixing of all solutions.

A blank test is also important to visualize if a reaction takes place without enzymes. Whereas a second “blank test”, beads without substrate, is useful to check if the beads cause a reaction with the pH-indicator.

As fluorophores often depend on the ionic strength of a solution, it is important to use the same buffer and stock solutions for a whole experiment.

5.4 Troubleshooting

The cap of the microtiter plate can fog, especially when the plate has already been preheated and stays outside the temperate plate reader for a while. The result would be a lower intensity in the first few minutes until the cap is not steamed up any more. To avoid this problem minimize the time where the plate is outside the reader and do not put the plate nor the cap on a cold surface.

6 References

6.1 Corresponding documents

7 Notations

8 Status of the standard operation procedure

Author	**5.1.1**
Checked by	**5.1.1**
Approved by	**5.1.1**
Replaces document No.	**5.1.1**
Date printed	**5.1.1** **21.09.2011 12:52:40**
Filename and path	**5.1.1** **Dokument1**

5.1.1

9 Modification

5.1.1

5.1.1

5.1.1

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