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DISSERTATION

Titel der Dissertation

Contributions to the development of biomimetic,
optical biosensors – feasibility studies and
optimization of prototypes

Verfasserin

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angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer. nat)

Wien, Juli 2009

Studienkennzahl lt. Studienblatt: A 091 419

Dissertationsgebiet lt.
Studienblatt: Chemie

Betreuerin / Betreuer: Univ. Prof. Dr. Fritz Pittner

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Abstract

There is a wide variety of natural products such as a food or any kind of cosmetics and pharmaceutical products, which are offered to consumers.

Even if these natural products are produced, handled and stored according to the official guidelines for the mentioned steps, the period, during which they can be used by consumers, is limited due to spoilage processes. Therefore, so called expiration dates are typically assigned for these products.

However, expiration dates are not necessarily reliable factors since the quality of products seems to vary to a large extent. Furthermore, in case of improper handling (e.g. during production, transportation or storage), spoilage of the products may occur prior to the expiration date given.

Spoilage of products can mainly be attributed to microorganisms, which “live on the product”, i.e. which are able to utilize and/or metabolize certain components and/or molecules of the product.

Since the consumer is not able to detect the microorganisms associated with the product simply by looking at the product, there is the risk of using a contaminated product and accordingly of being infected by microorganisms in case the immune system is unable to handle the number and/or aggressiveness of microorganisms.

The aim this work was to develop and optimize sensor prototypes indicating the quality of a product “in real time”, i.e. at that point in time, when the consumer is looking at the product and not only after several days upon incubation of the product in order to test for spoilage-causing microorganisms (a standard test, which can be done in order to analyze spoilage by microorganisms).

The design of the presented sensors relates to optical phenomena, such as anomalous absorption and remission of light. The crucial point of presented

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sensor prototypes is a layer sensitive to the analyte, a polymer degradable by enzymes produced by the respective microorganisms.

After incubation of the sensor setup with contaminated products, the lytic enzymes released from decaying cell material change the thickness of the polymer layer and generate an with naked eye visible color change. The production of presented sensor prototypes is very simple, cheap and they could be successfully integrated into product packaging.

Zusammenfassung

Es ist eine Vielzahl von Produkten aus dem Lebensmittelbereich, dem Kosmetiksektor oder dem pharmazeutischen Bereich im Handel erhältlich. Auch wenn diese Produkte entsprechend den offiziellen Richtlinien hergestellt, verpackt und gelagert werden, kann Verderb eintreten. Daher werden diese Produkte auch mit Ablaufdaten versehen.

Allerdings sind solche Ablaufdaten nicht immer verlässliche Angaben, da die Qualität der Produkte doch ziemlich schwanken kann. Besonders, wenn es während der Herstellung, dem Transport oder der Lagerung zu Unregelmäßigkeiten kommt, ist auch ein Verderb vor dem Ablaufdatum möglich.

Dieser Verderb liegt meist an bakteriellem Befall der Produktoberflächen. Die Mikroorganismen nutzen als Nahrungsquellen verschiedene Komponenten des Produkts, welche dadurch abgebaut werden.

Da der Konsument diese Mikroorganismen am Produkt meist nicht direkt sehen kann, besteht die Gefahr einer bakteriellen Infektion, falls das Immunsystem mit der Anzahl oder der Aggressivität der Mikroorganismen überfordert ist.

Das Ziel dieser Arbeit war die Entwicklung und Optimierung von Sensor-Prototypen, die die Qualität dieses Produktes in „Echtzeit“ sichtbar machen, d.h. zu dem Zeitpunkt, zu dem der Konsument das Produkt verwenden möchte und nicht erst nach mehrtägiger Inkubation hinsichtlich Verderbnis erregender Mikroorganismen, wie die derzeit verwendeten Standardtests auf Verderbnis durchgeführt werden.

Das Design des hier vorgestellten Sensors beruht auf optischen Phänomenen wie anomaler Absorption und Remission des Lichts. Das Herzstück des Sensors ist eine biomimetische Polymerschicht, die durch von den betreffenden Mikroorganismen sezernierte Enzyme angegriffen werden kann. Wenn der Sensor mit bakteriell belasteten Produkten in Kontakt kommt, wird diese Schicht

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abgebaut und durch die Schichtdickenänderung kommt es zu einer mit bloßem Auge erkennbaren Farbveränderung der Sensoroberfläche. Die Herstellung des hier präsentierten Sensor-Prototyps ist so einfach und billig, dass er auch leicht in die Verpackungen der betreffenden Produkte integriert werden könnte.

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1. Biosensors

Conventional tests for the detection and identification of food borne pathogens and the detection of microbial food deterioration give very reliable results. But they need conventional culturing techniques and thus are time consuming, need sophisticated lab equipment and well trained staff. Thus only testing of random samples is possible and contaminated probes might be detected too late.

The current tendency to carry out field monitoring has driven the development of biosensors as new analytical tools able to provide fast, reliable and sensitive measurements with lower cost. These biosensors for the moment do not compete with official analytical methods, but they can be used both – by regulatory authorities and by industry to provide enough information for routine testing and screening of samples [1].

The main area of biosensor application and potential application is in health care. Measurements of blood, gases, ions and metabolites are often necessary to show the patients metabolic state.

Biosensors can be used also in control of industrial processes in three ways:

- off- line in a laboratory,
- off- line, but close to the operation,
- on-line, in real time.

Such monitoring could result in improved product quality, increased product yields and increased health care and safety for a consument.

1.1 General Definition

Biosensors combine the power of microelectronics with the selectivity and sensitivity of biological components such as whole cells, organelles or biomolecules, e.g. antibodies, receptors, enzymes or nucleic acids. They are used to detect individual substances in the environment, such as industrial emissions that originate, for instance, from the textile, cellulose or pharmaceutical industry as well as from agricultural activities.

Biosensor can be defined as a device incorporating a biological element connected to a transducer. A transducer converts an observed change (physical or chemical) into a measurable signal, usually an electronic signal, whose magnitude is proportional to the concentration of a specific analyte. The biological elements provide the major selective element in biosensors. They must be substances that can attach themselves to one particular analyte but not to others.

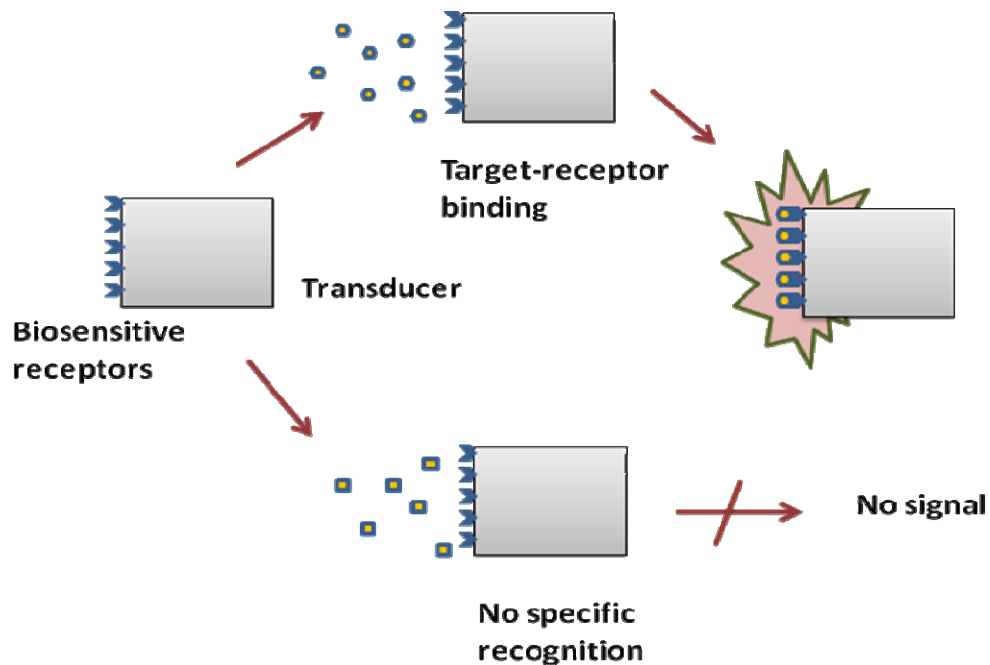


Figure 1 Principe of a generic biosensor. The binding-event of a target analyte to the biosensitive receptor gives rise to the occurrence of a distinct signal, depending on the kind of transducer.

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Biosensors may be classified according to the biological specificity-conferring mechanism or, alternatively, to the mode of physicochemical signal transduction. They may be further classified according to the analytes or reactions that they monitor:

- *direct* monitoring of analyte concentration or of reactions producing/consuming such analytes;
- alternatively, *indirect* monitoring of an inhibitor or activator of the biological recognition element. [2]

According to the **biorecognition principle**, biosensors are classified into immunochemical, enzymatic, non-enzymatic receptor, whole-cell, and DNA biosensors.[3]

Immunosensors present the advantages of great sensitivity and selectivity, but in contrast uncover troubles derived from the regeneration of the immuno-surface, and cross-reactivity, although a certain degree of cross-reactivity is often desirable in order to determine different congeners of the same family.

Enzymes are the most regularly used biological elements for biosensors. They are suitable to act as recognition elements because of their specificity and the wide range of enzymes available. The general *modus operandi* to quantify the decrease of activity of the immobilized enzyme in the presence of the target analyte as the easiest way of measurement is the selective inhibition of specific enzymes by different classes of compounds.

Biosensors based on *natural receptors* can be built by integrating the specific receptor within a membrane and coupling it to a transducing device. These natural receptors are proteins of non-catalytic or non-immunogenic origin, which can specifically bind to certain compounds, thus causing a change in structure or activity of an associated enzyme.

Whole cells of living organisms, such as bacteria, plant and animal cells, or even tissue slices have been used as the recognition component by interrogating their general metabolic status. These biosensors are useful to determine whether a substance is toxic to certain cells. Another application of whole-cell biosensors is the determination of the biological oxygen demand (BOD).

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In the case of *DNA biosensors* two strategies are applied to detect pollutants: one is the hybridization detection of nucleic acid sequences from infectious microorganisms, and the other one, the monitoring of small pollutants interacting with the immobilized DNA layer. [4]

On the basis of the **transducing element**, biosensors can be categorized as piezoelectric, thermal, electrochemical and optical sensors.

In the following chapters I want to go further into detail about this kind of taxonomy, starting with the *piezoelectric biosensors*, which have been proven to be versatile tools for application in quality assurance and process control in many different industrial sectors.

Furthermore, I want to give a brief overview of *thermal* – or also referred to as *calorimetric* – *biosensors* that are based on the measurement of heat transfer first described by Robert Gardon. [5]

The *electrochemical biosensors*, and among them the amperometric and potentiometric ones, are the best described in literature, not just because of their leading position on the biosensor-market.

Those sensors based on *optical principles* are the next most commonly used transducers. The various types of optical transducers exploit properties such as simple light absorption, fluorescence/phosphorescence, bio/chemiluminescence, reflectance, Raman scattering, and refractive index. Surface plasmon resonance (SPR) as a further transduction mechanism shows a main advantage over most optical biosensors, because of the direct determination of the analyte without the use of labeled molecules. [3]

1.2. Piezoelectric Biosensors

The Curie brothers discovered in 1880 that anisotropic crystals (those having no centre of symmetry) such as quartz, tourmaline and Rochelle salt, give out an electrical signal when mechanically stressed. Conversely, if an electrical signal is applied to such crystals, they will deform mechanically. Thus, with an oscillating electrical potential the crystal will oscillate mechanically.

Each crystal has a natural resonant frequency of oscillation, which can be modulated by its environment. The usual frequency is in the 10 MHz range. The actual frequency is dependent on the mass of the crystal together with any other material coated on it. The change in resonance frequency (Δf) resulting from the adsorption of an analyte on the surface can be measured with high sensitivity, resulting in sensors with detection limits in the picogram range.

The frequency of this oscillation (f) depends on thickness and cut of the crystals, each possessing a characteristic resonance frequency, which changes as molecules ad- or desorb from the surface of the crystal, obeying the relationship freely adapted from Sauerbrey:

$$\Delta f = -C_f \Delta m$$

$$C_f = \frac{2f_0^2}{\sqrt{\rho\mu}A}$$

... where Δf is the change in resonance frequency (Hz) correlating with Δm , the change in mass, and a correlation coefficient C_f , a constant for the particular crystal. It depends on such factors as density (ρ), shear modulus (μ), resonance frequency of the fundamental mode of the crystal (f_0) and the adsorbing surface area A (cm²). For any piezoelectric crystal, the change in frequency is proportional to the mass of absorbed material, up to about a 2% change.

Most gravimetric biosensors use thin piezoelectric quartz crystals, either as resonating crystals (quartz crystal microbalance), or as bulk/surface acoustic wave devices. [6]

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In a *quartz crystal microbalance* (QCM) the sensing element consists of an AT-cut quartz crystal with a gold (or silver) electrode deposited on its surface. The QCM will detect change in the mass of material at the electrode, such as :

- Ad- / desorption of species at the monolayer level
- Electrodeposition/ electrodisolution at the electrode due to Faradaic redox processes and
- Transfer of species between the solution and a surface immobilised film.

A typical QCM is made from a 10 MHz AT-cut quartz crystal giving a sensitivity of $4 \text{ ng cm}^{-2} \text{ Hz}^{-1}$.

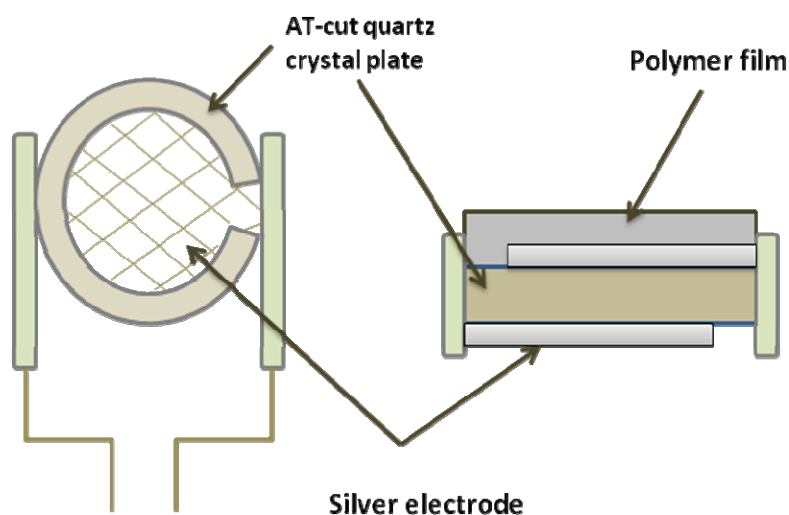


Figure 2: The Quartz Crystal Microbalance is an extremely sensitive mass sensor, capable of measuring mass changes in the nanogram range. QCMs are piezoelectric devices fabricated of a thin plate of quartz with electrodes affixed to each side of the plate.

A *surface acoustic wave* (SAW) is travelling along the surface of a material having some elasticity, with amplitude that typically decays exponentially with the depth of the substrate. This kind of wave propagates through the substrate and is then converted back to an electric field for measurement. As the acoustic wave propagates through or on the surface of the material, any changes to the characteristics of the propagation path affect the velocity and/or amplitude of the wave. Changes in velocity can be monitored by measuring the frequency or

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phase characteristics of the sensor and then be correlated to the corresponding physical quantity.

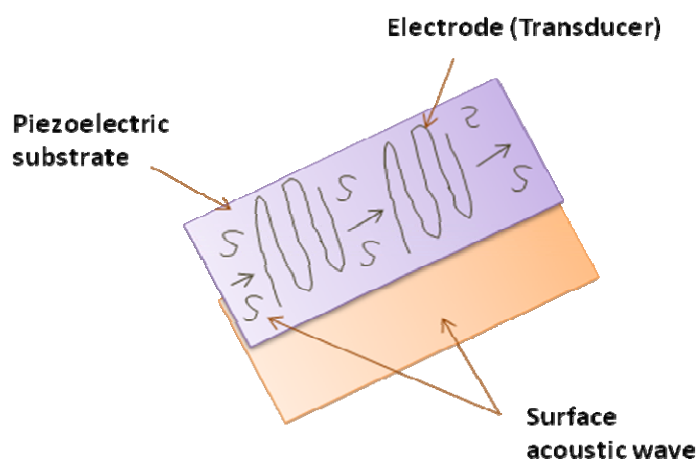


Figure 3: A typical acoustic wave device consists of two sets of interdigital transducers. One transducer converts electric field energy into mechanical wave energy, the other one converts mechanical energy back into an electric field.

In both cases, deposition or attachment of an analyte on the surface of the sensor causes a change in mechanical resonance frequency of the sensor. This principle makes them ideally suited for sensing immunogens, which deposit on immunoglobulin immobilized on a quartz crystal. Another reference sensor could be integrated to track frequency drifts due to temperature inconstancies or non-specific depositions.

The advantages of gravimetric sensors are that electro-active products do not interfere with the signal, and in the process of sensing, their output is a frequency modulated digital signal that can be easily interfaced to a digital device like a microprocessor. [7]

1.3. Calorimetric Biosensors

Biochemical reactions involving enzymes, in common with most other reactions, generate heat which is proportional to the amount of substrate that may be used as a basis for measuring rate of reaction and analyte concentration. This represents the most generally applicable type of biosensor.

The temperature changes are usually determined by means of thermistors at the entrance and exit of small packed bed columns containing immobilized enzymes within a constant temperature environment.

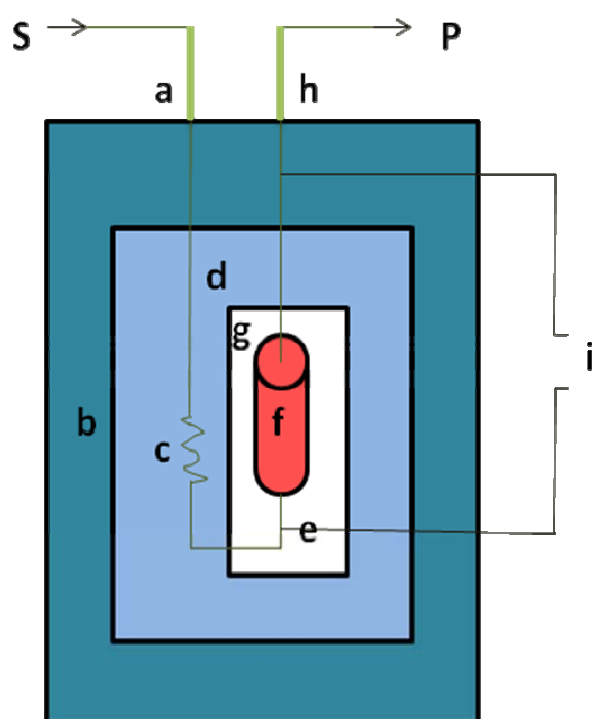


Figure 4: Schematic diagram of a calorimetric biosensor. The sample stream passes to the heat exchanger (c) within an insulating block (d) from where it flows past the reference thermistor (e) and into the packed bed bioreactor (f), where the reaction occurs. The change in temperature is determined by the thermistor (g) and the solution passed to waste (h). External electronics (i) determine the difference in the resistance, and hence temperature, between the thermistors.

There are three general types of sensors classified by the way of heat transfer between reaction vessel and surroundings:

- ❖ *Heat Conduction Calorimetry* is characterized by a fast heat transfer between the chamber and the isothermal heat sink surrounding, which is measured as voltage output of a thermoelectric sensor.
- ❖ *Isoperibol Calorimetry* is the most common method, also known under the synonym Isothermal Jacket Calorimetry. This method is not featured

by the measurement of heat transfer, because of the almost adiabatic condition, but the recording of a temperature change within the reacting solution, whereas the immediate cell environment remains at constant temperature.

- ❖ In the technique of *Isothermal Calorimetry* the temperature of the reaction chamber is kept constant by either heating or cooling. The degree of the energy required for this purpose gives information about the reaction components.

The main application fields of calorimetric biosensors are e.g. online industrial monitoring and process control in clinical chemistry(the detection of the glucose-level) as well as metabolites) in blood or of heavy metal and pesticides in the environment and enzyme linked immunoassays.

1.4. Electrochemical Biosensors

An electrochemical biosensor is defined by IUPAC (International Union of Pure and Applied Chemistry) as a “self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative information using a biological recognition element (biochemical receptor) retained in direct spatial contact with an electrochemical transduction element. (...) It is considered to be a chemically modified electrode (CME) as electronic conducting, semi-conducting or ionic conducting material coated with a biochemical film.” [2]

The diversity of molecular recognition systems and electrochemical transducers appears to be very broad. Nevertheless common features related to their operating principles are significant:

- ❖ Because of the nature of their operational principle, amperometric sensors, including biocatalytic-amperometric sensors, alter the concentration of the analyte in their vicinity. These sensors may reach a steady-state but never equilibrium. Knowledge of the rate-limiting step of their response, i.e. mass transport rate versus analyte consumption reaction rate, is very important for the understanding of their operational characteristics.

- ❖ Potentiometric as well as sensors based upon bio-complexing usually operate at or near equilibrium and are not subject to such transport limitations. On the other hand the magnitude of their apparent equilibrium constant and kinetics will define the continuity of the sensor response and the necessity for reagent introduction. If these sensors operate without requiring reagent addition and are capable of rapid and reproducible regeneration, they are referred to as multiple-use biosensors.

Conventional electrochemical systems employ three electrodes: (1) a working, (2) a reference, and (3) a counter electrode. The monitored reaction takes place at the working electrode. The function of the reference electrode is to measure the potential at the interface of the working electrode and the sample as accurately as possible. In an ideal situation, no current passes through the reference electrode. The task of the counter electrode is to ensure that the correct potential difference between the reference and working electrode is being applied, assumed to be the same as the desired potential at the working electrode.

For technical reasons many commercially available electrochemical biosensors use only two electrodes. Precise control of the voltage difference between the working and the reference electrode must be maintained, but such control is difficult to achieve in a two-electrode system. [8]

A dual-purpose reference/counter electrode combines the properties of both electrodes. Although ideally it ought to maintain a steady potential, still a low current passes through which results in a shift in potential, mostly small enough so that the response at the working electrode is not significantly affected.

1.4.1. Potentiometric Biosensors

Potentiometry measures the changes in the distribution of charges causing the development of an electrochemical potential. This is achieved by determining the potential difference between either an indicator or a reference electrode or two reference electrodes separated by a semi permeable membrane, when there is no significant current flowing between them.

The transducer-unit may be an ion-selective electrode (ISE), which is an electrochemical sensor based on thin films or selective membranes as recognition elements. [9]

The sensor's specificity entirely depends on the membrane, which means that for each analyte there has to be used a special kind of sensor – multi-analyte detections are therefore not possible. The most common potentiometric devices are pH-electrodes.

But this is not the only important task, since there are several other ion- or gas selective electrodes available, as e.g. for F^- , I^- , Na^+ , K^+ , Ca^{2+} , NH_4^+ , CO_2 and NH_3 . The potential difference between indicator and reference electrodes is proportional to the logarithm of the ion activity or concentration or gas fugacity.

When a biocatalyst layer is placed adjacent to the potentiometric detector, one has to take into account the following points that might interfere with an accurate result:

- (i) the transporting-time of the substrate to be analyzed to the biosensor surface;
- (ii) the analyte diffusion to the reacting layer;
- (iii) its reaction in the presence of a biocatalyst; and
- (iv) the diffusion of the reaction product towards both, the detector and the bulk solution. [2]

1.4.2. Amperometric Biosensors

Amperometry is based on the measurement of the current resulting from the electrochemical oxidation or reduction of an electro-active species. It is usually performed by maintaining a constant potential at a Pt-, Au- or C-based working electrode or an array of electrodes with respect to a reference electrode, which may also serve as the auxiliary electrode, if currents are low (10^{-9} to 10^{-6} A). The resulting current is directly correlated to the bulk concentration of the electro-active species or its production or consumption rate within the adjacent biocatalytic layer. [2]

Despite the high number of biosensors under development and the amount of research literature on this area, few practical systems are currently enjoying market acceptance.

The first successful commercial biosensor was the “glucose pen”, launched by Exactech in 1987. Nowadays, 90 % of sales come from glucose-detecting biosensors for medical applications. [10]

The simplest amperometric biosensors in common usage involve the Clark oxygen electrode. It consists of a platinum cathode where oxygen is reduced and a silver/silver chloride reference electrode. A potential is applied between the central platinum cathode and the annular silver anode. This generates a current which is carried between the electrodes by means of a saturated solution of KCl. This electrode compartment is separated from the biocatalyst by a thin plastic membrane, permeable only to oxygen.

The efficient reduction of oxygen at the surface of the cathode causes the oxygen concentration there to be effectively zero. The rate of electrochemical reduction therefore depends on the rate of diffusion of the oxygen from the bulk solution, which is dependent on the concentration gradient and hence the bulk oxygen concentration. A typical application for this simple type of biosensor is the determination of glucose concentrations by the use of an immobilised glucose oxidase membrane. A major driving force for biosensor development is the need for reliable glucose sensors as an adjunct to maintenance of normoglycemia in diabetic patients. This requires regular and in some cases,

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continuous monitoring because major fluctuations can occur in a very short time.

The responsibility for carrying out such measurements lies with the patient, and therefore the sensor must be user friendly and must provide a clear improvement in the patient's quality of life. This need has been principally met by self-monitoring blood glucose (SMBG) meters, consisting of meter readout and a single use strip employing a drop of blood taken from the finger.[11]

A novel type of potentially implantable glucose biosensors for continuous monitoring of glucose levels in diabetic patients has been developed. This glucose biosensor is based on an amperometric oxygen electrode and glucose oxidase immobilized on carbon powder held in a form of a liquid suspension. The enzyme material can be replaced (the sensor recharged) without sensor disassembly. [12]

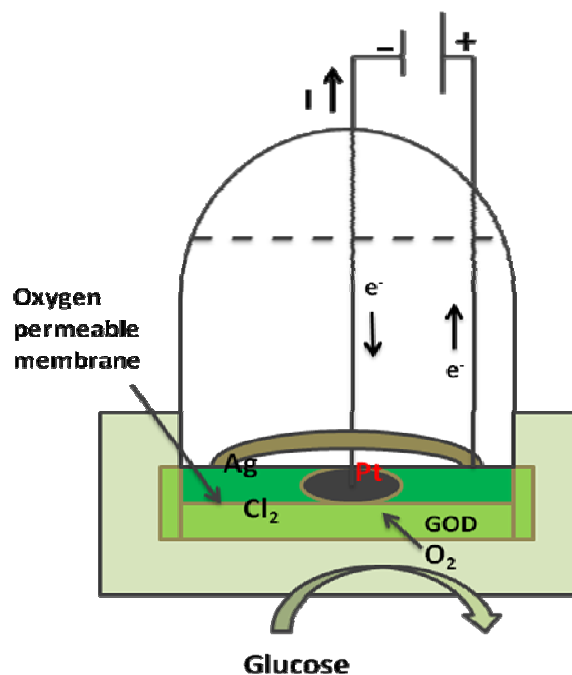


Figure 5: Schematic diagram of a simple amperometric biosensor

Introduction

The main advantage of this class of transducer is its low cost in manufacturing, which leads to a high rate of one-way electrodes depending on this technique. The high reproducibility that is possible for these electrodes eliminates the exhausting requirement for repeated calibration. The type of instrument used for these measurements can be obtained easily, and is inexpensive and compact; this allows the possibility of on-site measurements.

Limitations for this transducer include potential interferences to the response as several electro-active compounds in the analyte generate false current values. These effects have been eliminated, for clinical applications through the use of selective membranes which carefully control the molecular weight or the charge of compounds which have access to the electrode. [13]

1.4.3. Conductometric Biosensors

Enzyme reactions which produce or consume ionic species will, depending on the total ionic strength of the media, change the conductance or capacitance of the solution.

Many enzyme reactions, such as that of urease, and many biological membrane receptors are monitored by this technique. In the case of urease interdigitated microelectrodes measure the increasing conductivity in the solution due to ammonium and bicarbonate ions produced by the hydrolysis reaction of urea. [14]

Thin film conductometric electrodes can also be used to detect heavy metals. Glucose oxidase, alcohol oxidase and butyryl oxidase are immobilized on transducer surfaces and used as bioactive elements for detection of Ag^+ , Hg^{2+} and Pb^{2+} . Thus, dynamic concentration ranges of 1-100 μM can be obtained. [13]

Because the sensitivity of the measurement is hindered by the parallel conductance of the sample solution, usually a differential measurement is performed between a sensor with enzyme and an identical one without enzyme. [2]

Further boundaries are set due to the temperature-sensitivity of conductance. That is the reason for the necessary use of differential methods with internal controls.

The primary advantage of this technique is in the application of inexpensive, reproducible and disposable sensors.

The main disadvantage might be that ionic species produced must significantly change the total ionic strength to obtain a reliable measurement. This requirement increases the detection limit to unacceptable levels and results in potential interferences from variability in the ionic strength of the sample.

1.5. Optical Biosensors

Optical sensors are by definition of IUPAC able to transform changes of optical phenomena, which are the result of an interaction of the analyte with the receptor part of the sensor. [15]

This group of sensors may be subdivided further according to the type of optical properties applied. To mention some detection methods in this field I want to list the most common optical sensor principles in a table:

Principle

<i>Absorption</i>	Measured in transparent media caused by the absorptive itself or by a reaction with suitable indicator
<i>Reflectance</i>	Measured in non-transparent media, using an immobilized indicator
<i>Luminescence</i>	Measurement of the intensity of light emitted by a chemical reaction in the receptor system
<i>Fluorescence</i>	Measured as the positive emission effect caused by irradiation. Also, selective quenching of fluorescence may be the basis of such devices
<i>Refractive index</i>	Measured as the result of a change in solution composition

Table 1: Examples of detection principles used in optical sensing

Introduction

These detection principles can be summarized in two main groups: in the observation of fluorescence - labeled systems and in direct optical detection in the heterogeneous phase.[16]

Direct optical detection can be measured by remission (absorption of reflected radiation, optodes) by measuring micro - reflectivity, or measuring interference. In the last case either Mach - Zehnder interferometers or measurement of changes in the physical thickness of the layer (measuring micro - reflectivity) caused e.g. by swelling effects in polymers due to interactions with an analyte or in bioassays (due to affinity reactions) also play an important role.

The development of optical fiber sensors during recent years is related to two of the most important scientific advances of the 1960's: the laser and modern low - cost optical fiber. [17]

During the late 1960's and early 1970's some of these low - loss optical fibers were used in the development of the first chemical sensors, since then their application has continued to progress and spread to very different areas, e.g. clinical, environmental, industrial, food and military applications. [18]

The great success of optical - fiber sensors is their use to tackle difficult measurement situations where use of conventional sensors is not appropriate.

The advantages of optical devices are:

- No „reference electrode“ is needed- but a reference source is useful
- There is no electrical interference;
- An immobilized reagent does not have to be in contact with optical fibers. It can be replaced easily;
- Some analytes, such as oxygen, can be sensed in equilibrium;
- They are highly stable with respect to calibration, especially if one can measure the ratio of intensities at two wavelengths;
- They can respond simultaneously to more than one analyte using multiple immobilised reagents with different wavelenghts for response, e.g. O₂ and CO₂;
- Multi-wavelength measurements can be made to monitor changes in the state of the reagent

- They have a potential for higher information content than electrical transducers.

Their disadvantages are as follows:

- They will only work if appropriate reagent phases can be developed.
- They have a limited dynamic range compared with electrical sensors- typically 10^2 compared with 10^6 - 10^{12} for ion-selective electrodes.
- They are extensive devices, dependent on the amount of reagent, hence they are difficult to miniaturize.
- They show problems concerning the long-term stability of the reagents under incident light.
- Response times can be slow because of the time of mass transfer of analytes to the reagent phase.

While most optical sensor development tries to benefit from the merits of micro - electronics my work is aiming at developing a visual sensor translating the analyte concentration into a signal visible with naked eye directly, though only semi quantitatively.

1.5.1. Multiplexing Optical Biosensors

After the introduction into the field of biosensors in general a deeper description of optical methods in biosensing and their applications shall be given. Optical methods have recently attracted interest especially for detection of biomolecular interactions and in highly parallelized systems. The properties of electromagnetic radiation can be characterized by amplitude, frequency (wavelength), phase, polarisation state, and time dependence. In optical spectroscopy usually absorbance (or transmittance) is monitored. Especially for the detection of biochemical interactions like hybridization of two complementary DNA molecules or protein - protein - interactions the fluorescence based assays play a big role.

1.5.1.1. Thick Film Sensors

The need for a novel gas sensor material capable of providing reliable operation in harsh environments is now higher than ever. Such sensors find a range of applications, including the monitoring of traffic pollutants or food quality in specially designed electronic noses. Gas sensors based on metal - oxides are commonly used in the monitoring of toxic pollutants and can provide the necessary sensitivity, selectivity and stability required by such systems.

Commonly used oxides include, zinc oxide, titanium dioxide, iron oxide, tungsten oxide and tin oxide.[19]

These materials have been employed successfully to detect a range of gas vapours, particularly ethanol, methanol and propanol. Thick film technology is often used to fabricate such sensors and possesses many advantages, for example, low cost, simple construction, small size and good sensing properties. In addition, this approach provides reproducible films consisting of a well - defined microstructure with grains and grain boundaries that can be studied easily. Thick film sensors are - in most cases - oxide semiconductor gas sensors and have been extensively studied in order to understand or improve their sensing properties to inflammable and toxic gases. [20]

Among a variety of oxide semiconductors, tin dioxide (SnO_2) has proven itself to be one of the most attractive materials for gas sensor applications from a viewpoint of gas sensitivity as well as chemical stability. The gas sensing properties of SnO_2 sensors has been shown to be influenced by many intrinsic as well as extrinsic factors. The extrinsic factors include additives, grain size and microstructure, among which the latter two are important for sensor devices using SnO_2 . Sensor response to reducing gases (H_2 and CO) begins to increase sharply as the grain size of SnO_2 decreases to a critical value (6 nm). This corresponds to twice the thickness of space charge layer (grain size effect). On the other hand, the effect of microstructure has been recognized recently. The adsorption of gas on the surface of a semiconductor can bring about a significant change in the electrical resistance of the materials; there has been a sustained and successful effort to make use of this change for purpose of gas detection. Detection of toxic and flammable gases is a subject of growing

importance in both domestic and industrial environments. Some metal oxides such as SnO_2 and TiO_2 are well known for their high sensitivity to change in the surrounding gas atmosphere. They operate at moderate temperatures of about 300 - 400 °C, and are considered as “surface sensors” because the gas–solid interaction leading to the physical adsorption and chemisorptions modify electron/hole density in a relatively shallow region near the surface. Such thick film sensors show an enormous effect of diffusion from the liquid to the physical point of interaction of the analyte with the sensor matrix. This fluid solid interface though macroscopically flat, on a molecular level may be strongly structured and therefore have either beneficial or negative influence on the sensoric behaviour.

1.5.2. Thin Film Sensors

First optical chemical thin - film sensors were based on the measurement of changes in absorption spectrum and were developed for the measurement of CO_2 and O_2 concentration. Since then a large variety of optical methods has been used in chemical sensors and biosensors including ellipsometry, spectroscopy (luminescence, phosphorescence, fluorescence, Raman), interferometry (white light interferometry, modal interferometry in optical waveguide structures), spectroscopy of guided modes in optical waveguide structures (grating coupler, resonant mirror), and surface resonance. In these sensors a desired quantity is determined by measuring the refractive index, absorbance and fluorescence properties of analyte molecules or a chemo - optical transducing medium.

1.5.2.1. Luminescence

In luminescence the change in intensity or lifetime of fluorescent sample molecules called luminophores or fluorophores are sensed. The evanescent field excites sensor-particles and may force excited particles to release their radiation in guided modes improving sensitivities.[21]

The institution of ion-sensitive fluorescent dyes, which are designed to show a high affinity to specific ions, has allowed fluorescence to be used for measurement of even non-fluorescent substances. In 1980 the first fluorescent dye specific to calcium was reported, nowadays there are numerous dyes enabling detection of for example sodium, potassium, and magnesium ions by means of fluorescence. Chromophores exist that are functionalized for labeling a wide range of biomolecules including proteins and DNA.

Fluorescent biolabels are designed for excitation by red lasers (He/Ne; 633 nm) and diode lasers (635–650 nm). They can be used for covalent coupling to proteins and other biomolecules containing primary amino groups like amino-modified DNA oligomers, amino-modified biotin and proteins. Labeling makes sensing possible in a competitive format for binding between a biomolecule, biomolecule's complementary form and either the biomolecule or its labeled form. The number of fluorescent labels on the sensor surface quantifies the concentration of the biomolecule.

A major application of fluorescence measurements is the detection of low levels of analytes by fluorescence *quenching* of either intensity or decay-lifetime. The sensitivity of the luminescence is the % quenched intensity or % decrease in luminescent decay- lifetime caused by a given analyte concentration. Long lifetimes are required to distinguish sensor luminescence from relatively short-lived luminescence of interfering organic compounds.

Organometallic complexes of transition metals formed in conjunction with a fluorophore have both relatively high emission intensity and long lifetimes (milliseconds) permitting relatively simple and low cost measurement systems. Miniaturization of luminescent probes led to the production of oxygen sensors of diameter <1 mm and micrometer scale sensors for the detection of ions.

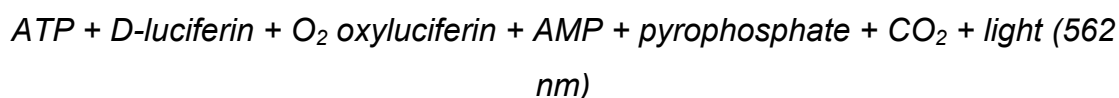
Introduction

The London South Bank University presented a promising biosensor involving luminescence that uses firefly luciferase to detect the presence of bacteria in food or

clinical samples.

Therein bacteria are specifically lysed and the ATP released (roughly proportional to

the number of bacteria present) reacted with D-luciferin and oxygen in a reaction which produces yellow light in high quantum yield.



The light produced may be detected photometrically by use of rather expensive high voltage photomultiplier tubes of high sensitivity or cheap low-voltage photodiode systems.

1.5.2.2. Resonance Enhanced Absorption (REA)

Light absorbing metal clusters, deposited only few nanometers from a reflecting surface (mirror) generate a strong color effect, REA. Absorption of light at defined wavelengths is intensified by an interference mechanism, „anomalous absorption“ is observed. At a specific distance between mirror and cluster (and fixed angle of light reflection) only part of the reflected lights is in phase with the oscillating field around the clusters. The almost complete absorption of these light waves and the high reflectivity at other wavelengths results in a visible color response. This color is a measure for the distance between cluster and mirror can be used to display alterations in the nanoworld. One field of REA application is biochip technology with DNA or protein arrays that are used in various fields: from DNA diagnostics to drug- and antigen screening.

Although the principle of Resonance Enhanced Absorption is quite simple, several different assay principles can be distinguished:

- Changes in number of cluster per area unit influence the intensity of the resulting color and so simple capture assays are possible.
- Changes in the distance between mirror and cluster results in a change of color and this approach can be used for the production of active polymer biochips.
- Packing density or spatial arrangement of the cluster layer can be changed to alter REA colors. This assay is of interest when using elongated clusters.

Colloids and clusters are found in everyday life: silver grains in photographic films, the red color of glass, aerosols, precipitates or dust particles etc. Fine tuning of cluster composition, size, solubility, plasmon effects, scattering and stability has received much attention. The state of single clusters was described by Faraday, Mie, Svedberg, Zsigmondy and others.

Background colors (mirror surface, distance layer) and thin film colors are additional effects that modify REA spectra. Since a maximum of dynamic range is desirable the device has to be built from layers of balanced refractive indices. The color response is tuned to compete with the optical background either by adjusting the thickness of the distance layer or by changing the path of light. Thin film colors are caused by interference and depend on refractive indices of the material applied in device design. Using of quality mirrors and distance layers with low refractive indices minimizes thin film colors.

1.5.2.3. Metal Clusters

Metal clusters are nanometric crystals or amorphous particles and bear unique physical and chemical behaviour. Clusters combine properties of metals and semiconductors including surface phenomena, interesting electronic and optical resonance. In literature metal clusters are also referred to as nanoparticles, nanoislands or precipitates.

Atoms in metal cluster material are bound by the same forces as in bulk material, but the surface to volume ratio is quite different and therefore surface effects dominate metal cluster behaviour. Even clusters built of about thousand

Introduction

or more atoms contain up to 25% of surface confined atoms. In general surface confined energy transduction is very inefficient but in nanometer size clusters the boundary region reaches deeply beyond the surface or even penetrates the whole surface.

To describe the behaviour of clusters classical electrodynamics are employed. Macroscopic metal objects clearly differ to nanometric metal objects in term of electrons. Electrons in macroscopic metal materials move being unconfined by material borders. This free movement of the electron gas results in strong and unspecific reflectivity. For colloidal particles the optical properties are defined not by a freely moving electron gas but by the oscillation of the electron gas. This collective swinging motion of electrons within the cluster is also a so-called particle plasmon.

Metal clusters with a band-gap in the visible range are excited by the electromagnetic field of a light wave. Most of the novel phenomena are based on the excitation of plasmons in the visible or infrared part.

For simple consideration, the behaviour of plasmon oscillations is modelled using a quasistatic regime. It can be assumed, that clusters of 10-20 nm in size behave like simple dipoles. For larger clusters and high numbers of clusters the situation becomes more complicated and the Mie function includes all electronic and atomic behaviour of the colloidal particle, which means that multipolar and magnetic modes of absorption and scattering are added compared to the simple dipolar model for small clusters.

Clusters can be formed from a wide variety of materials, but their application is limited by chemical stability in air, water or biological environment, especially stability against oxidation and thiol modification is a problem for many forms off clusters, Clusters of only a few atoms in size do not exhibit resonant behaviour in the visible range of the spectrum. Metal nano-cluster technology is based on the fact that electrons are moving freely and are excited by an external field. Under these conditions it is clear, that metals with a high amount of free electrons show the best optical effects and for theoretical studies alkali metals are ideal but they are very unstable in air and in aqueous solutions and so only noble metal clusters can be used in plasmon studies.

Introduction

Noble metals are the best material for resonant cluster technology. Ag and Au deviate from free electron behaviour because of interband transitions in the visible spectrum. The plasmon energy shifts from the free electron value of the bulk metal (10 eV) to 14 eV. As a consequence, optical resonance peaks of silver and gold are around 300-400 nm and 520 nm. Gold cluster solutions are orange, red or red-violet, depending on the method of preparation and thus on size, shape and aggregation state.

While chemically synthesized clusters are closer to the ideal model, clusters deposited by sputter coating or vacuum deposition are neither round nor free of crystal defects. To produce layers of metal nanoclusters (from materials such as silver and gold) sputter coating or slow thermal evaporation are applied. Films of a wide variety of materials are deposited within seconds or minutes. Sputter coating is usually done in argon plasma at 10^{-2} to 10^{-1} mbar. The thickness of the metal layer is adjusted by variation of sputter time, pressure and current. At high interaction energies a layer by layer growth mode is observed, resulting in a film instead of clusters. If the interaction between neighbouring metal atoms exceeds the interaction with the surface, an island-cluster growth is the result. Sputter coated clusters are small, flat and asymmetric. Heating to 100-350°C (Au up to 600°C) melts clusters as well as layers, resulting in defined nanocluster films of round shape. Possible materials, sputter coating settings and the resulting thin-film strongly depend on the sputter coater itself. Sputter coating is the method of choice for deposition of metallic layers. Both mirror- and clusters layers are produced by this technique.

On the one hand chemical clusters are closer to the ideal model as mentioned before, but on the other hand sputter coating is reproducible, quick and cheap method for the production of metal clusters and has been used throughout those thesis.

1.5.2.4 Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) is an optical biosensor technique that measures molecular binding events at a metal surface by detecting changes in the local refractive index. The phenomenon of anomalous diffraction on diffraction gratings due to the excitation of surface plasma waves was first described in the beginning of the twentieth century by Wood. Since then, surface plasmons have been intensively studied and their major properties have been assessed. When an optical beam is incident on a metal-coated glass prism its energy and momentum can be transferred into the surface of the metal to create a surface plasmon, which is a collective oscillation of free electrons that can give rise to very intense scattering and forms an electron density wave. [22]

The latter propagates along the glass–metal surface, but decays over about one wavelength into both the glass and metal media as evanescent wave.[21]

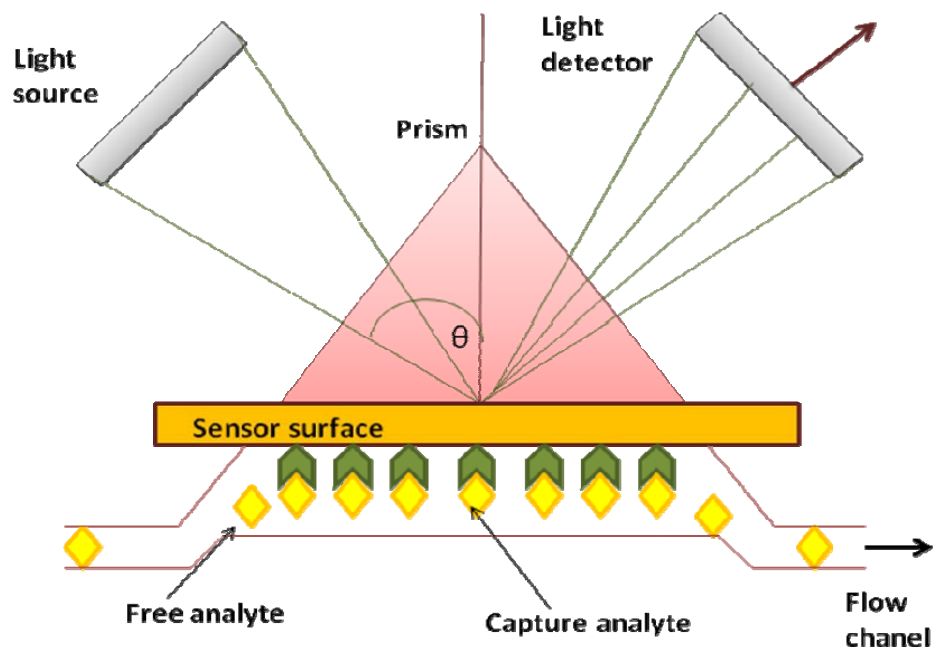


Figure 6: Surface Plasmon Resonance (SPR) technology: Upon binding of analyte molecules a change in refractive index can be measured.

Introduction

There are several instrumental formats that can be used in SPR experiments that can be roughly categorized into three types of measurements: *scanning angle SPR*, *scanning wavelength SPR*, and *SPR imaging*. [23]

For all SPR formats the reflectivity of light incident on a metal/dielectric interface is monitored and correlated to changes in the local index of refraction of the dielectric layer adjacent to the metal film. The most widely used format for an SPR experiment is the *scanning angle technique*, in which the reflectivity of monochromatic incident light upon a metal film is monitored as a function of the incident angle. The wavelength is fixed and the incident angle is swept until resonance is observed as a dip in the reflectivity curve.

The popularity of the scanning angle technique can be partially attributed to the existence of commercially available instrumentation from Biacore. Commercial application has made it possible to use SPR as a detection method for many purposes, including basic life science research, drug discovery, environmental monitoring and process analysis. Especially the possible real-time monitoring of macromolecular interactions is of paramount importance and a great benefit.

In case of Biacore SPR is evoked when light is totally internally reflected from a dielectric–dielectric interface (glass–solution) into which a very thin film (10 nm) of gold (or other such metal that obeys the free electron model, e.g., silver, aluminium) is interposed. The decaying evanescent wave at the interfacial region can collectively excite regions of charge in the gold film to form a surface charge density wave (the surface plasmon) parallel to the metal dielectric interface. Excitation or resonance of these surface plasmons within the metal occurs when the parallel wave vector component of the incident light becomes equal to the wave vector for the propagating surface plasmon. Resonance results in a decrease of the reflected light intensity applied as the measured parameter in this SPR device. For light of constant wavelength the condition for resonance is a function of the light's incident angle and effective refractive index of the surface layer; thus, an SPR experiment involves varying the angle of incident light until a drop in intensity is observed. [24]

If the incident angle is fixed near resonance and the wavelength swept until resonance is observed as a dip in the reflectivity curve, this technique is called *scanning wavelength measurement*.

Introduction

Both the scanning angle and scanning wavelength measurements typically provide only one or a few data points at a time. In contrast, *SPR imaging* measurements use the changes in reflectivity from a gold thin film that occur upon adsorption to generate difference images to simultaneously monitor tens, hundreds, or more interactions in a parallel manner. The high-throughput capabilities of SPR imaging have made it an attractive tool for screening biomolecular interactions. For example, SPR imaging has been used in an array format to study the hybridization of DNA and RNA to nucleic acid arrays fabricated on gold films. [25]

SPR offers several advantages over conventional techniques such as fluorescence or ELISA (enzyme-linked immunosorbent assay). First, because the measurements are based on refractive index changes, detection of an analyte is label free and direct. The analyte does not require any special characteristics (scattering bands) or labels (radioactive or fluorescent) and can be detected directly, without the need for multistep detection protocols. Second, the measurements can be performed in real time, allowing the user to collect kinetic data, as well as thermodynamic data. Last, SPR is a versatile technique, capable of detecting analytes over a wide range of molecular weights and binding affinities.

Although SPR has historically been limited by its throughput, new methods are emerging that allow for the simultaneous analysis of many thousands of interactions. When coupled with new protein array technologies, high-throughput SPR methods give users new and improved methods to analyze pathways, screen drug candidates and monitor protein–protein interactions. [26] The following main detection approaches have been commonly used in SPR sensors:

1. Measurement of the intensity of the optical wave near the site of resonance
2. Measurement of the resonant momentum of the optical wave including angular analysis.

1.5.2.5. Interferometry

Interferometry is the result of superposing two or more light waves and was first explained by Thomas Young (1773-1829).

In the case of interferometry biosensors the waves originate from the same source, either of them takes a path through a sample, the other follows a reference path. After passing through the sample arm, the two light beams are brought together to interfere and produce a set of interference fringes. The position of the fringe, and thus, the phase, shifts due to changes in the sample's dielectric constant-value.

A *Mach–Zehnder interferometer* is the most frequently employed system due to its ease of use, flexibility, and separate reference arm, providing accessibility and appropriateness for the scheme to be further miniaturized into integrated optics. Its bulk set-up is shown in Figure 7. The source beam is split by a semi silvered mirror, named “beam splitter”, and the two resulting waves travel down separate paths, until each of them reaches a totally reflecting mirror. The two beams then pass a second semi silvered mirror and enter the detector. A slight tilt of one of the beam splitters will result in a path difference and a change in the interference pattern.

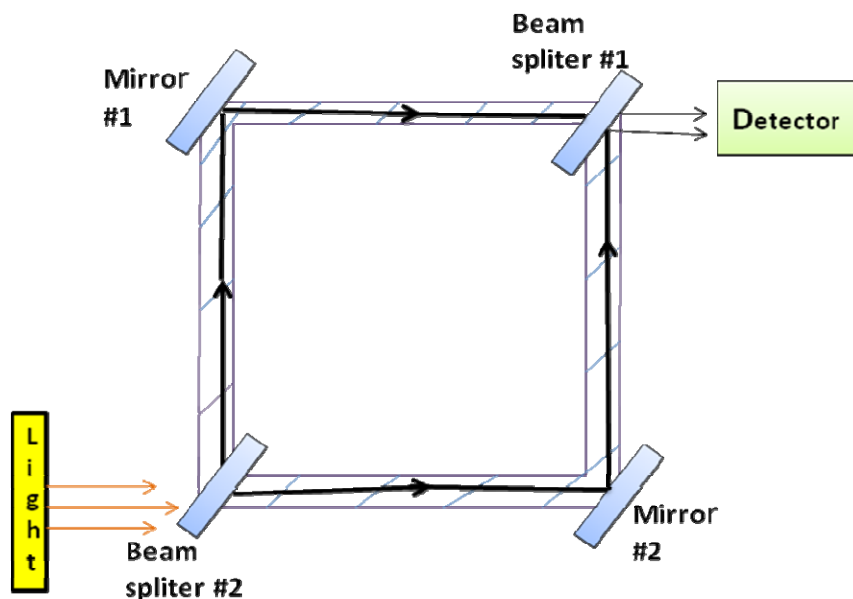


Figure 7: Top view of the interferometer introduced by Mach and Zehnder.

Positioning a sample arm in the way of one of the light beams makes it possible to measure the changes in the concentration of molecules, which induces a slightly changed dielectric constant of the material in the sensing region.

The dielectric constant is a characteristic quantity of each substance. In general, it is a complex constant, with the real part giving reflective surface properties, and the imaginary part giving the absorption coefficient. [21]

Two types of sensing are possible:

- *Homogeneous or 'bulk' sensing* where the measurement is homogeneously spread in the evanescent field region causing a homogeneous change in the refractive index of the sample material. The sensing is non-selective and thus the sensor cannot differentiate between the diverse analytes.
- *Surface sensing*, where a very thin sensitive layer called an adlayer on top of the core layer selectively interacts with a given analyte. The adlayer has a distinct refractive index, whose change should be measured.

The sensitivity of biosensors based on interferometry depends on the mode propagating and the refractive index distribution within the evanescent field region. Furthermore, it also depends on the resolution with which effective index changes can be measured.

1.5.2.6. Fabry - Perot Interferometer

A Fabry - Perot - Interferometer or etalon is typically made of a transparent plate with two reflecting surfaces, or two parallel highly reflecting mirrors. (Technically the former is an etalon and the latter is an interferometer, but the terminology is often used inconsistently.) The transmission spectrum as a function of wavelength exhibits peaks of large transmission corresponding to resonances of the etalon. It is named after Charles Fabry and Alfred Pérot. 'Etalon' is from the French hybrid, meaning 'measuring gauge' or 'standard'. The varying transmission function of an etalon is caused by interference between the multiple reflections of light between the two reflecting surfaces. Constructive interference occurs if the transmitted beams are in phase, and this corresponds to a high - transmission peak of the etalon. If the transmitted

beams are “out of phase”, destructive interference occurs and this corresponds to a transmission minimum. Whether the multiply - reflected beams are “in phase” or not, depends on the wavelength (λ) of the light, the angle the light travels through the etalon (θ), the thickness of the etalon (l) and the refractive index of the material between the reflecting surfaces (n).

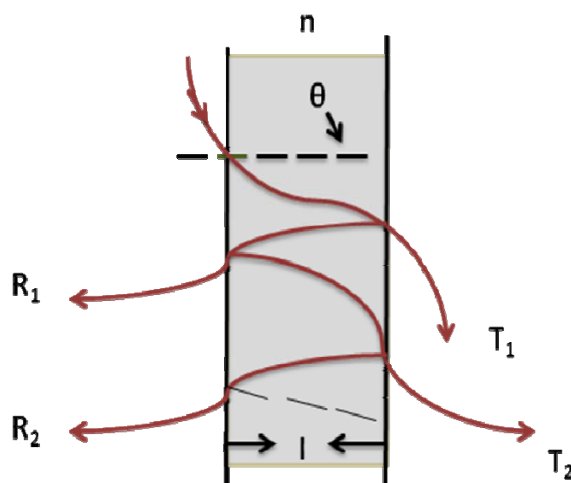


Figure 8: Scheme of Fabry – Perot interferometer

1.5.2.6. Anomalous Absorption

The combination of the two phenomena plasmon absorption and optical interference is generally called “anomalous absorption”.

Anomalous absorption is a strong, broad-band absorption in the visible range, which is referred to a special assembling involving a bulk metal film in near spatial separation from nanometric metallic particles.

Ensembles of nanoparticles display unique optical and electrical properties that are distinct from their respective bulk properties and from average measurements of collections of widely spaced particles. [27]

The semi-free electrons in such clusters react like resonators upon excitation, due to their spatial confinement. These cluster films show a weak metallic reflection but appear to be strongly colored during transmission. Owing to the

spatial confinement of the free electron plasma in the metal particle, the light-field-driven plasma oscillations show resonances in the visible in contrast to the plasma oscillations in an extended metal, where no light driven resonances are possible, since only surface regions are excited by the light field and surface plasmons are formed. In an extended metal, the unconfined electron movement is responsible for strong, unspecific reflectivity, well-known as metallic glance.[28]

Recapitulating, the conduction electrons in clusters act like an oscillator system whereas those in a bulk metal act as a relaxator system. The scientific interest in cluster films strongly increased in the 1980's when surface-enhanced optical phenomena were discovered. It was found, as a result of the resonantly oscillating charges inside a metal particle, that the local electric field strength outside the particle is strongly increased over the original field irradiated by the light source. Several new surface-enhanced optical effects were observed when molecules were positioned close to the cluster film's surface: *Surface enhanced absorption, surface enhanced luminescence and surface enhanced photochemistry*. If a cluster film is positioned close to a smooth metal surface it can be observed that the spectral reflectivity minimum strongly depends on the interlayer thickness and thus a thickness change will be visible upon illumination with white-light by the altered colour impression of the reflected light. This absorbing thin-film positioned at a defined distance to a metal mirror represents a special kind of reflection interference filter. At an appropriate distance of the absorbing layer to the mirror, fields reflected by the mirror are in the same phase at the position of the absorbing layer as the incident fields and by this feedback mechanism the effective absorption coefficient of the absorbing layer is strongly enhanced. The molar extinction coefficients are up to 1000 times higher compared to conjugated chromophores. As for a given interlayer distance the optimum phase is only given for a defined wavelength, the system is characterized by a spectrally narrow reflection minimum, whose spectral position shifts sensitively with the interlayer thickness. [29]

A number of applications of this phenomenon have already been patented.

1.6. Contamination and Spoilage of Natural Products

There is a wide variety of natural products such as food or any kind of cosmetics and pharmaceutical products, which are offered to consumers.

Even if these natural products are produced, handled and stored according to the official guidelines for the mentioned steps, the period, during which they can be used by consumers, is limited due to spoilage process. For this reason, so called expiration dates are typically assigned for these products.

However, expiration dates are not necessarily reliable factors since the quality of products seems to vary to a large extent. Furthermore, in case of improper handling (e.g. during production, transportation or storage), spoilage of the products may occur prior to the expiration date given.

Spoilage of products can mainly be attributed to microorganisms, which “live on the product”, i.e. which are able to utilize and/or metabolize certain components and/or molecules of the product.

Since the consumer is not able to detect the microorganisms associated with the product simply by looking at the product, there is the risk of using a contaminated product and accordingly of being infected by microorganisms in case the immune system is unable to handle the number and/or aggressiveness of microorganisms.

Thus, there is the need for a device indicating the quality of a product “in real time”, i.e. at that point in time, when the consumer is looking at the product and not only after several days upon incubation of the product in order to test for spoilage-causing microorganisms (a standard test, which can be done in order to analyze spoilage by microorganisms). Such a device should have a simple setup. Furthermore, the device should be cost-efficient in terms of the materials needed. Clearly, any risk of contaminating the product by the device (in terms of

e.g. harmful chemicals or the like) needs to be avoided. Finally, the production of such a device should be easy, fast and cost-efficient.

Thus, there is a need for a cost-efficient “real time” sensor indicating the quality of a product which can be produced simply (e.g. in terms of materials needed) and fast.

1.7. Setup and Application of Optical Three-Layer Sensor

Optical properties of mirror and metal clusters can be used to produce biomimetic devices of extraordinary functionality. There are two different designs which are described in this work:

1. The design of the sensor relates to the phenomenon of “anomalous absorption”, which can best be described as a thin film enhanced absorption. A metal cluster film positioned at a well defined distance to a smooth metal surface shows that the minimum of spectral reflectivity strongly depends on the thickness of the interlayer: This setup represents a special kind of reflection interference filter. In such a sensor setup we have integrated a biodegradable polymer which is degraded by the same enzymes and at the same rate as food decay will happen. The degradation of the polymer results in reduction of the film thickness and thus in a specific change of the color. [30]
2. The second possibility is a Fabry-Perot sensor setup that includes two reflecting metal layers and polymer interlayer between them.

1.8. Setup and Application of Optical Two-Layer Sensor

During this work it could be shown that a device comprised of two layers (a reflective layer and biodegradable polymer layer) only, can be used for the analysis of the quality and/or age of products such as medical products, cosmetics and foods.

Typically, degradation occurs via the cleavage of covalent bonds within the polymer. Polymer degradation is caused by lytic enzymes secreted from either the product to be tested or from a microorganism associated with product or from both. Examples for such enzymes are phospholipases, pronases, proteinases, hydrolases, lipases, esterases. Thus, the process of degradation is irreversible resulting in a definite “signal” of the device in form of a irreversible color change.

The color of the device is defined by remission of incident light by the reflecting layer in combination with the properties of the polymer layer. Thus, the polymer layer may display different characteristics as long as it is biodegradable and optically transparent such that incident light is able to contact the reflecting layer and remitted light is able to pass the polymer layer (wherein the polymer layer does, however, influence the remission).

The intensity/degree of color change is in this respect proportional to the degree of change in the polymer layer. One major factor influencing the characteristics of the polymer layer is obviously the thickness of the polymer layer; another factor relates to the refractive index of the polymer layer, which is characterizing the optical thickness of the layer.

Incident light is remitted by the reflecting layer as described above and ultimately leads in combination with the optical properties of the polymer layer to a specific color of the device which may appear to the human eye as e.g. a red, white, blue or green color.

Most preferably, the biodegradable polymer is comprised of a biomimetic polymer, i.e. a polymer with characteristics mimicking a natural material, which has already been approved and extensively tested for its behaviour when

exposed to a natural product and/or a living organism. Poly (lactic-co-glycolic acid) (PLGA), which was preferably used as biodegradable polymer, is approved for *in vivo* use.

Further polymers, which can be used in sensor setup described above, may be selected from the group of polymers comprising polylactic acid (PLA), poly-L-lactic acid (PLLA), polyhydroxybutyrate (PHB) and polyvinylcaprolactame (PVCL) or any other polymer, which can be classified as a polymer degradable by biomolecules as defined above. Preferably, biodegradable synthetic polymers can be used. This may also comprise synthetic polymers of gelatine, agarose, dextrose, lipids, cellulose, starch, chitin, polyhydroxyalkanoates, poly(-caprolactone) (PCL) or PCL-systems, poly(ethylene/butylenes) succinate, poly(ethylene/butylenes) adipate or polynucleic acids.

The biodegradable polymer layer may additionally comprise a cross-linking agent. This cross-linking agent may be a bifunctional agent, such as e.g. diisocyanate, glutardialdehyde or Desmodur (Desmodur 2460 M, Bayer) products based on diphenylmethane diisocyanate (MDI), toluene diisocyanate (TDI), hexamethylene diisocyanate (HDI) and isophorone diisocyanate (IPDI) may also be used.

The sensor setup is in general based on the remission of light by a reflecting layer; thus, the color of the device is mainly dependent on the reflecting layer used and thus the nature of the reflecting material. Furthermore, however, the polymer layer, which is positioned on the reflecting layer, also influences the process of remission. Thus, the color of the device also depends on the presence of the polymer layer and the optical properties and/or characteristics of the polymer layer. [31]

If e.g. a polymer layer with a thickness of 100 nm comprising PLGA is positioned on a reflecting layer, the device will show a specific color resulting from the cumulative effect of remission and the optical properties of the PLGA polymer. Obviously, the device displays a different color in case the PLGA layer is absent since the effect is then dependent on the remission by the reflecting

layer only. Thus, such a device comprised of a reflecting layer only displays a different color than the device comprising polymer layer as well.

2. Microbiology

2.1. Microorganisms under Stress Conditions

Within any population of microorganisms surviving exposure to preservation treatments or environmental stress there exist individual cells that are regarded as sub lethally injured. Stress treatments that cause injury include heating, refrigeration, irradiation, freezing, high salt, acid or alkali levels, preservatives, desiccation, starvation or nutrient limitation. Individually, cells subjected to these conditions will suffer differing degrees of injury. Some will remain fully intact, exhibiting no signs of damage, some will suffer sufficient damage to lead to a loss of viability and the remainder will be sub lethally injured the latter suffering damage that will lead to a temporary change in physiology. From a methodological point of view injury manifests itself in three important ways:

- a.) sensitivity to the selective agents used traditionally in microbiological culture media;
- b.) sensitivity to low levels of reactive oxygen species that can exist in microbiological culture media and
- c.) extended lag phase during which repair of damage takes place.

Despite being in a debilitated physiological state, injured cells can pose a significant spoilage and public health risk. The main concern is that injured cells might undergo resuscitation either in the food or following ingestion. In Gram-negative enteric organisms and pseudomonads, resistance to lipophilic dyes, bile salts, and many lipophilic antibiotics is due to their restricted penetration through the outer membrane combined with active efflux through multidrug

transporter system. If the outer membrane is damaged, cells are enabling to grow on selective media containing bile salts or dyes. [32]

2.2. Spoilage of Food

There are many important factors influencing the ability of microorganisms to grow on/in food products. These factors are the same as for general bacterial growth. Temperature is perhaps the most important one, which can be estimated in range from 0 °C to 60 °C. It was shown that enzymes of most microorganisms are inactivated at 60 °C, due to rapid denaturation. However, the activity of many enzymes increases 2 fold with an increase of 10 °C. The pH of the growth medium is the second important factor that influences microbial growth on food products such as a meat. Optimal pH for the most of enzymes lies at a pH around 7. Lowering the pH by pickling (vinegar or other acids) can prevent food from spoiling. Another factor is oxygen supply. Reducing the amount of oxygen can greatly influence bacterial growth through completely inhibiting growth of microorganisms or changing the flora that develops on the food products. Furthermore, the reduction of oxygen is a very useful method to inhibit or completely stop the growth of the worst food poisoning bacteria. Supply with water plays here also an important role. Optimal water activity occurs at $a_w = 0.99$. Food is a huge sources of carbon, nitrogen or phosphate the nutrient requirements for bacterial growth. The first energy source for microorganisms on food products will be freely diffusible glucose. Glucose affects the ability of microorganisms to develop high populations. The main catabolic pathways used by food borne bacteria to utilize glucose are the Embden-Meyerhof-Parnas pathway, Entner-Doudoroff pathway and the heterofermentative pathway. When glucose levels are exhausted, bacteria begin to utilize other components as energy source. The breakdown of proteins and fatty acids will only start if no more glucose is available. Defects which are often associated with the spoilage of food include: color changes, changes in texture, off flavours, off odours and slime. Generally most microbiologists agree that

spoilage becomes obvious when the total bacterial count reaches numbers as high as 10^7 CFU/mL.

Redox potential should also be mentioned here. Biodeterioration is not confined to highly aerobic situations but may also occur in anaerobic clays, sewer pipes, and large reservoirs of stagnant cutting fluids. Bacteria play a prominent part in these situations. The exclusion of oxygen can be used as a control measure in preventing growth of obligate aerobes. The use of an atmosphere of 10-20 per cent CO₂ is sufficient to retard or inhibit the growth of many bacteria and fungi. When used in conjunction with lowered temperature the effect is enhanced and can thus be used to transport fruit which would be affected by the CO₂ or lower temperatures if either was used independently.

2.3. *Pseudomonas* species

The genus *Pseudomonas* has undergone taxonomic changes in recent years, with species being removed and placed in the genera *Xanthomonas*, *Stenotrophomonas*, *Burkholderia*, *Comamonas* ect.

Pseudomonas spp. belong to a group of organisms of great importance as pathogens and as spoilage organisms. *Pseudomonas* spp. are ubiquitous and have been isolated from a variety of sources including drinking water, animals, humans, plants and therefore also from various food. They are Gram-negative rods, catalase positive, oxidase positive or negative and typically motile. They are aerobic, although nitrate in some cases can be used as an alternative electron acceptor, allowing growth to occur anaerobically, e.g. in vacuum-packed meat products. The predominant spoilage organisms in proteinaceous foods with high a_w belong to genus *Pseudomonas*. In food and environmental samples *Pseudomonas* spp. may only be present in low concentration and are often accompanied by a competing microflora which is physiologically similar.

2.4. Microbial Enzymes

Enzymes have been used by man throughout the ages, either in the form of vegetable or animal tissues rich in enzymes, or in the form of microorganisms utilized for a variety of purpose. However, it was not until the middle of the nineteenth century that one began to get an understanding of what enzymes are and why they work the way they do. Probably, the first pure enzyme preparation was done by French researchers Payen and Persoz, who in 1833 isolated the enzyme complex diastase from malt. The first microbial enzyme preparation was made by Japanese scientist Takamine who, using traditional oriental techniques, developed an industrial process for the manufacture of fungal amylase by growth of a strain of *Aspergillus oryzae* on moist rice or wheat bran.

Besides the detergent industry microbial enzymes have found applications in several other industries. In starch processing a number of different amylases have to a large extent replaced acid as a catalyst for starch hydrolysis, whereby a much broader range of products and higher yields have been achieved. The latest development in this field is the introduction of glucose isomerase, an enzyme capable of catalyzing the conversion of glucose to fructose. Fructose is about twice as sweet as glucose, and enzymatic isomerization has made it possible to convert starch to a sweetener equal to cane sugar in sweetness and energy content.

Of the more than 2,000 enzymes known today fewer than 50 are produced in commercial quantities. Some of these are made from plant materials or animal organs, but the majority is produced from microorganisms. Furthermore, almost all new enzyme preparations developed are of microbial origin. There are several reasons for this:

(i) microorganisms grow fast and are well suited for cultivation under industrial conditions;

- (ii) their growth substrates are relatively inexpensive and generally consist of common agricultural products available almost anywhere in the world-thus microbial enzymes can be made in almost unlimited quantities; and
- (iii) furthermore, the choice of suitable microorganisms is very wide and can easily be increased, e.g. by using mutation techniques, so that it is possible to develop microorganisms suited for the production of virtually any enzyme desired. [33]

2.5. Bacterial Fermentation

The optimal bacterial medium should provide an energy source as well as carbon and nitrogen sources and any special growth requirements, such as essential amino acids or vitamins. However, good growth is not sufficient to ensure a high enzyme yield. The presence of specific components in the medium may inhibit or promote enzyme production. A well-known repressant for certain enzymes e.g. amylases, is glucose. In such cases the glucose concentration should be kept low, either by adding the sugar incrementally during the fermentation or by using a slowly metabolizable sugar, such as lactose or partly hydrolyzed starch.

The medium composition should be balanced so as to keep the pH within the desired limits during fermentation. This can be achieved by adding a buffer system such as phosphate or calcium carbonate to the medium. Alternatively substances which, when metabolized, give rise to the formation of either acid or base may be added. Thus salts of organic acids will tend to increase the pH, ammonium salts will tend to lower the pH. Sometimes inducers are necessary for the formation of enzymes in high yield. This was also the case in this work, where by addition of glucose, oil or phosphate a higher bacterial excretion of lytic enzymes occurred.

The subsequent recovery process should also be taken into account when formulating the medium. The composition should be such that at the end of the

fermentation the dry matter content is as low as possible, and any materials which could hamper separation of solid and liquid phases should be absent.

2.6. Microbial Interactions

This aspect although widely studied using abstracted groups of microorganisms is still little understood in considering *in situ* populations of bacteria and fungi in biodeterioration problems. Many types of bacterial interaction have been described such as direct hyphal interaction, chemical antagonism (volatile or non-volatile), and the complex competition for nutrients. All of these processes are important in biodeterioration problems. Fungi which attack wood are known to produce volatile inhibitors which depress the growth of other potential colonizers.

Many situations in biodeterioration studies consist of complex nutrient formulations made up of simple inorganic and larger organic molecules. The availability of these potential colonizers will vary initially and after attack has commenced. Those one microorganism may have an initial selective advantage as far as the available nutrients are concerned but may then be replaced or added to by other species giving rise to a succession. All of above environmental factors will figure to varying extents in the three environments-marine, freshwater, and terrestrial- all of which have their own specific biodeterioration problems. A complete understanding of the organism-substrate-environmental interactions or the ecology of biodeterioration is necessary. The reliance upon one method of control in a situation not fully understood may result in developing an even worse situation. With knowledge of the environmental factors, the microorganisms present, and the substrates the biodeterioration is able to predict a problem, identify the agents responsible, and recommend the correct preventive or curative measure.

Materials and Methods

1. Microbiology

1.1. Agars for the Isolation and Identification of Gram Negative Bacteria

1.1.1. Plate Count Agar

Plate count agar is a nonselective microbiological growth medium for the monitoring of total bacterial counts from testing samples. The investigated sample containing the bacteria has to be diluted in Ringer Solution before it is plated onto the agar. It is recommended to prepare dilutions in the range from 10^{-1} to 10^{-9} and plate those from 10^{-3} to 10^{-9} (depending on the total bacterial count expected).

1.1.2. ÖNÖZ Agar

ÖNÖZ agar is a selective medium for the isolation and identification of *Salmonella* and some other Enterobacteriaceae (*bacterial identification depends on the morphology of the colony as listed in Table 2*).

Microorganisms	Appearance of colonies
<i>Pseudomonas</i>	Glossy, dirty yellow to greenish; culture medium is yellow
<i>Enterobacter</i>	Large, mucoid, bluish or reddish, slight precipitation ring around the colonies
<i>Proteus</i>	Rust-colored, culture medium surrounding the colonies of same color, if growth is too dense, dark brown to black
<i>Salmonella</i>	Yellow, medium size; 1 st day: black dots start to develop on the yellow colonies 2 nd day; black dots clearly visible on the yellow colonies; Culture medium surrounding the colonies is yellowish

Table 2: Characterization of microorganisms

1.1.3. GSP Agar

GSP agar is a *Pseudomonas/Aeromonas* selective agar acc. to KIELWEIN (base) for microbiology. This is an agar containing glutamate and starch as its only nutrients and phenol-red as a pH indicator. Starch can be degraded by *Aeromonas* producing acid (color change of phenol-red to the yellow, but not by *Pseudomonas*). For improvement of selectivity it is possible to add some selective inhibitors such as penicillin or the antimycotic pimarin (it was not necessary in our case).

1.2. Liquid Media for Bacterial Growth and Enzyme Excretion

Liquid media listed in this work were used not only for bacterial growth but also for inducing enzyme production. For example, some of them such as LM-Medium contain phosphate in form of $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$. Phosphate is an important inducer and activator of lytic enzymes such as aminopeptidase and phospholipase. The main goal was to find media suitable to simulate food decay conditions at the meat surface and a certain spoilage grade.

1.2.1. Peptone Water

Peptone water is a liquid growth medium commonly used for fermentation studies and the recovery of bacterial cells after long exposure to environmental stress. It contains a phosphate buffer.

1.2.2. LM- and EM-Media

LM-and EM-Medium are liquid media for promotion of bacterial growth and induction of fermentation processes.

1.2.3. Ringer Solution

Ringer solution was used for dilutions of bacterial cultures for detection of bacterial counts.

1.3. Comparison of Bacterial Growth in LM and EM Media

To study the behavior of *Pseudomonas* and *Enterobacter* in two different growing media, both were isolated from pork meat (ÖNÖZ Agar). Stock cultures were prepared in 200 ml of Liquid Medium and incubated at 37°C. Every 1 to 2 hour/s, the optical density of 1 ml of aliquot is measured at 578 and 600nm.

The total bacterial count was also determined with Plate Count Agar. All dilutions were made with Ringer Solution. Five dilutions (10^{-3} - 10^{-7}) per sample were plated and incubated for 72 hours at 30°C.

In parallel, *Pseudomonas* and *Enterobacter* were incubated for 6 hours at 30°C, in order to compare in detail growth behavior and in correlation the enzyme activity.

1.4. Culture Preparation for Maximal Enzyme Production

Many protocols were developed during this work in a way to gain the highest lipase yield level. The main bacterial culture (100 ml) containing the bacteria from homogenized pork meat was used as a starter culture and incubated for 6 hours (Culture a) and 24 hours (Culture b) in a water bath at 15 °C. After these periods of bacterial growth, the cultures showed the total bacterial counts of $\sim 10^7$ CFU/mL Culture a, and $\sim 10^8$ CFU/mL for Culture b. Starter culture was prepared in LM medium, with $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ as a phosphate source.

Then the bacterial cultures were centrifuged for 3-5 minutes at 5000 rpm in a top table centrifuge. It is important to mention that a bacterial centrifuge (at 4°C) and the supernatants (in 100-500 µl aliquots) were frozen in liquid nitrogen and stored at -20°C or even better at -80°C.

1.5. Determination of the Rate of Meat Spoilage

1.5.1. Sample preparation

Fresh pork meat was bought by a local retailer (Spar). Half of the meat was minced and the rest sliced. Minced meat (10g) was weighed in a glass Petri dish and infected with 1 mL bacterial cocktail (in LM-Medium inoculated bacterial strains: *Pseudomonas*, *Enterobacter*, *Proteus* and *Salmonella*) in a final concentration of 10^3 CFU/g meat. The samples were incubated at 4°C (cold room) and at 15°C (water bath) for 24 h, 48 h, 72 h, 96 h and one week (168h). Positive controls (10g of minced meat) were tested in parallel for meat deterioration under the same conditions.

Pork cutlets were sliced [m (4°C)=43,29 g, m (15°C)=44, 56 g], transferred to plastic Petri dishes and infected with 1 mL bacteria cocktail per 10 g of meat. The samples were incubated at 4°C (cold room) and 15°C (water bath) for four days and one week.

1.5.2. Homogenization of the Pork Meat

For homogenization 10 g of contaminated minced meat were added to 90 mL of LM-Medium and homogenized for 1 minute by 230 rpm using a Stomacher® 400 Circulator. Pork cutlets were added to 100 mL of LM-Medium and homogenized for 2 minutes by 230 rpm. On the whole, 19 samples were homogenized, also fresh frozen meat as a reference.

1.6. Screening of Enzymatic Activity

1.6.1. Qualitative detection of lytic enzymes

1.6.1.1. Tributyrin Agar Base (*Acila Sarl*)

Tributyrin Agar Base is a solid culture medium for testing the lipolytic capacity of microorganisms. Tributyrin is a very good lipid source for detecting lipolytic microorganism because some microorganisms able to hydrolyze tributyrin will not hydrolyze other triglycerides or fats containing longer chain fatty.

1.6.1.2. Spirit Blue Agar

Spirit blue agar contains Lipase Reagent (mixture of tributyrin and polysorbate 80) or some other lipid source for detection of lipolytic microorganisms. Spirit blue is a dye without any toxic effects and that makes it suitable for many lab protocols. It is mostly used for testing of milk and dairy products. Peptone is also a very important component of spirit blue agar, as a source of carbon, nitrogen and vitamins.

1.6.2. Quantitative Detection and Amount of Lytic Enzymes

1.6.2.1. Determination of Leucine Aminopeptidase Activity

Reagent A : 60mM Potassium Phosphate Buffer, pH 7.2 at 37°C.

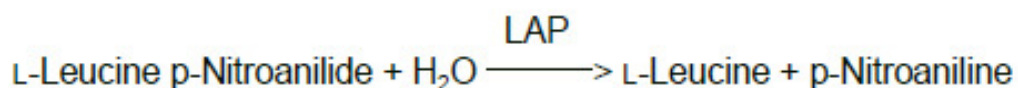
Reagent B: 1.66mM L-Leucine p-nitroanilide Solution (substrate, SIGMA ALDRICH).

Reagent C: 10mM Tris-HCl Buffer with 3mM Magnesium Chloride, pH 8.0 at 37°C (Activation Buffer).

Reagent D: Leucine Aminopeptidase solution (10U/mg), in cold deionized water (Non-activated).

Reagent E: Leucine Aminopeptidase solution (10/mg), in cold reagent C (activated) incubated at 37°C for 15 minutes to activate.

In order to determine the enzymatic activity of bacteria in meat, an enzymatic assay of leucine aminopeptidase (LAP) was carried out. At pH 7.2, the activity of the exopeptidase LAP, that selectively releases N-terminal amino acid residues from polypeptides and proteins, was examined.



In each cuvette, 2ml of freshly prepared 1.66mM L-Leucine p-nitroanilide solution (SIGMA-ALDRICH) served as a substrate. Since the temperature and the presence of divalent cations in the reaction center play an important role in the activity of an enzyme, two different conditions were examined.

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For the non-activated state, the enzyme was diluted in deionized water without any divalent cations added. For the activated state, the enzyme was incubated for 15 minutes at 37°C and diluted in reagent C. The absorbance of the samples was measured for 5 minutes at 405 nm.

To determine the activity of the enzyme, calculations were made as given below

$$\text{Units/ml enzyme} = \frac{(\Delta A_{405\text{nm}}/\text{min Test} - \Delta A_{405\text{nm}}/\text{min Blank}) (2.1)(\text{df})}{(9.62)(0.1)}$$

(2.1)= Total volume (in milliliters) of assay

df= Dilution factor

9.62= Millimolar extinction coefficient¹ of p-nitroaniline at 405 nm

0.1= Volume of enzyme used (in milliliters)

One enzymatic unit will hydrolyze 1 µM of L-leucine p-nitroanilide forming L-Leucine and p-nitroanilide per minute at pH 7.2 and 37°C. Potassium Phosphate Buffer (Reagent A) was used as a blank.

1.6.2.2. LAP Activity - Generation of Calibration Line

A calibration line was generated by means of increasing leucine aminopeptidase activities. Therefore a stock of leucine aminopeptidase with a specific activity of 10 U/mg was prepared.

Dilutions of enzyme solutions were made with either deionized water (for the non-activated state) or reagent C (for the activated state).

Dilutions of leucine aminopeptidase in the range of 10 to 1000 mU/ mg were used for generation of a calibration curve for the active state and dilutions in the range of 10 to 500 mU/mg for the inactive state.

1.6.2.3. LAP Activity in Test Samples

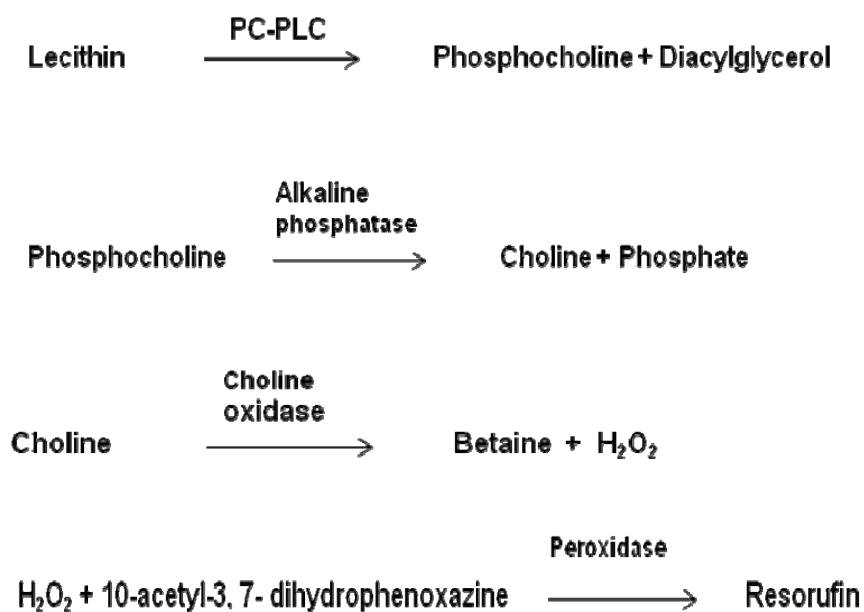
To determine the role of LAP activity in bacteria in LM and EM media and homogenate cultures for meat deterioration, samples were used as leucine aminopeptidase enzyme solution in reagent D and E. It is of high importance that samples were spun down and that only the supernatant was used for measurements in order to minimize the possible turbidity effect deriving from the high amount of bacteria or meat.

1.6.3. Determination of Phospholipase C Enzyme Activity

The Amplex® Red Phosphatidylcholine-Specific Phospholipase C Assay Kit provides a sensitive method for continuous monitoring of phosphatidylcholine-specific phospholipase C (PC-PLC) activity *in vitro* using a fluorescence microplate reader. In this enzyme-coupled assay, PC-PLC activity is monitored indirectly using 10-acetyl-3, 7-dihydrophenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H₂O₂. [34]

PC-PLC converts the phosphatidylcholine (lecithin) substrate to phosphocholine and diacylglycerol. Then by reaction with alkaline phosphatase, phosphocholine is hydrolyzed and the thus generated choline is oxidized by means of choline oxidase to betaine and H₂O₂. [35]

In the final step H₂O₂, reacts with Amplex Red reagent in the presence of horseradish peroxidase in a 1:1 stoichiometry to generate the highly fluorescent product, resorufin. [34, 36].



Materials and Methods

Materials:

Component A: Amplex Red reagent (10-acetyl-3,7-dihydrophenoxazine MW =257 g/mol)

Component B: Dimethylsulfoxide (DMSO)

Component C: Horseradish peroxidase 200 U (1 unit is defined as amount of enzyme that will form 1 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C)

Component D: Hydrogen peroxide (H₂O₂)

Component E: 5x Reaction Buffer (28 mL of 250 mM Tris-HCl, pH 7.4, 70 mM NaCl, 50 mM dimethylglutarate and 10 mM CaCl₂)

Component F: Choline oxidase from *Alcaligenes sp.* 12 U (1 unit is defined as the amount of choline oxidase that will form 1 µmole of H₂O₂ due to oxidation of 1 µmole of choline to betaine aldehyde per minute at pH 8.0 at 37°C)

Component G: Alkaline Phosphatase from calf intestine 500 U (1 unit is defined as the amount of enzyme that will hydrolyse 1µmole of 4-nitrophenyl phosphate per minute in 1M diethanolamine buffer, pH 9.8 at 37°C)

Component H: 129 mM Phosphatidylcholine (lecithin) solution in ethanol

Component I: Phosphatidylcholine-specific phospholipase C from *Bacillus cereus*, 2 U (1 unit is defined as the amount of enzyme that will liberate 1 µmole of water-soluble organic phosphorus from L-α-phosphatidylcholine per minute at pH 7.3 at 37°C).

Materials and Methods

Reagent 1: 20 mM stock solution of the Amplex Red reagent: Allow one vial of the Amplex Red reagent (Component A) and DMSO (Component B) to warm to room temperature. Just prior to use, dissolve the contents of the vial of Amplex Red reagent (1 mg) in 200 μ L DMSO. Each vial of Amplex Red reagent is sufficient for approximately 100 assays of 200 μ L each.

Reagent 2: 1X working solution of Reaction Buffer by adding 5 mL of 5 X Reaction Buffer stock solutions (Component E) to 20 mL of deionized water (dH₂O).

Reagent 3: 200 U/mL stock solution of horseradish peroxidase (HRP): dissolve the contents of the vial of HRP (Component C) in 1.0 mL of 1X Reaction Buffer.

Reagent 4: 20 mM H₂O₂ working solution: mix 23 μ L of 3.0% H₂O₂ with 977 μ L of dH₂O. 20 mM H₂O₂ working solution is instable and should be prepared freshly.

Reagent 5: 20 U/mL stock solution of choline oxidase : dissolve the contents of the vial of choline oxidase (Component F) in 600 μ L of 1X Reaction Buffer.

Reagent 6: 400 U/mL stock solution of alkaline phosphatase: dissolve the contents of the vial of alkaline phosphatase (Component G) in 1.25 mL of 1X Reaction Buffer.

Reagent 7: 10 U/mL stock solution of *B. cereus* PC-PLC: dissolve the contents of the vial of PC-PLC (Component I) in 200 μ L of 1X Reaction Buffer.

1.6.3.1. PC-PLC activity - Generation of a Calibration Line

A calibration line was generated with increasing Phosphatidylcholine-specific phospholipase C activities. For calibration line we used a stock of Phosphatidylcholine-specific phospholipase C with specific activity from 10 U/mL.

Dilutions of enzyme solutions were made with 1X working solution of Reaction Buffer.

1.6.3.2. PC-PLC Assay

The following protocol describes the assay of PC-PLC in a total volume of 200 μ L per microplate well. The volumes recommended here are sufficient for about 100 assays.

2.1 Dilute the PC-PLC-containing samples in 1X Reaction Buffer. A volume of 100 μ L will be used for each reaction.

2.2 Prepare a positive control by diluting the 10 U/mL PC-PLC stock solution (prepared in step 1.7) into 1X Reaction Buffer to produce a 0.1 U/mL PC-PLC solution. Use 1X Reaction Buffer without PC-PLC as a negative control. A volume of 100 μ L will be used for each reaction.

2.3 Prepare another positive control by diluting the 20 mM H_2O_2 working solution to 10 μ M in 1X Reaction Buffer.

2.4 Pipette 100 μ L of the diluted samples and controls into separate wells of a microplate.

2.5 Master mix of Amplex Red reagent was prepared containing: 200 μ L of Amplex Red reagent stock solution (400 μ M), 100 μ L of horseradish peroxidase (2U/mL), 200 μ L of alkaline phosphatase stock solution (8U/mL), 100 μ L of

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choline oxidase stock solution (0,2 U/mL and 78µL lecithin (1mM) to 9,32 mL of 1x Reaction Buffer.

Reaction was started by adding 100 µL of Master Mix to each microplate well containing the samples and controls (positive control 10 U/mL of PC-PLC and as negative control 1x Reaction Buffer).

The microplate with samples was incubated for 1 hour at 37°C, protected from light. The emitted fluorescence was measured in a fluorescence microplate reader using excitation by 560 nm and emission detection at 590 nm.

1.7. Preparation of LM- Bacteria

In order to induce growth of spoiling bacteria in the meat juice, the meat juice was incubated for 5 h at 37°C. The contaminated meat juice was then incubated on media plates (GSP- and ÖNÖZ Salmonella-Agar) at 37°C. Following this incubation, the colonies of the aerobe gram negative bacteria of the strains *Pseudomonas*, *Enterobacter*, *Proteus* and *Salmonella* were identified according to their morphology, and picked.

For the cocktail, 3 ml selective liquid media were inoculated with the four strains mentioned above and incubated in at 37°C for 24 h under shaking at 180 rpm. Following this incubation, 0.5 ml of this culture were used to inoculate 100 ml liquid media with phosphate (phosphate was used in order to induce the excretion of enzymes). The bacteria were then incubated at 30°C for 16 h (180 rpm). Following this incubation, the bacterial cocktail was aliquoted in 1 ml aliquots and frozen in liquid nitrogen and stored at -80°C, representing LM-bacteria.

1.8. Preparation of Bacterial Cocktail

Gram-negative aerobic bacterial strains (*Pseudomonas*, *Enterobacter*, *Proteus*, *Salmonella*) were isolated from spoilt meat juice (incubated for 5 h at 37°C) using Salmonella Agar acc. to ÖNÖZ (Merck).

A colony picked from the selective media was inoculated in 3 mL peptone water (peptone 0.1%, NaCl 0.5%, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 0.9% and KH_2PO_4 0.15% all in dd H_2O) and allowed to grow overnight at 37°C. Then 2 mL of the bacterial pre-culture was inoculated in 200 mL peptone water and incubated at 37°C for 24 h. Aliquots (1 mL) were frozen in liquid nitrogen and stored at -80°C. After incubation (up to 3 days at 30°C) bacterial counts were determined by plating 100 μL bacterial cultures onto Plate Count Agar (Fluka). The culture dilutions (10^{-1} to 10^{-9}) were prepared in Ringer Solution.

1.9. Preparation of the Meat Homogenate

Meat samples (pork cutlets) were supplied from the local supermarket (Spar). Meat samples of 10 g were placed in Petri dishes, infected with bacterial cocktail D (prepared as outlined above, used in a bacterial concentration of 1000 CFU/g) and incubated as follows: for meat homogenate 1, the samples were incubated for 96 hours at 4 °C, and for homogenate 2, the samples were incubated for 48 hours at 15°C.

The contaminated meat was then transferred into 90 ml liquid media in Stomacher Bags and homogenized using a Stomacher® 400 Circulator, 1 min by 230 rpm. The homogenates were then aliquoted in 1 ml aliquots, frozen in liquid nitrogen and stored at - 80 °C.

1.10. Preparation of the Contaminated Pharmaceutical Product

Samples (50 µl) of both, *Pseudomonas* and *Enterobacter* strains (grown separately in liquid media as mentioned above) were added to 50 ml of Acumed, a solution for contact lenses (4Care AG, Kiel, Germany). The resulting contaminated solution was incubated for 24 h at room temperature corresponding to the sample “contaminated solution for contact lenses” as mentioned above.

1.11. Preparation of the Contaminated Cosmetic

10 g of “hand & nail cream Kamill” (Burnus, Darmstadt, Germany) were mixed with 50 ml Ringer-solution and thoroughly vortexed. To the resulting suspension, 50 µl of both, *Pseudomonas* and *Enterobacter* strains (grown separately in liquid media as mentioned above) were added. The resulting contaminated suspension was incubated for 24 h at room temperature corresponding to the sample “contaminated hand cream” as mentioned above.

1.12. Correlation with Standard Test Systems - Microbial Detection of Bacterial Growth

One of the most important steps towards a functional sensor setup was the correlation with microbial standard test. This procedure is essential to adapt the sensitivity at the required conditions for a real - time optical freshness sensor for food.

Foodstuffs of animal and plant origin may present intrinsic hazards, due to microbiological contamination. Microbiological criteria are tools that can be used in assessing the safety and quality of foods. Due to reasons related to sampling, methodology and uneven distribution of microorganisms, microbiological testing of finished food products done alone is however insufficient to guarantee the safety of a foodstuff tested. The safety of the foodstuffs must principally be ensured by a more preventative approach, such as product and process design and the application of Good Hygiene and Manufacturing Practices (GHP, GMP) and the Hazard Analysis Critical Control Point (HACCP) principles.

In the EU exist own laws about food safety and their control written in the "COMMISSION REGULATION" (EC) No 2073/2005 from 15 November 2005; Article 2:

Foodstuffs should not contain microorganisms or their toxins or metabolites in quantities that present an unacceptable risk for human health. [37]

In Germany and Austria meat is considered to be fresh, when the results of microbial analysis of meat sample have a total bacterial count of 5 Mio CFU/g (colony forming units). The EU regulation of a zero tolerance of bacterial counts is not implemented in Austrian law.

2. Techniques of surface coating

In the following chapters the techniques for production of meat freshness probes are described. Since it displays a thin-film sensor, it was necessary to use coating methods capable of generating surfaces of probably only a few nanometers thickness.

2.1. Gravure Printing

For the biosensor setup, the biomimetic polymer solution (PLA or PLGA) is directly transferred onto the Inconel layer via Gravure Printing.



Figure 9 . K Printing Proofer Gravure (ERICHSEN)

The mirror layer is first attached to the rubber which covers the impression roller. The distance between the impression roller, the doctor blade and the printing plate is of high importance for the amount of liquid printed onto the mirror layer and thus, the thickness of the polymer layer and the visual perception of the sensor surface.

After application of the polymer solution onto the printing plate, the impression roller can be run over the plate resulting in a defined layer with the desired thickness over the mirror layer. During the printing procedure, the excess of polymer solution is scraped off by the doctor blade. Between printing procedures plate, impression roller and doctor blade have to be cleaned thoroughly with 2-butanol then trichloromethane and finally with 2-butanol, again.

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After printing, the foils were dried at 80°C for 10 minutes and then washed in a water bath for 10 minutes under shaking. After air-drying, the foils were then put into the incubator at 80°C for 10 minutes. Then, the biosensors can be cut into appropriate pieces for testing their reaction on bacteria and enzyme solutions.

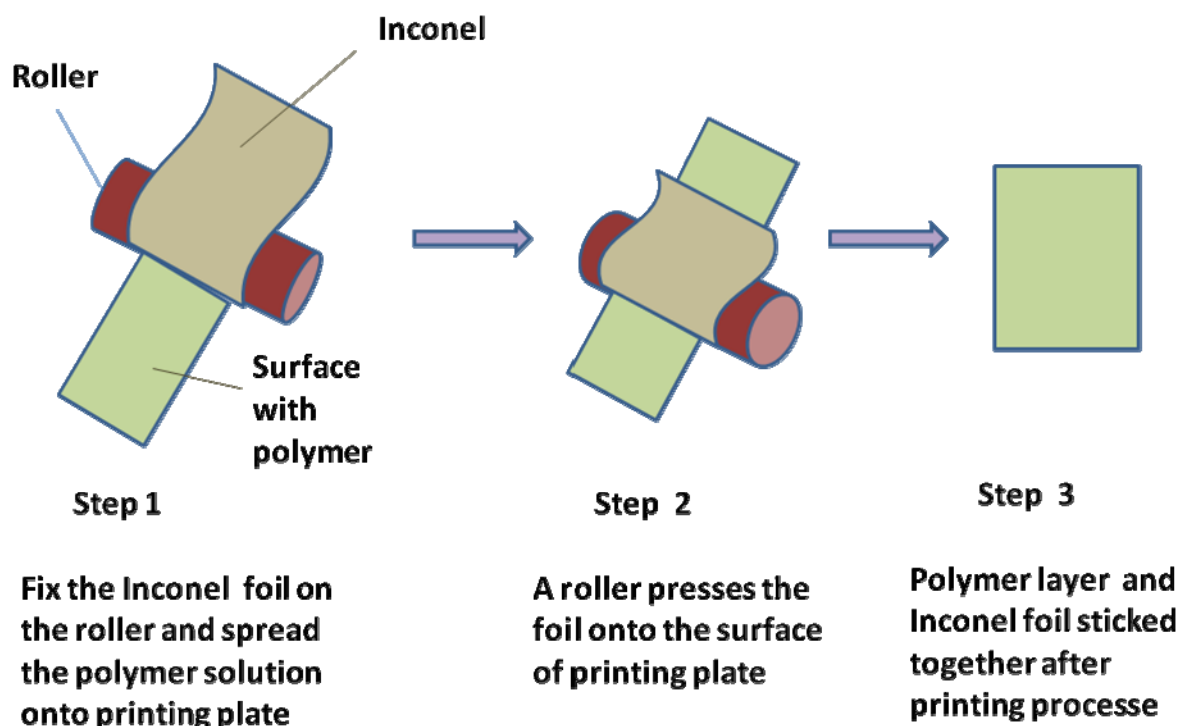


Figure 10: Schematic illustration of the gravure printing process

2.2. Sputter Coating

During this work nanoparticles were applied by sputter coating, generating the anomalous absorption and visualizing the homogeneity of the printed layers. Using an Agar Sputter Coater the following metals can be deposited: gold, palladium, silver, copper, tin and alike ones. Those metals have rather low evaporation temperatures and can therefore be sputtered even at low energies. These metals are deposited as atoms, which assemble into islands on the surface. These islands usually are small, rather flat, and asymmetric and interconnected directly after sputtering. Upon heating to 100 to 350 °C the islands disconnect and can acquire an almost spherical shape. The sputter process itself is carried out with the help of argon plasma. A foil of one of the metals mentioned above is inserted into the target holder and the chip is placed on the table inside the vacuum chamber of the Agar Sputter Coater. The chamber is evacuated to a vacuum of at least 0.03 mbar and after washing the chamber extensively with argon at a pressure of 1 mbar, the pressure is adjusted to 0.08 mbar. Next the maximum electrical field of 5 kV is applied which results in the starting of a plasma. The metal is deposited on top of the table and on the chips positioned there. Most metals can be deposited in a highly controlled manner by adjusting the field applied and the time during which the plasma reaction is allowed to proceed.

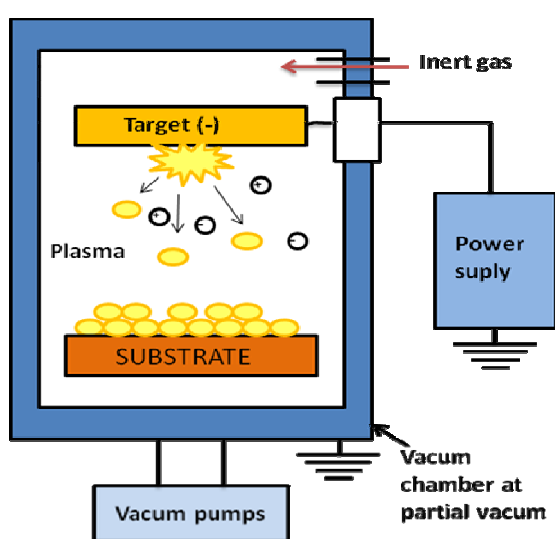


Figure 11: Sputtering Vacuum Deposition Process

Sputter coated metal island films are different in order of the shape of nano-sized structures in comparison to the metal island films created by evaporation or by chemical synthesis. For many applications in life science it is very useful to have noble metal nanoparticles in a nearly spherical shape.

This is important for a covalent attachment of biomolecules like proteins or DNA at the nanoparticles, because if the metal island film consists of nanoparticles with a more or less spherical shape, more binding sites for the attachment of the biomolecules are available.

2.3. General Setup of Biosensors

Perishable products are very often predisposed for bacterial degradation. Such microbial deterioration can be caused by different gram negative bacteria, e.g. *Salmonella*, *Pseudomonas* or *Enterobacter*. These bacteria secrete various enzymes during their growth causing deterioration of products.

There is a real need for obtaining microbial test results in a very short time but traditional and standard detection methods may take up to several days to yield an answer. [38]

This is clearly unsatisfactory, even if these conventional methods show high selectivity and sensitivity. Biosensors have the potential to shorten up the time for analysis and simultaneously to maintain a high level of sensitivity.

Especially when a consumer wants to get information on the quality of the food he is buying in the shop, a quick real time test integrated into the package would be of high interest. This is also true for the producer as he can show the hygienic level of his production line by means of such a device.

The biosensors that have been created provide numerous advantages compared to standard microbiological tests.

As these tests are reversible and also are time consuming until results can be gained, they are not capable of providing information on the microbial status of food that fast and furthermore the results can be manipulated.

Materials and Methods

In contrast, the here presented biosensors have the ability to display bacterial infection of, for example, nutrients by the principles of optical phenomenon and they carry the advantage of a memory effect that cannot be manipulated.

The sensor prototypes developed and optimized during this work are able to detect bacteria that are contained in perishable natural products and to show contamination of those products by these pathogenic bacteria. Besides meat and other food, cosmetic products may also be contaminated with microorganisms which can lead to infections that cause inflammation.

The biosensor contains a polymer that is degradable by lytic enzymes that are secreted by microorganisms during decay of products. As the temperature is increasing, the bacteria will grow and this will also lead to an increased release of enzymes that cause deterioration of products.

The biosensors presented here are based on the optical phenomenon, which will be visible as the polymer is degraded.

Therefore, the biosensors can be used as new tools for the analysis of natural products deterioration and they provide fast information on product decay that can easily give information on the quality of production, transport and storage.

2.3.1. Two Layer Sensor Setup

Two layer sensor setup consists of a reflective layer that is made up of Ni- Cr Alloy which is then covered with a polymer layer. This distance layer is a polymer that is generally made up of PLGA (= poly (lactic-co-glycolic acid)) dissolved in ethylacetate containing varying amounts of Desmodur. Desmodur serves as a linker of the PLGA molecules, influences its viscosity, composition and viscosity. When lytic enzymes come into contact with the polymer layer as e.g. secreted by bacteria, the polymer will be degraded and as a consequence this results in a color change of the biosensor for example from green to red. Bacteria causing food deterioration secrete such lytic enzymes.

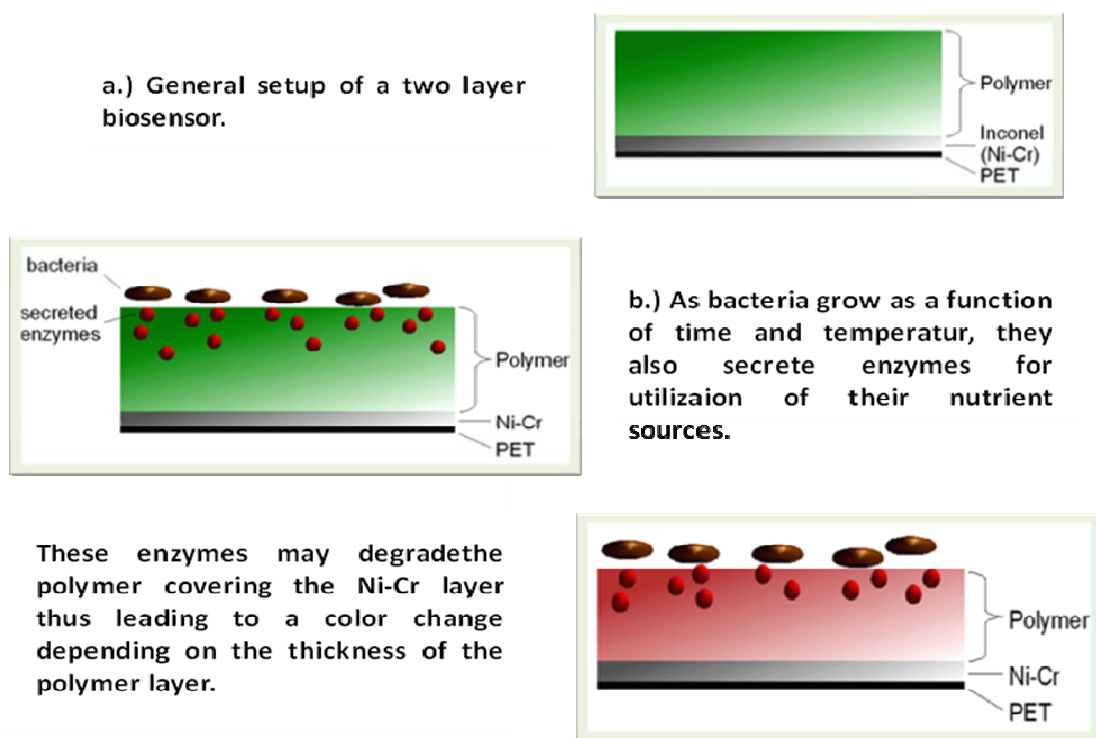


Figure 12: Degradation of biomimetic (PLGA) layer by excreted lytic enzymes.

2.3.2. Three Layer Sensor Setup

The three layer sensor is an optical thin-layer system consisting of a metal mirror, a transparent biodegradable interlayer and a metal island film. Due to the special optical behavior of a nanoparticle-film and due to the special layer design this system shows characteristic spectral reflection behavior, strongly dependent on the thickness of the transparent interlayer. [39]

The optical property of metal nanoparticle films, necessary for our application is the so called “anomalous absorption”, a strong, broad-band absorption in the visible.

Depending on the thickness of the distance layer the sensor shows a specific color, hence by changing the gauge of the interlayer a bright color change can be observed.

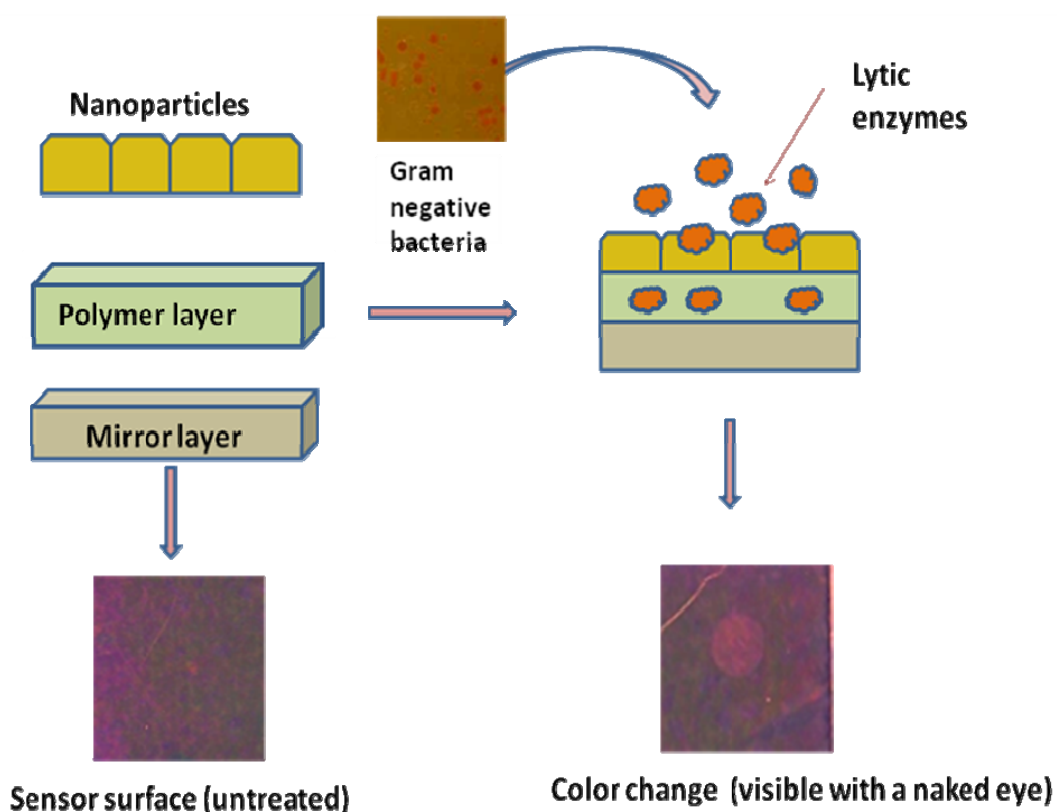


Figure 13. Three layer sensor setup. The sensor shows high sensitivity (right) by contact with bacteria which are secreting lytic enzymes after certain time of incubation (up to 4-6 hours).

This novel sensor system is very easy to use and designed to meet customer requirements.

2.3.3. Mirror Layer

There are great demands on the mirror layer, such as stability in biological media, the need for a smooth surface and the compatibility with tested products. The smoothness of the mirror-layer regulates the brilliance of the color. Only smooth surfaces are able to interact with the nanoparticles in a definite distance that results in brilliant colors. Nanoparticle layer and mirror layer have to be very similar in their oxidation state. During this work two different mirrors were used: Inconel- a Ni-Cr alloy with good oxidation resistance and gold mirror (35 nm), kindly provided by Hueck Folien.

2.3.4. Biomimetic Polymer Layer

Regarding the functional efficiency of sensor setups as described above, the distance layer, a biodegradable polymer layer, is one of the most crucial points. This analyte sensitive layer has mimetic, shrinking and swelling properties and must have the ability to react to various microbial and cellular lytic enzymes.

There is a wide range of valuable polymers showing this characteristic trait, but only two of them were tested in this work: PLA (poly lactic acid) and PLGA (poly lactic-co-glycolic acid).

Poly lactic acid belongs to the family of aliphatic polyesters commonly made from α - hydroxy acids. PLA is a thermoplastic, high-strength, high- modulus polymer that can be made from renewable resources to yield objects for use in either the industrial packaging field or the biocompatible medical device market. It is one of the few polymers whose stereochemical structure can easily be modified by polymerizing a controlled mixture of the L- or D-isomers to yield

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high-molecular-weight amorphous or crystalline polymers that can be used for food contact and are generally recognized as safe. [40]

PLA can be synthesized in a wide range of molecular weights by following two processes:

1. Direct polycondensation reaction of lactic acid which is readily obtained by the controlled fermentation of starches, usually from corn. When water is cleaved off from lactic acid, an oligomer is formed – an ester consisting of 10 to 30 lactic acid units. This oligomer is in equilibrium with the corresponding dilactide – an ‘internal’ ester consisting of two molecules of 2-hydroxypropanoic acid;
2. Ring-opening polymerization of cyclic dimers, i.e. Dilactide, in the presence of metal catalysts to synthesize high molecular weight polymers. [41]
- 3.

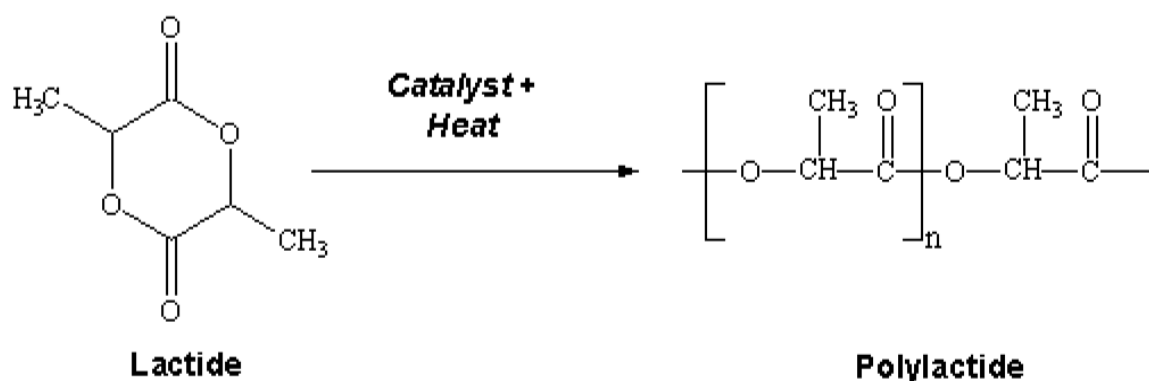


Figure 14 : Ring-opening polymerization (ROP) of dilactide provides polylactic acid

The degradation of poly lactic acid results from simple hydrolysis of the ester bonds.

When poly (L-lactic acid) (PLLA) materials are exposed to the environment, they are hydrolytically degraded to L-lactic acid and its oligomers. Especially the

Materials and Methods

PLA degradation caused by enzymes like proteinase K was investigated with great effort in the last years. [42]

PLGA (poly-lactic-co-glycolic acid) is a co- polyester of lactic and glycolic acid. It can be dissolved in dichloromethane, chloroform, acetone, ethyl-acetate, tetrahydrofurane, dioxane, DMSO and toluene. PLGA is biodegradable and often used as a biomaterial for medical and pharmaceutical applications. Hydrolysis of the polymer chain leads to PLGA degradation and results in lactic and glycolic acid which can be metabolized in the Krebs- cycle to CO_2 and H_2O . For our application we used Resomer RG 503 H from Boehringer Ingelheim. RG 503 H with a viscosity of 0,38 dl/g and a molecular mass of 50 000 Da and contain a big part of acid carboxyl group. Resomer RG 503 H contains D, L- lactic acid and glycolic acid in equal amount, ideal ratio of both components for fast degradation in human body.

PLGA was approved in the year 2000 by FDA (Food and Drug Administration) for application in drug targeting.[43, 44]

In the sensor setup, described above, the poly lactide interlayer is decomposed by enzymes that derive from food spoilage and can originate either as lytic enzymes produced by bacteria that are always present on the surface of biological materials, or arise from cell lysis itself.

Nevertheless both PLA and PLGA were examined due to their possible application in the sensor setup.

2.3.5. Linker

As mentioned in the previous chapters, it is possible to tune the sensor's sensitivity by changing the linkage of the PLA- and PLGA- strands. The longer these strands are and the higher the interlinkage between them, the greater is the challenge for the enzymes to crack them. There might be a lot of different ways to extend or even to cross-link the polymer threads. In our case methylene diphenyl diisocyanate-(MDI), ($C_{15}H_{10}N_2O_2$) was applied, the most common aromatic diisocyanate worldwide, accounting for 61.3 % of the global market in the year 2000. [45]

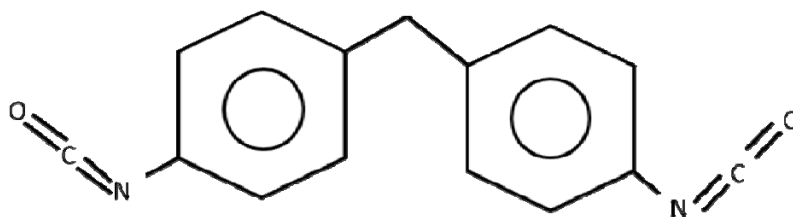


Figure 15: 4,4'-methylene diphenyl diisocyanate (MDI)

The major application of Desmodur lies in the production of polyurethane, whose products range from cellular materials, such as flexible foams for seating applications and rigid insulating foams, to coatings, adhesives, sealants and elastomers. Isocyanates are often used as building blocks for agricultural and pharmaceutical chemicals, and they are also useful blocking agents for functional groups, which can be of interest in peptide chemistry. *“The future of this vibrant field with endless possibilities for new and unique applications remains bright.”* [46]

In this work Desmodur® 2460 M was preferably used, a monomeric diphenylmethane diisocyanate with high 2,4'-isomer content, and also triphenylmethane- 4, 4', 4''- triisocyanate. The big advantage of this isomer

compared to the 4,4'-MDI is that the storage and handling are greatly simplified because at room temperature it is a liquid.

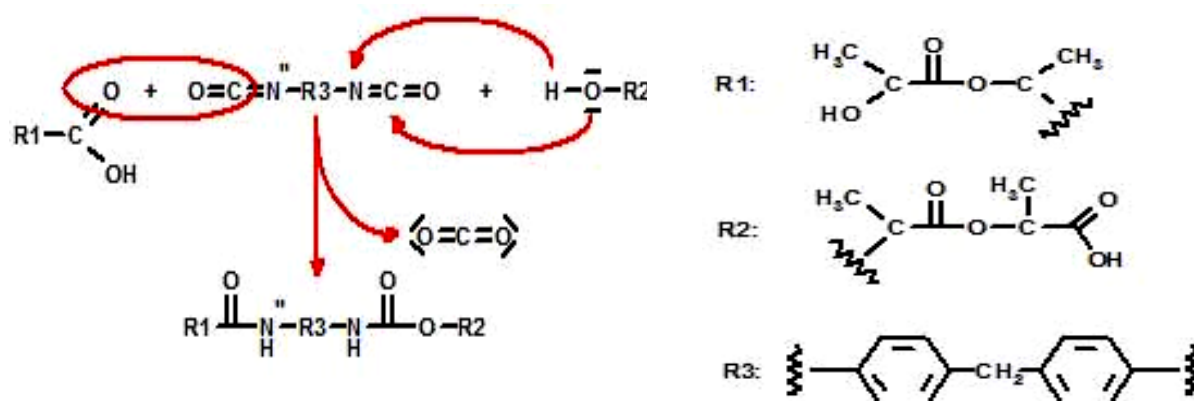


Figure 16: Diisocyanates linking two PLA-strands

Figure 16 shows the linkage reaction between two PLA-strands with the aid of MDI. Generally MDI may link the tails of two PLA threads or connect two ends of one and the same PLA-strand, thus resulting in huge cyclic molecule.

The outcome of the former are elongated polymer-strands, and this leads to an increase in molecular weight, which results in a decline of the sensor's sensitivity. This provides an excellent opportunity to adjust the sensor's response towards lytic enzymes that are capable of hydrolyzing the ester- and as well the carbamate-bonds.

2.3.6. Nanoparticle layer

Mainly gold was used for formation of nanoparticles because it was the most prudential answer within the means of our laboratorial equipment.

The coating with gold nanoparticles worked satisfying and could even be regulated by changing the term of plasma persistence. For example a period of 10 seconds under a sputter current of 40 mA and an argon pressure of 0.08 mbar implicates a nanoparticle layer thickness of about 5 nm. It is obvious that with an extension of the sputtering time the layer thickness can be increased.

Furthermore, it was observed that an increase in sputtering time came along with intensification of the sensor's brilliance. So it was possible to produce nanoparticle layers with desired traits.

2.4. Sensitivity Test for Three-Layer Sensor

2.4.1. Degradation of PLA by Protease-Mix

As already described in literature PLA polymer can be degraded by lytic enzymes as e.g. proteases and lipases. [42]

In order to simulate this reaction, PLA sensors were incubated with a mixture of 3 commercially available proteolytic enzymes. This Protease-mix contains Proteinase K, Chymotrypsin and Trypsin in an equal amount. Enzyme dilutions were prepared in 0,1 M Tris HCl Puffer pH 8.2. The polymer PLA was dissolved in 2, 2, 2- trifluoroethanol to a final concentration in the range of 3 % to 8 % w/v. The linking agent was applied in different concentrations to figure out the selectivity and the limit of detection. The mirror layer consists of Inconel- Ni-Cr alloy, evaporated on top of a PET film as a support. After the gravure printing process the sensors were incubated at 80 °C for 10 min and gold island film was sputter coated on top using argon plasma of 0.08 mbar for 10, 15 and 20 sec.

The pipetting scheme was as follows:

Protease concentration		
1.	20 mg/ mL	5 mg/ mL
2.	15 mg/ mL	2,5 mg/mL
3.	12,5 mg/ mL	0, 1 M Tris-HCl buffer pH 8.2
4.	10 mg/ mL	ddH ₂ O

Table 3: Pipetting scheme

The Protease–Mix dilutions in 0.1 M Tris - HCl pH 8.2 and negative controls were applied to the sensor surface in 1 μ l dots like described in the pipetting scheme.

Then the sensors were incubated in a humidity chamber at 4°C, room temperature and 37 °C for 4 hours and overnight. The reaction was stopped by washing with ddH₂O and drying under an air stream.

2.4.2. Degradation of PLA and PLGA by meat juice

A Fabry-Perot sensor setup includes two reflecting metal layers and a polymer interlayer in between. The first reflecting layer for a PLA setup consists of 35 nm Au on 36 μ m PET foil that was kindly provided by Hueck Folien, for a PLGA setup Inconel coated PET foil was used. The biomimetic polymer PLA was dissolved in 2,2,2-trifluoroethanol to a final concentration of 10 % w/v, polymer, PLGA was dissolved in 2,2,2-trifluoroethanol to a final concentration between 20% and 35% (w/v).

The linking agent Desmodur was applied in a concentration range of 10^{-3} to 10^{10} % for PLA- and in a concentration range of 10^{-8} to 1 % for a PLGA setup to figure out the selectivity and the limit of detection.

After the gravure printing process the sensors were incubated at 80 °C for 10 min and a second reflecting Au metal layer was sputter coated on top with argon plasma of 0.08 mbar for 120 sec. for PLA- and for 45 sec for PLGA setup.

The first experiments with meat juice were carried out with meat which was bought in a typically packaging with protecting gas for improvement of shelf life. The meat was bought by local retailer like Billa. Mostly, the meat juice was found directly in the packaging. Dilution series with 0.1 M Tris- HCl buffer pH 8.2 from the meat juice were prepared. For a functional assay, “stock solutions” of fresh (10^5 CFU/mL) and spoiled meat juice (after 5 hours incubation by 37°C and total bacterial count of 10^7 CFU/mL) were prepared.

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For determination of the possible range for a functional assay different solutions were prepared as described in the scheme:

	fresh meat juice	spoiled meat juice
1.	Stock 10^5 CFU/mL	Stock 10^7 CFU/mL
2.	1:2 dilution with buffer	1:2 dilution with buffer
3.	1:4 dilution with buffer	1:4 dilution with buffer
4.	0.1 M Tris- HCl buffer pH 8.2	0.1 M Tris- HCl buffer pH 8.2
5.	ddH ₂ O	ddH ₂ O

Table 4: Pipetting scheme

On top of the sensor surface 1 μ L of every solution was pipetted and then the sensors incubated in a humidity chamber for 4 hours and over night both at 4°C and also at room temperature. After the incubation time the sensor surface was washed with ddH₂O and dried under an airstream.

2.5. Sensitivity Test for Two- layer Sensor Setup

2.5.1. Degradation of PLGA and Proof of Principle

To describe a degradation behavior of PLGA, functionality tests with PLGA sensors printed on Inconel were prepared.

A NiCr-Alloy (Inconel by CPFilms, OD 2.2) was used as a reflective layer. Onto this layer, a polymer layer of 21 % (w/v) PLGA and 0.5 % (v/v) Desmodur in ethyl-acetate was printed, followed by incubation for 10 minutes by 80°C).

An enzyme mix of the proteases proteinase K, trypsin and chymotrypsin (in a ratio of 1:1:1) was prepared in 0.1 M Tris/HCl, pH 8.2 to a final protein concentration of 20 mg/ml. Starting from this solution, different concentrations as indicated below were obtained by corresponding dilution steps in 0.1 M Tris/HCl, pH 8.2.

The proteases (as well as Tris-buffer and water as negative controls) were pipetted onto the biodegradable polymer layer in different concentrations as indicated below. After an incubation of 2 h, 4 h and overnight at 4 °C and also at room temperature, the device was washed with ddH₂O, dried under an airstream and scanned from the polymer layer-side to make the color change visible.

Protease concentration		
1.	20 mg/ mL	5 mg/ mL
2.	15 mg/ mL	2.5 mg/mL
3.	12,5 mg/ mL	0.1 M Tris-HCl buffer pH 8.2
4.	10 mg/ mL	ddH ₂ O

Table 5: Pipetting scheme

2.5.2. PLGA Setup- Addition of Substrates

As described before, biosensors with different concentrations of PLGA, different additives and also different concentrations of Desmodur were prepared to test the functionality and sensitivity of the sensors. Most of the chosen additives were mimicking agar components in order to provide a nutrimental environment for the bacteria and to trigger the release of lipolytic enzymes which are consecutively responsible for the degradation of the polymer layer.

The biomimetic polymer PLGA was solved in ethyl acetate to a final concentration between 20% and 27% (w/v). The linking agent Desmodur was applied in a concentration range between $5 \cdot 10^{-8}$ % to figure out the selectivity and the limit of detection.

After the gravure printing process the sensors were incubated at 80 °C for 10 min.

The optimization of the sensor properties for the “real life”, which means detection of the freshness of the food, was carried out for bacterial cultures consisting of *Pseudomonas*, *Enterobacter*, *Proteus* and *Salmonella* and for pork – meat homogenate.

For all tested sensor setups, test for sensitivity and selectivity at 4 °C, room temperature and also at 37 °C was carried out.

The sensors were incubated at these three temperatures respectively for 6 h and over night.

PLGA samples containing the following substrates were prepared:

Materials and Methods

PLGA concentration in % (w/v)	Substrates
22 % PLGA	oil with increasing concentrations of Desmodur *
20-22 % PLGA	glycerol tributyrat with increasing concentrations of Desmodur *
21/23/25 %PLGA	tryptone and glucose with increasing concentrations of Desmodur *
20-22% PLGA	glycerol tributyrat and oil with increasing concentrations of Desmodur *
20-22% PLGA	glycerol tributyrat and oil without Desmodur
21/23% PLGA	glucose and oil with increasing concentrations of Desmodur *
20/21% PLGA	tryptone and oil with increasing concentrations of Desmodur *
23/24% PLGA	tryptone and oil with increasing concentrations of Desmodur *
20/21% PLGA	glycerol tributyrat with increasing concentrations of Desmodur *
25/27% PLGA	tryptone and oil with increasing concentrations of Desmodur *
20/21% PLGA	glycerol tributyrat with increasing concentrations of Desmodur *
23/24% PLGA	glycerol tributyrat with increasing concentrations of Desmodur *
21/23% PLGA	lactose with increasing concentrations of Desmodur *
21/23% PLGA	glucose, lactose and oil with increasing concentrations of Desmodur *
21/23% PLGA	tween 20 with increasing concentrations of Desmodur *
21/23% PLGA	glucose, glycerol tributyrat, oil with increasing concentrations of Desmodur *
21/23% PLGA	milk powder with increasing concentrations of Desmodur *
26 % PLGA	sodium-citrate with increasing concentrations of Desmodur*
21/23 %PLGA	Na ₂ HPO ₄ with increasing concentrations of Desmodur*

* Different concentrations of Desmodur alternatively were used: $5 \cdot 10^{-8}\%$; $5 \cdot 10^{-6}\%$; $5 \cdot 10^{-5}\%$; $5 \cdot 10^{-4}\%$; $5 \cdot 10^{-3}\%$; $5 \cdot 10^{-2}\%$; $5 \cdot 10^{-1}\%$; 0.5%; 0.7%; 0.8%; 1%; 1.2%; 1.4%; 1.6%; 2.2%; 2.4%; 2.6%; 3%.

Table 6: Overview of used PLGA concentration in combination with different substrates

2.5.3. Application of PLGA for Analysis of Natural Products

The general protocol for preparing the devices was: a NiCr-alloy (Inconel by CPFilms, OD 2.2) used as reflective layer. Onto this layer, a polymer layer of PLGA with weight-percent as indicated below and Desmodur with weight-percent as indicated below in ethylacetate was printed, followed by incubation for 10 minutes at 80°C.

The following devices were prepared and tested for their ability to detect the above mentioned samples:

A) 21% PLGA with 0.5% Desmodur

420 mg PLGA and 200 μ l 100% Desmodur were mixed in 3.8 ml ethyl acetate and allowed to react at room temperature under stirring.

B) 21% PLGA with 1.0% Desmodur

420 mg PLGA and 400 μ l 100% Desmodur were mixed in 3.6 ml ethyl acetate and allowed to react at room temperature under stirring.

C) 23% PLGA with 5×10^{-8} % Desmodur

420 mg PLGA and 1 μ l of a 10^{-4} % Desmodur solution were mixed in 3.99 ml ethyl acetate and allowed to react at room temperature under stirring.

The sensors were then incubated with meat homogenate, bacterial cocktail, contaminated solution for contact lenses and contaminated hand cream to test their ability to identify contamination and spoilage of the natural products. The dots are applied according to the scheme in Table 7:

Materials and Methods

Column 1	Column 2	Column 3
1. Meat homogenate 1* 10 ⁶ CFU/mL		9. 4 Bacteria – LM
2. Meat homogenate 2** 10 ⁷ CFU/mL	6. Culture a (10 ⁷ CFU/ml)	10. 4 Bacteria –LM 10 ⁶ CFU/mL
3. Acumed ¹ 10 ⁸ CFU/mL	7. Culture b (10 ⁸ CFU/ml)	11. Phospholipase 10 mg/mL
4. Kamill ² 10 ⁶ CFU/mL	8. Culture b (10 ⁶ CFU/mL)	12. Ringer solution

Table 7: Pipetting scheme

*- pork cutlets after 96 hours at 4°C

¹- Acumed –solution for contact lenses

** - minced meat after 48 hours at 15°C

²- Kamill- hand &nail creme

Finally, the sensors were incubated at 37°C for 6 hours and then scanned for documentation of the color changes.

Results and Discussion

1. Microbiology

1.1. Isolation, Identification and Quantification of Gram Negative Bacteria

Pork meat juice was aliquoted into 100 µL Eppendorf-tubes and spoiled by incubation for 5 hours at 37°C.

Dilutions were prepared in Ringer Solution (pH 8,2) and tested on ÖNÖZ Agar (Merck). Due to table 2 it was possible to identify four of bacterial strains which were responsible for spoilage of the pork meat: *Pseudomonas*, *Enterobacter*, *Proteus* and *Salmonella*.

Microorganisms	Appearance of colonies
<i>Pseudomonas</i>	Glossy, dirty yellow to greenish; culture medium is yellow
<i>Enterobacter</i>	Large, mucoid, bluish or reddish, slight precipitation ring around the colonies
<i>Proteus</i>	Rust-colored, culture medium surrounding the colonies of same color, if growth is too dense, dark brown to black
<i>Salmonella</i>	Yellow, medium size; 1 st day: black dots start to develop on the yellow colonies 2 nd day; black dots clearly visible on the yellow colonies; Culture medium surrounding the colonies is yellowish

Table 2: Characterization of microorganisms

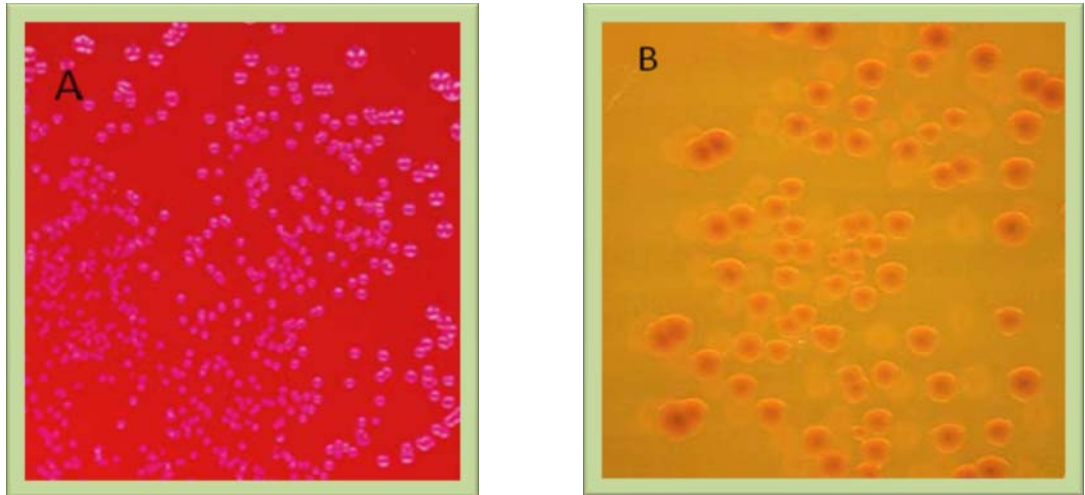


Figure 17. Identification of *Pseudomonas* with two selective media: A. GSP-Agar and B. ÖNÖZ-Agar. *Pseudomonas* bacteria onto GSP-Agar appear as blue/violet large colonies with diameter of ~3 mm, surrounded by a red-violet zone.

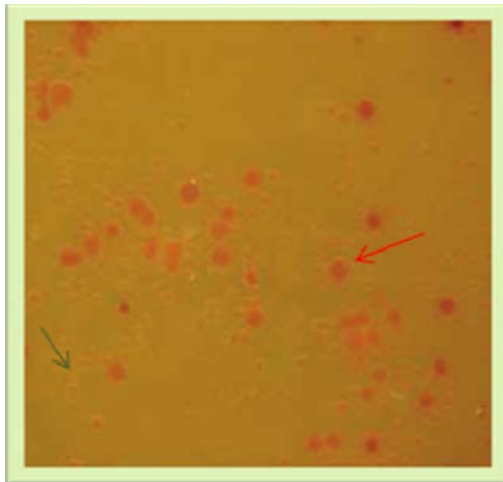


Figure 18. ÖNÖZ-Agar. Aerobic gram negative bacteria, *Enterobacter* (red arrow) and *Proteus* (green arrow), isolated from a spoiled pork meat juice (incubated for 5 hours at 37 °C). Dilutions were prepared with Ringer Solution (pH 8,2) and tested on the ÖNÖZ agar plates

1.2. Comparison of Bacterial Growth in LM and EM media

As already mentioned before, *Pseudomonas spp.* are the most common spoilage organisms. [47, 48]

Under aerobic conditions, they are more abundant than other bacterial strains. Though, under anaerobic conditions as e.g. in vacuum packs, Enterobacteriaceae rather take the lead. [47, 49]

Since the environmental conditions have an important influence on the secretion of lytic enzymes, we examined the growth behavior of *Pseudomonas* and *Enterobacter* in two different media. Growth curves of both bacteria strains were generated for LM and EM medium and the optical density measured regularly at 578 and 600nm. Enterobacter is expected to grow better in EM medium.

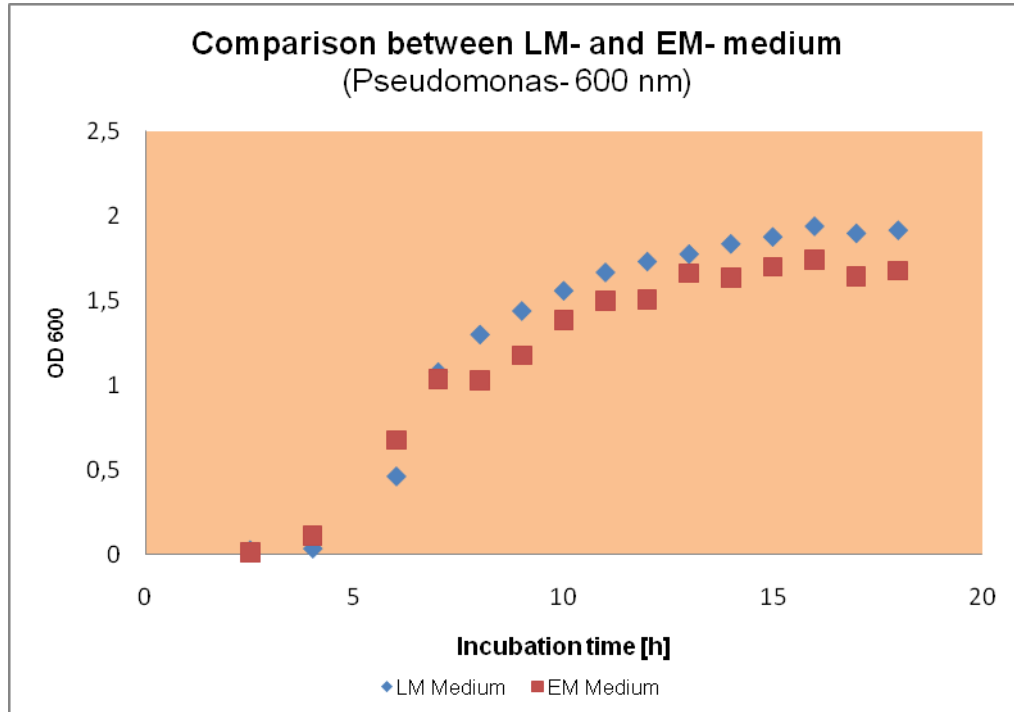


Diagram 1: Comparison of *Pseudomonas* growth in LM- and EM-Medium by measuring of optical density at 600 nm

Results and Discussion

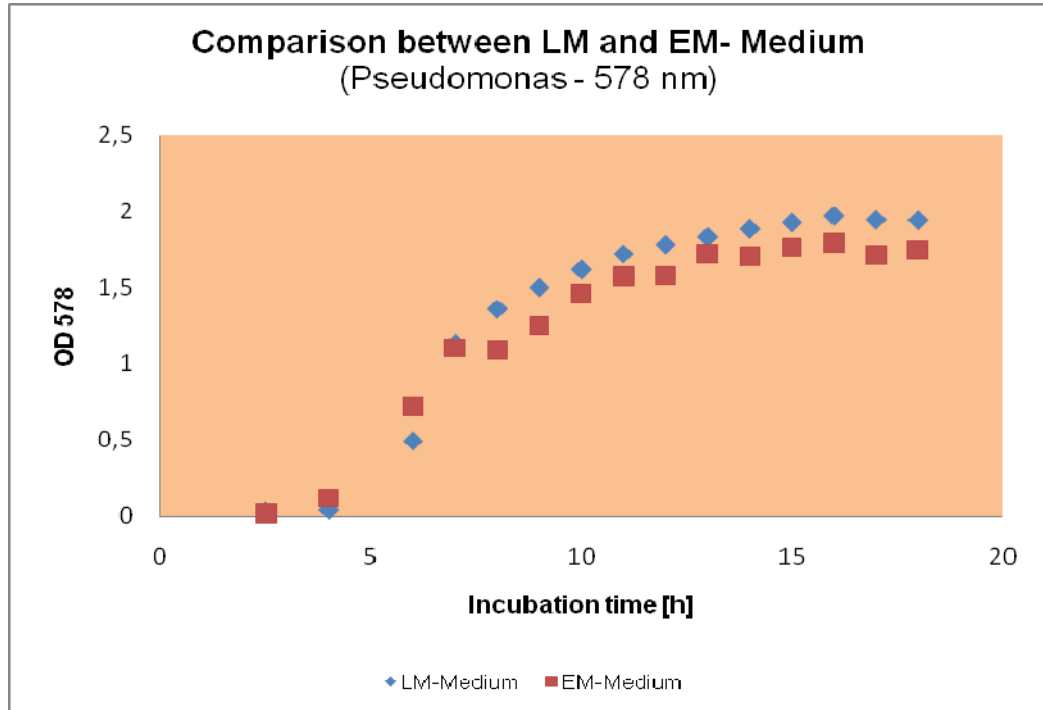


Diagram 2: Comparison of *Pseudomonas* growth in LM- and EM-Medium by measuring of optical density at 578 nm

Diagrams 1 and 2 show the growth curves of *Pseudomonas* in LM and EM media. The optical density was measured at 578 and 600nm. The lag phase seems to end after about 5 to 6 hours of incubation at 37°C. The log phase ranges over 4 hours (incubation time 6 to 10 hours) and is identifiable by the optical density between 0.46 and 1.61. The stationary phase is reached after 16 hours of incubation at 37°C. Comparing both growth curves and the optical density data, we can deduce that *Pseudomonas* slightly grows better in LM media than in EM media.

For *Enterobacter*, the same procedure was carried out in LM and EM media. Again, the optical density was measured at 578 and 600nm.

Results and Discussion

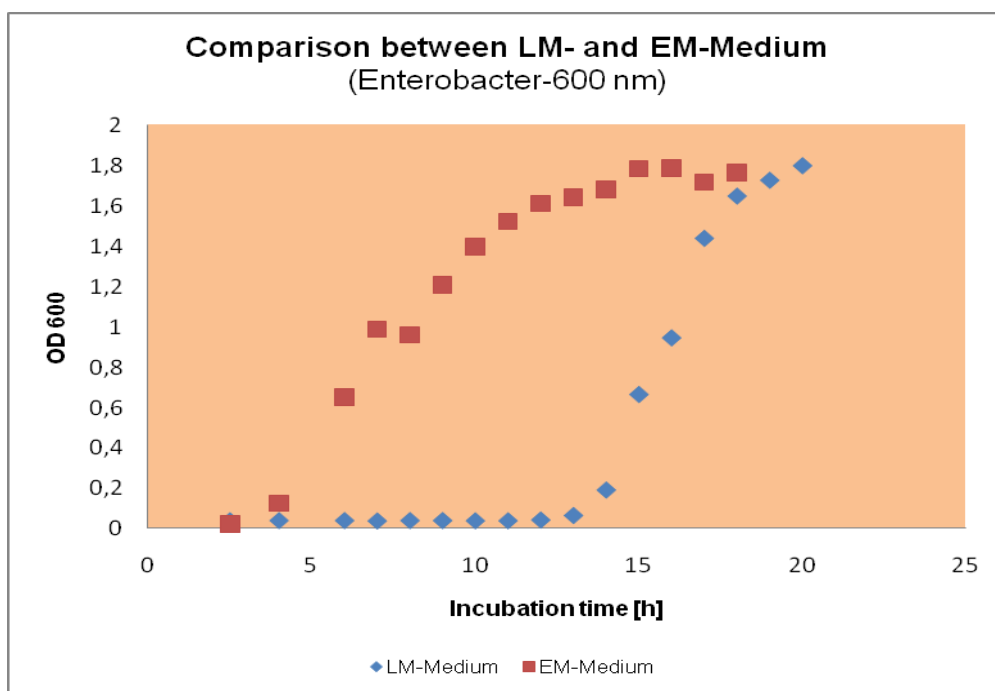


Diagram 3: Comparison of *Enterobacter* growth in LM- and EM-Medium by measuring of optical density at 600 nm

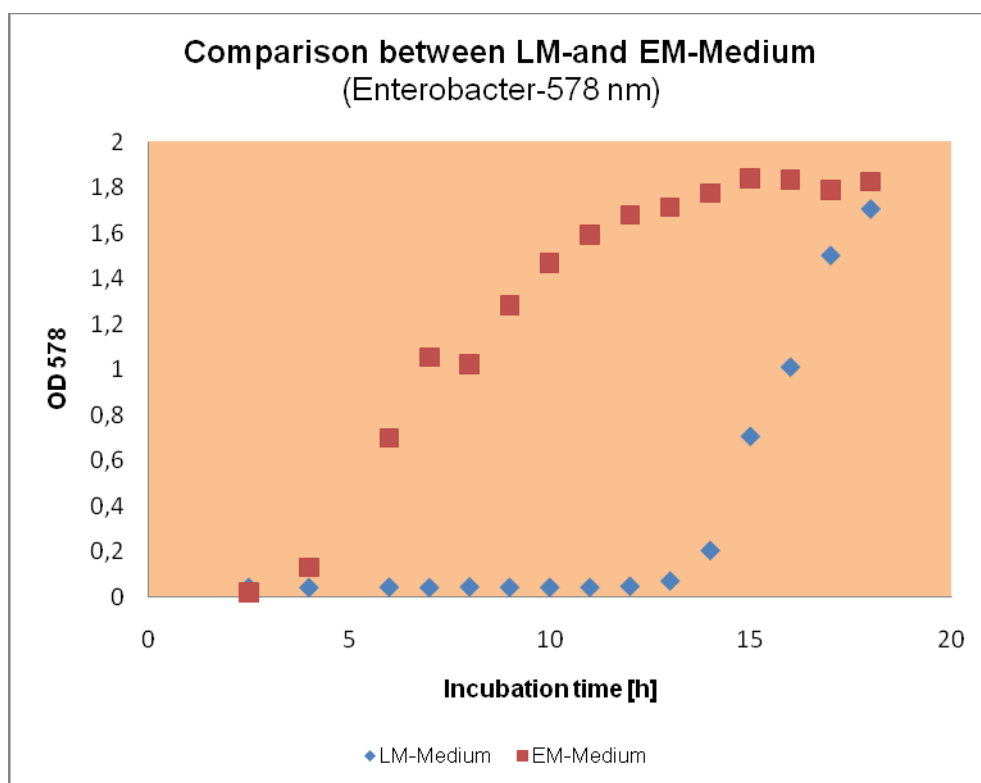


Diagram 4: Comparison of *Enterobacter* growth in LM- and EM-Medium by measuring of optical density at 578 nm

Results and Discussion

In LM medium, the lag phase seems to end after only about 13 to 14 hours of incubation at 37°C. The log phase ranges over 4 to 5 hours (incubation time 14 to 18 hours) and is identifiable by the optical density between 0.18 and 1.70.

In EM medium in contrary, the lag phase already ends after 5 to 6 hours of incubation at 37°C, OD values are between 0.65 and 1.59.

In LM medium, the stationary phase is reached after 20 hours of incubation at 37°C. In EM medium, this phase is already reached after 15 hours of incubation at 37°C.

Comparing both growth curves and the optical density data shows that *Enterobacter* grows better in EM media than in LM media.

1.2.1. Determination of total bacteria count with Plate Count Agar

To focus on the bacterial growth in log phase in two different media, the total bacteria count was also determined.

Pseudomonas in log phase and in LM medium shows 2.0×10^6 CFU/ml (after 6 hours of incubation) up to 1.00×10^{10} CFU/ml (after 16 hours of incubation).

Results and Discussion

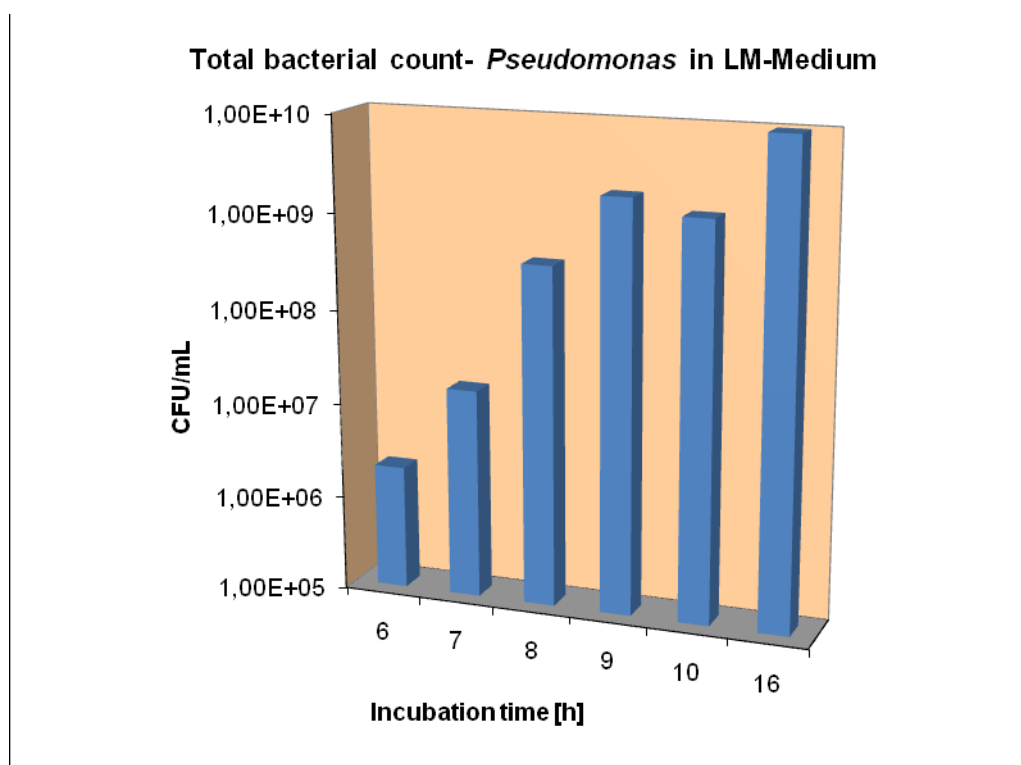


Diagram 5: Total bacterial count of *Pseudomonas* in *log*- phase in LM- Medium

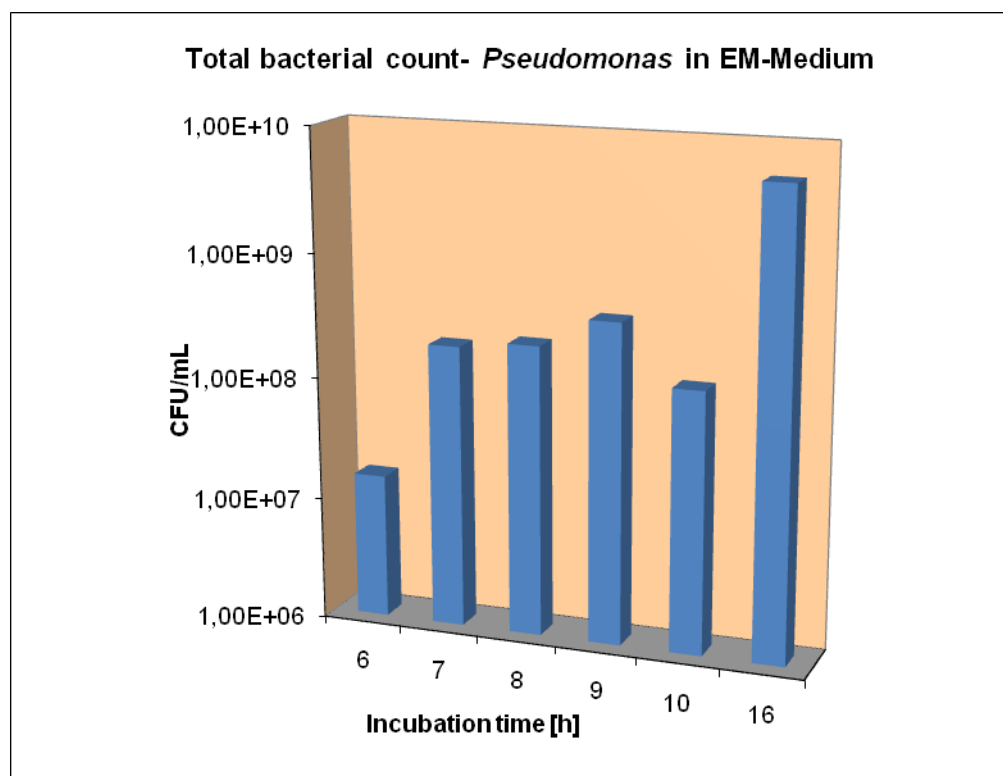


Diagram 6: Total bacterial count of *Pseudomonas* in *log*- phase in EM- Medium

Results and Discussion

Pseudomonas in log phase and in EM media, shows 1.50×10^7 CFU/ml (after 6 hours of incubation) up to 5.50×10^9 CFU/ml (16 hours of incubation).

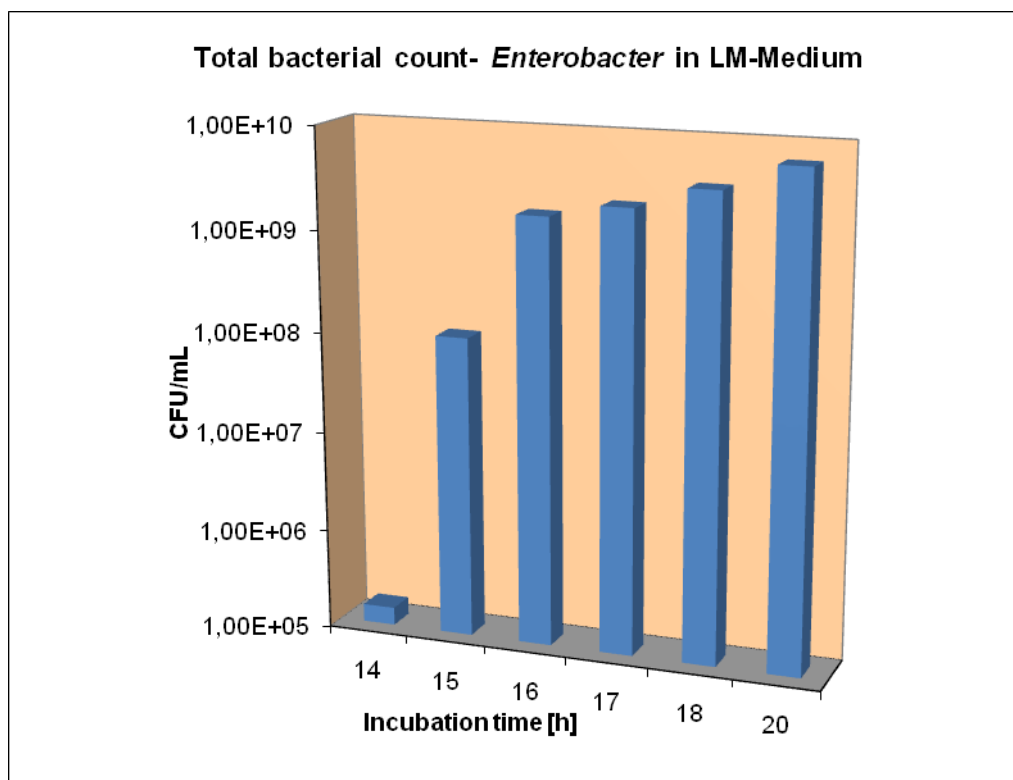


Diagram 7: Total bacterial count of *Enterobacter* in log- phase in LM- Medium

Enterobacter in the log phase and LM medium shows 1.50×10^5 CFU/ml (after 14 hours of incubation) up to 6.70×10^9 CFU/ml (after 20 hours of incubation).

Results and Discussion

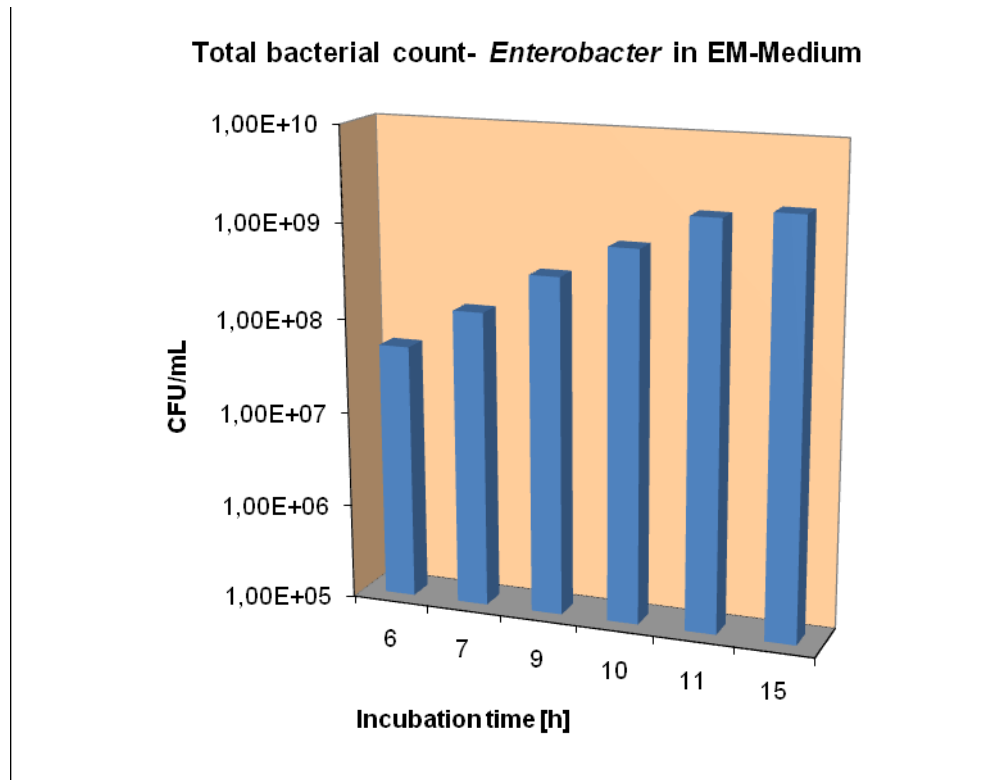


Diagram 8: Total bacterial count of *Enterobacter* in *log*- phase in EM- Medium

In comparison in EM Medium *Enterobacter* grows in *log* phase from 5.0×10^7 CFU/ml (after 6 hours of incubation) to 2.20×10^9 CFU/ml (after 15 hours of incubation).

1.3. Determination of the Rate of Meat Spoilage

To investigate the correlation between total bacterial count and grade of meat deterioration, pork meat was incubated for varying times and temperatures and its spoilage level was determined.

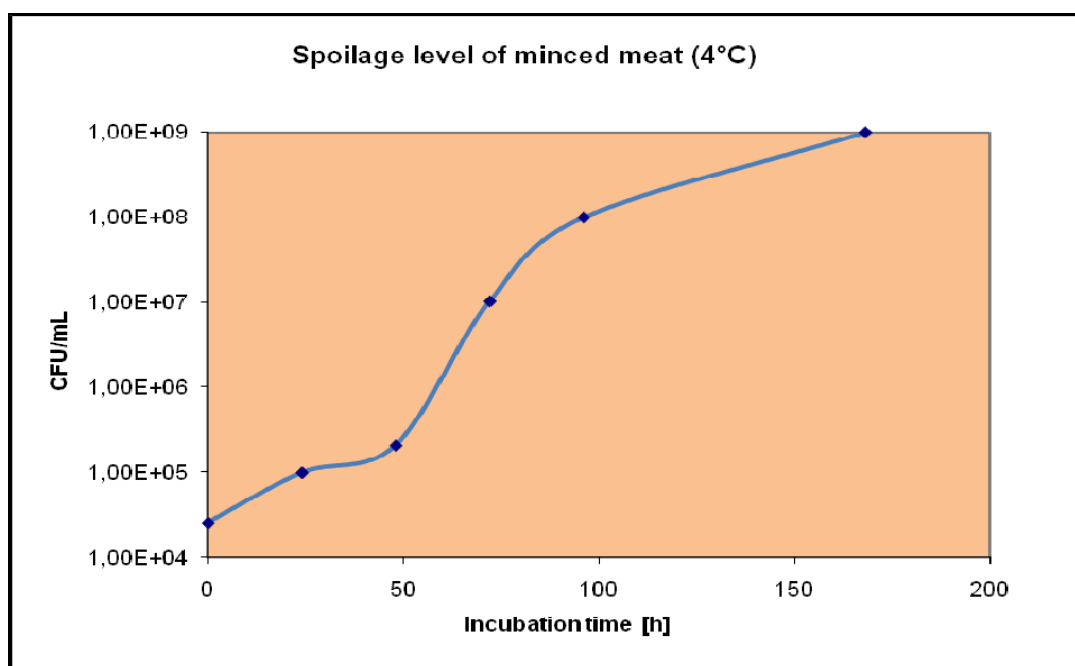


Diagram 9: Spoilage level of minced meat at 4°C

Minced meat was incubated at 4°C in the cold room for various times: 0 h (directly frozen, reference), 24h, 48h, 72h, 96h and 168h. It is shown in diagram 9 that the total bacterial count rises with the incubation time. Freshly minced meat frozen at -20°C (0 h, reference) contained about 2.58×10^5 CFU/ml.

The total bacterial count increases up to 1.0×10^9 CFU/ml after 168 hours. According to guidelines and this diagram, it is obvious that minced meat incubated at 4°C for 72 hours, should definitely not be consumed any more.

Results and Discussion

Minced meat was also incubated at 15°C in a water bath for the same times as above. The total bacterial count rises again with the incubation time. Here we can clearly see that already at the beginning of incubation the total bacterial count increases very abruptly in comparison to the incubation at 4°C.

Freshly minced meat frozen at -20°C (0 h, reference) contained about 2.58×10^5 CFU/ml. Here, the total bacteria count increases up to 7.75×10^9 CFU/ml after 168 hours. Diagram 10 shows according also to guidelines that minced meat incubated at 15°C should not be consumed any more after about 10 to 15 hours.

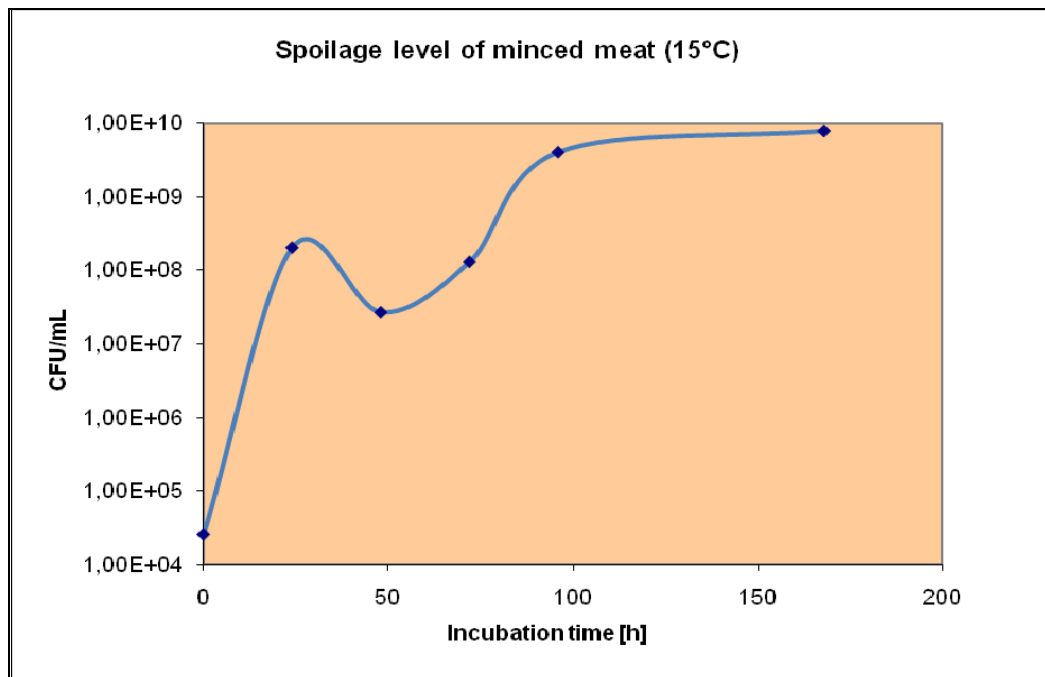


Diagram 10: Spoilage level of minced meat at 15°C

The total bacterial count was also determined for samples with pork cutlets.

Results and Discussion

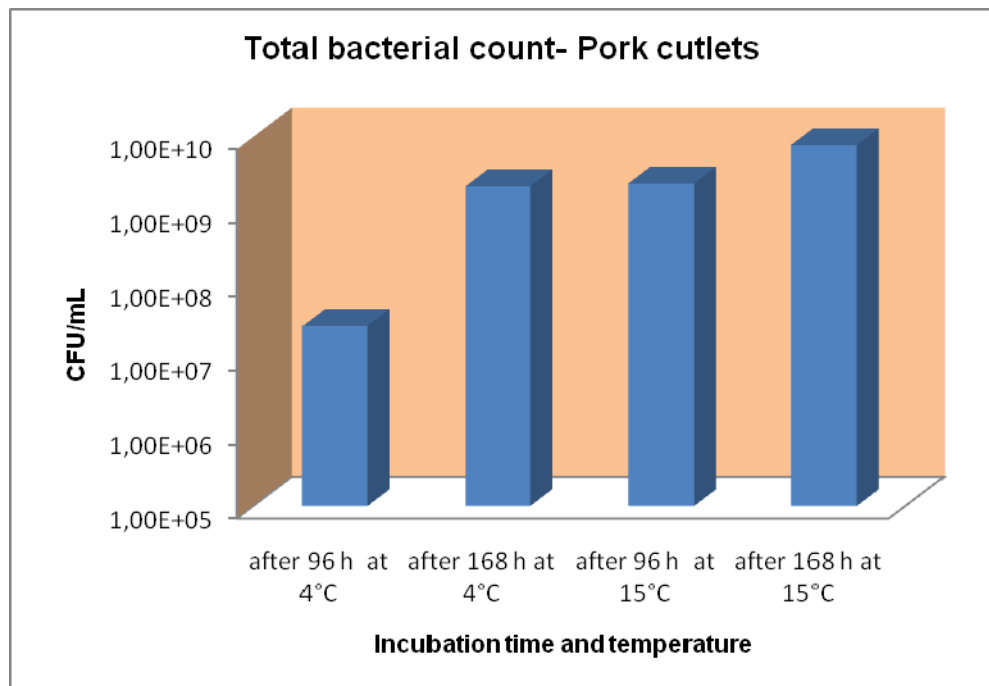


Diagram 11: Total bacterial count- Pork cutlets

II Screening of Enzymatic Activity

In meat spoilage, the total bacteria count and also the amount of secreted lytic enzymes are of high importance. Gram-negative bacteria as e.g. *Pseudomonas* often secrete proteolytic and lipolytic enzymes which are major causes of food decay. [50]

It has already been reported that *Pseudomonas spp.* are able to produce lipases even at low temperatures. Enzymes of e.g. *Pseudomonas fluorescens* or *Pseudomonas aeruginosa* are active even at low temperatures between -2 and 7°C and are even capable of lipolysis and proteolysis at very low concentrations.

In our project, we focused on the leucine aminopeptidase and phospholipase C.

1.4. Qualitative Detection of Exogenous Lytic Enzyme Activity

1.4.1. Tributyrin Agar Base

Tributyrin Agar Base containing certain amount of glycerine-tributyrate and Spirit Blue Agar were used for the testing of four bacterial strains (*Pseudomonas*, *Salmonella*, *Enterobacter* and *Proteus*), isolated from pork meat, for the excretion of lytic enzymes. Searching for the optimal environmental conditions (temperature, culture media, ect.) for the maximal bacterial enzyme production it was possible to see that all four bacterial strains (growing in LM-medium) show the highest lipase yield in bacterial count of 10^6 cfu/mL and 10^8 cfu/mL, after incubation at 30°C and 37°C after 24 hours. First evidence for lipolytic activity of meat bacteria incubated at 4°C was observed after 120 hours.

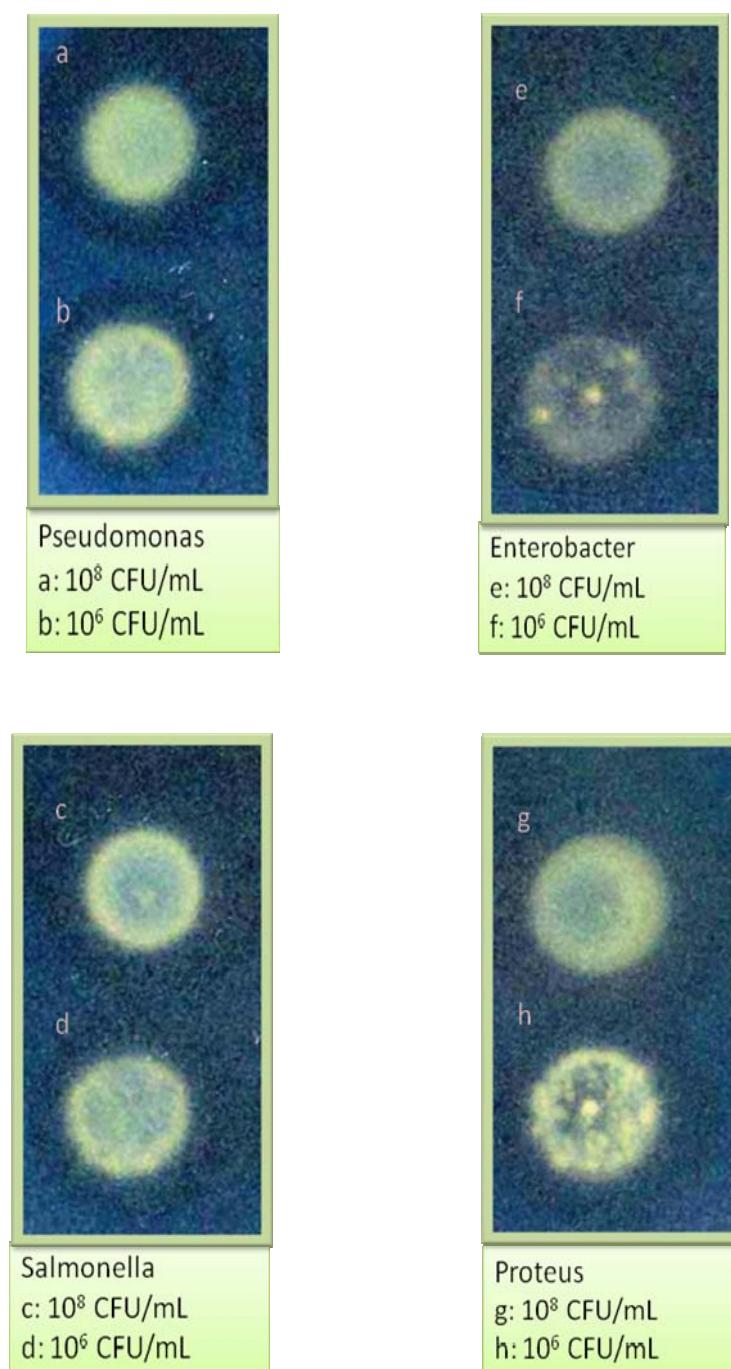


Figure 19. Tributyrin Agar Base and evidences of lytic enzyme production for *Pseudomonas*, *Enterobacter*, *Salmonella* and *Proteus*, after 24 hours at 37°C.

1.4.2. Lipase Activity for Bacterial Culture a and Culture b

These two bacterial cultures were growing in LM medium under above (see) optimized conditions and showed even by qualitative tests (Tributyryn Base Agar) an excellent enzyme secretion.



Figure 20. Tributyrin Agar Base. Evidences for the lytic enzymes occur due to a dark circle around the growing colonies of bacteria (yellow arrows). Incubation time: 120 hours at 4°C.

1.4.3 Spirit Blue Agar

1.4.3.1. Four Gram negative bacteria

Confirmation of lytic activity for aboved Gram negative bacteria was also done with Spirit Blue Agar.

Bacterial cultures were also incubated at three different temperatures (4°C, 30°C and 37°C) and for three different incubation times (24h, 48h and 120h). The bacteria showed best enzyme production after 24 hours at 30°C and 37°C, just like by experiments with Tributyrin Agar Base.

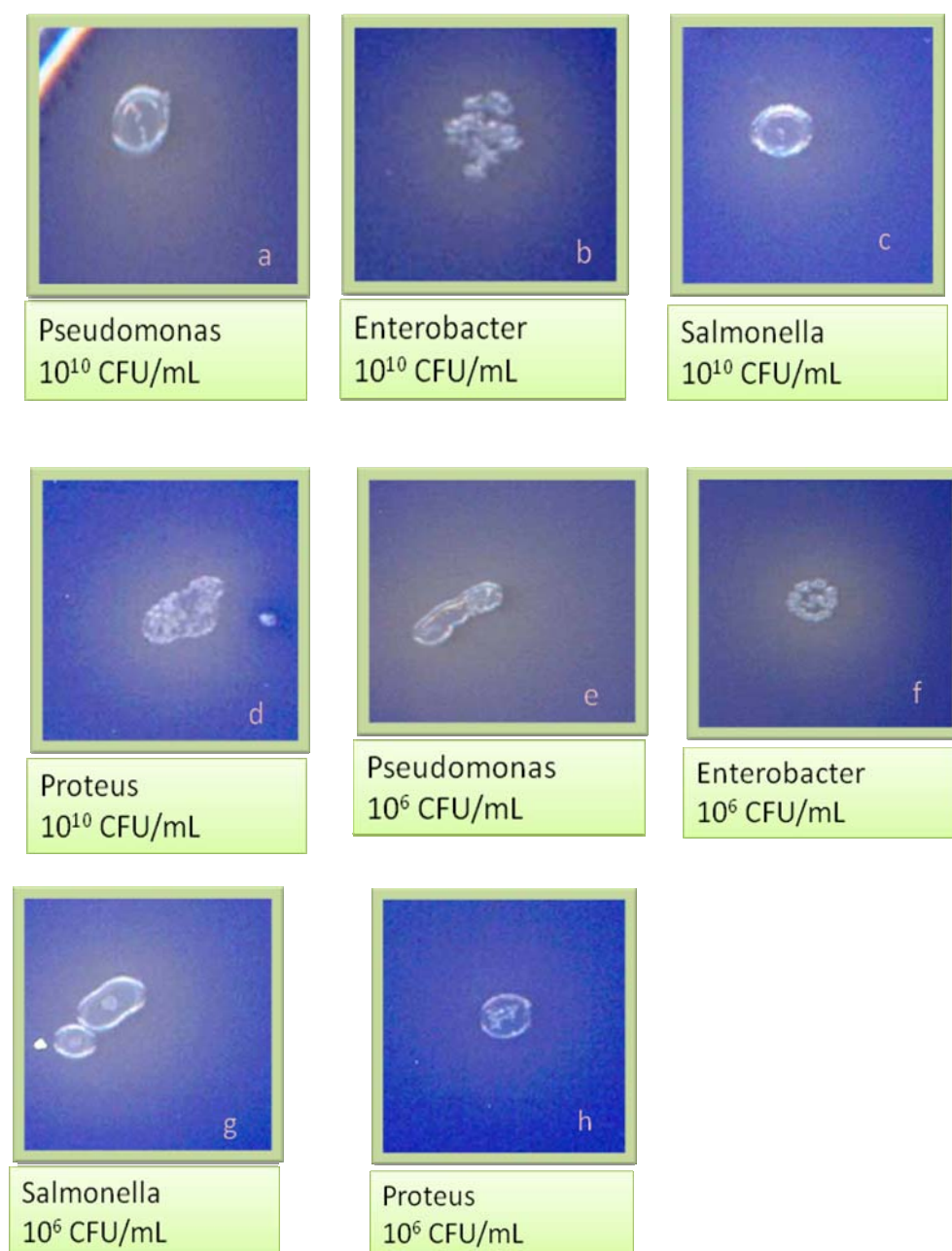
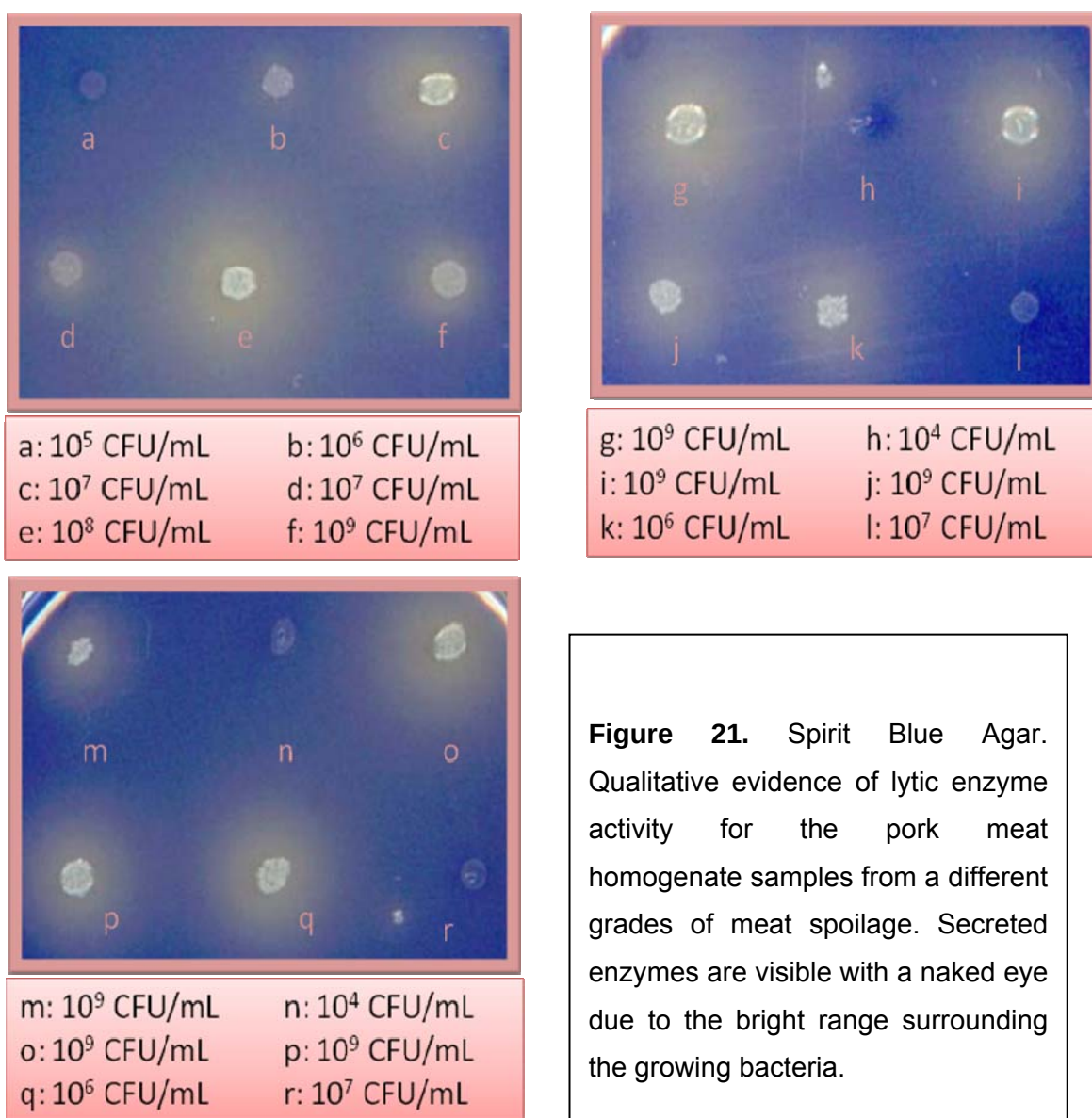


Figure 21. Spirit Blue Agar. Evidence of lytic enzyme activity for *Pseudomonas* (a,e), *Enterobacter* (b,f), *Salmonella* (c,g) and *Proteus* (d,h).

Results and Discussion

1.4.3.2. Grade of Meat Spoilage

Meat homogenate samples which were prepared from different pork samples and incubated at different temperatures and times were also tested for the enzyme activity. Tributyrin Agar Base and Spirit Blue Agar were used a test tool in this case, too. These media display the existence of lytic microorganisms as they do degrade the media as a result of their metabolism.



1.5. Quantitative Detection and Amount of Lytic Enzymes

1.5.1. Determination of Leucine Aminopeptidase Enzyme Activity

Aminopeptidases are exopeptidases cleaving the free N-terminus of polypeptides and proteins in order to liberate a single amino acid residue, in this case L-Leucine. [51, 52]

1.5.1.1. Generation of Calibration Line

Calibration lines for activated and non-activated cases were generated with a stock of Leucine aminopeptidase (10U/ mg). Dilutions of enzyme solutions were made in deionized water (for non-activated enzyme) and reagent C (Materials and Methods, for activated enzyme).

The following activities of Leucine aminopeptidase were used for the activated case: 10, 25, 50, 60, 75, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 mU/mg. The values of the measured absorption ranged from 0.001 up to 0.589.

For the non-activated case, the activities were the following: 10, 15, 20, 30, 40, 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 mU/mg. The values of the measured absorption ranged from 0,004 up to 0,725.

Results and Discussion

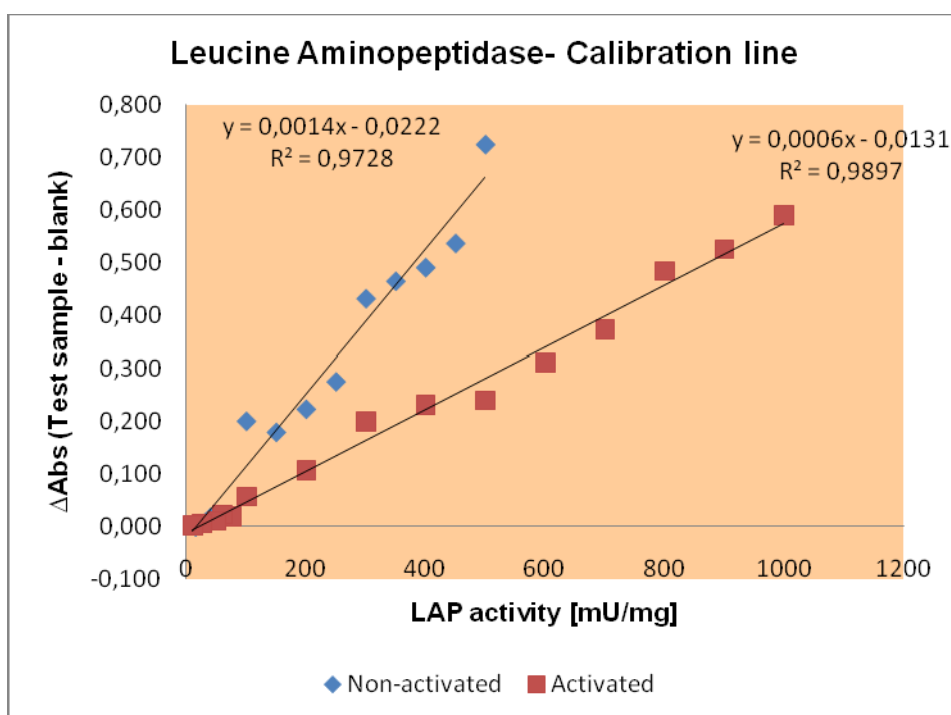


Diagram 12: Leucine Aminopeptidase-Calibration Line

1.5.1.2. Leucine Aminopeptidase (LAP) Activity in Bacterial and Homogenate Cultures

To correlate the total bacterial count in bacteria and homogenate samples used to test the functionality of the biosensors and the activity of the secreted enzymes, LAP activity was also determined for the following samples:

- ❖ *Pseudomonas* stock with 10^9 CFU/ml in LM (PS stock),
- ❖ *Pseudomonas* in LM diluted to 10^6 CFU/ml (PS 10^6),
- ❖ *Proteus* stock with 10^9 CFU/ml in LM (Pr stock),
- ❖ *Proteus* in LM diluted to 10^6 CFU/ml, (Pr 10^6)
- ❖ *Enterobacter* stock with 10^9 CFU/ml in LM (En stock),
- ❖ *Enterobacter* in LM diluted to 10^6 CFU/ml (En 10^6),
- ❖ *Salmonella* stock with 10^{10} CFU/ml in LM (Sal Stock),

Results and Discussion

- ❖ *Salmonella* in LM diluted to 10^6 CFU/ml (Sal 10^6)
- ❖ Fresh pork cutlet after 96h of incubation at 4°C (Sample 11)
- ❖ Infected minced meat after 48h of incubation at 15°C (Sample 16)

On first sight, we can clearly see that non-activated samples show overall lower LAP activity than activated samples.

Focusing on the samples of bacteria, most of the dilutions show higher LAP activity than the stock solutions. For the activated case, this is valid for *Pseudomonas* (PS), *Proteus* (Pr) and *Salmonella* (Sal) in concentration of 10^6 CFU/ml. For the non-activated case, this is valid for *Pseudomonas* (PS), *Proteus* (Pr) and *Enterobacter* (En) in concentration of 10^6 CFU/ml.

Pseudomonas and *Salmonella* seem to play an important role by excretion of the most active Leucine aminopeptidase: the highest activity was measured for the activated case with *Pseudomonas* in bacterial count of 10^6 CFU/ml, and for the nonactivated case with the *Salmonella* stock (10^{10} CFU/ml).

Focusing on the homogenate samples, we can see that sample 11 shows the highest LAP activity of 53.50 mU for the activated case. Comparing homogenate samples incubated at 15°C , the highest LAP activity of 35.17mU for the activated case is detected for sample 16 (incubation at 15°C for 48h).

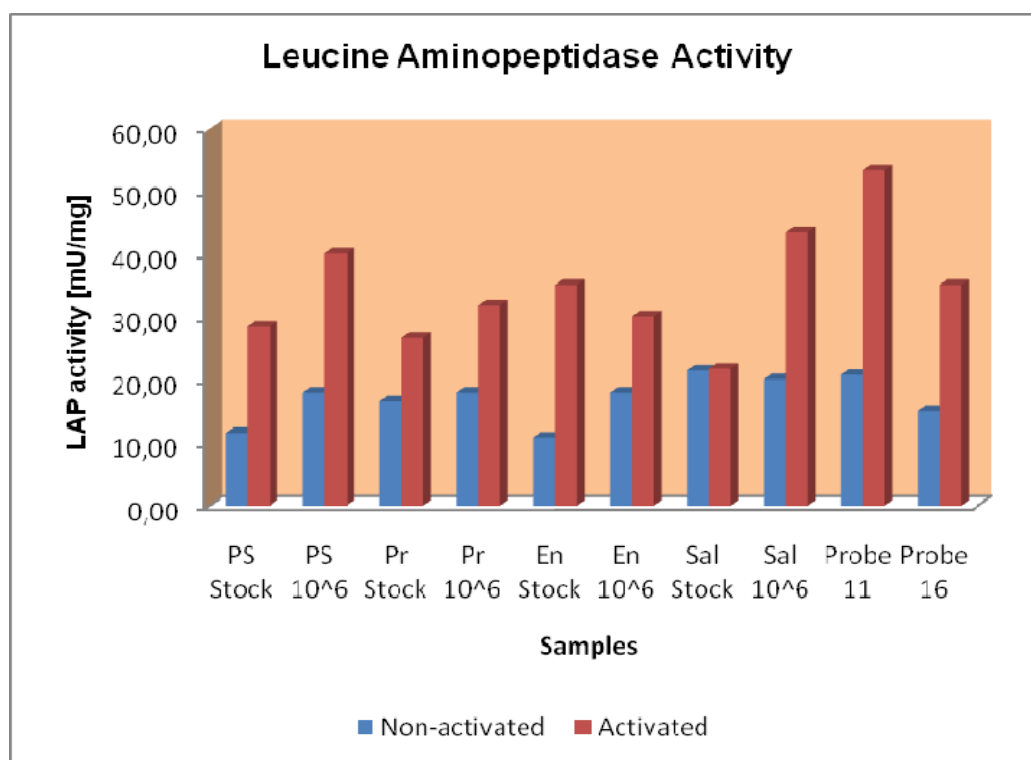


Diagram 13: Leucine Aminopeptidase Activity in Bacterial and Homogenate Cultures

1.5.1.3. LAP Activity in Combination with Meat Deterioration

To correlate grades of meat deterioration with the amount of secreted enzymes, the LAP activity was also measured for infected meat samples incubated for different times and different temperatures.

In diagram 14, we can see the LAP activity of different samples of infected and non-infected (control) pork cutlets. Comparing the pork cutlets incubated for 168 hours at -20°C and at 4°C, there is no big observable difference in LAP activity (non-activated and activated). Though, after 96 hours of incubation at 4°C, LAP activity is higher (48.83mU for activated and 25.5mU for non-activated) than after 168 hours at -20°C and even after 168 hours of incubation at 4°C.

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Pork cutlet was also incubated for different times at 15°C in the water bath. Comparing 96 hours and 168 hours of incubation, there is no significant difference in LAP activity to observe.

Comparing now the incubation at 15°C and 4°C for the same incubation time, it is obvious that the LAP activity is higher in samples incubated at 15°C (57.17mU for activated, 24.79mU for non-activated)

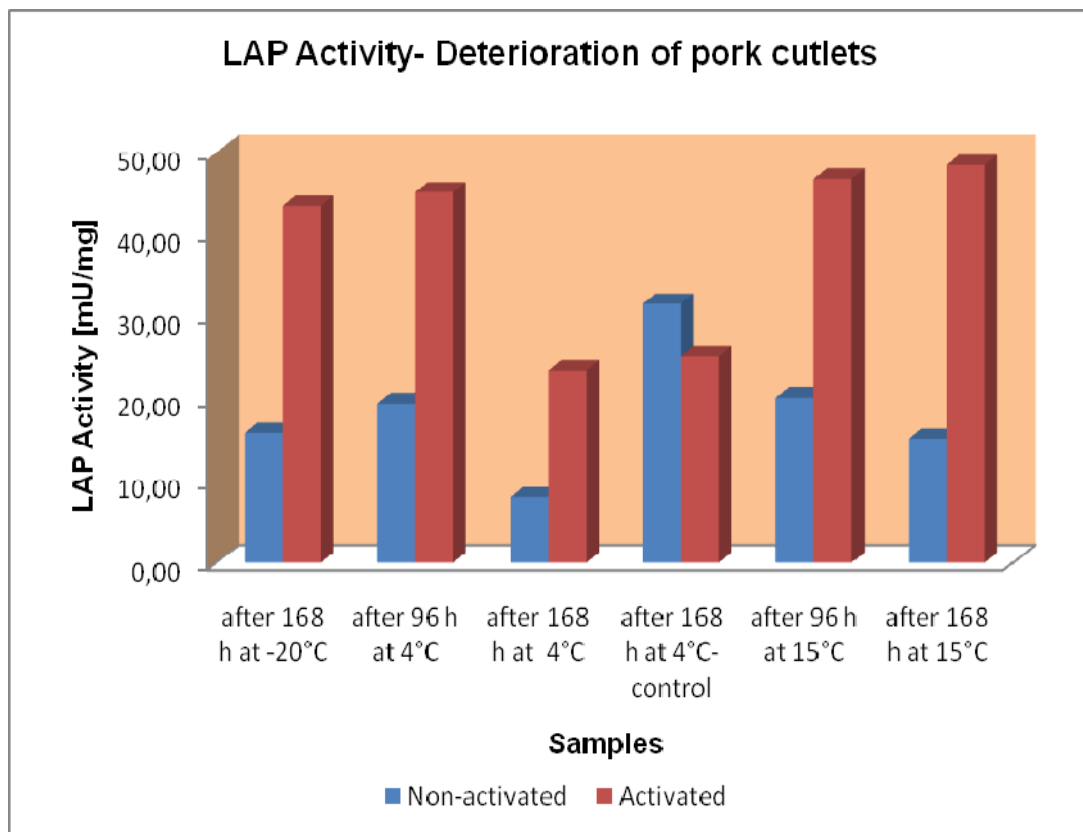


Diagram 14: Comparison of LAP Activity with deterioration of pork cutlets

In parallel to pork cutlets, minced pork meat was also examined for LAP activity. Generally, non-activated cases show again lower LAP activity than activated cases.

In the diagram 15, we can definitely see the rise in LAP activity with elevated incubation times at 4°C. The highest LAP activity (47.17mU for activated, 17.64

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for non-activated) was found after 72 hours of incubation at 4°C. At this time, the total bacterial count represented 1.03×10^7 CFU/ml and bacteria stood in the middle of their log phase.

Comparing the LAP activity of pork cutlets and minced meat, shows that e.g. after 96 hours of incubation at 4°C, a slightly higher LAP activity was measured with pork cutlets than with minced meat. Though, after 168 hours of incubation at 4°C, LAP activity was already higher with minced meat than with pork cutlets.

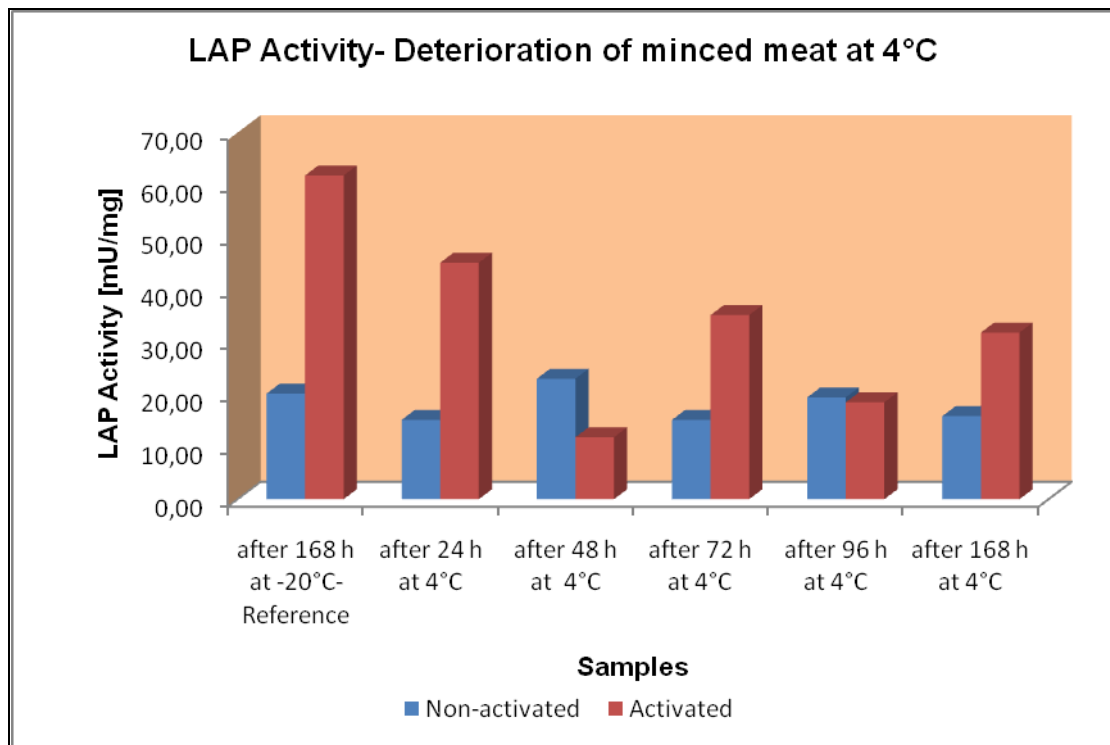


Diagram 15: Comparison of LAP Activity with deterioration of minced meat at 4°C

Minced pork meat was also incubated for different times at 15°C. Interestingly, in two cases, nonactivated samples seem to show higher LAP activity than activated samples (after 24h and 72h at 15°C).

Looking at the other incubation times, LAP activity seems to pace up and down. Highest LAP activity (42.17mU for activated) was measured for non-infected minced meat incubated for 168 hours at 15°C.

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Comparing the LAP activity of minced meat incubated at 4°C and at 15°C, in both progresses a slightly lower LAP activity was measured after an incubation of 96 hours than at 168 hours.

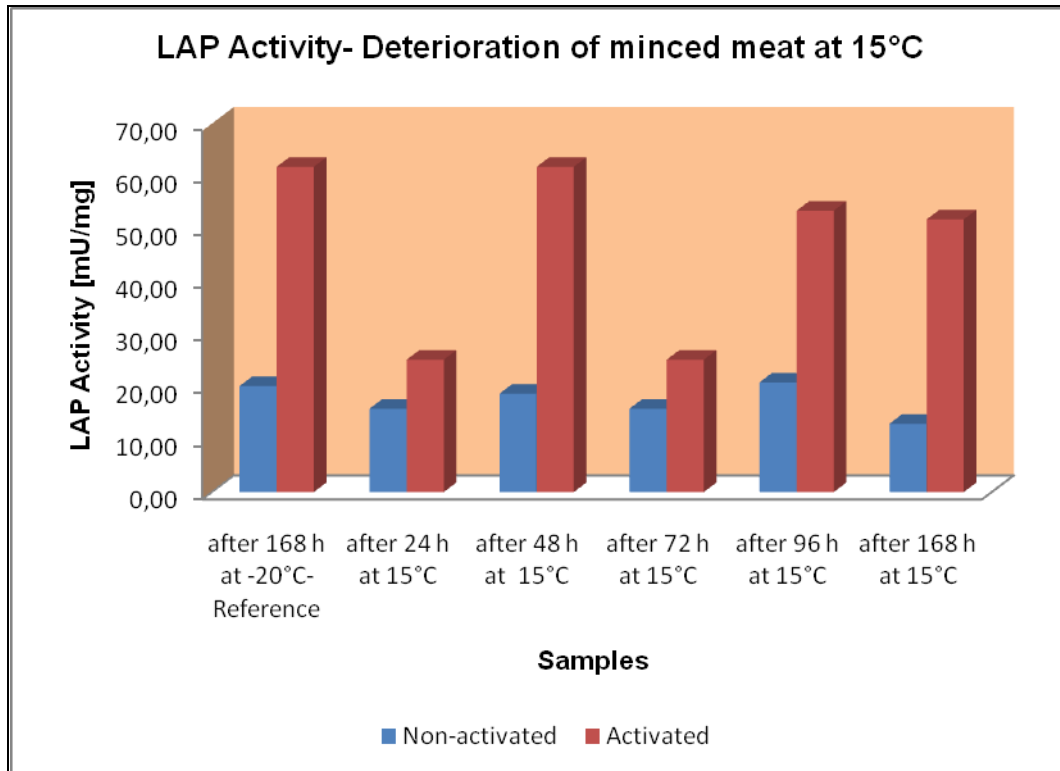


Diagram 16: Comparison of LAP Activity with deterioration of minced meat at 15°C

1.5.1.4. LAP Activity in Combination with Bacterial Growth in LM and EM Media

The environmental conditions have an important influence on the bacterial growth behavior.

Therefore, the LAP activity was also measured for bacteria grown in different media. Samples picked out are within the range of the log phase.

As shown in diagram 17, *Pseudomonas* grown in LM medium show the highest LAP activity (35.17mU for activated, 18.71mU for non-activated) after 7 hours of incubation at 37°C.

Referring to the totally determined bacterial count, the *Pseudomonas* after this incubation time are in the middle of their log phase (1.60×10^7 CFU/ml). On the beginning of the stationary phase, was also high LAP activity measured (30.17mU for activated, 20.86mU for non-activated).

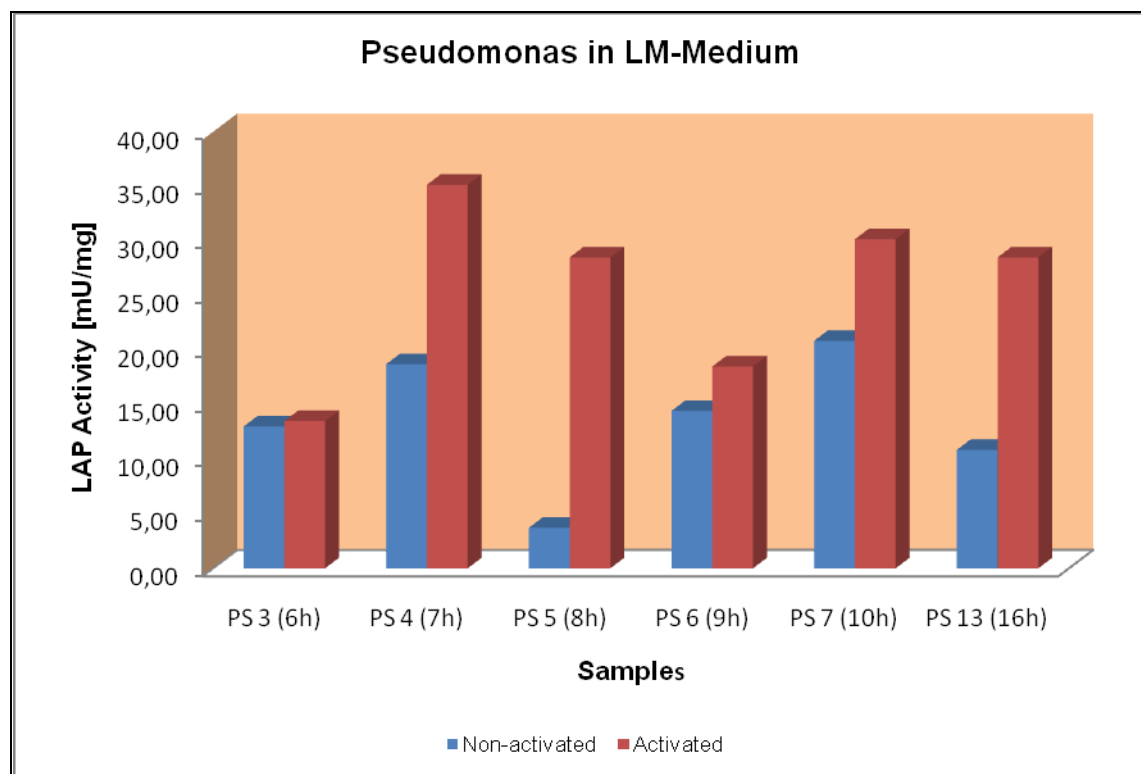


Diagram 17: LAP Activity of *Pseudomonas* in *log*- phase in LM- Medium

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In EM medium, *Pseudomonas* show a clear rise in LAP activity all over the log phase. For the activated cases, overall higher LAP activity is measured than for the non-activated cases. In diagram 18, we can clearly see that highest LAP activities are found around 9-12 hours of incubation at 37°C. The total bacterial count after 9 hours of incubation represents 4.00×10^8 CFU/ml. The highest LAP activity after 12 hours of incubation represents 38.5mU for the activated and 17.29mU for the nonactivated case. After 12 hours of incubation, an abrupt fall in LAP activity is measured. This is the point where the stationary phase begins.

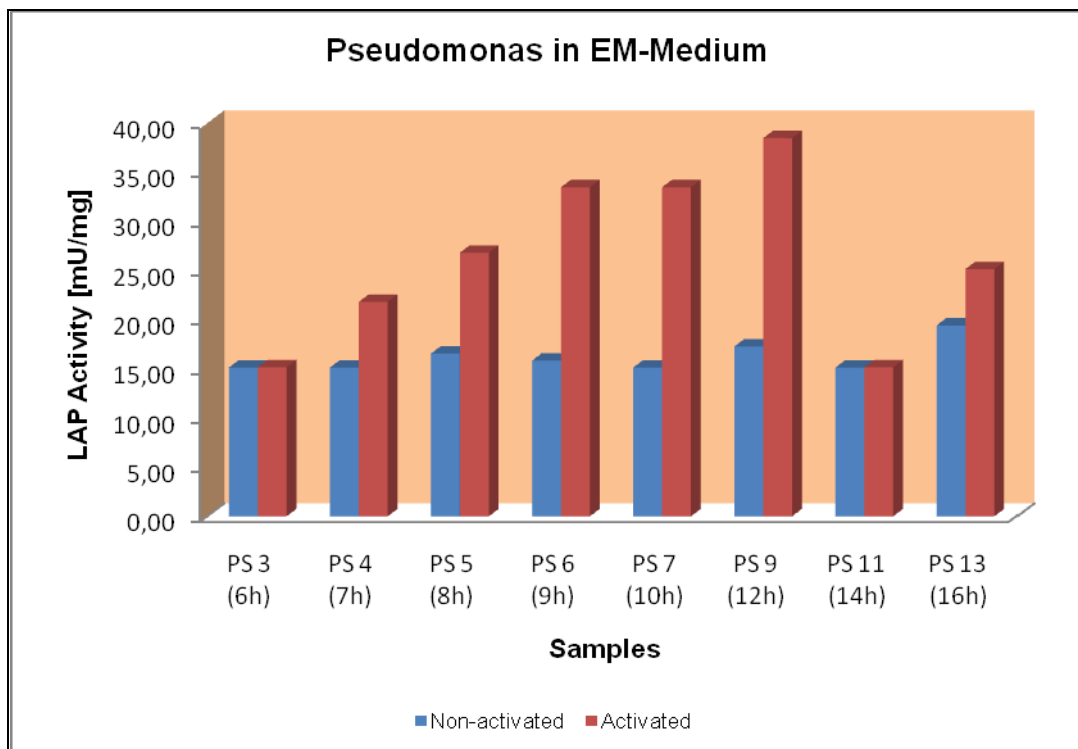


Diagram 18: LAP Activity of *Pseudomonas* in *log*- phase in EM- Medium

For *Enterobacter*, the same procedures were carried out.

In LM medium, *Enterobacter* interestingly shows high LAP activity after 14 hours of incubation, though the total bacterial count was not so high (1.5×10^5 CFU/ml). After 15 hours of incubation, LAP activity is (for the activated case)

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lower than 1 hour before. However, the total bacteria count already reached at this incubation time 1×10^8 CFU/ml. This suggests that even with high total bacterial count, the LAP activity does not have to rise compulsorily.

Highest LAP activity was found after 16 and 20 hours of incubation (36.83mU for activated).

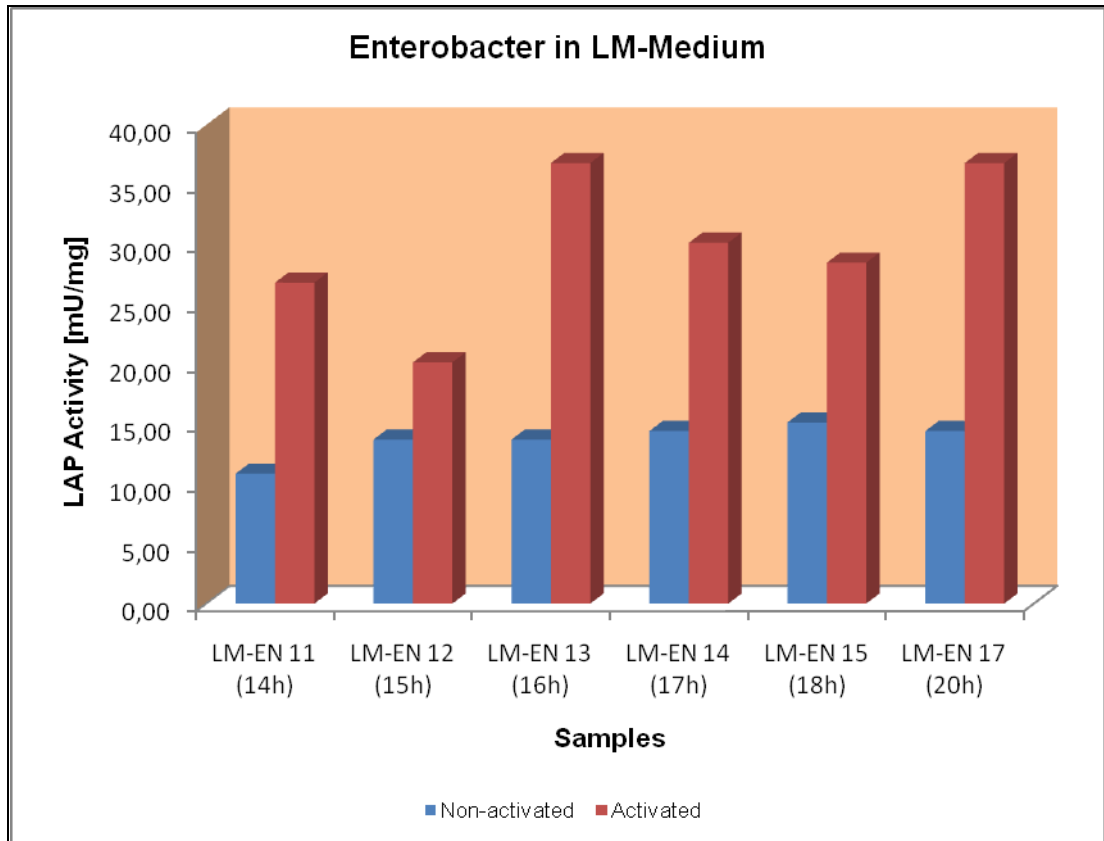


Diagram 19: LAP Activity of *Enterobacter* in *log*- phase in LM- Medium

Results and Discussion

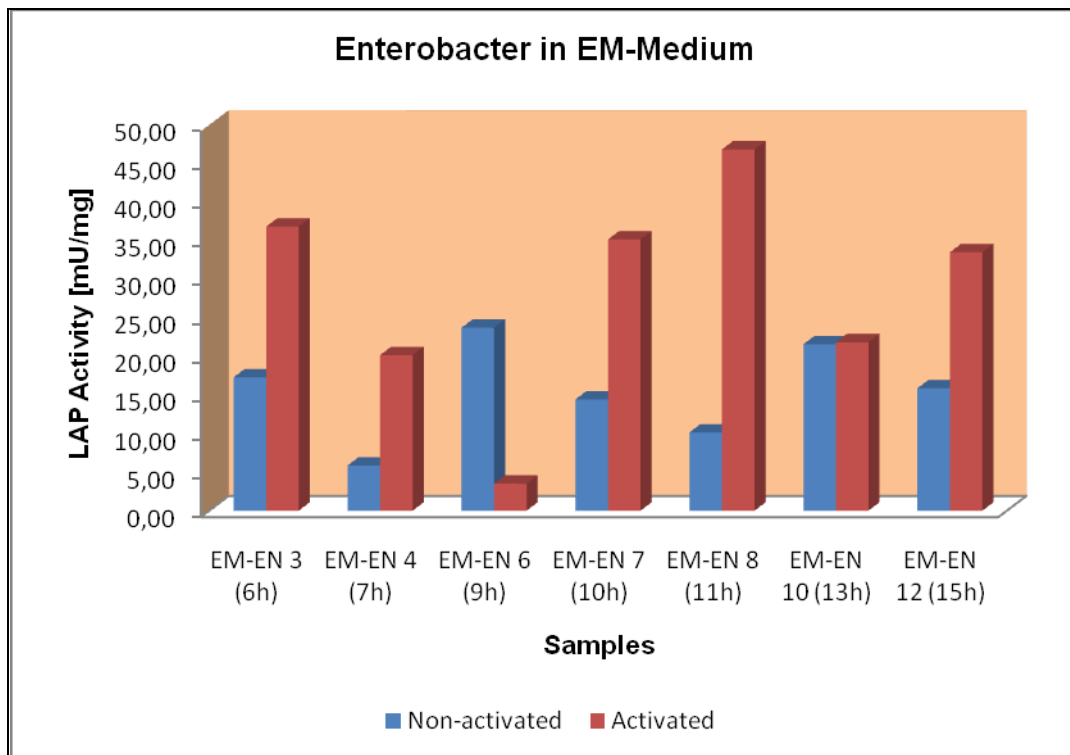


Diagram 20: LAP Activity of *Enterobacter* in *log*- phase in EM- Medium

In EM medium, *Enterobacter* seems to show a LAP activity that paces up and down. While the total bacterial count ranges over from 5.0×10^7 CFU/ml to 2.20×10^9 CFU/ml, a steady rise in LAP activity is not measured. Overall, the LAP activity for activated cases is higher than for the non-activated cases.

The highest LAP activity with *Enterobacter* in EM medium was measured after 11 hours of incubation (46.83mU for activated). At this point, the total bacterial count represents 1.8×10^9 CFU/ml.

1.5.1.5. LAP Activity in Combination with Bacterial Culture Preparation

The main bacterial culture (100 ml) containing the bacteria from homogenized pork meat was used as a starter culture and incubated for 6 hours (Culture a) and 24 hours (Culture b) in a water bath at 15 °C. After these periods of bacterial growth, the cultures showed the total bacterial counts of $\sim 10^7$ CFU/mL for Culture a, and $\sim 10^8$ CFU/mL for Culture b. It is important to say that the starter bacterial culture was prepared in LM medium, with $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ as a phosphate source. Afterwards, the bacterial cultures were centrifuged for 3-5 minutes at 5000 rpm in a top table centrifuge (at 4°C) and LAP activity in the supernatants was measured.

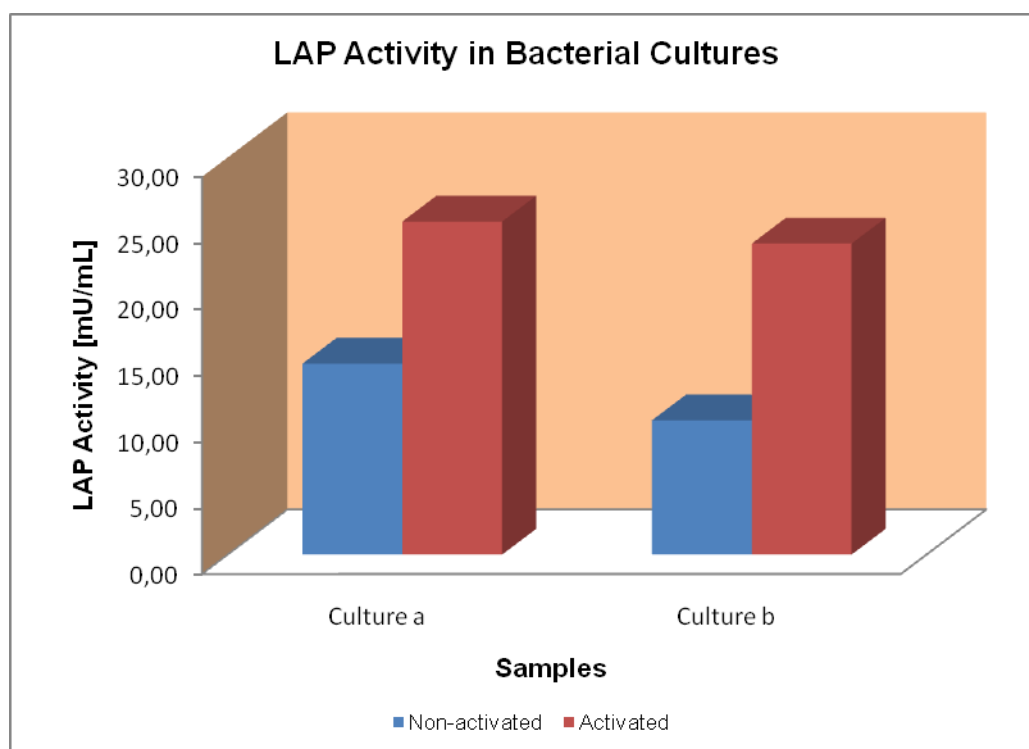


Diagram 21: LAP Activity in Bacterial Culture Preparation

On first sight, we can clearly see that non-activated samples show overall lower LAP activity than activated samples.

Culture a show slightly higher LAP activity (25.17 mU for activated) than *Culture b* (23.50mU for activated).

1.6. Determination of Phosphatidylcholine - Specific Phospholipase C Enzyme Activity

Phosphatidylcholine-specific Phospholipase C converts the phosphatidylcholine (lecithin) substrate to form phosphocholine and diacylglycerol. After the action of alkaline phosphatase, which hydrolyzes phosphocholine, choline is oxidized by choline oxidase to betaine and H_2O_2 .

Finally, H_2O_2 , in the presence of horseradish peroxidase, reacts with Amplex Red reagent in a 1:1 stoichiometry to generate the highly fluorescent product, resorufin.

1.6. 1. Generation of calibration line

A calibration line was generated with a stock of Phosphatidylcholine-specific Phospholipase C (10U/ mL). Dilutions of enzyme solutions were made with 1X working solution of Reaction Buffer (see Materials and Methods). The following activities were used for the Phosphatidylcholine-specific Phospholipase C: 25, 50, 75, 100, 150, 200, 250, 300, 400 and 500 mU/mL.

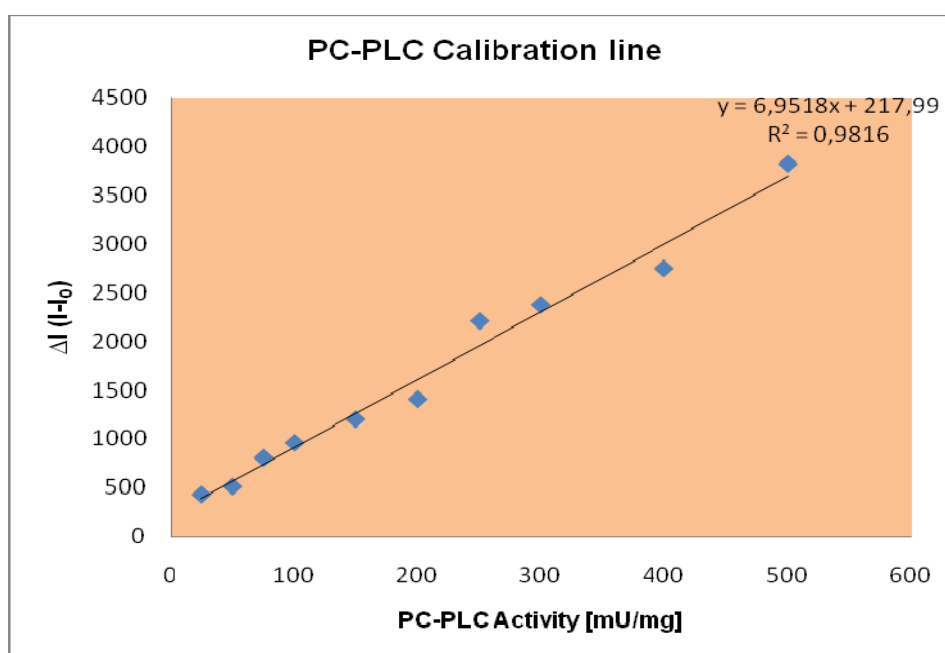


Diagram 22: Phosphatidylcholine-specific Phospholipase C- Calibration Line

Results and Discussion

The values of the measured fluorescence ranged from 425 up to 3818.

1.6.2. PC-PLC Activity in Bacterial Cultures

To compare the total bacterial counts of the bacteria which were used to test the functionality of the biosensors and the activity of the secreted lytic enzymes, the PC-PLC activity was determined for the following samples:

- ❖ *Pseudomonas* stock with 10^9 CFU/ml in LM (PS stock),
- ❖ *Pseudomonas* in LM diluted to 10^6 CFU/ml (PS 10^6),
- ❖ *Proteus* stock with 10^9 CFU/ml in LM (Pr stock),
- ❖ *Proteus* in LM diluted to 10^6 CFU/ml, (Pr 10^6)
- ❖ *Enterobacter* stock with 10^9 CFU/ml in LM (En stock),
- ❖ *Enterobacter* in LM diluted to 10^6 CFU/ml (En 10^6),
- ❖ *Salmonella* stock with 10^{10} CFU/ml in LM (Sal Stock),
- ❖ *Salmonella* in LM diluted to 10^6 CFU/ml (Sal 10^6)

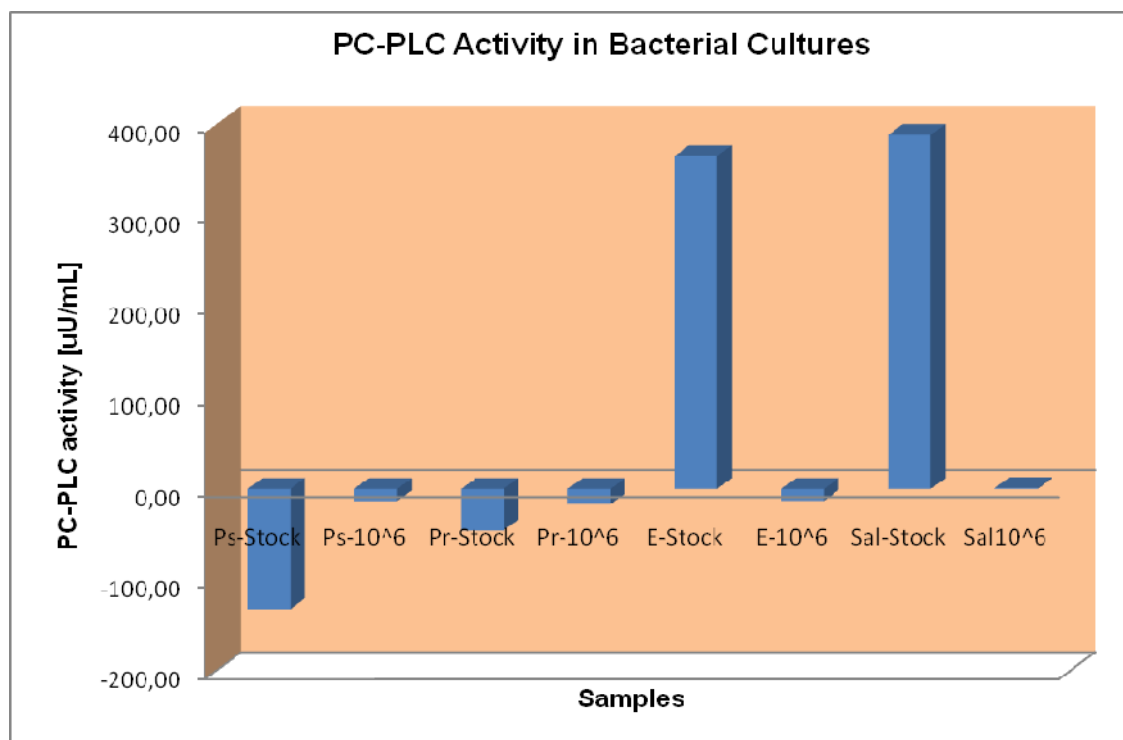


Diagram 23: PC-PLC Activity in Bacterial Cultures

Enterobacter and *Salmonella* seem to play important roles in secreting the most of active Phosphatidylcholine-specific Phospholipase C: the highest activity was measured for the *Enterobacter* stock and *Salmonella* stock.

1.6.3. PC-PLC Activity in Combination with Meat Deterioration

To correlate grades of meat deterioration with the amount of secreted lytic enzymes, the PC-PLC activity was also measured for infected meat samples incubated for different times and different temperatures.

In diagram 24, we can see the PC-PLC activity of different samples of infected and non-infected (control) pork cutlets. Comparing pork cutlets incubated for 168 hours at -20°C (494, 96 mU/mL) and at 4°C (642, 16 mU/mL) there is a significant difference in PC-PLC activity. Though, after 168 hours of incubation at 4°C, PC-PLC activity is higher (642,16 mU/mL) than after 168 hours at -20°C and after 96°C hours of incubation at 4°C.

Pork cutlet was also incubated for different times at 15°C in the water bath. The sample after 96 hours at 15°C showed PC-PLC activity (333, 15 mU/mL).

Comparing the meat incubation at 15°C and 4°C for the same incubation time, we can clearly see that the PC-PLC activity is higher in samples incubated at 4°C (642,16 mU/mL).

Results and Discussion

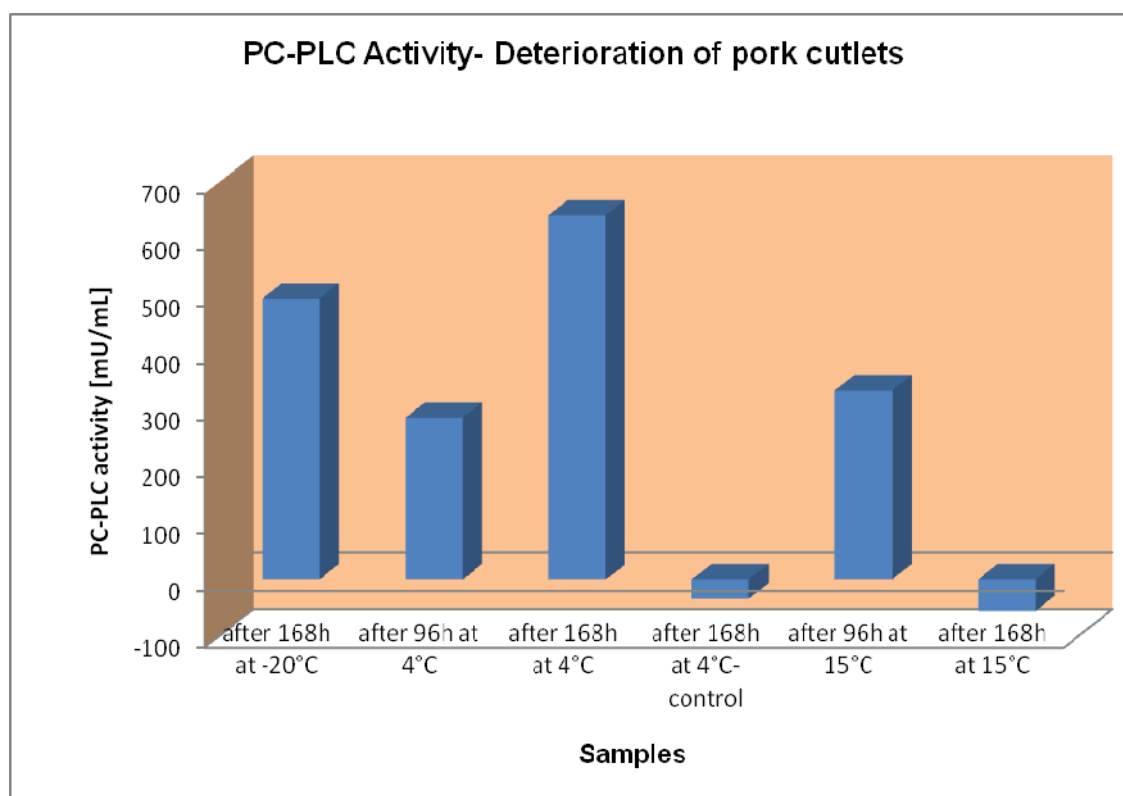


Diagram 24: Comparison of PC-PLC activity with deterioration of pork cutlets

In parallel to pork cutlets, minced pork meat was also examined for PC-PLC activity.

In diagram 25, we can see an irregular rise in PC-PLC activity with elevated incubation times at 4°C. The highest PC-PLC activity (514, 11 mU/mL) was found in a reference sample, after 168 hours of incubation at -20°C. At this time, the total bacterial count represented 2.58×10^4 CFU/ml and bacteria were at the beginning of their log phase (see Determination of meat deterioration).

Comparing the PC-PLC activity of pork cutlets and minced meat, we can say that e.g. in the reference sample, a higher PC-PLC activity was measured with minced meat than with pork cutlets. Though, after 168 hours of incubation at 4°C, PC-PLC activity was already higher with pork cutlets than with minced meat.

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Deterioration of minced meat at 4°C	
Samples	PC-PLC activity [mU/mL]
after 168 h at -20°C- reference	514,11
after 24 h at 4°C	476,86
after 48 h at 4°C	504,19
after 72 h at 4°C	434,28
after 96 h at 4°C	498,43
after 168 h at 4°C	308,7

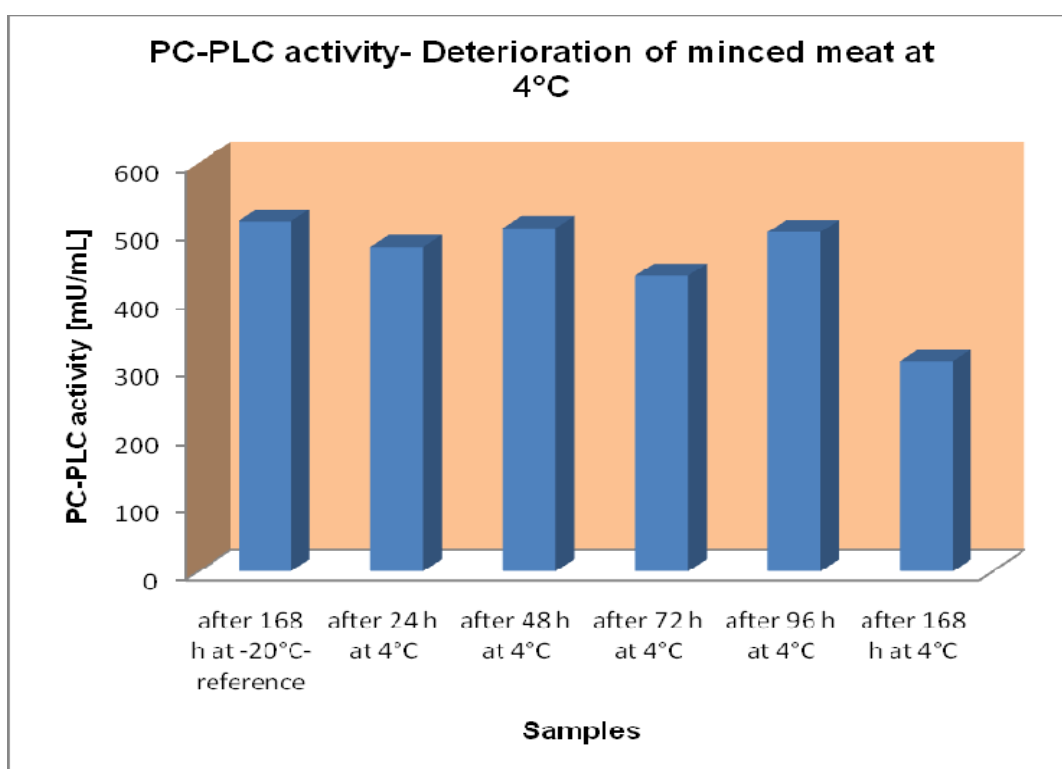


Diagram 25: Comparison of PC-PLC activity with deterioration of minced meat at 4°C

Minced pork meat was also incubated for different times at 15°C.

Highest PC-PLC activity (765,13 mU/mL) was measured for minced meat incubated for 168 hours at 15°C. Looking at other incubation times, PC-PLC activity seems to pace up and down.

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Deterioration of minced meat at 15°C	
Samples	PC-PLC activity [mU/mL]
after 168 h at -20°C- reference	514,11
after 24 h at 15°C	582,44
after 48 h at 15°C	732,33
after 72 h at 15°C	495,41
after 96 h at 15°C	532,53
after 168 h at 15°C	765,13

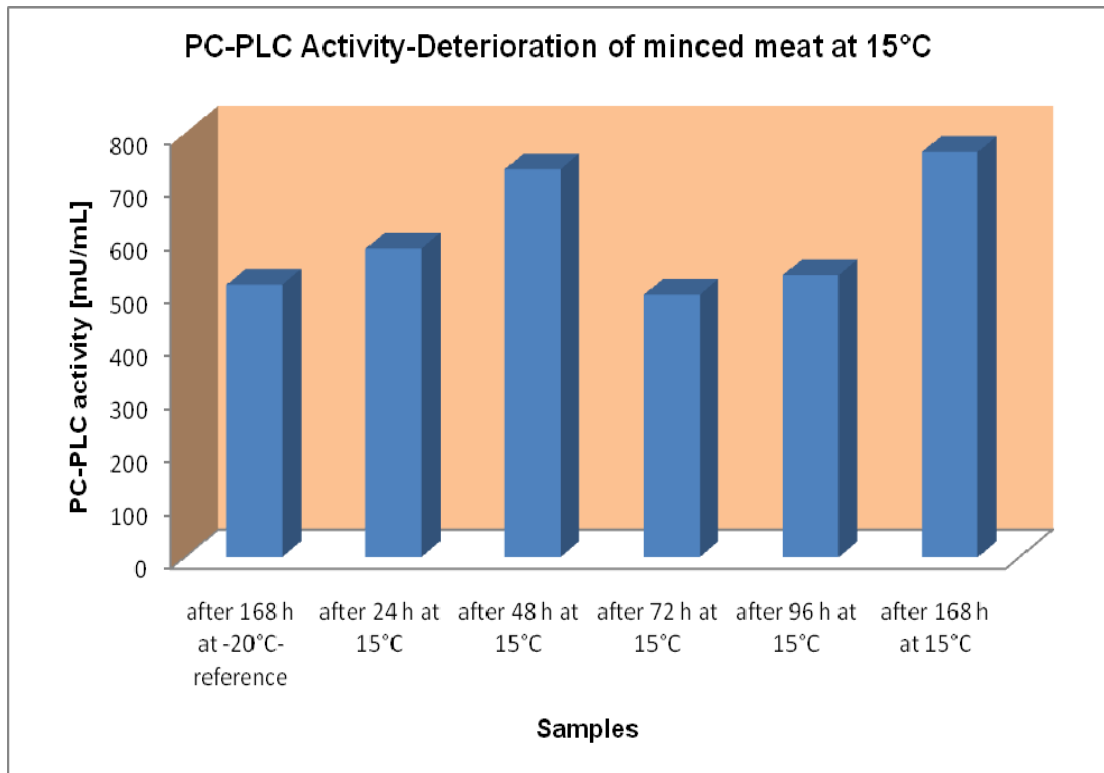


Diagram 26: Comparison of PC-PLC activity with deterioration of minced meat at 15°C

1.6.4. Phosphatidylcholine-specific Phospholipase C (PC-PLC) Activity in Combination with Bacterial Growth in LM and EM Media

PC-PLC activity was also measured for bacterial grown in different media. Samples are within the range of the log phase.

As we can see in diagram 27, *Pseudomonas* grown in LM medium show the highest PC-PLC activity (2,09 U/mL) after 7 hours of incubation at 37°C.

Referring to the total determined bacterial count, the *Pseudomonas* is in the middle of their log phase (1.60×10^7 CFU/ml) after this incubation time. On the beginning of the stationary phase high PC-PLC activity could also be measured (1,875 U/mL).

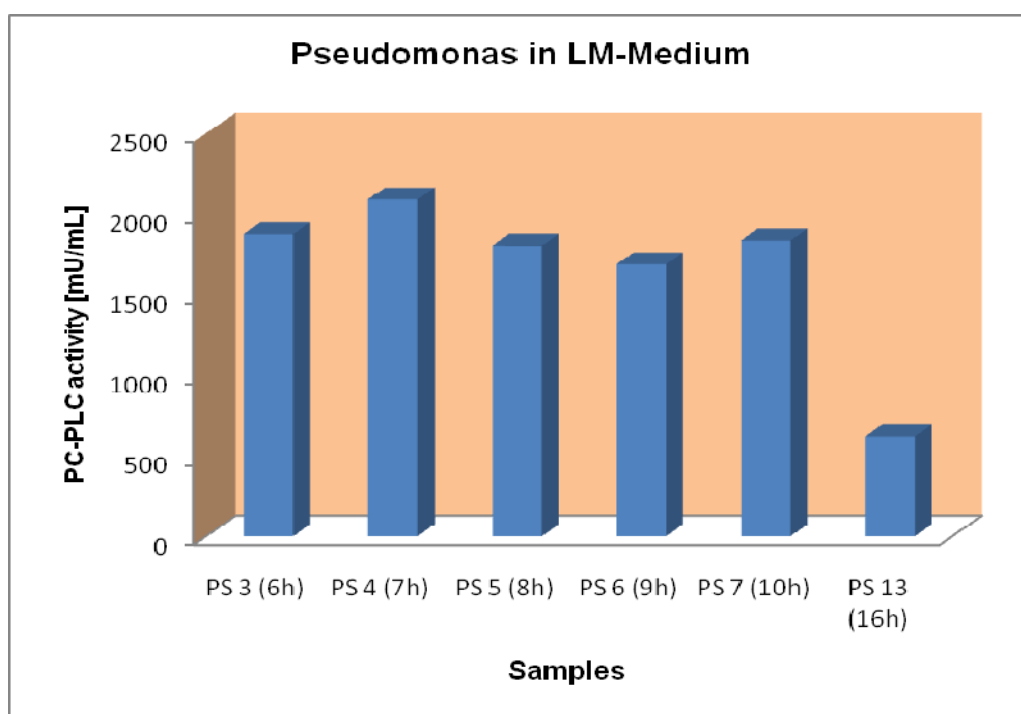


Diagram 27: PC-PLC Activity of *Pseudomonas* in log –Phase in LM-Medium

Results and Discussion

In EM medium, *Pseudomonas* show a clear rise in PC-PLC activity all over the log phase. Diagram 28 shows clearly that highest PC-PLC activities are found around 9-10 hours of incubation at 37°C. The total bacterial count after 9 hours of incubation represents 4.00×10^8 CFU/ml. The highest PC-PLC activity after 10 hours of incubation represents 2, 13 U/ mL. After 12 hours of incubation, an abrupt fall in PC-PLC activity is measured. This is the point where the stationary phase begins.

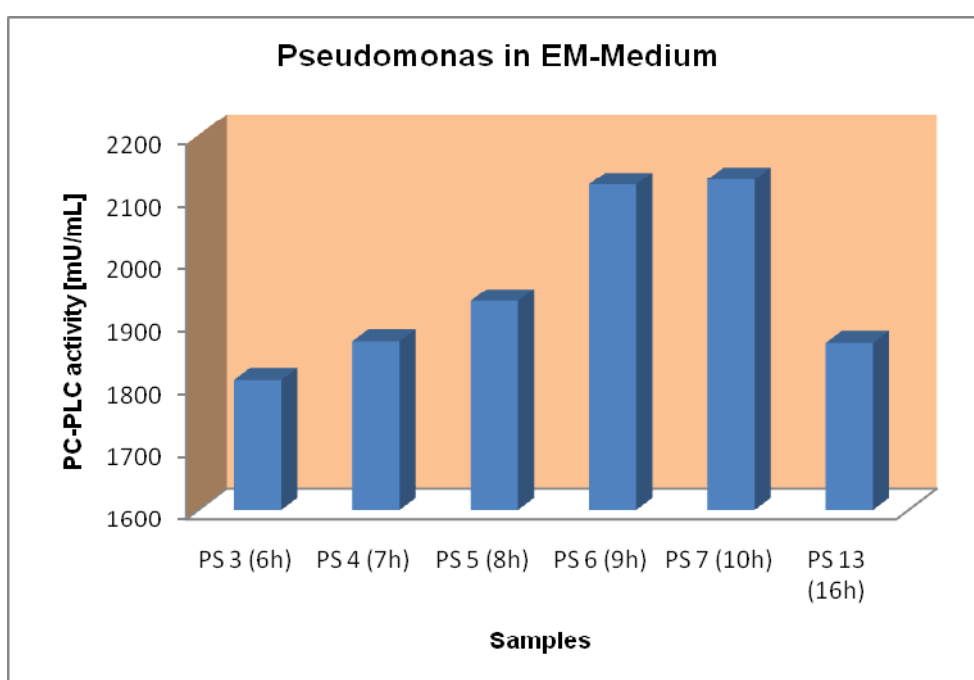


Diagram 28: PC-PLC Activity of *Pseudomonas* in log –Phase in EM-Medium

For *Enterobacter*, the same procedures were carried out.

In LM medium, *Enterobacter* interestingly shows high PC-PLC activity after 14 hours of incubation, though the total bacterial count was not so high (1.5×10^5 CFU/ml). After 15 hours of incubation, PC-PLC activity is lower than 1 hour before. However, the total bacterial count already reached at this incubation time 1×10^8 CFU/ml. This suggests that even with high total bacterial count, the PC-PLC activity does not have to rise compulsorily.

Results and Discussion

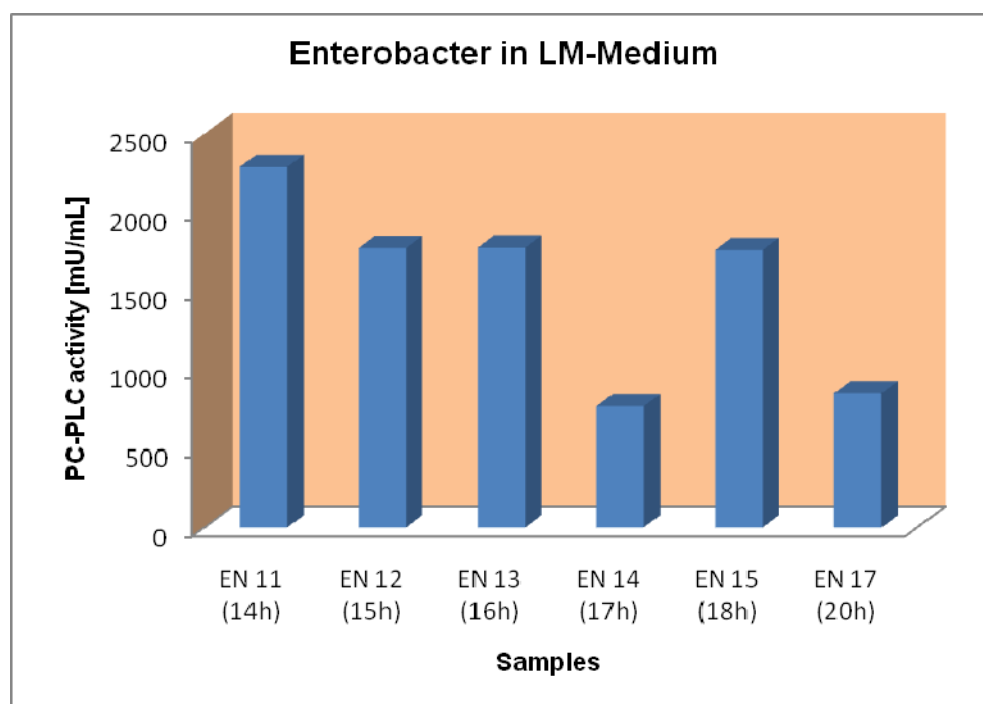


Diagram 29: PC-PLC Activity of *Enterobacter* in log –Phase in LM-Medium

In EM medium, *Enterobacter* seems to show a PC-PLC activity that paces up and down. While the total bacterial count ranges over from 5.0×10^7 CFU/ml to 2.20×10^9 CFU/mL, a steady rise in PC-PLC activity is not measured.

The highest PC-PLC activity with *Enterobacter* in EM medium was measured after 15 hours of incubation (2,15 U/mL). At this point, the total bacterial count represents 2.20×10^9 CFU/mL.

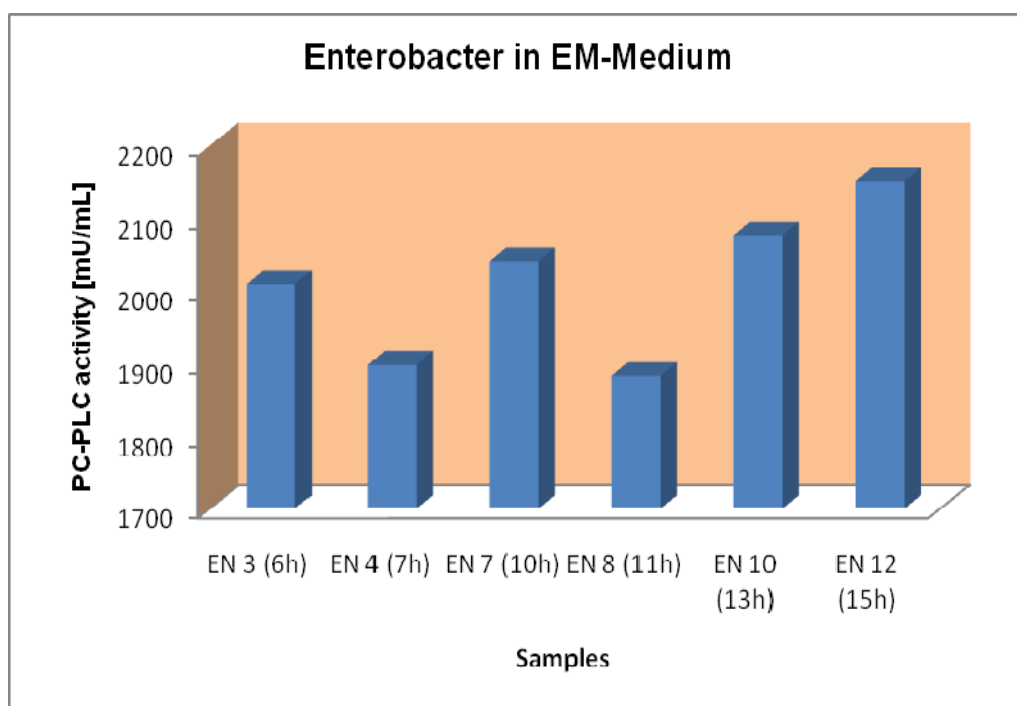


Diagram 30: PC-PLC Activity of *Enterobacter* in log –Phase in EM-Medium

1.6.5. PC-PLC Activity in Combination with Bacterial Culture Preparation

The main bacterial culture (100 ml) containing the bacteria from homogenized pork meat was used as a starter culture and incubated for 6 hours (Culture a) and 24 hours (Culture b) in a water bath at 15 °C. After these periods of bacterial growth, the cultures showed the total bacterial counts of $\sim 10^7$ CFU/mL for Culture a, and $\sim 10^8$ CFU/mL for Culture b. It is important to say that the starter bacterial culture was prepared in LM medium, with $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ as a phosphate source. Afterwards, the bacterial cultures were centrifuged for 3-5 minutes at 5000 rpm in a top table centrifuge (at 4°C) and PC-PLC activity in the supernatants was measured.

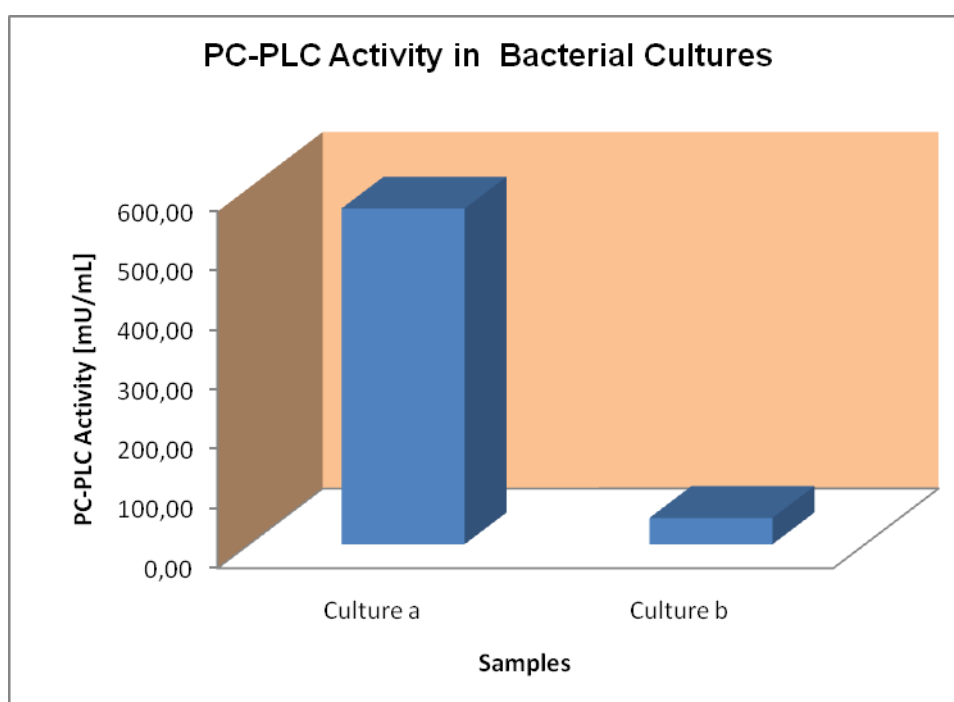


Diagram 31: PC-PLC Activity in Bacterial Culture Preparations

As we can see in Diagram 31, Culture a with total bacterial count from 10^7 CFU/mL shows high PC-PLC activity (565 mU/mL).

2. Biosensors

In the following section performance and properties of the novel devices are discussed in detail. Not all attempts to generate functional devices were successful, but there are many devices that should be analysed in more detail because they yielded functional and stable setup.

2.1. Inconel- Polymer-PLA

The examined sensors consist of an Inconel-coated PET-foil acting as a mirror, an interlayer with gravure printed biomimetic polymer (PLA polymer linked with Desmodur in 2,2,2-trifluoroethanol) and a gold-nanoparticle film.

2.1.1 Functionality Test

The following tables give an overview of the sensors setups with several combinations of the parameters such as: PLA concentration (w/v), Desmodur concentration (v/v) and the thickness of the gold island film, which can be optimized due to sputtering of a gold nanoparticles in a time - range between 10 sec and 45 sec.

All experiments have been carried out several times to assure reproducibility of the effect.

Sensors were incubated in humidity chamber for 4 hours by three different temperatures: 4°C, room temperature and 37°C as shown in the following tables.

Results for sensor sensitivity are presented with: (+): very strong signal and change of color; (+/-): signal is present but without observable color change; (-): there is no signal at all.

Results and Discussion



(+):very strong signal

(+/-):signal is present

(-): no signal

Figure 22: Examples for interpretation of results for sensor **sensitivity**

Sputter time: 10 sec (Au)					
PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C	PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
3%	5*10 ⁻¹ %	- / - / +/-	3,85 %	5*10 ⁻⁷ %	- / - / -
3%	5*10 ⁻² %	- / +/- / +/-	3,85 %	5*10 ⁻⁸ %	- / +/- / -
3,5 %	5*10 ⁻¹ %	- / - / -	4 %	5*10 ⁻⁷ %	- / +/- / -
3,5 %	5*10 ⁻³ %	+/- / + / +/-	4 %	5*10 ⁻⁸ %	+/- / +/- / -
3,5 %	5*10 ⁻⁶ %	+ / + / +	4,5 %	5*10 ⁻¹ %	- / +/- / +
3,75 %	5*10 ⁻¹ %	- / - / -	4,5 %	5*10 ⁻⁴ %	- / - / -
3,75 %	5*10 ⁻² %	- / +/- / -	4,5 %	5*10 ⁻⁵ %	- / - / -
3,75 %	5*10 ⁻⁴ %	- / +/- / -	4,5 %	5*10 ⁻⁶ %	+ / - / -
3,75 %	5*10 ⁻⁶ %	- / +/- / -	4,5 %	5*10 ⁻⁷ %	- / - / -
3,75 %	5*10 ⁻⁹ %	- / +/- / -	4,5 %	5*10 ⁻⁸ %	- / - / -

Table 8a . Sensor sensitivity test for PLA concentrations in a range from 3% to 4,5%. Trifluoroethanol was used as a solvent. Gold sputtering: 10 sec.

Results and Discussion

The first tests were carried out with Protease mixture: Proteinase K, Trypsin and α -Chymotrypsin in 0,1 M Tris-HCl, pH 8.2.

The different concentrations of PLA were printed on top of a metalized PET film. After this gravure printing process an Au island film was sputtered on top using argon plasma of 0.08 mbar for 10 sec.

A dilution series of the Protease-Mix in 0.1 M Tris - HCl pH 8.2 and buffer and ddH₂O as a negative control were applied to the sensor surface.

For the following sensors the dots are applied according to scheme in Table 8b:

Protease concentration		
1.	20 mg/ mL	5 mg/ mL
2.	15 mg/ mL	2,5 mg/mL
3.	12,5 mg/ mL	0, 1 M Tris-HCl buffer pH 8.2
4.	10 mg/ mL	ddH ₂ O

Table 8b: Pipetting scheme



3,5% PLA	4 % PLA	4% PLA	6% PLA
5*10⁻⁶% Desmodur	5*10⁻⁶% Desmodur	5*10⁻⁸% Desmodur	0,05% Desmodur
After 4h at 4°C	After 4h at 4°C	After 4h at 4°C	After 4h at rt

Figure 23. PLA degradation by Protease mix at 4°C and room temperature

Results and Discussion

Figure 23 shows clearly that – independently of incubation time and/or temperature – in most cases the protease mix led to a visible degradation of the biomimetic PLA layer.

Sputter time: 10 sec (Au)					
PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT	PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT
5%	$5 \cdot 10^{-1}$ %	+/- / -	5,5 %	$5 \cdot 10^{-5}$ %	- / -
5%	$5 \cdot 10^{-2}$ %	- / -	5,5 %	$5 \cdot 10^{-6}$ %	- / -
5 %	$5 \cdot 10^{-3}$ %	- / -	6 %	$5 \cdot 10^{-1}$ %	- / +/-
5 %	$5 \cdot 10^{-4}$ %	+/- / -	6 %	$5 \cdot 10^{-2}$ %	+/- / +/-
5 %	$5 \cdot 10^{-5}$ %	- / -	6 %	$5 \cdot 10^{-3}$ %	+/- / -
5 %	$5 \cdot 10^{-6}$ %	- / -	6 %	$5 \cdot 10^{-4}$ %	- / -
5 %	$5 \cdot 10^{-7}$ %	- / -	6 %	$5 \cdot 10^{-5}$ %	- / -
5 %	$5 \cdot 10^{-8}$ %	- / -	6 %	$5 \cdot 10^{-6}$ %	- / -
5,5 %	$5 \cdot 10^{-1}$ %	- / +/-	6 %	$5 \cdot 10^{-7}$ %	- / -
5,5 %	$5 \cdot 10^{-2}$ %	- / -	8 %	$5 \cdot 10^{-4}$ %	- / -
5,5 %	$5 \cdot 10^{-3}$ %	- / -	8 %	$5 \cdot 10^{-5}$ %	- / -
5,5 %	$5 \cdot 10^{-4}$ %	- / -	8 %	$5 \cdot 10^{-6}$ %	- / -

Table 9a . Sensor sensitivity test for PLA concentrations in a range from 5% to 8%.
Trifluoroethanol was used as a solvent. Gold sputtering: 10 sec.

Results and Discussion

Column 1	Column 2	Column 3
1. Bacterial Cocktail A 10^8 CFU/mL	5. HII2 or HII1	9. 4 Bacteria – LM
2. Bacterial Cocktail A 10^6 CFU/mL	6. Culture a (10^7 CFU/ml)	10. 4 Bacteria –LM 10^6 CFU/ml
3. Homogenate I 10^8 CFU/mL	7. Culture b (10^8 CFU/ml)	11. Phospholipase 10 mg/mL
4. Homogenate I 10^6 CFU/mL	8. Culture b 10^6 CFU/mL	12. Ringer solution

Table 9b: Pipetting scheme



Figure 24. PLA (3,5 %) with $5 \cdot 10^{-3}\%$ Desmodur, Au 10 sec, after 6 hours at 37°C

Experiments with bacterial and meat solutions yield color changes of the biosensor. In the selected biosensor presented in Fig. 24, bacterial cocktail A, homogenate I, HII2 and bacterial mix in LM medium were applied and resulted in degradation of the PLA layer.

Results and Discussion

Sputter time: 15 sec (Au)					
PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C	PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
3 %	$5 \cdot 10^{-1}$ %	- / + / +	3,75 %	$5 \cdot 10^{-9}$ %	- / - / -
3 %	$5 \cdot 10^{-2}$ %	- / +/- / +/-	3,75 %	$5 \cdot 10^{-10}$ %	- / - / -
3 %	$5 \cdot 10^{-3}$ %	- / +/- / +/-	3,85 %	$5 \cdot 10^{-6}$ %	- / - / -
3 %	$5 \cdot 10^{-4}$ %	- / +/- / +/-	3,85 %	$5 \cdot 10^{-7}$ %	+/- / +/- / -
3,5 %	$5 \cdot 10^{-1}$ %	+ / + / +	3,85 %	$5 \cdot 10^{-8}$ %	+/- / +/- / -
3,5 %	$5 \cdot 10^{-2}$ %	+/- / +/- / +/-	3,85 %	$5 \cdot 10^{-9}$ %	- / - / -
3,5 %	$5 \cdot 10^{-3}$ %	+/- / + / +	3,85 %	$5 \cdot 10^{-10}$ %	- / - / -
3,5 %	$5 \cdot 10^{-4}$ %	+/- / +/- / +/-	4 %	$5 \cdot 10^{-6}$ %	+/- / +/- / -
3,5 %	$5 \cdot 10^{-6}$ %	+/- / + / +	4 %	$5 \cdot 10^{-7}$ %	+/- / + / +/-
3,75 %	$5 \cdot 10^{-1}$ %	- / + / -	4 %	$5 \cdot 10^{-8}$ %	- / - / -
3,75 %	$5 \cdot 10^{-2}$ %	+/- / +/- / -	4 %	$5 \cdot 10^{-9}$ %	- / - / -
3,75 %	$5 \cdot 10^{-3}$ %	+/- / - / -	4,5 %	$5 \cdot 10^{-1}$ %	- / +/- / +
3,75 %	$5 \cdot 10^{-4}$ %	+/- / - / -	4,5 %	$5 \cdot 10^{-4}$ %	- / - / -
3,75 %	$5 \cdot 10^{-5}$ %	- / - / -	4,5 %	$5 \cdot 10^{-5}$ %	- / - / +
3,75 %	$5 \cdot 10^{-6}$ %	+ / - / -	4,5 %	$5 \cdot 10^{-6}$ %	- / +/- / -
3,75 %	$5 \cdot 10^{-7}$ %	- / - / -	4,5 %	$5 \cdot 10^{-7}$ %	- / +/- / -
3,75 %	$5 \cdot 10^{-8}$ %	- / - / -	4,5 %	$5 \cdot 10^{-8}$ %	- / - / -

Table 10a. Sensor sensitivity test for PLA concentrations in range from 3% to 4,5%. Trifluoroethanol was used as a solvent. Gold sputtering: 15 sec.

Results and Discussion

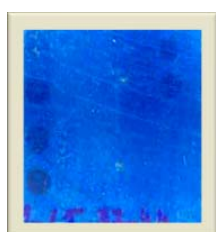
The functionality tests were carried out with Protease mixture: Proteinase K, Trypsin and α -Chymotrypsin in 0,1 M Tris-HCl pH 8.2.

Different PLA concentrations were printed on top of metallic PET film. Then a gold island layer was applied by sputtering at 0,08 mbar for 15 sec. A dilution series of the Protease-Mix in 0.1 M Tris - HCl - pH 8.2, buffer and ddH₂O as a negative control were applied to sensor surface.

Protease concentration		
1.	20 mg/ mL	5 mg/ mL
2.	15 mg/ mL	2,5 mg/mL
3.	12,5 mg/ mL	0, 1 M Tris-HCl buffer pH 8.2
4.	10 mg/ mL	ddH ₂ O

Table 10b: Pipetting scheme

For the following sensors, the dots were applied according to the scheme in Table 10b. Again, the Protease is responsible for a color change.



4,5%PLA
0,5%Desmodur
After 4h at 37°C



3,5% PLA
0,5%Desmodur
after 16h at rt

Figure 25: PLA degradation by Protease-mix, Au 15

Results and Discussion

Sputter time: 15 sec (Au)		
PLA concentration (w/v)	Desmodur (v/v)	Sensor sensitivity 4°C / RT
5 %	$5 \cdot 10^{-1}$ %	- / +
5 %	$5 \cdot 10^{-2}$ %	- / -
5 %	$5 \cdot 10^{-3}$ %	- / -
5 %	$5 \cdot 10^{-4}$ %	- / -
5 %	$5 \cdot 10^{-5}$ %	- / -
5 %	$5 \cdot 10^{-6}$ %	- / -
5 %	$5 \cdot 10^{-7}$ %	- / -
5 %	$5 \cdot 10^{-8}$ %	- / -
5 %	$5 \cdot 10^{-9}$ %	- / -
5,5 %	$5 \cdot 10^{-1}$ %	- / +/-
5,5 %	$5 \cdot 10^{-2}$ %	- / -
5,5 %	$5 \cdot 10^{-3}$ %	- / -
5,5 %	$5 \cdot 10^{-4}$ %	- / -
5,5 %	$5 \cdot 10^{-5}$ %	- / -
5,5 %	$5 \cdot 10^{-6}$ %	- / +/-
6 %	$5 \cdot 10^{-1}$ %	- / +
6 %	$5 \cdot 10^{-2}$ %	- / +
6 %	$5 \cdot 10^{-3}$ %	+/- / -
6 %	$5 \cdot 10^{-4}$ %	- / -
6 %	$5 \cdot 10^{-5}$ %	- / -
6 %	$5 \cdot 10^{-6}$ %	- / -
6 %	$5 \cdot 10^{-7}$ %	- / -
8 %	$5 \cdot 10^{-4}$ %	- / -
8 %	$5 \cdot 10^{-5}$ %	- / -
8 %	$5 \cdot 10^{-6}$ %	- / -

Table 11a . Sensor sensitivity test for PLA concentrations in range from 5% to 8%. Trifluoroethanol was used as a solvent. Gold sputtering: 15 sec.

Results and Discussion

Sputter time: 20 sec (Au)					
PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT / 37°C	PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C/RT/ 37°C
3%	$5 \cdot 10^{-1}$ %	- / +/- / +/-	3,75 %	$5 \cdot 10^{-9}$ %	+/- / + / +
3%	$5 \cdot 10^{-2}$ %	- / +/- / +/-	3,75 %	$5 \cdot 10^{-10}$ %	- / - / -
3 %	$5 \cdot 10^{-3}$ %	- / +/- / +/-	3,85 %	$5 \cdot 10^{-6}$ %	- / - / -
3 %	$5 \cdot 10^{-4}$ %	- / +/- / +/-	3,85 %	$5 \cdot 10^{-7}$ %	+/- / + / +/-
3,5 %	$5 \cdot 10^{-1}$ %	+/- / + / +	3,85 %	$5 \cdot 10^{-8}$ %	+/- / + / +/-
3,5 %	$5 \cdot 10^{-2}$ %	+/- / +/- / +/-	3,85 %	$5 \cdot 10^{-9}$ %	- / - / -
3,5 %	$5 \cdot 10^{-3}$ %	+/- / + / +	3,85 %	$5 \cdot 10^{-10}$ %	- / - / -
3,5 %	$5 \cdot 10^{-4}$ %	+/- / +/- / +/-	4 %	$5 \cdot 10^{-6}$ %	- / - / -
3,5 %	$5 \cdot 10^{-6}$ %	+ / + / +	4 %	$5 \cdot 10^{-7}$ %	+/- / + / +/-
3,75 %	$5 \cdot 10^{-1}$ %	- / - / -	4 %	$5 \cdot 10^{-8}$ %	- / +/- / -
3,75 %	$5 \cdot 10^{-2}$ %	- / - / -	4 %	$5 \cdot 10^{-9}$ %	- / - / -
3,75 %	$5 \cdot 10^{-3}$ %	- / - / -	4,5 %	$5 \cdot 10^{-1}$ %	- / +/- / +
3,75 %	$5 \cdot 10^{-4}$ %	+/- / + / +	4,5 %	$5 \cdot 10^{-4}$ %	- / - / +/-
3,75 %	$5 \cdot 10^{-5}$ %	- / - / -	4,5 %	$5 \cdot 10^{-5}$ %	- / - / -
3,75 %	$5 \cdot 10^{-6}$ %	- / - / -	4,5 %	$5 \cdot 10^{-6}$ %	+/- / - / -
3,75 %	$5 \cdot 10^{-7}$ %	- / - / -	4,5 %	$5 \cdot 10^{-7}$ %	- / - / -
3,75 %	$5 \cdot 10^{-8}$ %	- / - / -	4,5 %	$5 \cdot 10^{-8}$ %	- / - / -

Table 12a. Sensor sensitivity test for PLA concentrations in range from 3% to 3,75%. Trifluoroethanol was used as a solvent. Gold sputtering: 20 sec.

Results and Discussion

Functionality tests were carried out with a protease mix and meat juice. The meat was bought by a local retailer like Billa. The meat juice was found directly in the packaging.

Dilution series with 0.1 M Tris- HCl buffer pH 8.2 from the meat juice were prepared. For a functional assay, “stock solutions” of fresh (10^5 CFU/mL) and spoiled meat juice (after 6 hours incubation by 37°C and a total bacterial count of 10^7 CFU/mL) were prepared. Dilution series with 0.1 M Tris- HCl buffer pH 8.2 from the meat juice were prepared as described in the scheme:

	fresh meat juice	spoiled meat juice
1.	Stock 10^5 CFU/mL	Stock 10^7 CFU/mL
2.	1:2 dilution with buffer	1:2 dilution with buffer
3.	1:4 dilution with buffer	1:4 dilution with buffer
4.	0.1 M Tris- HCl buffer	0,1 M Tris- HCl buffer
5.	ddH ₂ O	ddH ₂ O

Table 12b: Pipetting scheme



After 16h at 4°C

Figure 25: 6% PLA with 0, 5% Desmodur, Au 20 sec,

Results and Discussion

A dilution series of the Protease-Mix in 0.1 M Tris - HCl - pH 8.2 and buffer and ddH₂O as a negative control were also applied onto the sensor surface.

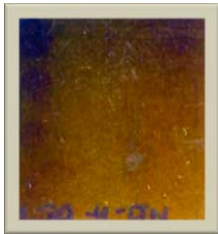
Protease concentration				
1.	20 mg/ mL	5 mg/ mL	 After 16h at 4°C	 after 16h at
2.	15 mg/ mL	2,5 mg/mL		
3.	12,5 mg/ mL	0, 1 M Tris-HCl buffer		
4.	10 mg/ mL	ddH ₂ O		
			Figure 26: 3,75% PLA with 0,5% Desmodur	Figure 27: 4,5% PLA with 5*10 ⁻⁶ % Desmodur

Table 12c: Pipetting scheme

The color changes shown in Fig.25-27 are a result of enzymatic degradation of the biomimetic polymer layer by lytic enzymes. As can be seen with the naked eye, undiluted spoiled meat juice and dilutions showed strong signals at 4°C. Negative controls showed no signals.

Results and Discussion

Sputter time: 20 sec (Au)		
PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT
5 %	$5 \cdot 10^{-1} \%$	- / +
5 %	$5 \cdot 10^{-2} \%$	- / -
5 %	$5 \cdot 10^{-3} \%$	- / -
5 %	$5 \cdot 10^{-4} \%$	- / -
5 %	$5 \cdot 10^{-5} \%$	- / -
5 %	$5 \cdot 10^{-6} \%$	- / -
5 %	$5 \cdot 10^{-7} \%$	- / -
5 %	$5 \cdot 10^{-8} \%$	- / -
5 %	$5 \cdot 10^{-9} \%$	- / -
5,5 %	$5 \cdot 10^{-1} \%$	- / +
5,5 %	$5 \cdot 10^{-2} \%$	- / -
5,5 %	$5 \cdot 10^{-3} \%$	- / -
5,5 %	$5 \cdot 10^{-4} \%$	- / -
5,5 %	$5 \cdot 10^{-5} \%$	- / -
5,5 %	$5 \cdot 10^{-6} \%$	- / -

Table 13 a. Sensor sensitivity test for PLA concentrations in the range from 5% to 5,5%. Triflouroethanol was used as a solvent. Gold sputtering: 20 sec.

Results and Discussion

Sputter time: 20 sec		
PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT
6 %	$5 \cdot 10^{-1}$ %	- / +
6 %	$5 \cdot 10^{-2}$ %	- / +/-
6 %	$5 \cdot 10^{-3}$ %	- / +/-
6 %	$5 \cdot 10^{-4}$ %	- / -
6 %	$5 \cdot 10^{-5}$ %	- / -
6 %	$5 \cdot 10^{-6}$ %	- / -
6 %	$5 \cdot 10^{-7}$ %	- / -
8 %	$5 \cdot 10^{-2}$ %	- / -
8 %	$5 \cdot 10^{-3}$ %	+/- / -
8 %	$5 \cdot 10^{-4}$ %	- / -
8 %	$5 \cdot 10^{-5}$ %	+/- / +/-
8 %	$5 \cdot 10^{-6}$ %	- / -

Table 13b. Sensor sensitivity test for PLA concentrations in the range from 6% to 8%. Trifluoroethanol was used as a solvent. Gold sputtering: 20 sec.

Results and Discussion

Sputter time: 30 sec (Au)		
PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
3%	$5 \cdot 10^{-1} \%$	- / +/- / +/-
3%	$5 \cdot 10^{-2} \%$	- / - / +/-
3 %	$5 \cdot 10^{-3} \%$	- / +/- / +/-
3 %	$5 \cdot 10^{-4} \%$	- / +/- / +/-
3,5 %	$5 \cdot 10^{-1} \%$	- / +/- / +/-
3,5 %	$5 \cdot 10^{-2} \%$	+/- / +/- / +/-
3,5 %	$5 \cdot 10^{-3} \%$	- / +/- / +/-
3,5 %	$5 \cdot 10^{-4} \%$	- / +/- / +/-
3,75 %	$5 \cdot 10^{-1} \%$	- / + / +
3,75 %	$5 \cdot 10^{-2} \%$	- / - / -
3,75 %	$5 \cdot 10^{-3} \%$	- / - / -
3,75 %	$5 \cdot 10^{-4} \%$	- / - / -
3,75 %	$5 \cdot 10^{-5} \%$	- / - / -
3,75 %	$5 \cdot 10^{-6} \%$	- / +/- / +/-
3,85 %	$5 \cdot 10^{-6} \%$	- / +/- / +/-
3,85 %	$5 \cdot 10^{-7} \%$	+/- / - / -
3,85 %	$5 \cdot 10^{-8} \%$	- / - / -
3,85 %	$5 \cdot 10^{-9} \%$	- / - / -
3,85 %	$5 \cdot 10^{-10} \%$	- / - / -
4 %	$5 \cdot 10^{-6} \%$	- / +/- / +/-
4 %	$5 \cdot 10^{-7} \%$	- / - / -
4,5 %	$5 \cdot 10^{-6} \%$	- / - / -
4,5 %	$5 \cdot 10^{-7} \%$	- / - / -

Table 14a. Sensor sensitivity test for PLA concentrations in the range from 3% to 4,5%. Triflouroethanol was used as a solvent. Gold sputtering: 30 sec.

Results and Discussion

Sputter time: 30 sec (Au)		
PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT
5 %	$5 \cdot 10^{-1}$ %	- / +
5 %	$5 \cdot 10^{-2}$ %	- / -
5 %	$5 \cdot 10^{-3}$ %	- / -
5 %	$5 \cdot 10^{-4}$ %	- / -
5 %	$5 \cdot 10^{-5}$ %	- / +/-
5 %	$5 \cdot 10^{-6}$ %	- / -
5 %	$5 \cdot 10^{-7}$ %	- / -
5 %	$5 \cdot 10^{-8}$ %	- / -
5,5 %	$5 \cdot 10^{-1}$ %	- / +
5,5 %	$5 \cdot 10^{-2}$ %	- / +/-
5,5 %	$5 \cdot 10^{-3}$ %	- / +/-
5,5 %	$5 \cdot 10^{-4}$ %	- / -
5,5 %	$5 \cdot 10^{-5}$ %	- / -
5,5 %	$5 \cdot 10^{-6}$ %	- / -
6 %	$5 \cdot 10^{-1}$ %	- / +/-
6 %	$5 \cdot 10^{-2}$ %	- / +
6 %	$5 \cdot 10^{-3}$ %	- / -
6 %	$5 \cdot 10^{-4}$ %	- / -
6 %	$5 \cdot 10^{-5}$ %	- / -
6 %	$5 \cdot 10^{-6}$ %	- / -
8 %	$5 \cdot 10^{-2}$ %	- / +/-
8 %	$5 \cdot 10^{-3}$ %	+/- / -
8 %	$5 \cdot 10^{-4}$ %	- / -
8 %	$5 \cdot 10^{-5}$ %	- / +/-

Table 14b . Sensor sensitivity test for PLA concentrations in the range from 5% to 8%. Triflouroethanol was used as a solvent. Gold sputtering: 30 sec

Results and Discussion

Functionality tests were carried out with meat juice. The meat was bought by a local retailer like Billa. The meat juice was obtained directly from the package. Dilution series with 0.1 M Tris- HCl buffer pH 8.2 from the meat juice were prepared. For a functional assay, “stock solutions” of fresh (10^5 CFU/mL) and spoiled meat juice (after 6 hours incubation by 37°C and total bacterial count of 10^7 CFU/mL) were prepared.

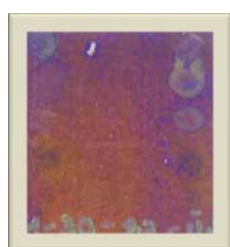
For the following sensors the dots are applied according to the scheme in Table 14c :

	fresh meat juice	spoiled meat juice
1.	Stock 10^5 CFU/mL	Stock 10^7 CFU/mL
2.	1:2 dilution with buffer	1:2 dilution with buffer
3.	1:4 dilution with buffer	1:4 dilution with buffer
4.	0.1 M Tris- HCl buffer	0,1 M Tris- HCl buffer
5.	ddH ₂ O	ddH ₂ O

Table 14 c: Pipetting scheme



Figure 28: 5%PLA with $5 \cdot 10^{-4}\%$ Desmodur, Au 30 sec, after 4h at 37°C



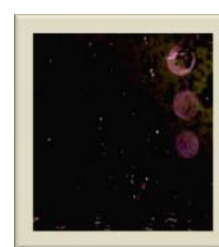
$10^{-2}\%$ Desmodur

After 16h at 37°C



$5 \cdot 10^{-3}\%$ Desmodur

after 16h at 4°C



$5 \cdot 10^{-4}\%$ Desmodur

after 16h at 4°C

Figure 29: Degradation of 6%PLA , Au 30 sec

Results and Discussion

As can be seen in Fig. 29, excreted lytic enzymes are responsible for degradation of the biomimetic polymer layer resulting in a color change visible with naked eye .

Au sputter time: 45 sec		
PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
3%	$5 \cdot 10^{-1} \%$	- / - / +/-
3%	$5 \cdot 10^{-2} \%$	- / - / +/-
3 %	$5 \cdot 10^{-3} \%$	- / - / +/-
3 %	$5 \cdot 10^{-4} \%$	- / - / +/-
3 %	$5 \cdot 10^{-5} \%$	- / - / +/-
3,75 %	$5 \cdot 10^{-1} \%$	- / - / -
3,75 %	$5 \cdot 10^{-2} \%$	- / - / -
3,75 %	$5 \cdot 10^{-6} \%$	- / - / -
3,75 %	$5 \cdot 10^{-9} \%$	- / - / -
3,85 %	$5 \cdot 10^{-6} \%$	- / - / -
3,85 %	$5 \cdot 10^{-7} \%$	- / - / -
4 %	$5 \cdot 10^{-1} \%$	- / +/- / +
4 %	$5 \cdot 10^{-2} \%$	- / - / -
4 %	$5 \cdot 10^{-3} \%$	- / - / -
4 %	$5 \cdot 10^{-4} \%$	- / - / -
4 %	$5 \cdot 10^{-5} \%$	- / + / -
4 %	$5 \cdot 10^{-7} \%$	- / - / -
4 %	$5 \cdot 10^{-8} \%$	- / - / -
4,5 %	$5 \cdot 10^{-6} \%$	- / - / -
4,5 %	$5 \cdot 10^{-7} \%$	- / - / -

Table 15a. Sensor sensitivity test for PLA concentrations in the range from 3% to 4,5%. Trifluoroethanol was used as a solvent. Gold sputtering: 45 sec.

Results and Discussion

Sputter time: 45 sec (Au)		
PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT
5 %	$5 \cdot 10^{-1}$ %	- / +
5 %	$5 \cdot 10^{-2}$ %	- / -
5 %	$5 \cdot 10^{-3}$ %	- / -
5 %	$5 \cdot 10^{-4}$ %	- / -
5 %	$5 \cdot 10^{-5}$ %	- / -
5 %	$5 \cdot 10^{-6}$ %	+/- / -
5 %	$5 \cdot 10^{-7}$ %	- / -
5 %	$5 \cdot 10^{-8}$ %	- / -
5,5 %	$5 \cdot 10^{-1}$ %	- / +
5,5 %	$5 \cdot 10^{-2}$ %	- / -
5,5 %	$5 \cdot 10^{-3}$ %	+/- / -
5,5 %	$5 \cdot 10^{-4}$ %	+/- / +/-
5,5 %	$5 \cdot 10^{-5}$ %	- / -
5,5 %	$5 \cdot 10^{-6}$ %	+/- / +/-
6 %	$5 \cdot 10^{-1}$ %	- / +
6 %	$5 \cdot 10^{-2}$ %	- / +/-
6 %	$5 \cdot 10^{-3}$ %	- / -
6 %	$5 \cdot 10^{-4}$ %	- / -
6 %	$5 \cdot 10^{-5}$ %	- / -
6 %	$5 \cdot 10^{-6}$ %	- / -
8 %	$5 \cdot 10^{-2}$ %	- / +/-
8 %	$5 \cdot 10^{-3}$ %	- / +/-
8 %	$5 \cdot 10^{-4}$ %	- / -
8 %	$5 \cdot 10^{-5}$ %	- / -

Table 15b . Sensor sensitivity test for PLA concentrations in the range from 5% to 8%.

Trifluoroethanol was used as a solvent. Gold sputtering: 45 sec.

Results and Discussion

For the functionality tests meat was bought by a local retailer like Billa. The meat juice was found directly in the packaging. Dilution series with 0.1 M Tris-HCl buffer pH 8.2 from the meat juice were prepared. For a functional assay, “stock solutions” of fresh (10^5 CFU/mL) and spoiled meat juice (after 6 hours incubation by 37°C and total bacterial count of 10^7 CFU/mL) were prepared.

The dots on the sensor surface were applied according to the scheme in Table 15c:

	fresh meat juice	spoiled meat juice
1.	Stock 10^5 CFU/mL	Stock 10^7 CFU/mL
2.	1:2 dilution with buffer	1:2 dilution with buffer
3.	1:4 dilution with buffer	1:4 dilution with buffer
4.	0.1 M Tris- HCl buffer	0,1 M Tris- HCl buffer
5.	ddH ₂ O	ddH ₂ O

Table 15c: Pipetting scheme



5% PLA

**5*10⁻⁵% Desmodur
Desmodur**

after 16h at rt



6% PLA

0,5 % Desmodur

after 16h at 37°C



6% PLA

5*10⁻²%

after 16h at 4°C

Figure 29: Degradation of PLA sensors with a Au island film sputtered for 45 sec

In Figure 29, we can clearly see that – independently of incubation time and/or temperature – in most cases the samples of spoiled meat resulted in a visible degradation of the biomimetic PLA layer.

2.1.2 Conclusion

According to an overview of all experiments with PLA, which was used as a biomimetic interlayer for the sensor setup described above, and looking at all interpreted results for sensor sensitivity and functionality, it was possible to define and select “best of” combinations for polymer-, linker –concentration and sputter time.

The strongest signal and the highest sensitivity of the sensors could be found in sensor setups with 3,5% w/v , 4,5% w/v and 5% w/v PLA and 0,5% v/v Desmodur (diphenylmethanediisocyanate) as a crosslinker. Optimal sputter times for gold nanoparticles were 15 sec and 20 sec.

2.2 Fabry-Perot Setup

2.2.1 Functionality test with PLA

Fabry-Perot sensor setup includes two reflecting metal layers and an intermediate polymer interlayer. The first reflecting layer consists of 35 nm Au on 36 μm PET foil that was kindly provided by Hueck Folien. The biomimetic polymer PLA was dissolved in 2,2,2-trifluoroethanol to a final concentration of 10 % w/v. The linking agent Desmodur was applied in a concentration range from 10^{-3} – 10^{-10} % to figure out the selectivity and the limit of detection.

After the gravure printing process the sensors were incubated at 80 °C for 10 min and a second reflecting Au metal layer was sputter coated on top with argon plasma of 0.08 mbar for 120 sec.

The first experiments with meat juice were carried out with meat bought in a typically packaging with protecting gas for improvement of shelf life. The meat was bought by a local retailer like Billa. In most cases, the meat juice could be found directly in the packaging. Dilution series with 0.1 M Tris- HCl buffer pH 8.2 from the meat juice were prepared. For a functional assay, “stock solutions” of fresh (10^5 CFU/mL) and spoiled meat juice (after 5 hours incubation by 37°C and total bacterial count of 10^7 CFU/mL) were prepared. Dilution series with 0.1 M Tris- HCl buffer pH 8.2 from the meat juice were prepared as described in the scheme in Table 16:

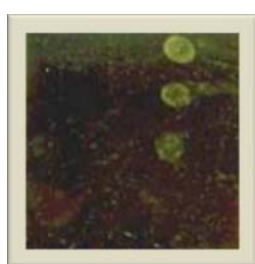
	fresh meat juice	spoiled meat juice
1.	Stock 10^5 CFU/mL	Stock 10^7 CFU/mL
2.	1:2 dilution with buffer	1:2 dilution with buffer
3.	1:4 dilution with buffer	1:4 dilution with buffer
4.	0.1 M Tris- HCl buffer	0,1 M Tris- HCl buffer
5.	ddH ₂ O	ddH ₂ O

Table 16: Pipetting scheme

Results and Discussion

On top of the sensor surface 1 μL of every solution was pipetted and then the sensors incubated in a humidity chamber for 4 hours and over night at 4°C and also at room temperature. After the incubation time the sensor surface was washed with ddH₂O and dried under an airstream.

The colour changes shown in Fig. 30 and Fig. 31 are the result of enzymatic degradation of the biomimetic polymer layer by lytic enzymes.. The strongest signal and the highest sensitivity of the sensors could be seen in sensor setup with 10 % w/v PLA and 10⁻⁴% v/v Desmodur (diphenylmethanediisocyanate). As can be seen with the naked eye, undiluted spoiled meat juice at 4°C shows the strongest signal after 4 hours. Negative controls showed no signals.



at 4°C

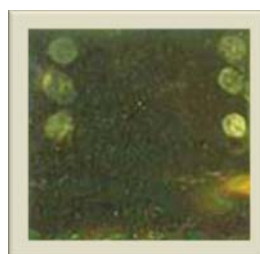


at RT

Figure 30: Incubation for 4 hours



at 4°C



at RT

Figure 31: Incubation overnight

2.2.2 Functionality test with PLGA

A Fabry-Perot sensor setup with two reflecting metal layers and PLGA as a polymer interlayer was prepared. The first reflecting layer consists of Inconel coated PET foil. The biomimetic polymer PLGA was dissolved in 2,2,2-trifluoroethanol to a final concentration between 20% and 35% (w/v). The linking agent Desmodur was applied in a concentration range between 10^{-8} -1 % to figure out the selectivity and the limit of detection. After the gravure printing process the sensors were incubated at 80 °C for 10 min and a second reflecting Au metal layer was sputter coated on top with argon plasma of 0.08 mbar for 45 sec.

For the functionality test, the dots were applied according to the scheme in Table 17.

Fresh meat juice		Spoiled meat juice
1.	Stock 10^5 CFU/mL	Stock 10^7 CFU/mL
2.	1:2 dilution with buffer	1:2 dilution with buffer
3.	1:4 dilution with buffer	1:4 dilution with buffer
4.	0.1 M Tris- HCl buffer pH 8.2	0.1 M Tris- HCl buffer pH 8.2
5.	ddH ₂ O	ddH ₂ O

Table 17: Pipetting scheme



Untreated sensor

after 4h at 4°C

after 4h at room

after 4h at 37°C

temperature

Results and Discussion

Parallel to functionality tests a series of stability tests were performed to define a possible application of PLGA for Fabry-Perot setup.

However, this setup did not show the necessary stability and the whole setup dissolved after incubation with bacterial solutions and after washing with ddH₂O. The main reasons for setup instability are the conditions of the sputtering process (vacuum, heat etc.) which were too aggressive for PLGA.

2.3 Two layer sensor

The two- layer sensor setup consists of a metal layer that is made up of Ni and Cr which is then covered with a polymer layer. This polymer is dissolved in an organic solvent containing varying amounts of Desmodur. Desmodur serves as a linker of the polymer molecules, influences its composition, stability and viscosity. When lytic enzymes come into contact with the polymer layer as e.g. secreted by bacteria, the polymer will be degraded and as a consequence this results in a colour change of the biomimetic sensor.

Regarding the functional efficiency of the described sensor setup the polymer layer is one of the most crucial points. In the sensor setup the biomimetic layer is decomposed by enzymes that derive from meat spoilage and can originate either as lytic enzymes produced by bacteria that are always present on the surface of biological materials, or arise from meat cell lysis itself.

There is a wide range of valuable polymers that show characteristic trait, necessary for our application, but only two of them were tested in this work: PLA (poly lactic acid) and PLGA (poly-lactic-co glycolic acid). PLA and PLGA have similar chemical properties, both of them are biodegradable and approved by FDA. They contain a variety of chemical bonds that can be cleaved easily by lytic enzymes.

2.3.1 PLA Setup

As already described in literature a PLA polymer can be degraded by lytic enzymes as e.g. proteases and lipases.[42]

In order to simulate this reaction, unsputtered PLA sensors, directly after the printing process, were incubated with a mixture of 3 commercially available proteolytic enzymes in a humidity chamber for 4 hours and overnight at 4°C and as well at room temperature. This Protease-mix contains Proteinase K,

Results and Discussion

Chymotrypsin and Trypsin in equal amounts. Enzyme dilutions were prepared in 0,1 M Tris HCl Puffer pH 8.2.

The classification of the results has been made in the same way as described above. It has to be mentioned that for interpretation of results we defined: (+): very strong signal and change of color; (+/-): signal is present but without observable color change; (-) there is no signal at all.

PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity after 4 h incubation 4°C / RT	Sensor sensitivity after incubation overnight 4°C / RT/ 37°C
3 %	$5 \cdot 10^{-1}$ %	- / -	- / +/-
3 %	$5 \cdot 10^{-2}$ %	- / +/-	- / +/-
3 %	$5 \cdot 10^{-3}$ %	- / +/-	- / +/-
3 %	$5 \cdot 10^{-4}$ %	- / +/-	- / +/-
3,5 %	$5 \cdot 10^{-1}$ %	- / +/-	- / +/-
3,5 %	$5 \cdot 10^{-2}$ %	- / -	- / -
3,5 %	$5 \cdot 10^{-3}$ %	- / -	- / -
3,5 %	$5 \cdot 10^{-4}$ %	- / -	- / -
3,5 %	$5 \cdot 10^{-6}$ %	- / -	- / -
3,75 %	$5 \cdot 10^{-1}$ %	- / -	- / -
3,75 %	$5 \cdot 10^{-2}$ %	- / -	- / -
3,75 %	$5 \cdot 10^{-4}$ %	- / -	- / -
3,75 %	$5 \cdot 10^{-6}$ %	- / -	- / -
3,85 %	$5 \cdot 10^{-6}$ %	- / +/-	- / +/-
3,85 %	$5 \cdot 10^{-7}$ %	- / +/-	+/- / +/-
3,85 %	$5 \cdot 10^{-8}$ %	- / -	- / -

Table 18a . Sensor sensitivity test for PLA concentrations in the range from 3% to 3,85%

Results and Discussion

PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity after 4 h incubation 4°C / RT	Sensor sensitivity after incubation overnight 4°C / RT
4 %	$5 \cdot 10^{-6}$ %	- / -	- / -
4 %	$5 \cdot 10^{-7}$ %	- / -	- / -
4,5 %	$5 \cdot 10^{-6}$ %	- / -	- / -
4,5 %	$5 \cdot 10^{-7}$ %	- / -	- / -
5 %	$5 \cdot 10^{-1}$ %	- / -	- / +/-
5 %	$5 \cdot 10^{-2}$ %	+/- / +	+/- / +
5 %	$5 \cdot 10^{-3}$ %	+/- / +	+ / +/-
5 %	$5 \cdot 10^{-4}$ %	- / +/-	- / -
5 %	$5 \cdot 10^{-5}$ %	- / +/-	+ / +/-
5 %	$5 \cdot 10^{-6}$ %	- / +/-	+ / +/-
5 %	$5 \cdot 10^{-7}$ %	- / +/-	+ / +/-
5 %	$5 \cdot 10^{-8}$ %	+/- / +/-	+ / +/-

Table 18b . Sensor sensitivity test for PLA concentrations in range from 4% to 5%



Figure 32: Inconel- 5% PLA with $5 \cdot 10^{-2}$ % Desmodur after incubation with Protease- mix for 4 hours at 4°C and at room temperature

Results and Discussion



Figure 33: Inconel- 5% PLA with $5 \cdot 10^{-3}$ and $5 \cdot 10^{-5}$ % Desmodur after overnight incubation with Protease-mix at 4°C and at room temperature

PLA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity after 4 h incubation 4°C / RT	Sensor sensitivity after incubation overnight 4°C / RT
5,5 %	$5 \cdot 10^{-1}$ %	- / -	- / +
5,5 %	$5 \cdot 10^{-2}$ %	- / -	+/- / +/-
5,5 %	$5 \cdot 10^{-3}$ %	- / +/-	+ / +/-
5,5 %	$5 \cdot 10^{-4}$ %	- / +/-	+/- / +/-
5,5 %	$5 \cdot 10^{-5}$ %	- / +/-	- / +/-
5,5 %	$5 \cdot 10^{-6}$ %	- / +/-	- / +/-
6 %	$5 \cdot 10^{-1}$ %	- / +	- / -
6 %	$5 \cdot 10^{-2}$ %	+/- / +	+/- / +
6 %	$5 \cdot 10^{-3}$ %	- / +/-	+ / +/-
6 %	$5 \cdot 10^{-4}$ %	+/- / +	+ / +/-
6 %	$5 \cdot 10^{-5}$ %	+/- / +	+ / +/-
6 %	$5 \cdot 10^{-6}$ %	- / +/-	+/- / +/-
6 %	$5 \cdot 10^{-7}$ %	+/- / +/-	+/- / +/-
8 %	$5 \cdot 10^{-4}$ %	+/- / +/-	+ / -
8 %	$5 \cdot 10^{-5}$ %	+ / +/-	+ / +/-

Table 18 c . Sensor sensitivity test for PLA concentrations in range from 5% to 8%

Results and Discussion

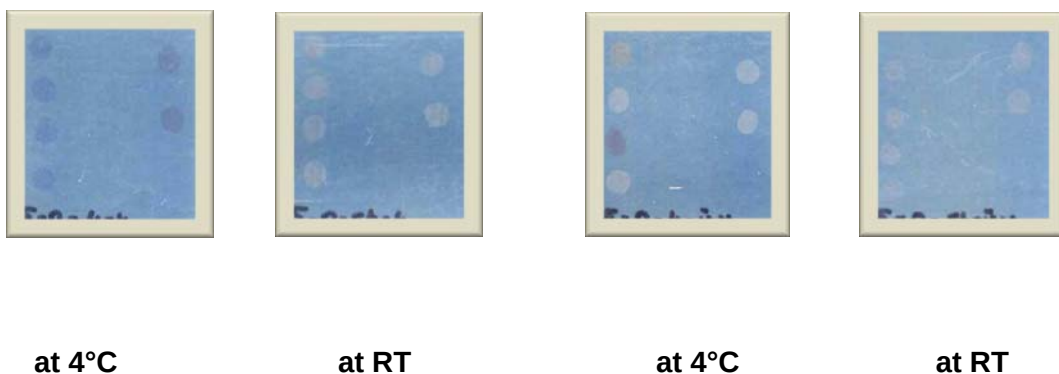


Figure 34: Inconel- 8% PLA with 5×10^{-5} % Desmodur after incubation with Protease- mix for 4 hours and overnight at 4°C and at room temperature

As can be see in Fig.32-34, the degradation of the polymer network obviously took place and could be easily observed by the naked eye, although the colour shifted only from dark blue to light grey.

2.3.2 PLGA Setup

PLGA sensor setup consists of two layers: a metal layer that is made up of Ni and Cr which gets covered with a polymer layer. This layer is a polymer PLGA (= poly (lactic-co-glycolic acid)) dissolved in ethylacetate containing varying amounts of Desmodur.

2.3.2.1 PLGA Concentration Range

For the functionality of the above described sensor setup it was important from the beginning to define a possible concentration range of polymer, which can be printed onto the surface of the metal layer. Therefore, the concentrations between 10% and 40% PLGA were printed. The PLGA solutions with concentrations lower than 20% showed very low viscosity and printed foils had a poor quality. Optimal concentration range of printing could be estimated between 20% PLGA up to 35% PLGA, printed foils showed very good quality, brilliant colors and reproducibility.

2.3.2.2 Degradation of PLGA and Proof of Principle

To describe a degradation behavior of PLGA, functionality tests with PLGA printed onto Inconel were prepared. PLGA sensors show a green or red colour which depends of the thickness and optical properties of the polymer layer. The different colour stripes on the sensor surface result from inhomogenities during the printing process, as the used gravure printer cannot compete with industrially used devices.

The first tests were carried out with Protease mixture: Proteinase K, Trypsin and α -Chymotrypsin in 0,1 M Tris-HCl pH 8.2.

Results and Discussion

The different concentrations of PLGA were printed on top of a metallized PET film. A dilution series of the Protease-Mix in 0.1 M Tris - HCl - pH 8.2 and buffer and ddH₂O as a negative control were applied to the sensor surface.

Protease concentration		
1.	20 mg/ mL	5 mg/ mL
2.	15 mg/ mL	2,5 mg/mL
3.	12,5 mg/ mL	0, 1 M Tris-HCl buffer pH 8.2
4.	10 mg/ mL	ddH ₂ O

Table 19 : Pipetting scheme for proof of principle of PLGA sensors



Figure 35: Inconel- 21% PLGA with 0.5% Desmodur in ethyl acetate
Signal intensity after incubation for 2 h, 4 h and overnight at 4°C



Figure 36: Inconel- 21% PLGA with 0.5% Desmodur in ethyl acetate
Signal intensity after incubation for 2 h, 4 h and overnight at room temperature

Results and Discussion

After incubation times of 2 h, 4 h and over night, the reaction was stopped by washing with ddH₂O, and then followed by drying under an air stream.

The color changes shown in Fig. 35 and Fig. 36 are the result of enzymatic degradation of the biomimetic polymer layer by lytic enzymes. The degradation is highly selective and clearly visible with naked eyes. The signal intensity correlates with increasing incubation time. It is generally known, that temperature is one of the important factors that affects a catalytic activity of enzyme. Thus it's not surprising that the sensors incubated at room temperature (~25°C) showed stronger signals as those which were incubated at 4°C.

2.3.3 PLGA Setup: Addition of Substrate

The optimization of the sensor properties for the “real life”, which means detection of the freshness of the food, was carried out for bacterial cultures consisting of *Pseudomonas*, *Enterobacter*, *Proteus* and *Salmonella* and for pork – meat homogenate.

As already described, biosensors with different concentrations of PLGA, different additives and also different concentrations of Desmodur were prepared to test the functionality and sensitivity of the sensors. Most of the chosen additives were mimicking agar components in order to provide a nutrimental environment for the bacteria and to trigger the release of lipolytic enzymes which are consecutively responsible for the degradation of the polymer layer.

Previous experiments showed that best functionality of sensors is achieved when using PLGA concentrations between 20 and 25%, dissolved in ethyl acetate. Furthermore, the higher the concentration of Desmodur, the higher was the cross-linking. This results in a slower degradation of the polymer by lipolytic enzymes and in a slower change in color of the sensor.

Comparing the schemes of the applied dots and the color changes of the biosensors, consequences can be taken.

For all the tested sensor setups, tests for sensitivity and selectivity were performed at 4 °C, room temperature and at 37 °C.

The sensors were incubated at these three temperatures for 6 h and over night.

Results for sensor sensitivity are presented with: (+): very strong signal and change of color; (+/-): signal is present but without observable color change; (-): there is no signal at all.

Results and Discussion

2.3.3.1. Addition of Glucose and Tryptone

Addition of Tryptone and Glucose		
PLGA concentration (w/v)	Desmodur (diisocyanate) concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
21%	$5 \cdot 10^{-3}$ %	- / +/- / +
21%	$5 \cdot 10^{-6}$ %	- / +/- / +
21 %	$5 \cdot 10^{-8}$ %	- / +/- / +
23 %	$5 \cdot 10^{-3}$ %	- / - / +
23 %	$5 \cdot 10^{-6}$ %	- / - / +
23 %	$5 \cdot 10^{-8}$ %	- / - / +
25 %	$5 \cdot 10^{-3}$ %	- / - / +
25 %	$5 \cdot 10^{-6}$ %	- / - / +
25 %	$5 \cdot 10^{-8}$ %	- / - / +

Table 20a. Results of experiments with tryptone and glucose stock solution (50 mg tryptone and 10 mg glucose in 10 ml trifluorethanol). PLGA was added later and dissolved at room temperature

Results and Discussion

Column 1	Column 2	Column 3
1. Bacterial Cocktail A 10^8 CFU/mL	6. Homogenate I 10^5 CFU/mL	11. Protease- Mix* 20 mg/mL
2. Bacterial Cocktail A 10^6 CFU/mL	7. Homogenate II 10^8 CFU/mL	12. Protease- Mix* 15 mg/mL
3. Bacterial Cocktail A 10^5 CFU/mL	8. Homogenate II 10^6 CFU/mL	13. Protease- Mix* 10 mg/mL
4. Homogenate I 10^8 CFU/mL	9. Homogenate I 10^5 CFU/mL	14. Protease- Mix* 5 mg/mL
5. Homogenate I 10^6 CFU/mL	10. Bacterial Cocktail C 10^6 CFU/mL	15. Ringer solution



Figure 37:
Inconel-21%
PLGA with $5 \times 10^{-6}\%$
Desmodur, after
16h at room
temperature

Table 20b. Pipetting scheme. * Equal amounts of
Proteinase K, Chymotrypsine and Trypsine.

This experiment showed that all microbiological solutions applied onto the sensor surface, showed only slight degradation. Only the Protease-Mix concentration gradient showed a visible color change.

Results and Discussion

Addition of Tryptone and Glucose		
PLGA concentration (w/v)	Desmodur (triisocyanate) concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
21%	$5 \cdot 10^{-3} \%$	- / + / +
21%	$5 \cdot 10^{-4} \%$	- / +- / +
21 %	$5 \cdot 10^{-5} \%$	+ / + / +
21 %	$5 \cdot 10^{-6} \%$	+ / + / +
21 %	$5 \cdot 10^{-8} \%$	+ / + / +
23 %	$5 \cdot 10^{-3} \%$	+ / + / +
23 %	$5 \cdot 10^{-4} \%$	+ / + / +
23 %	$5 \cdot 10^{-5} \%$	+ / + / +
23 %	$5 \cdot 10^{-6} \%$	+ / + / +
23 %	$5 \cdot 10^{-8} \%$	+ / + / +
25 %	$5 \cdot 10^{-3} \%$	+ / + / +
25 %	$5 \cdot 10^{-4} \%$	+ / + / +
25 %	$5 \cdot 10^{-5} \%$	+ / + / +
25 %	$5 \cdot 10^{-6} \%$	+ / + / +
25 %	$5 \cdot 10^{-8} \%$	+ / + / +

Table 21a: Results of experiments with tryptone and glucose stock solution (50 mg tryptone and 10 mg glucose in 10 ml ethyl acetate). PLGA was added later and dissolved at room temperature

Results and Discussion

Column 1	Column 2	Column 3
1. Bacterial Cocktail A 10^8 CFU/mL	6. Homogenate I 10^5 CFU/mL	11. Protease- Mix* 20 mg/mL
2. Bacterial Cocktail A 10^6 CFU/mL	7. Homogenate II 10^8 CFU/mL	12. Protease- Mix* 15 mg/mL
3. Bacterial Cocktail A 10^5 CFU/mL	8. Homogenate II 10^6 CFU/mL	13. Protease- Mix* 10 mg/mL
4. Homogenate I 10^8 CFU/mL	9. Homogenate I 10^5 CFU/mL	14. Protease- Mix* 5 mg/mL
5. Homogenate I 10^6 CFU/mL	10. Bacterial Cocktail C 10^6 CFU/mL	15. Ringer solution

Table 21b. Pipetting scheme. * Equal amounts of Proteinase K, Chymotrypsine and Trypsine.

For the following sensors, the dots were applied according to the scheme in Table 21b. The bacterial cocktail A clearly shows degradation of the polymer layer at 4°C, the signal was stronger and more intense at higher temperature



After 16 hours at 4°C



after 16 hours at
room temperature



after 16 hours at 37°C

Figure 38: Inconel- 23% PLGA with 5×10^{-5} % Desmodur

Results and Discussion

Column 1	Column 2
1. H II 2 10 ⁹ CFU/mL	3. Ringer solution
2. H II 2 10 ⁶ CFU/mL	

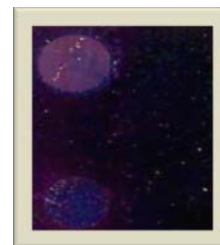


Table 21c: Pipetting scheme

after 4 h at 4°C

after 4h at rt

Application of homogenates (HII2) results in a highly selective color change.

2.3.3.2. Addition of sunflower oil as a bacterial nutrient source

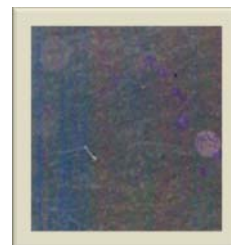
Gram-negative bacteria, especially *Pseudomonas*, are able to excrete lipases able to hydrolyze animal fat (e.g. pork and beef) and vegetable oil (e.g. sunflower).

Addition of Sunflower Oil		
PLGA concentration (w/v)	Desmodur (triisocyanate) concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
21%	5*10 ⁻⁸ %	- / + / +
22%	5*10 ⁻³ %	- / + / +
22 %	5*10 ⁻⁶ %	- / + / +
22 %	5*10 ⁻⁸ %	- / + / +
23 %	5*10 ⁻⁶ %	+ / + / +
23 %	5*10 ⁻⁸ %	- / + / +
25 %	5*10 ⁻³ %	+ / + / +
25 %	5*10 ⁻⁶ %	+ / + / +

Results and Discussion

Table 22a: Results of experiments with sunflower oil (1-20 μ L/ in 2 mL ethylacetate)

Column 1	Column 2	Column 3
4 Bacteria Stock- 10^8 CFU/ml	Homogenate Stock 10^8 CFU/ml	Protease-Mix * 20 mg/ml
4 Bacteria – 10^6 CFU/ml	Homogenate - 10^6 CFU/ml	Protease-Mix* 10 mg/ml
GSP- 10^6 CFU/ml		Ringer Solution



4 hours



16 hours

Figure 39: Inconel- 22% PLGA with
 5×10^{-3} % Desmodur- at 37°C

Table 22 b. Pipetting scheme. * Equal amounts of
Proteinase K, Chymotrypsine and Trypsine

As can be seen from Fig. 39, degradation of PLGA is visible for bacteria cocktail stock (*Pseudomonas*, *Enterobacter*, *Proteus* and *Salmonella*) and Protease-mix.

2.3.3.3. 22% PLGA and oil with increasing concentrations of Desmodur

($5 \cdot 10^{-3} \%$ - 3 %)

Addition of Sunflower oil		
PLGA concentration (w/v)	Desmodur (triisocyanate) concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
22 %	$5 \cdot 10^{-3} \%$	- / - / -
22 %	$5 \cdot 10^{-2} \%$	- / - / -
22 %	$5 \cdot 10^{-1} \%$	- / - / -
22 %	1 %	- / - / +/-
22 %	1,2 %	- / - / +/-
22 %	1,4 %	- / - / +/-
22 %	1,6 %	- / - / +/-
22 %	1,8 %	- / - / +/-
22 %	2,2 %	- / - / +/-
22 %	2,4 %	- / +/- / +/-
22 %	2,6 %	- / - / +/-
22 %	3 %	- / - / +/-

Table 23a: Results of experiments with sunflower oil (1-20 μL in 2 mL ethylacetate) and increased triisocyanate concentration

Results and Discussion

Column 1	Column 2	Column 3
4 Bacteria Stock- 10^8 CFU/ml	Homogenate Stock- 10^8 CFU/ml	Protease-Mix * 20 mg/ml
4 Bacteria - 10^6 CFU/ml	Homogenate - 10^6 CFU/ml	Protease-Mix* 10 mg/ml
GSP- 10^6 CFU/ml		Ringer Solution



after 16h at 4°C



after 4h at 37°C

Table 23 b. Pipetting scheme; * Equal amounts of Proteinase K, Chymotrypsine and Trypsine

These experiments show that – independently of incubation time and/or temperature – in most cases the enzymes mix (20 and/or 10mg/ml) resulted in a visible degradation of the sensor. The second sensor listed here is composed of 22% PLGA with 1.6% of Desmodur. Here, degradation is also visible for the bacteria cocktail stock (*Salmonella*, *Proteus*, *Enterobacter*, and *Pseudomonas*) and for the homogenate stock (10^8 CFU/ml).

Results and Discussion

2.3.3.4. PLGA and Vegetable Butter as a Lipid Source

Addition of butter					
PLGA concentration (w/v)	Triisocyanate concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C	PLGA concentration (w/v)	Triisocyanate concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
21 %	1 %	- / +/- / +/-	23 %	1 %	- / +/- / -
21 %	5*10 ⁻¹ %	- / +/- / +/-	23 %	0,8 %	- / +/- / +/-
21 %	5*10 ⁻² %	- / +/- / +/-	23 %	0,7 %	- / +/- / +/-
21 %	5*10 ⁻³ %	- / +/- / +/-	23 %	5*10 ⁻⁴ %	- / +/- / +/-
21 %	5*10 ⁻⁴ %	- / +/- / +/-	23 %	5*10 ⁻⁵ %	- / +/- / -
21 %	5*10 ⁻⁵ %	- / +/- / +/-	23 %	5*10 ⁻⁶ %	- / + / +
21 %	5*10 ⁻⁶ %	+/- / +/- / +	23 %	5*10 ⁻⁷ %	- / +/- / +/-
21 %	5*10 ⁻⁸ %	+/- / +/- / +	23 %	5*10 ⁻⁸ %	- / +/- / +/-

Table 24a. Experiments with vegetable fat (from sunflower, canola and corn) dissolved in ethylacetate at room temperature (55 mg butter/10 ml ethylacetate).

Column 1	Column 2	Column 3
4 Bacteria Stock- 10 ⁸ CFU/ml	Homogenate Stock- 10 ⁸ CFU/ml	Protease-Mix * 20 mg/ml
4 Bacteria -10 ⁶ CFU/ml	Homogenate - 10 ⁶ CFU/ml	Protease-Mix* 10 mg/ml
GSP-10 ⁶ CFU/ml		Ringer Solution



After 4 hours at 37°C

Figure 40: Inconel- 21% PLGA with 5*10⁻⁶ % Desmodur

Table 24b. Pipetting scheme; * Equal amount of Proteinase K, Chymotrypsine and Trypsine

Results and Discussion

In Figure 40, the Protease- mix shows a clearly visible degradation of the polymer layer for both concentrations. Homogenate solutions also result in a color change at 37°C.

2.3.3.5. PLGA and glycerin tributyrate with increasing concentration of Desmodur (triisocyanate)

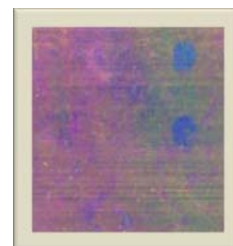
Glycerin tributyrate is a main component of butter and often used trigger for identification of microorganisms able to produce lytic enzymes.

Addition of glyceryl tributyrate		
PLGA concentration (w/v)	Desmodur (triisocyanate) concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
20 %	5*10 ⁻¹ %	- / - / -
20 %	5*10 ⁻³ %	- / - / +
20 %	5*10 ⁻⁵ %	- / + / +
21 %	5*10 ⁻¹ %	+/- / + / +
21 %	5*10 ⁻³ %	- / + / + / -
21 %	5*10 ⁻⁵ %	- / - / + / -
22 %	5*10 ⁻¹ %	- / + / +
22 %	5*10 ⁻³ %	- / - / -
22 %	5*10 ⁻⁵ %	- / - / + / -

Table 25a. Experiment with glyceryl-tributyrate and increasing concentration of desmodur (triisocyanate), using ethylacetate as a solvent

Results and Discussion

Column 1	Column 2	Column 3
4 Bacteria Stock- 10^8 CFU/ml	Homogenate Stock- 10^8 CFU/ml	Protease-Mix * 20 mg/ml
4 Bacteria - 10^6 CFU/ml	Homogenate - 10^6 CFU/ml	Protease-Mix* 10 mg/ml
GSP- 10^6 CFU/ml		Ringer Solution



After 4h at room temperature

Figure 41: Inconel- 21 %PLGA with 0, 5% Desmodur

Table 25b. Pipetting scheme; * Equal amount of Proteinase K, Chymotrypsine and Trypsine

As can be seen in Figure 41, both concentrations of Protease- mix show a very intensive colour change as a result of the degradation of PLGA.

2.3.3.6. PLGA and glyceryl tributyrate with increasing concentration of Desmodur (diisocyanate)

Addition of glyceryl tributyrate		
PLGA concentration (w/v)	Desmodur (diisocyanate) concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
20 %	$5 \cdot 10^{-1}$ %	+ / + / +
20 %	$5 \cdot 10^{-3}$ %	- / + / +
20 %	$5 \cdot 10^{-5}$ %	- / - / +/-
21 %	$5 \cdot 10^{-1}$ %	+ / + / +
21 %	$5 \cdot 10^{-3}$ %	+ / + / +
21 %	$5 \cdot 10^{-5}$ %	+ / + / +
22 %	$5 \cdot 10^{-1}$ %	+ / + / +
22 %	$5 \cdot 10^{-3}$ %	+ / + / +
22 %	$5 \cdot 10^{-5}$ %	+ / + / +

Table 26a. Experiment with glyceryl-tributyrate and rising concentrations of desmodur (diisocyanate), using ethylacetate as a solvent

Results and Discussion

Column 1	Column 2	Column 3
1. Bacterial Cocktail A 10 ⁸ CFU/mL	5. PS I 10 ⁶ CFU/mL	9. Protease- Mix* 20 mg/mL
2. Bacterial Cocktail A 10 ⁶ CFU/mL	6. PS II 10 ⁶ CFU/mL	10. Protease- Mix* 10 mg/mL
3. Homogenate 10 ⁸ CFU/mL	7. H II 1 10 ⁶ CFU/mL	11. Ringer solution
4. Homogenate 10 ⁶ CFU/mL	8. H II 2 10 ⁶ CFU/mL	

Table 26b. Pipetting scheme; * Equal amounts of Proteinase K, Chymotrypsine and Trypsine

The following biosensors also show degradation of the polymer layer by the enzyme.- mix at higher temperatures.

In addition, *Pseudomonas* in LM (PS 1 and PS 2, in log phase) is even capable of producing lipolytic enzymes at low temperatures. It is often observed that at higher temperatures, the applied dots are salted out and deposits remain on the sensor, even after washing, bacteria stock and bacteria diluted to 10⁶ CFU/mL)

Results and Discussion



After 4h at room temperature



after 4h at 37°C



after 16h at 4°C

Figure 42: Inconel- 20% PLGA with 0, 5%; $5 \cdot 10^{-3}\%$ and $5 \cdot 10^{-5}\%$ Desmodur



After 16h at room temperature



after 16h at 37°C

Figure 43: Inconel- 21% PLGA with 0.5% and 0.005% Desmodur

Results and Discussion

2.3.3.7. PLGA, glyceryl tributyrat and oil without Desmodur

Addition of Glycerol Tributyrat	
PLGA concentration (w/v)	Sensor sensitivity 4°C / RT/ 37°C
20 %	- / + / +
20 %	+/- / + / +
20 %	- / + / +
21 %	+/- / + / +
21 %	+/- / + / +
21 %	+/- / + / +
22 %	+/- / + / +
22 %	+ / + / +
22 %	+ / + / +

Table 27a. Experiments with glyceryl-tributyrat and vegetable oil,
but without linker . Ethylacetate was used as a solvent

In all cases the Protease- mix (20mg/ml) gives a good degradation of the polymer layer.

Column 1	Column 2	Column 3
1. Bacterial Cocktail A 10 ⁸ CFU/mL	4.Homogenate I 10 ⁸ CFU/mL	7. Culture b 10 ⁶ CFU/mL
2. Bacterial Cocktail A 10 ⁶ CFU/mL	5. H II 2 10 ⁶ CFU/mL	8. Protease- Mix* 20 mg/mL
3. Homogenate I 10 ⁸ CFU/mL	6. Culture a 10 ⁶ CFU/mL	9. Ringer solution



After 16h at 37°C

Figure 44: Inconel- 20% PLGA

Table 27 b. Pipetting scheme; * Equal amount of
Proteinase K, Chymotrypsine and Trypsine

Results and Discussion

2.3.3.8. PLGA, Tryptone and oil with increasing concentrations of

Desmodur (5×10^{-8} - 1 %)

Addition of Tryptone and Oil					
PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C	PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
20 %	1 %	+/- / + / +	21 %	1 %	+ / + / +
20 %	5×10^{-2} %	+/- / + / +	21 %	5×10^{-2} %	+ / + / +
20 %	5×10^{-3} %	+/- / + / +	21 %	5×10^{-3} %	+ / + / +
20 %	5×10^{-6} %	- / + / +	21 %	5×10^{-6} %	+/- / +/- / +
20 %	5×10^{-8} %	+/- / + / +	21 %	5×10^{-8} %	+/- +/- / +/-
PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C	PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
23 %	1 %	+ / + / +	24 %	1 %	+ / + / +
23 %	5×10^{-2} %	+ / + / +	24 %	5×10^{-2} %	+ / + / +
23 %	5×10^{-3} %	+/- / + / +/-	24 %	5×10^{-3} %	+ / + / +
23 %	5×10^{-6} %	+/- / + / +	24 %	5×10^{-6} %	+ / + / +
23 %	5×10^{-8} %	+/- / + / +	24 %	5×10^{-8} %	- / + / +
PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C	PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
25 %	1 %	- / + / +	27 %	1 %	+ / + / +
25 %	5×10^{-2} %	- / + / +	27 %	5×10^{-2} %	+ / + / +
25 %	5×10^{-3} %	+/- / + / +	27 %	5×10^{-3} %	+ / + / +
25 %	5×10^{-6} %	+/- / + / +	27 %	5×10^{-6} %	+ / + / +
25 %	5×10^{-8} %	+/- / + / +	27 %	5×10^{-8} %	+ / + / +

Table 28a. Experiments with tryptone and sunflower oil in a stock of 50 mg tryptone in 10 ml ethylacetate dissolved at 50°C (~2 hours) and prepared a day before,

Results and Discussion

Experiments with tryptone and oil as additives also yield color changes of the biosensor. In the selected biosensor shown above, application of homogenate (HII2) and bacteria mix in LM medium led to degradation of the polymer layer.

Column 1	Column 2	Column 3
1. Bacterial Cocktail A 10 ⁸ CFU/mL	5. HII2 or HII1	9. 4 Bacteria – LM
2. Bacterial Cocktail A 10 ⁶ CFU/mL	6. Culture a (10 ⁷ CFU/ml)	10. 4 Bacteria –LM 10 ⁶ CFU/ml
3. Homogenate I 10 ⁸ CFU/mL	7. Culture b (10 ⁸ CFU/ml)	11. Phospholipase 10 mg/mL
4. Homogenate I 10 ⁶ CFU/mL	8. Culture b (10 ⁶ CFU/mL)	12. Ringer solution



After 16h at room temperature

Figure 45: Inconel- 20 % PLGA with 5*10⁻⁸ % Desmodur

Table 28b: Pipetting scheme

For the following sensors, the dots were applied according to the scheme in Table 24b. The best results with tryptone and oil were achieved with application of homogenate HII2 and the bacteria mix in LM medium. Color changes were also obtained for homogenate Culture b (10⁸ CFU/ml) and the bacteria mix in LM medium diluted to 10⁶ CFU/ml.



After 4h at 37°C



after 16h at room temperature

Figure 46: Inconel- 23 % PLGA with 5*10⁻⁸ % Desmodur

Results and Discussion

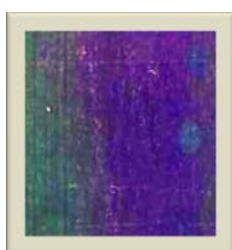
2.3.3.9. PLGA and Sodium-Citrate

Addition of sodium-citrate		
PLGA concentration (w/v)	Desmodur (diisocyanate) concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
26 %	1,2 %	- / + / +
26 %	$5 \cdot 10^{-3}$ %	- / + / +
26 %	$5 \cdot 10^{-6}$ %	- / + / +
26 %	$5 \cdot 10^{-8}$ %	- / + / +

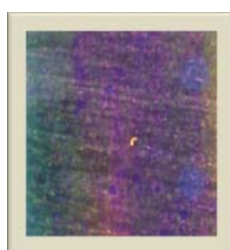
Table 29 a. Experiments with tri-sodium-citrate x 2H₂O as a substrate, solved in ddH₂O (22,8 mg tri-sodium-citrate x 2H₂O/55 µl ddH₂O).

Column 1	Column 2	Column 3
Homogenate Stock-10 ⁸ CFU/ml	GSP-10 ⁶ CFU/ml	Protease-Mix * 20 mg/ml
Homogenate - 10 ⁶ CFU/ml	Ringer Solution	Protease-Mix* 10 mg/ml

Table 29 b. Pipetting scheme; * Equal amounts of Proteinase K, Chymotrypsine and Trypsine



5*10⁻⁶% Desmodur
after 4h at rt



5*10⁻⁸% Desmodur
after 16h at rt



1,2%Desmodur
after 16h at 37°C

Figure 47: Degradation of 26% PLGA Sensors

Results and Discussion

In Fig. 47 above, the Protease- mix shows for both concentrations a clearly visible degradation of the polymer layer. Homogenate solutions also result in a color change at 37°C.

2.3.3.10. PLGA, glucose and oil with increasing concentrations of Desmodur ($5 \cdot 10^{-8}$ - 1 %)

Addition of glucose and oil					
PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C	PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
21 %	1 %	+ / + / +	23 %	1 %	+ / + / +
21 %	$5 \cdot 10^{-2}$ %	+ / + / +	23 %	$5 \cdot 10^{-2}$ %	+ / + / +
21 %	$5 \cdot 10^{-3}$ %	+ / + / +	23 %	$5 \cdot 10^{-3}$ %	+ / + / +
21 %	$5 \cdot 10^{-6}$ %	+ / + / +/-	23 %	$5 \cdot 10^{-6}$ %	- / + / +
21 %	$5 \cdot 10^{-8}$ %	+ / + / +/-	23 %	$5 \cdot 10^{-8}$ %	- / + / +

Table 30a. Experiments with glucose and oil. Stock of glucose and sunflower oil were prepared in ethyl acetate (7 mg glucose in 10 ml solvent).

PLGA sensors with glucose and oil as a substrate showed the best signal even at 4°C, after only 4 hours.

Results and Discussion

Column 1	Column 2	Column 3
1. Bacterial Cocktail A 10 ⁸ CFU/mL	4. Homogenate I 10 ⁶ CFU/mL	7. Culture b 10 ⁶ CFU/mL
2. Bacterial Cocktail A 10 ⁶ CFU/mL	5. H II 2 10 ⁶ CFU/mL	8. Protease- Mix* 20 mg/mL
3. Homogenate I 10 ⁸ CFU/mL	6. Culture a 10 ⁶ CFU/mL	9. Ringer solution

Table 30 b. Pipetting scheme; * Equal amount of Proteinase K, Chymotrypsine and Trypsine

For the following sensors, the dots were applied according to the scheme in Table 30 b. Again, the Protease mix (20mg/ml) is responsible for a color change. Also homogenate solutions (Homogenate stock, Homogenate-10⁶ CFU/ml, HII2, Culture a) and Culture b) are able to degrade the polymer layer at all given temperatures and incubation times.



23 % PLGA
1% Desmodur
Desmodur
After 4 hours



23 % PLGA
5*10⁻³ % Desmodur
after 4 hours



21% PLGA
5*10⁻⁶ %
after 16 hours

Figure 48 : PLGA sensors after incubation for 4 and 16 hours at 37°C

Results and Discussion

2.3.3.11. PLGA and Tween 20 with increasing concentrations of Desmodur (5×10^{-8} - 1 %)

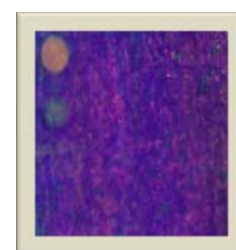
Addition of Tween 20					
PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C	PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
21 %	1 %	+ / +/- / +	23 %	1 %	- / +/- / +
21 %	5×10^{-2} %	+ / +/- / +	23 %	5×10^{-2} %	- / + / +
21 %	5×10^{-3} %	- / - / +	23 %	5×10^{-3} %	+ / + / +
21 %	5×10^{-6} %	+ / +/- / +	23 %	5×10^{-6} %	+ / + / +
21 %	5×10^{-8} %	- / - / +	23 %	5×10^{-8} %	- / + / +

Table 31a. Tween 20 (1:100) dilution was prepared in ethyl acetate

Column 1	Column 2	Column 3
1. Bacterial Cocktail A 10^8 CFU/mL	5. HII2 or HII1	9. 4 Bacteria – LM
2. Bacterial Cocktail A 10^6 CFU/mL	6. Culture a (10^7 CFU/ml)	10. 4 Bacteria –LM 10^6 CFU/ml
3. Homogenate I 10^8 CFU/mL	7. Culture b (10^8 CFU/ml)	11. Phospholipase 10 mg/mL
4. Homogenate I 10^6 CFU/mL	8. Culture b (10^6 CFU/mL)	12. Ringer solution



After 6 hours
hours



after 16

Figure 49: PLGA (21% with 5×10^{-6} % and 5×10^{-8} % Desmodur) sensors at 37°C

Table 31 b: Pipetting scheme

Results and Discussion

Experiments with Tween 20 show color changes of the biosensor for the application of the bacteria mix in peptone water, its dilution to 10^6 CFU/ml and the homogenate stock.

2.3.3.12. Glucose, lactose and oil as a Trigger in PLGA Sensors

Addition of Sugars and Oil					
PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT / 37°C	PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT / 37°C
21 %	1 %	+ / + / +	23 %	1 %	+ / + / +
21 %	$5 \cdot 10^{-2}$ %	+ / + / +	23 %	$5 \cdot 10^{-2}$ %	+ / + / +
21 %	$5 \cdot 10^{-3}$ %	- / + / +	23 %	$5 \cdot 10^{-3}$ %	+ / + / +
21 %	$5 \cdot 10^{-6}$ %	+ / +/- / +	23 %	$5 \cdot 10^{-6}$ %	+ / + / +
21 %	$5 \cdot 10^{-8}$ %	+/- / + / +/-	23 %	$5 \cdot 10^{-8}$ %	+ / + / +

Table 32 a. Experiments with disaccharide and sunflower oil

A combination of glucose, lactose and sunflower oil showed excellent results after 6 hours.

For the following sensors, the dots were applied according to the scheme in Table 32b.

Results and Discussion

Column 1	Column 2	Column 3
1. Bacterial Cocktail A 10 ⁸ CFU/mL	5. HII2 or HII1	9. 4 Bacteria – LM
2. Bacterial Cocktail A 10 ⁶ CFU/mL	6. Culture a (10 ⁷ CFU/ml)	10. 4 Bacteria – LM 10 ⁶ CFU/ml
3. Homogenate I 10 ⁸ CFU/mL	7. Culture b (10 ⁸ CFU/ml)	11. Phospholipase 10 mg/mL
4. Homogenate I 10 ⁶ CFU/mL	8. Culture b (10 ⁶ CFU/mL)	12. Ringer solution

Table 32 b: Pipetting scheme

Degradation of the polymer layer was achieved with bacteria stocks, diluted to 10⁶ CFU/ml in peptone water, HII and the stock in LM medium.



21% PLGA
PLGA

5*10⁻⁶% Desmodur
Desmodur



21% PLGA

5*10⁻⁸% Desmodur



23% PLGA

0,5% Desmodur



23%

0,05%

Figure 50: PLGA Sensors after
6 h at 37°C

Figure 51: PLGA Sensors after
16 h at 37°C

Results and Discussion

2.3.3.13. PLGA, glucose, glycerol tributyrate and oil with increasing concentrations of Desmodur ($5 \cdot 10^{-8}$ - 1 %)

Addition of glucose, glycerol tributyrate and oil					
PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C	PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
21 %	1 %	+ / + / +	23 %	1 %	+/- / + / +
21 %	$5 \cdot 10^{-1}$ %	- / + / +	23 %	$5 \cdot 10^{-1}$ %	- / + / +
21 %	$5 \cdot 10^{-3}$ %	- / + / +	23 %	$5 \cdot 10^{-3}$ %	+ / + / +
21 %	$5 \cdot 10^{-6}$ %	- / + / +	23 %	$5 \cdot 10^{-6}$ %	+ / + / +
21 %	$5 \cdot 10^{-8}$ %	- / +/- / +	23 %	$5 \cdot 10^{-8}$ %	+ / + / +

Table 33 a. Overview of experiments with glucose, glycerol tributyrate and sunflower oil in 1:1:1 ratio.

Column 1	Column 2	Column 3
1. Meat homogenate 1* 10^6 CFU/mL	5. HII2 or HII1	9. 4 Bacteria – LM
2. Meat homogenate 2** 10^7 CFU/mL	6. Culture a (10^7 CFU/ml)	10. 4 Bacteria –LM 10^6 CFU/ml
3. Homogenate I 10^8 CFU/mL	7. Culture b (10^8 CFU/ml)	11. Phospholipase 10 mg/mL
4. Homogenate I 10^6 CFU/mL	8. Culture b (10^6 CFU/mL)	12. Ringer solution

Table 33b: Pipetting scheme

*- pork cutlets after 96 hours at 4°C

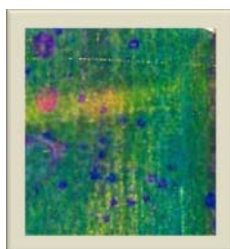
** - minced meat after 48 hours at 15°C

Results and Discussion

Experiments with glucose, glycerol tributyrat and oil as additives result in color changes for meat homogenate 1 and 2. Both are samples from the experiments of meat deterioration (see Microbiology- part 1.3). It is important to know that these samples are homogenates with 10^6 to 10^7 CFU/ml. Application of the homogenate HII stock also results in a color change on the sensor.



23% PLGA
 $5 \cdot 10^{-3}\%$ Desmodur
After 6h at 37°C



21% PLGA
 $5 \cdot 10^{-3}\%$ Desmodur
after 6 h at 37°C



21% PLGA
 $5 \cdot 10^{-1}\%$ Desmodur
after 16 h at 37°C

Figure 52: Degradation of PLGA

Results and Discussion

2.3.3.14. PLGA and Steamed Milk

Steamed Milk was used in PLGA sensors as an activator of bacterial induction and lipase production.

Addition of steamed milk					
PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C	PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
21 %	1 %	+ / + / +	23 %	1 %	+ / + / +
21 %	5*10 ⁻¹ %	+/- / + / +	23 %	5*10 ⁻¹ %	+ / + / +
21 %	5*10 ⁻³ %	+/- / + / +	23 %	5*10 ⁻³ %	- / + / +
21 %	5*10 ⁻⁶ %	+/- / +/- / +	23 %	5*10 ⁻⁶ %	+ / + / +
21 %	5*10 ⁻⁸ %	- / +/- / +	23 %	5*10 ⁻⁸ %	+ / + / +

Table 34a: Overview of experiments with steamed Milk

Column 1	Column 2	Column 3
1. Meat homogenate 1* 10 ⁶ CFU/mL	5.HII2 or HII1	9. 4 Bacteria – LM
2. Meat homogenate 2** 10 ⁷ CFU/mL	6. Culture a (10 ⁷ CFU/ml)	10. 4 Bacteria –LM 10 ⁶ CFU/ml
3. Homogenate I 10 ⁸ CFU/mL	7. Culture b (10 ⁸ CFU/ml)	11. Phospholipase 10 mg/mL
4. Homogenate I 10 ⁶ CFU/mL	8. Culture b (10 ⁶ CFU/mL)	12. Ringer solution

Table 34b: Pipetting scheme

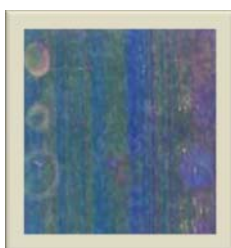
*- pork cutlets after 96 hours at 4°C

** - minced meat after 48 hours at 15°C

Results and Discussion

Again, meat homogenate 1 and 2 deliver a visible reaction with the polymer layer. Application of the homogenate HII stock or/and its dilution to 10^6 CFU/ml and Phospholipase also result in a color change on the sensor.

As for the sensor incubated at 37°C overnight, the dilution of the homogenate I stock and 10^6 CFU/mL dilutions, could also yield an answer.



23% PLGA

5×10^{-8} % Desmodur

After 6 hours



23% PLGA

1% Desmodur

after 16 hours

Figure 53: PLGA Sensors after incubation at 37°C

Results and Discussion

2.3.3.15. PLGA and NaH₂PO₄

Addition of NaH ₂ PO ₄					
PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C	PLGA concentration (w/v)	Desmodur concentration (v/v)	Sensor sensitivity 4°C / RT/ 37°C
21 %	1 %	+ / + / +	23 %	1 %	+ / + / +
21 %	5*10 ⁻¹ %	+ / + / +	23 %	5*10 ⁻¹ %	+ / + / +
21 %	5*10 ⁻³ %	+ / + / +	23 %	5*10 ⁻³ %	+ / + / +
21 %	5*10 ⁻⁶ %	+ / + / +	23 %	5*10 ⁻⁶ %	+ / + / +
21 %	5*10 ⁻⁸ %	- / + / +	23 %	5*10 ⁻⁸ %	+ / + / +

Table 35 a: Overview of experiments with NaH₂PO₄

Column 1	Column 2	Column 3
1. Meat homogenate 1* 10 ⁶ CFU/mL	5.HII2 or HII1	9. 4 Bacteria – LM
2. Meat homogenate 2** 10 ⁷ CFU/mL	6. Culture a (10 ⁷ CFU/ml)	10. 4 Bacteria –LM 10 ⁶ CFU/ml
3. Homogenate I 10 ⁸ CFU/mL	7. Culture b (10 ⁸ CFU/ml)	11. Phospholipase 10 mg/mL
4. Homogenate I 10 ⁶ CFU/mL	8. Culture b 10 ⁶ CFU/mL	12. Ringer solution

Table 35 b: Pipetting scheme

*- pork cutlets after 96 hours at 4°C

** - minced meat after 48 hours at 15°C



37°C

After 6 hours at

Figure 54: Inconell- 23 %
PLGA with 5*10⁻⁶ % Desmodur

Results and Discussion

For this combination, only incubation at 37°C led to degradation of the polymer and therefore, a signal was visible. Application of the meat homogenate 1 and 2 and HII stock result in a signal that was visible after 6 hours of incubation.

2.4 Application of PLGA Sensors for Analysis of Natural Products

PLGA two layer sensors may also be used in order to analyze if the production steps and/or storage of medical products, cosmetics or foods have been made correctly. Since most of the microorganisms responsible for the spoilage preferably proliferate at 37°C, natural products are stored and transported at temperatures below 37°C, preferably at 4°C or even frozen at –20°C to maintain an unfavourable temperature range for such microorganisms. Failure to comply with said conditions may result in contamination, which can be analyzed for by the present device.

The general protocol for preparing the devices was: Inconel- Ni Cr alloy coated PET foil was used as reflective layer. Onto this layer, a polymer layer of PLGA with Desmodur in ethylacetate was printed, followed by incubation for 10 minutes by 80°C.

The sensors were then incubated with meat homogenate, bacteria cocktail, contaminated solution for contact lenses and contaminated hand cream to test their ability to identify a contamination and spoilage of natural products. The dots are applied according to the scheme in Table 36:

Column 1	Column 2	Column 3
1. Meat homogenate 1* 10 ⁶ CFU/mL		9. 4 Bacteria – LM
2. Meat homogenate 2** 10 ⁷ CFU/mL	6. Culture a (10 ⁷ CFU/ml)	10. 4 Bacteria –LM 10 ⁶ CFU/ml
3. Acumed ¹ 10 ⁸ CFU/mL	7. Culture b (10 ⁸ CFU/ml)	11. Phospholipase 10 mg/mL
4. Kamill ² 10 ⁶ CFU/mL	8. Culture b 10 ⁶ CFU/mL	12. Ringer solution

Table 36: Pipetting scheme

Results and Discussion

*- pork cutlets after 96 hours at 4°C

¹- Acumed –solution for contact lenses

** - minced meat after 48 hours at 15°C

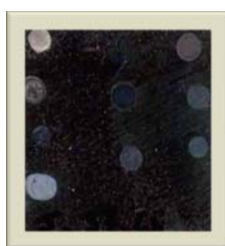
²- Kamill- hand & nail cream

Finally, the sensors were incubated at 37°C for 6 hours and then scanned for documentation of the colour changes.



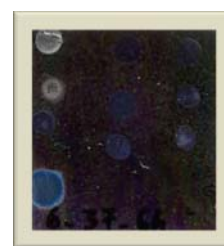
21% PLGA

0,5% Desmodur



21% PLGA

1% Desmodur



23% PLGA

5*10⁻⁸% Desmodur

Figure 55: Degradation of PLGA layer

Phospholipase was used as a positive control and resulted in a colour change.

The negative control Ringer-solution did not show a signal.

A concentration dependent effect could be observed for the colour change upon the incubation with bacterial cocktail and meat homogenate: a higher concentration seems to result in a more pronounced colour change, i.e. towards a brighter color.

The brightest color could be observed upon the incubation with contaminated hand crème; however, incubation with the contaminated solution for contact lenses also resulted in a clearly visible color change.

In general, the use of sensor with 21% PLGA shows the strongest signal and highly selective and brilliant color change as a result of an enzymatic degradation of polymer layer.

Conclusion

The aim of this thesis was to develop sensor chip prototypes able to detect a certain amount of lytic enzymes which are produced by food-borne pathogens. The meat decay is observable by an irreversible color change in real-time and on-site.

Usually, meat products in the store are kept on 4°C. Though, customers do not know exactly under which conditions meat was kept right after preparation. In this case, the presence of a biosensor could help out and give the customer the quality information about the product.

Temperature plays an important role in this relation. As we have seen before, a temperature difference of 11°C can influence the shelf-life of a meat product drastically (Meat deterioration).

Temperature also has an influence on the activity of the enzymes produced by food-borne pathogens. As we have seen before, a higher total bacteria count does not always mean that there is a higher activity of lytic enzymes such as leucine aminopeptidase and phospholipase (Bacterial Growth in LM and EM media).

Conventional microbiological tests are reversible and on the other hand results are obtained only after a long duration time, they are not capable of providing information on the microbial status of products very fast and the results can be manipulated.

In contrast, during this work were developed and optimized some biosensor prototypes with the ability to display bacterial infection of, for example, nutrients by the principles of an optical phenomenon and they carry the advantage of a memory effect that cannot be manipulated

Conclusion

Looking at this project, best results were achieved with biosensors containing PLGA as an analyte sensitive layer and tryptone, glucose or/and oil as a trigger to stimulate bacterial metabolism.

Additional substrates applied into the polymer layer led to an induction of microorganisms for better excretion of lytic and lipolytic enzymes, but conserving the total bacterial count. The sensor sensitivity was remarkably increased in that way showing a stronger signal but without influence on the stability of the polymer layer.

A possible disadvantage could be that in several cases, degradation of the polymer layer was better detectable at room temperature and at 37°C than at 4°C.

In the development of biosensors, it is very important that they keep their stability during usage. Homogeneity of the polymer layer should also be existent in order to assure reproducibility.

In addition, the biomimetic biosensor gives the possibility to adapt its polymer layer by different additives and after further research it could also be used for many other types of products, for example, in cosmetics or also for pharmaceutical products.

Future Aspects

Through this work it could be shown that the choice of polymer plays an extremely important role in the design of sensors. However, you could also notice that different combinations of polymers (PLA and PLGA) and additives hugely influence the metabolism of the bacteria. The microbiological protocols developed or optimized during this work showed an existence of correlation between bacterial counts and the amount of produced enzymes. I think that this could be a great starting point for development of new microbiological tests, using only these bacterial characteristics and their reactions in contact with certain polymers, additives or both. It is already known that bacteria during their growth and under enzyme secretion may change the pH value of the growing media. Here presented results and observations of bacterial behavior, above all for Gram negative bacteria, show that polymers such as PLGA or PLA could also be a good growing medium. The possible induction of microorganisms for better enzyme excretion by addition of different agents or even through their genetic modification could also be used for production of enzymes needed in daily scientific research when their costs are too high using conventional methods.

Appendix

Agars

Plate Count Agar

Composition (g/L): Tryptone 5,0; Yeast extract 2,5; Dextrose 1,0; Agar 9,0;

pH=7,0 ± 0,2.

Suspend 17,5 g of the Plate Count Agar in 1 liter demineralized water and autoclave for 15 minutes at 121°C. In this way prepared plates can be stored for three weeks at 4°C. Total bacterial count is possible to be seen first after 48-72 hours of incubation at 30 °C.

ÖNÖZ Agar (Merck)

Composition (g/L): Meat extract 6,0; Yeast extract 3,0; Peptone from meat 6,8; Lactose 11,5; Sucrose 13,0; Bile salt mixture 3,825; tri-Sodium citrate 5,5-hydrate 9,3; Sodium thiosulfate 5-hydrate 4,25; Sodium green 0,00166; Neutral red 0,022; Aniline blue 0,25; Metachrome yellow 0,47, di-Sodium hydrogen phosphate 2-hydrate 1,0; L-Phenylalanine 5,0; Iron (III) citrate 0,5; Magnesium sulfate 0,4; Agar-agar 15,0; pH=7,1± 0,2.

Suspend 80.5 g of the Salmonella Agar in 1 liter of demineralized water by heating in a boiling water bath. Incubate the medium plates, containing a pure bacterial culture, at 35-37 °C for 24-48 hours.

Appendix

GSP Agar (Merck)

Composition (g/L): Sodium-L(+) glutamate 10,0; Starch, soluble 20,0; Potassium dihydrogen phosphate 2,0; Magnesium sulfate 0,5; Phenol red 0,36; Agar-agar 12,0; pH=7,0 $\pm$ 0,2

Suspend 45 g of the GSP Agar in 1 liter of demineralized water and autoclave for 15 minutes at 121 °C. *Incubation time: 72 hours at 28 °C.*

Tributylin Agar (Acila Sarl)

Composition (g/L): Peptone 5,5; Yeast extract 3,5; Agar 15,0; pH 7,5 $\pm$ 0,2

Suspend 24 g of powder in 1L ddH₂O and heat to dissolve completely, add 10 mL tributyrin. Sterilize by autoclaving at 121°C for 10 minutes, mix and pour very hot into plates. *Incubation time: 24 hours at 30°C and 37°C and 120 hours at 4°C*

Spirit Blue Agar (Difco)

Composition (g/L): Pancreatic Digest of Casein 10,0; Yeast extract 5,0; Agar 20,0; Spirit Blue 0,15; pH 6,8 $\pm$ 0,2

Suspend 35 g of the powder in 1 L of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121°C for 15 minutes. Cool to 50-55°C. Aseptically add 30 mL lipid source and mix thoroughly. *Incubation time: 24 hours at 30°C and 37°C and 120 hours at 4°C*

Liquid Culture Media

Peptone Water

Composition (g/L): Tryptone 10.0; sodium chloride 5.0; di- sodium hydrogenphosphate x 2 H₂O 9.0; Kalimdihydrogenphosphate 1,5.

Dissolve all named components in demineralised water and autoclave for 10 minutes at 121 °C. (pH=7,2±0,2).

LM Medium

Composition (g/L): Tryptone 10.0; yeast extract 10.0; di-sodium hydrogenphosphate x 2H₂O 0.4.

Dissolve all components in demineralised water and autoclave for 10 minutes at 121 °C.

EM Medium

Composition (g/L): Tryptone 12.0; yeast extract 0.1; NaCl 0.5.

Dissolve all above components in demineralised water and autoclave for 10 minutes at 121 °C

Ringer Solution

Composition (g/L): sodium chloride 2.25; potassium chloride 0.105; calcium chloride x 6H₂O 0.12; sodium hydrogen carbonate 0.05; pH=7.0±0.2

Dissolve all above components in distilled water and autoclave for 15 minutes at 121 °C.

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Publications and Patents

Patents

- December 2007: „Device comprising two reflecting layers and a polymer layer for analyzing the age and/or quality of natural products“, U7098/DB, PCT/EP2007/063895, Patent pending.
- November, 2008: Ibrisimovic N., Barth M, Bohrn U., Ibrisimovic M., Pittner F. (2009) “Device comprising a polymer layer and a reflecting layer”, U 7145/ DB: patent pending

Publications

- „Sensor for real-time monitoring of food degradation“ **N. Ibrisimovic**, M. Bauer, F. Pittner, „Nanobiotechnology“, June 2008
- „Biomimetic sensor chip monitoring real-time food degradation: correlating chemical deterioration with microbiological status“ **N. Ibrisimovic**, M. Barth, U. Bohrn, M. Ibrisimovic, F. Pittner, *Chemical Monthly*, 2009

Poster presentations

- “Direct detection of the degradation of food by a specific colour change”, **N. Ibrisimovic**, M. Bauer, F. Pittner, „3rd International Symposium on Recent Advances in Food Analyses“ , Prague, Czech Republic, November 2007
- „Sensors for real-time monitoring of food degradation“ **N. Ibrisimovic**, M. Barth, M. Bauer, F. Pittner, „ Nanotech 2008“ Boston USA, June 2008

Curriculum Vitae

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1989 -1992: Elementary School „Nasto Nakic“ , Brcko, Bosnia and Herzegovina

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June 11th, 2001: High School final examination and selection for the best student of the generation

Curriculum Vitae

October 1st, 2001: Start of my Studies in Chemistry (major subjects: biochemistry and analytic chemistry) at University of Vienna

November, 2006 - June, 2007: Master thesis: „Quantification of biodegradable nanoparticles from PLGA (poly-lactic-co-glycolic acid)“, under supervision of Univ. Prof. Dr. Fritz Pittner (Department of Biochemistry and Molecular Cell Biology; University of Vienna) and Univ. Prof. Dr. Franz Gabor (Department of Pharmaceutical Technology and Biopharmacy; University of Vienna).

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July 2nd, 2007 - July, 2009: Ph.D. in Biochemistry, University of Vienna.

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Work experience

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German: fluent spoken and written

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Selected Publication

- „Biomimetic sensor chip monitoring real-time food degradation: correlating chemical deterioration with microbiological status” **N. Ibrisimovic**, M. Barth, U. Bohrn, M. Ibrisimovic, F. Pittner, *Chemical Monthly*, 2009

Biomimetic sensor chip monitoring real-time food degradation: correlating chemical deterioration with microbiological status

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Keywords: biomimetic sensor chip, freshness sensor, meat decay monitoring, metal nanocluster sensor chip

Abstract

Conventional tests, currently in use for the detection and identification of food borne pathogens and also of microbial food deterioration are time consuming as they are based on conventional culturing techniques or monitoring environmental conditions like the increase of temperature or change in pH. These parameters do not reflect the real quality of the meat to be tested.

The aim of our approach was to create a simple and cheap sensor providing reasonable sensitivity and selectivity to indicate the bacterial infection in real time monitoring combined with a memory effect that cannot easily be corrupted.

Thus an optical thin film sensor chip was developed able to detect bacterial decay of food through a specific colour change. The design of the sensor relates to the phenomenon of “anomalous absorption”, which can best be described as a thin film enhanced absorption. A metal nanoparticle layer positioned at a well defined distance to a smooth metal surface shows that the minimum of spectral reflectivity strongly depends on the thickness of the

distance layer. This setup represents a special kind of reflection interference filter. In such a sensor setup a biomimetic polymer is integrated which is degradable by lytic enzymes excreted by microorganisms in food decay. Meat deterioration under controlled conditions is correlated to the amount of enzymes secreted by microorganisms and the bacterial count. Thus, after incubation of the sensor setup with standard meat preparations, the enzymes released from decaying cell material change the thickness of the polymer layer and generate an easily visible colour change. This setup would be useful for integration into meat packaging.

Introduction:

Standard methods for the detection and identification of food degradation show high accuracy and low detection limits, but are generally expensive, time-consuming and require the use of highly trained personnel and laboratory equipment. The current tendency to carry out field monitoring has driven the development of biosensors as new analytical tools able to provide fast, reliable and sensitive measurements with lower cost. These biosensors for the moment do not compete with official analytical methods, but they can be used both – by regulatory authorities and by industry to provide enough information for routine testing and screening of samples [1].

Development and improvement of food processes is driven by the need for healthier, safer, more convenient, competitively superior and seasonally invariant foods and also more efficient processing plants with reduced waste. Online optical instruments such as refractometers, spectrophotometers, turbidity meters etc. that may be used to assess food composition have been developed. However, their applicability is often limited because of the complex composition of food interfering with the measurement and much stress has to be laid on correct sample preparation.

Available quick tests for food decay using parameters such as time, temperature or pH change are not really correlating to bacterial contamination.

Monitoring of pH gives only indirect information and most tests are reversible, and therefore generally unapt to contribute to the safety of the meat supply chain. Microbiological tests concerning bacterial counts or strains are very time consuming as they rely on conventional culturing techniques and thus are far from real time monitoring. Especially when a consumer wants to get information on the quality of what he is buying in the shop, a quick real time test integrated into the package would be of high interest, as well as also the producer can show the hygienic level of his production line by such a device.

Meat decay is caused mainly by microorganisms excreting lytic enzymes. Both, rate of bacterial growth and enzymatic action are functions of time and temperature. Increasing temperature raises the amount of microorganisms and also the activity of the secreted enzymes, thus enhancing enzymatic deterioration of meat tremendously. (Fig. 1)

<Figure 1>

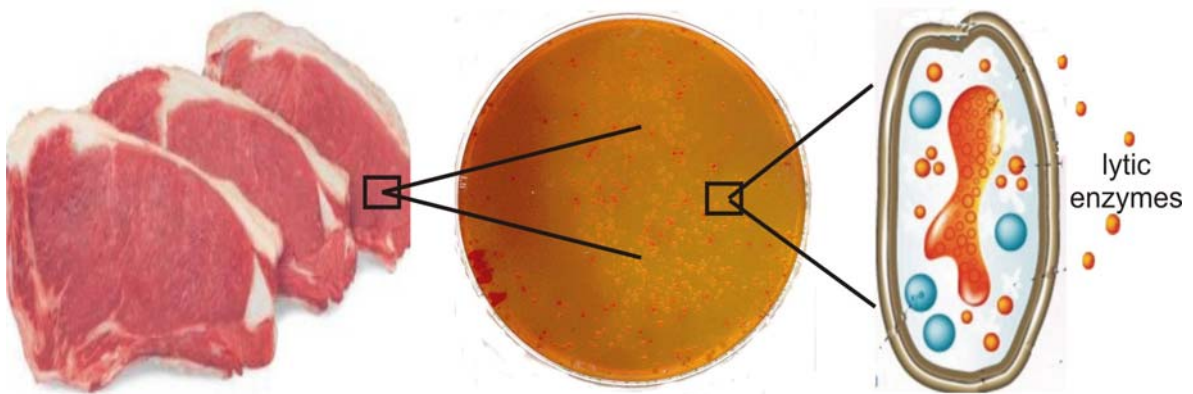


Fig. 1. Bacterial growth on meat surface causes excretion of lytic enzymes

In the last decades, the combination of knowledge in electrochemistry, biochemistry, physics and integrated circuit silicon technology made it possible

to provide highly specific, sensitive, selective, accurate and reliable microsensors [2 – 6].

Due to the special optical behaviour of a metal island film and due to the thin film set up, this system shows characteristic spectral reflection behaviour, strongly dependent on the thickness of the transparent distance layer [7], in our case formed by the biomimetic polymer. The optical property of metal island films, necessary for our application, is the so called “Plasmon absorption”, a strong, broad-band absorption in the visible, due to the confinement of the conduction electron plasma in nanometric particles. This is in contrast to the unconfined electron movement in an extended metal, responsible for strong, unspecific reflectivity, well known as metallic glance. An absorbing thin film positioned at a defined distance to a metal mirror represents a special kind of reflection interference filter. At an appropriate distance of the absorbing layer to the mirror, fields reflected by the mirror have the same phase at the position of the absorbing layer as the incident fields and, thus, by this feedback mechanism the effective absorption coefficient of the absorbing layer is strongly enhanced. This combination of the two phenomena plasmon absorption and optical interference is generally called “anomalous absorption”.

Fig. 2 shows the visual impression obtained by observation of the reflected light upon diffuse white-light illumination of the layer system used in our sensor set up in the interlayer optical thickness range 0 – 490 nm (optical thickness = geometrical thickness x effective refractive index) and some corresponding, measured reflection spectra.

<Figure 2>

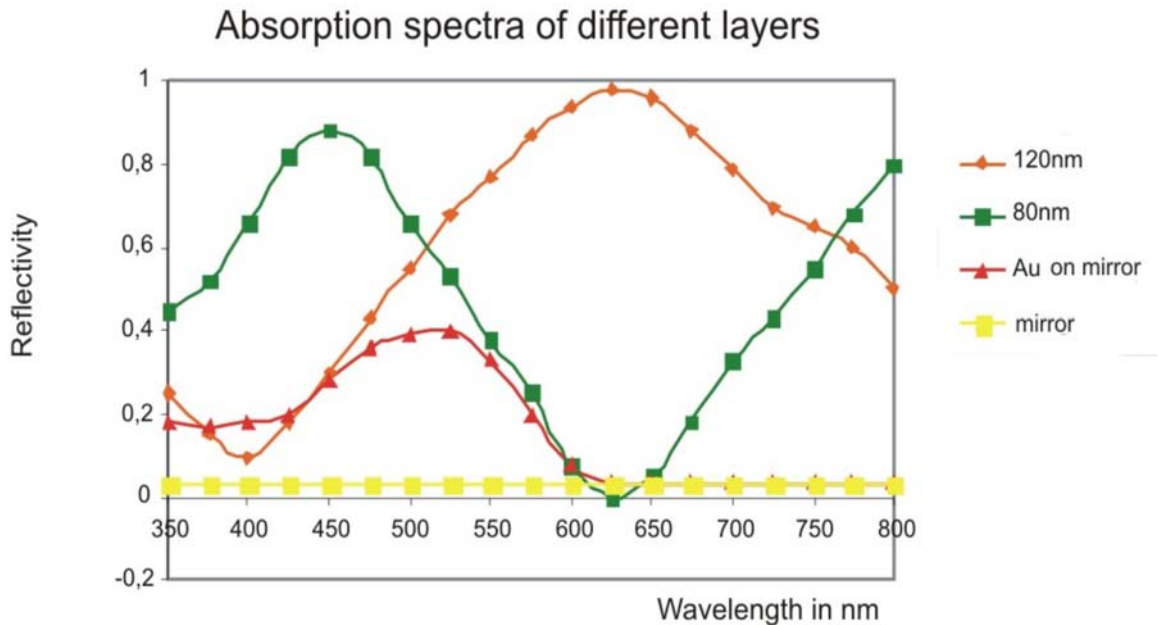


Fig. 2: Colour of the sensor as a function of thickness of the distance layer

Results and Discussion:

The setup of the sensor measuring bacterial decay of food is shown in Fig. 3 and assembled according to [8]. It has to be in permanent physical contact with meat or meat juice so that the lytic enzymes are able to diffuse through the highly permeable nanoparticle layer thus reaching the distance layer. It consists of a biomimetic polymer within a nanometric range and thus shows very fast response. The polymer chains are cleaved resulting in altered layer thickness and therefore a change in the colour can be observed by the naked eye. The thickness of the distance layer can be tuned by the viscosity and composition of the polymer solution and by the parameters of the printing process.

<Figure 3>

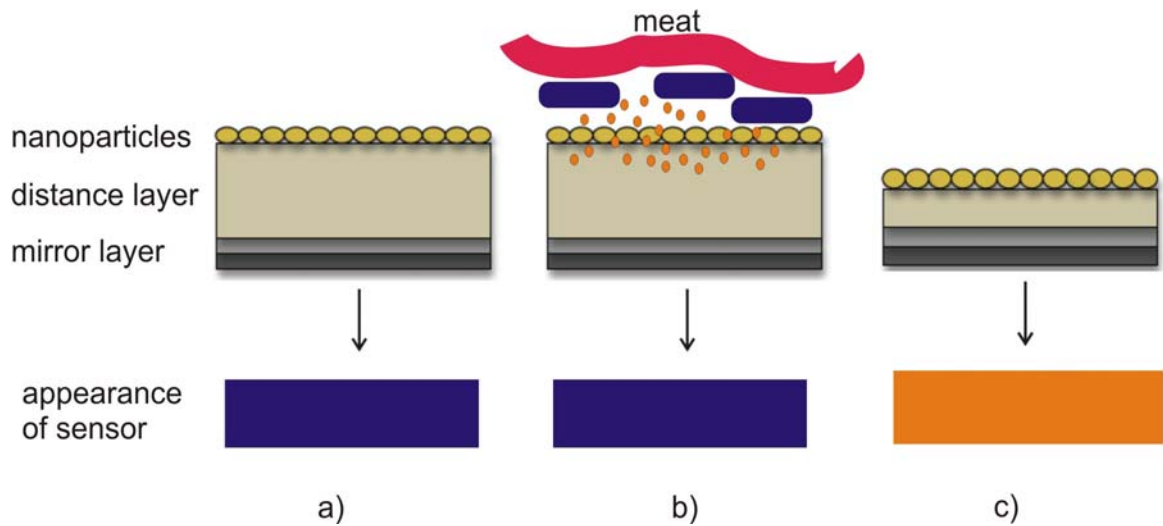


Fig. 3. Setup and function of the sensor: a) sensor setup b) sensor in contact with meat and bacteria c) sensor after degradation of the biomimetic polymer. The colours shown in the lower level are only examples and are dependent on the conditions of production.

The here presented biomimetic sensor for food is irreversible and therefore suitable for single use only. Another big advantage of this new sensor system is the cost efficiency for its production. Every step of the sensor production has been carried out on industrial testing equipment and with coating procedures like physical vapour deposition and gravure printing. These production methods guarantee very good economies of scale and thus low cost per unit.

Correlation of colour change and microbiological parameters:

For a functional assay different solutions were prepared as described in Table 1. The respective solutions (1 mm^3) were pipetted onto the sensor surface and then the sensors were incubated in a humidity chamber (over night at 4°C and in parallel also at room temperature). After incubation time the sensor surface was washed with ddH₂O and dried by an airstream.

<Table 1>

Table 1. Pipetting scheme:

	Column 1	Column 2	Column 3
1	Bacteria-mix* (10^8 CFU/mm^3)	Homogenate II (10^8 CFU/mm^3)	Enzyme-mix** (20 mg/mm^3)
2	Bacteria-mix* (10^6 CFU/mm^3)	Homogenate II (10^6 CFU/mm^3)	Enzyme-mix** (15 mg/mm^3)
3	Homogenate I (10^8 CFU/mm^3)	Pseudomonas (10^7 CFU/mm^3)	Enzyme-mix** (10 mg/mm^3)
4	Homogenate I (10^6 CFU/mm^3)	Pseudomonas (10^6 CFU/mm^3)	Enzyme-mix** (5 mg/mm^3)
5			Ringer Solution (neg. control)

* Equal amount of *Pseudomonas*, *Enterobacter*, *Proteus* and *Salmonella*.

**Equal amount of Proteinase K, Chymotrypsine and Trypsine.

Documentation of the results was done by scanning the sensors on a flat bed scanner (HP Scanjet 4890).

Fig. 4 shows the colour changes as a result of enzymatic degradation of the sensor after overnight incubation at room temperature. This degradation is highly selective and very sensitive; negative control by Ringer solution showed no degradation at all.

Pure bacteria mix and meat homogenate I (incubated for 40 h at 25°C with bacteria mix) with a concentration of 10^6 CFU/mm^3 as was dotted in column 1 did not give signals under these conditions, only meat homogenate I with 10^8

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CFU/mm³ (column 1, line 3) shows a barely visible signal. Homogenate II (incubated for 11 days at 4°C, 10⁶ CFU/mm³) and *Pseudomonas* (10⁷ CFU/mm³) in column 2 as well as all concentrations of enzyme mix (column 3) show signals clearly visible by the naked eye. The blue dot for the highest enzyme mix concentration results from higher degradation degree than performed by other samples, thus generating another colour.

What looks strange at the first glance in column 2 is the fact that homogenate II (10⁸ CFU/mm³) performed worse than that with the lower count (10⁶ CFU/mm³). The explanation is that serial bacteria levels in the meat juice beyond the logarithmic stage die off and lead to a lower response of the sensor chip than expected. This clearly shows that spot tests as performed here with meat juice containing bacteria in their logarithmic stage are only useful for quick testing of sensors to optimise their properties.

Preliminary experiments show that, for real time monitoring in situ, the sensors have to be kept closely in contact with the meat surface, since only there bacteria will grow freely without substrate depletion. The growing bacteria responsible for meat decay will alter the thickness of the interlayer by excreted lytic enzymes. As the gained signal is irreversible a colour change can be seen also in case of already died off microorganisms. Studies on this topic are already in progress and will be published soon in details.

<Figure 4>

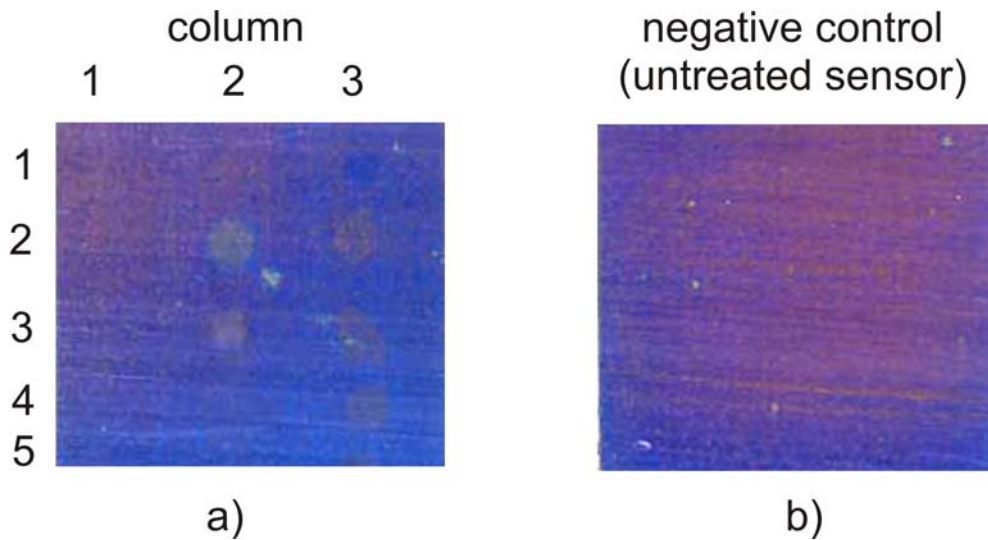


Fig. 4. Sensor response a) after overnight incubation at 25°C b) negative control (untreated sensor). The spots were applied according to the pipetting scheme as given in Table 1. The stripe pattern derives from the gravure printing process.

Conclusion: In this work we could present a biomimetic sensor prototype for monitoring cumulative impacts caused by temperature, bacterial growth and lytic enzyme action. The deterioration of meat can be followed via colour change visible for the naked eye in real time.

This sensor prototype may be adapted for different potential commercial applications as e.g.: facilitated quality control in store and also for shelf life. This gives the consumer insight on the actual freshness of the respective product and the provider the guarantee to having sold intact food. Beyond that the producer can proof optimum hygienic conditions during packaging with the help of the sensor.

Materials and Methods:

The sensor setup is performed according to [8].

Gravure Printing, also known as Intaglio printing, is characteristically used for long run, high quality printing producing a sharp, fine image (Fig. 5). This is accomplished by cutting or engraving and etching various sizes or depths of minute cells (or wells) below the surface of a plate or cylinder. The depth and size of each cell determines the amount of liquid, in our case the biomimetic polymer solution, that can be transferred to the print surface. The cells are flooded and loaded with this solution. The excess is scraped off the surface of the plate by a doctor blade, and what is left in the cells is transferred to the substrate. The nature of the process permits the formation of a layer with the desired thickness. A semi automatic gravure printer for laboratory use from Erichsen (Germany) was used. By this procedure the biomimetic polymer of the distance layer was printed onto the mirror coated substrate.

<Figure 5>

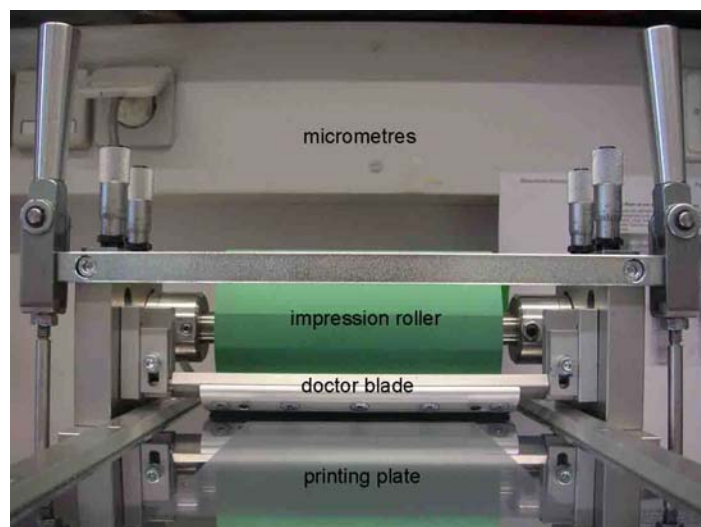


Fig. 5. Gravure Printer (K Printing Proofer 628 by Erichsen)

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Sputter Coating: Nanoparticles were applied by an Agar sputter coater as used for electron microscopy. In conventional SEM sputter coating techniques gold is bombarded with heavy gas atoms (Ar). Metal atoms ejected from the Au source by the ionized gas cross the plasma to deposit onto the sensor surface. Thus the nanoparticle layer responsible for the anomalous absorption was generated. This allowed also for visualizing the homogeneity of the printed layers, as changes in thickness of the polymer layer by imperfect printing procedures result in inhomogeneous colours of the sensor.

The whole sensor setup is shown in Fig. 3.

Sampling procedure, strain isolation and growth conditions

Gram-negative aerobic bacterial strains (*Pseudomonas*, *Enterobacter*, *Proteus*, *Salmonella*) were isolated from spoilt meat juice (incubated for 5 h at 37°C) using Salmonella Agar acc. to ÖNÖZ (Merck).

<Table 2>

Table 2. Characterisation of microorganism colonies

Microorganisms	Appearance of colonies
<i>Pseudomonas</i>	Glossy, dirty yellow to greenish; culture medium is yellow
<i>Enterobacter</i>	Large, mucoid, bluish or reddish, slight precipitation ring around the colonies
<i>Proteus</i>	Rust-coloured, culture medium surrounding the colonies of same colour, if growth is too dense, dark brown to black
<i>Salmonella</i>	Yellow, medium size; 1 st day: black dots start to develop on the yellow colonies; 2 nd day: black dots clearly visible on the yellow colonies; culture medium surrounding the colonies is yellowish

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A colony picked from the selective media was inoculated in 3 cm³ pepton water (peptone 0.1%, NaCl 0.5%, Na₂HPO₄·12H₂O 0.9% and KH₂PO₄ 0.15% all in dd H₂O) and allowed to grow overnight at 37°C. Then 2 cm³ of the bacterial pre-culture was inoculated in 200 cm³ pepton water and incubated at 37°C for 24 h. Aliquots (1 cm³) were frozen in liquid nitrogen and stored at -80°C. After incubation (up to 3 days at 30°C) bacterial counts were determined by plating 100 mm³ bacterial cultures onto Plate Count Agar (*Fluka*). The culture dilutions (10⁻¹ to 10⁻⁹) were prepared in Ringer Solution.

Preparation of homogenised infected meat

Pork cutlets (100 g) were infected with nine different bacterial strains that can be found in spoilt meat (*Serratia liquefaciens*, *Lactobacillus sakei*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides*, *Brochothrix thermosphacta*, *Acinetobacter Iwoffii*, *Pseudomonas lundensis*, *Pseudomonas fragi*, *Pseudomonas fluorescens* from DSMZ, the German Resource Center for Biological Materials), and incubated for 40 h at 25°C (homogenate I) or 11 days at 4°C (homogenate II). After these periods of incubation, the meat was homogenised in 200 cm³ Ringer Solution using a homogenizer-mixer. Bacterial cocktail was filtered with miracloth and then frozen by liquid nitrogen and stored at -80°C. Bacterial counts were performed, as described above, using the Plate Count Agar (*Fluka*).

Acknowledgements: We gratefully thank AGES (Austrian Agency for Safe Nutrition) for financial support, providing bacterial strains and giving feedback on microbiological problems.

Selected Publication

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