Master's Degree

Food Science and Technology Engineering

# Development of an enzymatic biosensor for nitrogen control for alcoholic fermentation


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**Budapest**

**2023**

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# Abstract

The aim of the present study has been to develop and optimize an electrochemical bienzymatic biosensor for the control of ammonium in must grape during alcoholic fermentation**.**

The biosensor has been developed with glutamate dehydrogenase (GLDH) and diaphorase (DP) enzymes immobilized on screen-printed carbon electrode (SPCE) modified with polyaniline/poly (2-acrylamido-2-methyl-1-propane sulfonic acid) (PAAMPSA).

The conductive polymer electrodeposited on the working electrode surface was able to increase the electrochemical properties of the biosensors and allowed the detection of NADH at low potential (0.1 V) in a linear range from 0.005 to 0.5 mM with a sensitivity of 1000.9 nA/mM and a limit of detection of 5 µM.

The electrochemical oxidation of NADH was used as an indicator reaction for the quantification of ammonium concentration. The bi-enzymatic biosensor has been optimized concerning GLDH/DP enzymatic unit ratio, as well as for NADH and alpha-ketoglutaric acid.

Under optimized conditions, ammonium was detected in a linear range from 1 µM to 200 µM (R2=0.9992), with a detection limit of 1 µM and a relative standard deviation (RSD) of 4,66 % (n=6 biosensors).

Finally, the biosensor has been applied to alcoholic solutions with three different amounts of ammonium. Based on these results, it is possible to assert that the biosensor can be considered a fast analytical technique for the control of ammonium in food samples.

# Introduction

Ammonia is a compound that is widely used in the agricultural, chemical, and automotive industries. It can also be found in nature in human and animal excretions, industrial waste, and wastewater treatment facilities [1]. It is recognized as a highly toxic compound where in excess it can alter the acidity of the soil and this will disrupt the nutrition cycle and the ecological balance [2]. Ammonia nitrogen is at trace level, and its concentration is easy to change [3]. It also occurs in significant quantities during alcohol production and fermentation, which can adversely affect quality or other production conditions. Therefore, the control of nitrogen during fermentation is an especially important parameter.

Alcoholic fermentation is the anaerobic conversion of sugars, primarily glucose and fructose, into ethanol and carbon dioxide. This overall reaction can be used to summarize the process.

$$C_6H_{12}O_6 \rightarrow 2\,CH_3CH_2\mathrm{OH} + 2CO_2$$

The alcohol fermentation is a much more complex process. Besides ethanol, several other compounds are produced throughout alcoholic fermentation such as higher alcohols, esters, glycerol, succinic acid, diacetyl, acetoin and 2,3-butanediol. During the process, various bacteria mainly divert pyruvate to produce ethanol to regenerate the $NAD^+$ consumed by glycolysis. This process, called alcoholic fermentation, is shown in Figure 1. [4].

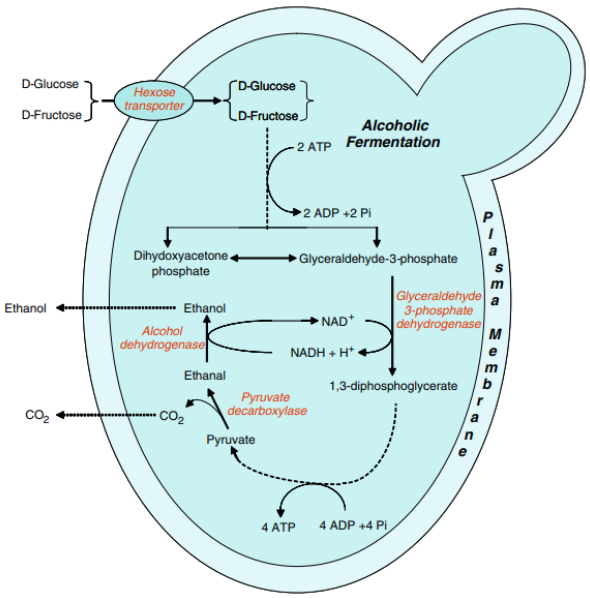


*Figure 1: alcoholic fermentation [4]*

Pyruvate is initially decarboxylated into ethanal by pyruvate decarboxylase. This enzyme needs magnesium and thiamine pyrophosphate as cofactors. Thereafter, alcohol dehydrogenase

reduces ethanal to ethanol, recycling the NADH to $NAD^+$. There are three isoenzymes of alcohol dehydrogenase in Saccharomyces cerevisiae, but isoenzyme I is chiefly responsible for converting ethanal into ethanol. Alcohol dehydrogenase uses zinc as cofactor. Both final products of alcoholic fermentation, ethanol and carbon dioxide, are transported outside the cell by simple diffusion [4].

Nitrogen is an essential nutrient for yeast during alcoholic fermentation. Nitrogen is involved in the biosynthesis of protein, amino acids, nucleotides, and other metabolites, including volatile compounds [5]. The importance of nitrogen control is very important which have been well described in winemaking. When *Saccharomyces cerevisiae* grows in grape juice it needs significant amounts of assimilable nitrogen to synthesize biomass. Numerous nitrogen compounds, including ammonia, amino acids, peptides, proteins, and others, are present in grape juice, but only some of them can be assimilated by Saccharomyces cerevisiae. Saccharomyces cerevisiae can only use ammonia and amino acids as assimilable sources of nitrogen when fermenting grape juice, with the exception of proline. Saccharomyces cerevisiae can assimilate proline, but only when aerobic conditions are present. This is why all ammonia and amino acids, with the exception of proline, have been referred to collectively as easily-assimilable nitrogen (EAN).

The formol index makes it easy to determine this EAN. Ammonia and amino acids are not found in big quantities in grape juice. As a result, in some circumstances, the yeasts' absorption of these substances may be a crucial step. At a low concentration of EAN the risk of stuck and sluggish fermentations is really high. For that reason, winemakers used to add ammonium salts to grape juice as a supplement. Depending on the yeast strain and the potential alcohol content, the EAN quantity is required to complete an alcoholic fermentation. Generally, it is considered that an EAN lower than 130 mg/l can seriously affect the correct development of alcoholic fermentation. In contrast, excessive nitrogen can lead to the presence of non-assimilated residual nitrogen in the wine, which is a factor in microbiological instability and can even favour the production of ethyl carbamate and biogenic amines. For that reason, nitrogen must be supplemented carefully and taking into account the initial EAN concentration of grape juice and its potential alcoholic degree [4, 5].

The first step in nitrogen assimilation is its transport inside the cell. Ammonium ion is transported inside the cell by facilitated diffusion. However, the intracellular pH causes ammonium ion to release a proton, which must be sent outside the cell via $H^+$-ATPase. Once inside the cytoplasm, ammonia is incorporated to the amino acid pool via glutamate

dehydrogenase or via glutamine synthetase, thus producing glutamate or glutamine respectively. On the other hand, amino acids are transported inside the cell by different transporters. The transport systems for amino acids in Saccharomyces cerevisiae are symport systems coupled to the entry of a proton. This proton must also be sent outside the cell in order to maintain the cellular homeostasis. Therefore, the uptake of ammonium and amino acids must be considered as active transport because it consumes ATP via H+-ATPase. Saccharomyces cerevisiae can use all amino acids, with the exception of proline, in the fermentation of grape juice. Proteins can be created directly from amino acids. However, the grape juice's amino acid composition may not exactly meet the needs of the cell. Because of this, yeasts must use the remaining amino acids to make up for the ones they don't have. In this case, ammonia is incorporated into other amino acids whereas the carbon skeleton is metabolized by the cell. For this reason, the lack of enough EAN can make the yeast use sulphur amino acids (cysteine and methionine), thus releasing hydrogen sulphite and mercaptans. Thus, supplementing with ammonium salts is advised to stop reduction off-odors as well as stuck and sluggish fermentations [4, 5].

The importance of enzyme based amperometric biosensor has increased during the past decade due to high selectivity of the bio-recognition element and the sensitivity of the electrochemical signal transduction. Amperometric quantifications with biosensor or bioassays using enzymes $NAD^+$/NADH dependent are generally based on the electrochemical oxidation of NADH produced during the enzymatic reaction. In theory, the direct oxidation of NADH at the electrode surface can be used as an indicative reaction and high over potential is required. However, this method will lead to electrode fouling as well as interferences from electroactive species. To avoid such undesirable phenomena and to increase the biosensor selectivity, electron transfer mediators have been used. Electron transfer mediator for the NADH oxidation can function as one electron oxidant, e.g., ferrocene and its derivatives. Enzymes that are frequently employed for catalyzing electron donation from NADH to several kinds of mediators including artificial dyes are diaphorase or lipoamide dehydrogenase [6]. The development of modified electrodes for NADH oxidation has been focused upon different strategies for the attachment of these mediators to the electrode surface, including the use of mediator groups covalently bound to various types of polymer backbone and coated onto the electrode surface, the use of mediators attached to hydrophobic groups designed to promote adsorption at the electrode surface, and the incorporation of the mediators into carbon paste electrodes [7].

# Chapter 1. Biosensor Technology

## 1. Definition

A biosensor is an analytical device containing a bioreceptor, that is an immobilized sensitive biological element (e.g., enzyme, antibody, antigen, organelles, DNA probe, cells, tissues or organic molecules) recognizing the analyte (e.g., enzyme substrate, complementary DNA, antigen) in contact with or integrated within a transducer, which ultimately converts a biological signal into a quantitatively measurable electrical signal (Fig. 2). The intensity of generated signal is directly or inversely proportional to the analyte concentration [8], [9]. Due to their special characteristic, inherent simplicity, relative low cost, quick response, and propensity for miniaturization, biosensors can be seen as complementary tools to traditional analytical methods (such as high performance liquid chromatography), allowing continuous monitoring [9].

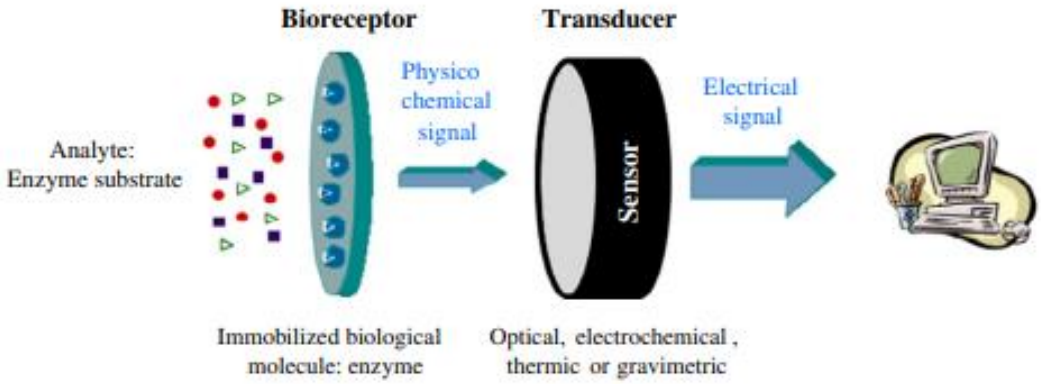


*Figure 2: Scheme of a biosensor*

## 2. Classification

Biosensors can be classified according to the mode of physicochemical transduction or the type of biorecognition element.

### 2.1 Classification according the transduction technique

Biosensors can be categorized as electrochemical, optical, calorimetric (thermometric), or piezoelectric depending on the transducer.

#### Electrochemical

Electrochemical biosensors are the most extensively investigated biosensors as they offer the advantage of low detection limit, specificity, simplicity of construction, and ease of operation [10]. These biosensors can be handheld devices for on-site monitoring or lab-on-chip

devices for in vivo monitoring thanks to recent advancements in electronic instrumentation [11]. Electrochemical biosensors can be further classified as amperometric biosensors, potentiometric biosensors, conductometric and impedimetric biosensors (Fig.3) [10].

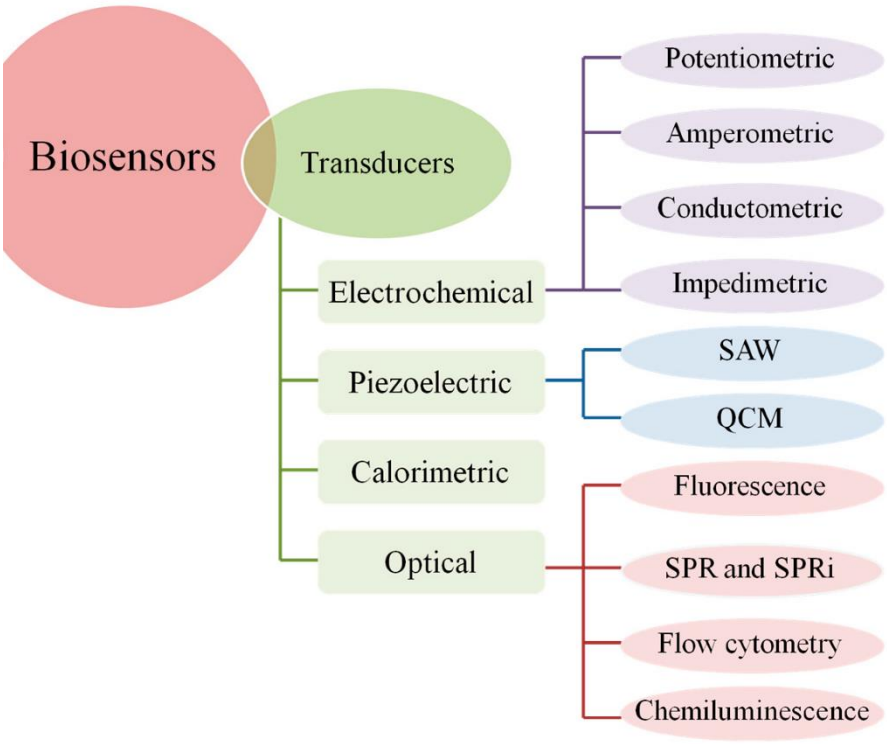


*Figure 3: Classification of biosensors*

## Amperometric biosensor

The most common type of biosensors is amperometric ones. The majority of biochemicals can now be detected and measured amperometrically through electro-oxidation or electroreduction that is catalyzed by an enzyme, hydrolysis or phosphorylation that is catalyzed by an enzyme and then electro-oxidation or electroreduction, or participation in a bio affinity reaction that promotes electro-oxidation or electroreduction. Compared to potentiometric biosensors, amperometric ones are much more sensitive and better suited for mass production [12]. The most investigated amperometric biosensor system is the glucose biosensor. In this system, glucose oxidase (GOx) catalyzes the reaction of glucose with oxygen to produce gluconolactone and hydrogen peroxide. The signal is usually depicted as current (Ampere) against concentration of glucose caused by a redox reaction of a mediator or hydrogen peroxide at the working electrode [13].

Typically, the working electrode is a noble metal or screen-printed layer covered by the bioelement. Other option is to use carbon nanotubes. At the applied working potential, conversion of electroactive species generated in the enzyme layer occurs at the electrode and the resulting current (typically from nA to μA range) is measured.

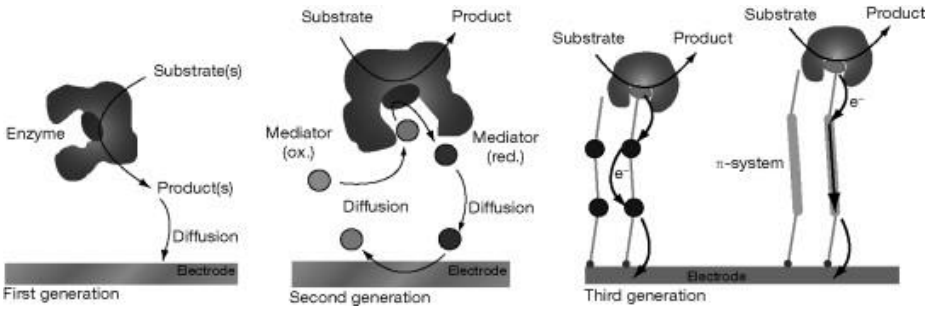


*Figure 4: Amperometric biosensor*

Amperometric biosensors use mediators, or molecules capable of transferring electrons, in their biochemical reaction. They can assist in a quicker electron transfer and take part in the redox reaction with the biological component. They can be described as a low molecular weight redox couple, that transports electrons from the enzyme's redox center to the surface of the indicator electrode. As a result, we can work with low potentials, thus the influences of oxygen (in case of oxidase) and of different interferants on response decrease. An ideal mediator should have reversible heterogeneous kinetics, be stable, react quickly with the target molecule, have a low overpotential for regeneration of the oxidized mediator, be pH independent, and not react with oxygen in its reduced form. Mediators allow to measure at low working potentials and to avoid the interference with unwanted species. Measurements are thus less dependent on oxygen concentration and if the electrochemical reaction does not involve protons, the enzyme electrode becomes relatively pH insensitive. Inorganic redox ions like ferricyanide, the organometallic compound ferrocene, or organic dyes like methylene blue, toluidine blue, or Prussian blue are frequently used as mediators [12].

## Potentiometric biosensor

This transducer measures difference in potential that is generated across an ion-selective membrane separating two solutions at virtually zero current flow [12]. The mechanism behind such biosensors is the change of the electrochemical potential of the electrode coating materials [13]. Almost all potentiometric sensors, including ion-selective electrodes, metal oxide-based sensors, and glass electrodes, are obtainable in the market. Additionally, by utilizing cutting-

edge contemporary silicon or thick-film technologies, they can be produced in large quantities in miniature formats [12].

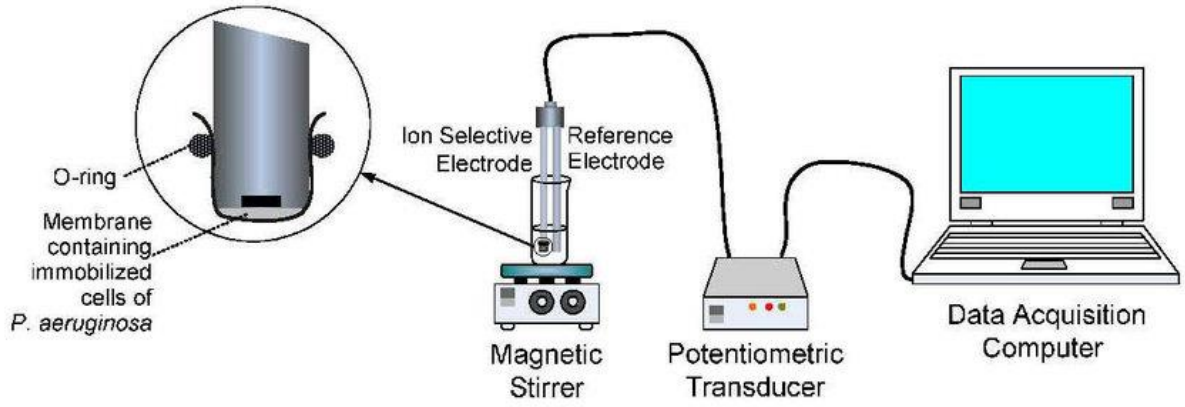


*Figure 5: Potentiometric biosensor*

## Conductometric biosensor

The conductometric measuring method can be used in enzyme catalysis to determine substance concentration and enzyme activity, selectivity in this case being provided by the enzymes, which catalyze only certain reactions [14]. The overall conductivity or resistivity of the solution changes when ions or electrons are generated as a result of a biochemical reaction. The electrical conductance/resistance of the solution is the parameter that is measured when using this transducer. Measurements of conductance are not extremely sensitive. When using sinusoidal voltage (AC), an electric field is created, reducing undesirable effects like concentration polarization, double layer charging, and Faradaic processes. Conductance is the term used to describe the inverse of resistance, which is why the term conductometric has been adopted [12]. Another disadvantage of conductometry is signal and noise level. The ratio between the level should not be lower than 2%. This makes the buffer and other ingredient concentrations that can be added to the reaction mixture crucial. If non-reacting ions are present in the solution, the method's sensitivity is decreased. However, low ionic strength buffers can be used to measure low concentrations until the signal to noise ratio is at the right level [14].

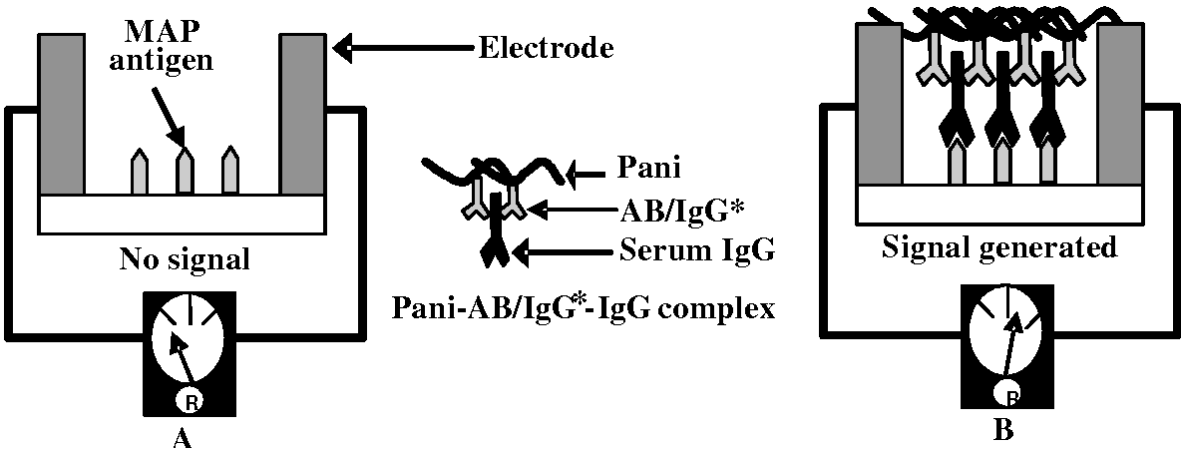


*Figure 6: Conductometric biosensor*

## Impedimetric

Electrochemical impedance spectroscopy (EIS) involves measuring resistive and capacitive changes caused by a biorecognition event. Typically, a small amplitude sinusoidal electrical stimulus is applied, causing current to flow through the biosensor. Typically, a conventional three-electrode system is made specific to the analyte by immobilizing a biorecognition element to the surface. A voltage is applied, and the current is measured. The interfacial impedance between the electrode and solution changes because of the analyte binding. An impedance analyzer can be used to control and apply the stimulus as well as measure the impedance changes.

## Optical

Optical biosensors technically fall within the larger category of electromagnetic detectors, but because of their significance and widespread application, they are sometimes regarded as a separate class of biosensitive devices [3].

Optical biosensors rely on measurement of light absorbed or emitted as a consequence of a biochemical reaction. Optical biosensors use a variety of optical techniques, including surface plasmon resonance (SPR), fluorescence, luminescence, absorption, etc. [10]. These biosensors can be created using electrochemiluminescence or optical diffraction [11].

Fluorescence is often used for biosensing due to its selectivity and sensitivity. A fluorescence-based device detects the change in frequency of electromagnetic radiation emission which is caused by previous absorption of radiation and also by generation of an excited state lasting for

a very short time. Single molecules may be repeatedly excited to produce a bright signal which can be measured even at single-cell level. Devices based on optical diffraction make use of silicon wafers that have been covalently covered with proteins. The wafer is exposed to UV light trough a photomask and the antibodies are thus inactivated in the exposed regions. Antigen-antibody bindings are formed in the active regions when wafer chips are incubated in an analyte. This allows creation of diffraction grating producing a diffraction signal when illuminated with a laser or other light source. Thus, obtained signal can be further amplified or directly measured. Fiber-optic biosensors (FOBS) use optical fibers for signal transduction and are dependable only on optical transduction mechanisms for detecting target biomolecules. Typical example of reliable and sensitive optical method is evanescent sensing. A majority of evanescent FOBS are tapered fiberoptic biosensors. SPR biosensors are optical sensors using special electromagnetic waves to monitor interactions between an analyte in solution and a bioelement immobilized on the SPR sensor surface [12].

The main application of this type of biosensors is the detection of biological analytes and analysis of biomolecular interactions where SPR biosensors provide advantage of label-free real-time analytical technology [12].

### Thermal

Thermal biosensors are based on measurement of the thermal changes occurring on biochemical recognition [10]. These biosensors are constructed by immobilization of biomolecules onto temperature sensors. The reaction heat, which is proportional to the analyte concentration, is detected after the analyte and the biocomponent come into contact. The molar enthalpy and total number of molecules involved in the reaction determine how much heat is generated or absorbed overall [11]. Most biological reactions result in a change in enthalpy, and sensitive thermistors can measure these heat changes [10]. Thermal biosensors don't need to be calibrated frequently and are insensitive to the optical and electrochemical properties of the sample. Calorimetric biosensors were employed for component analysis in the food, cosmetics, and pharmaceutical industries. [11].

### Piezoelectric

Piezoelectric biosensors involve measurement of mass change occurring as a result of biomolecular interaction. Piezoelectric crystals (usually a quartz-crystal coated with gold electrodes) [12] are employed to measure the mass change by correlating with the change in

oscillation frequency of the piezo crystal. Based on the type of biorecognition unit used, biosensors can be classified as enzymatic, nucleic acid–based, aptamer-based, antibody-based, whole cell–based biosensors [10].

The piezoelectric effect can be seen in a wide variety of materials, including quartz, tourmaline, lithium niobate or tantalate, orientated zinc oxide, and aluminum nitride. However, the properties of quartz are the main reason for its common usage for analytical applications. Piezoelectric transducers allow label-free detection of molecules [12].

With the application of an electrical signal with a specific frequency, these crystals can be made to vibrate at that frequency. Based on this, the mass of the crystal and the electrical frequency that is applied to it both affect the frequency of oscillation. With increasing of the mass due to binding of molecules, the oscillation frequency of the crystal is changed, and the resulting change can be measured electrically and finally used to determine the additional mass (both positive and negative one) of the crystal (mass-sensitive techniques). Since it was discovered that many opportunities for molecular sensing can be opened up once a suitable recognition layer or molecule is coated on the crystal, there has been a surge in interest in the application of piezoelectric devices. Additionally, piezoelectric biosensors demonstrated potential uses in the fields of food, the environment, and medicine [12].

### 2.2 Classification according the biorecognition element

Depending on the bioreceptor used, biosensors can broadly be classified into four classes: enzymes, antibody–antigen, nucleic acid/DNA and cells.

#### Enzymes

The first enzyme- based biosensor was reported by Updike & Hicks [15] using glucose oxidase for the measurement of glucose concentrations in biological solutions. In this kind of devices the enzymes are used for the selective detection of their competent substrate, in which the product obtained is directly determined using a transducer. These sensors are manufactured immobilizing enzymes on the working electrode of the sensor by techniques such as covalent binding, adsorption, entrapment, cross-linking, affinity or a combination of them. This step is important in order to build a device able to have high sensitivity, good storage stability, short response time and high reproducibility. Other factors that have influence on the performance of enzyme-based biosensors are the enzyme load, pH, temperature as well as the thickness of the enzyme layer on the electrode [9].

In general enzyme-based biosensors are the most common biological sensing devices due to the biomolecule specificity and biocatalytic activity. Examples of this are the commercially available glucometers (immobilized glucose oxidase or glucose dehydrogenase), where these devices represent about 90% of the global biosensor market [12].

### Antibodies

Antibodies also called immunoglobulins, are molecules composed by a severe number of amino acids and are involved in the defense mechanisms of the immune system against foreign substances called antigens. Antibodies have been extensively used as diagnostic tools in different fields, due to their high specificity to bind to a unique part (epitope) of their target molecule (antigen). This unique property is crucial for their use as immunosensors where only the specific analyte of interest fits in the antibody binding site [16].

The performance of an immunosensor is directly affected by the orientation of the immobilized antibody on the solid surface. This can be controlled by the interaction between specific reactive compounds on the electrode surface and the antibody, being possible to perform the functionalization of the sensor surface with specific groups such as glutaraldehyde, carbodiimide, etc. The favorable orientation of antibodies can directly contribute to optimum immunosensor performance [17]. Moreover, if the antibody is monoclonal (have affinity for the same antigen epitope), or polyclonal (affinity for the same antigen but different epitopes) the results and performance of the immunosensor change dramatically.

### Nucleic Acids

The sensitivity and selectivity of DNA and RNA based biosensors is given due to the strong base pair affinity between complementary sections of lined- up nucleotide strands. This kind of biosensors can be made by immobilizing single stranded DNA probes on different electrodes using electroactive indicators to measure the hybridization between DNA probes and their complementary DNA strands. The detection mainly relies on the conversion of the base- pair recognition event into a measurable electrical signal. Materials like carbon paste, pyrolytic graphite, glassy carbon among others are used for this kind of immobilization. These devices can be used in the environmental and food areas, in which the detection of specific DNA sequences can give information about the presence of pathogenic bacteria, fungus, genetically modified organism (GMO) etc. [18].

### Cells

Cell- based biosensors can be used for the detection of different parameters in physiological and biochemical process. This kind of devices are able to use an entire cell or a specific cellular component in order to detect and bind specifically to certain species present in the sample. The immobilization of the cells in the electrode surface can be done by using peptides, polylysine, collagen, laminin, etc., or using micro-contact printing, inkjet printing or a self-assembled monolayer which form micropatterns on the sensor surface, providing covalent linkages between the cells and the sensor surface [19]. One of the major advantages from this kind of bioreceptors is the low detection limit given by the signal amplification and their rapid response in real time bioassays, having numerous applications in biomedicine for the recognition of pathogens, toxins etc. [12].

## 3. Enzyme immobilization

Enzymes are globular proteins with catalytic activity with wide use in the food industry. Formulations developed to produce such biocatalysts as insoluble particles through enzyme immobilization have been developed to enhance their applicability and overcome some shortcomings toward cost-effective commercial processes. Immobilization can improve enzyme stability, specificity, and activity, when performed correctly. Additionally, immobilization facilitates downstream processing, expands the configuration of biocatalytic systems, and allows for continuous or repeated use of the biocatalyst. A carrier can be used to attach or entrap the enzyme, or it can be immobilized by cross-linking enzyme molecules to create a large, insoluble network. Therefore, the immobilization process as well as the carrier may be related to the properties of the immobilized enzyme formulations. As a result, the food industry has adopted a wide range of immobilized enzyme formulations and setups [20].

### Immobilization criteria

Immobilization of enzymes is an important feature in designing the biorecognition part of enzyme-based biosensors. There are several methods for enzyme immobilization. In order to be successfully immobilized, a biological component must meet the following requirements: (i) the biological component must retain substantial biological activity when attached to the sensor surface; (ii) the biological film must remain tightly associated with the sensor surface whilst retaining its structure and function; (iii) the immobilized biological film needs to have long-term stability and durability; and (iv) the biological material needs to have a high degree of specificity to a particular biological component [8].

The development of immobilization technologies for stabilizing biomolecules and tethering them to surfaces is a crucial component in the construction of biosensors. The usual goal is to create a thin film of biologically active material that is immobilized on or near the transducer surface and responds only to the presence of one or more materials or substances that need to be detected. The final performance of the biosensor is inextricably linked to the immobilization technique used, not only practical biosensor applications depend on the successful integration of several biological and physical technologies [8].

The biological films are assembled using repeatable procedures, and once formed, these films are adaptable to various environments, which are additional benefits of the immobilization method that are frequently sought after. The immobilized films, in particular, must maintain their stability and activity under a variety of physiological pHs, as well as changes in temperature, ionic strength, and chemical composition. In some circumstances, the capacity to co-immobilize more than one biologically active component is also desired. Sensitivity is a crucial consideration for a reliable and effective biosensor. The density of distribution and/or orientation of the biological material close to the sensor surface, as well as the sensitivity of the detection system, all affect how sensitive a biosensor is [8].

It has been proposed that only one site on the protein, far from the active site, should react on the sensor surface. Non-specific binding is an issue that affects a lot of biosensors. In a "serum" or solution containing many molecular species, the presence of a specific analyte is typically to be detected, and there is interference from other species in the solution. In order to reduce non-specific binding, blocking agents - such as bovine serum albumin (BSA) for immunosensing applications - are frequently added to the system. If precise information about particular biomolecule interactions is to be obtained, non-specific binding must be reduced. It has also been proposed that biological components should generally be attached through single sites on surfaces with low non-specific biomolecule adsorption. Another important consideration in biomolecule immobilization is to ensure that the biological component does not desorb while the biosensor is being used, especially when the biomolecules are being trapped using polymer trapping or membrane barriers [8].

### 3.1 Methods used for immobilization of enzymes

The incorporation of biological components in membrane structures was first described by Clark and Lyons (1962). Since that pioneering work, in which an enzyme-based glucose sensor was developed by the entrapment of glucose oxidase in a membrane enclosed sandwich, there have been a number of methods which have been described for the immobilization of enzymes

and proteins on surfaces and within various matrices [8]. The most popular immobilization methods for construction of biosensors are entrapment, adsorption, covalent binding, cross-linking and affinity (Fig.7) [12].

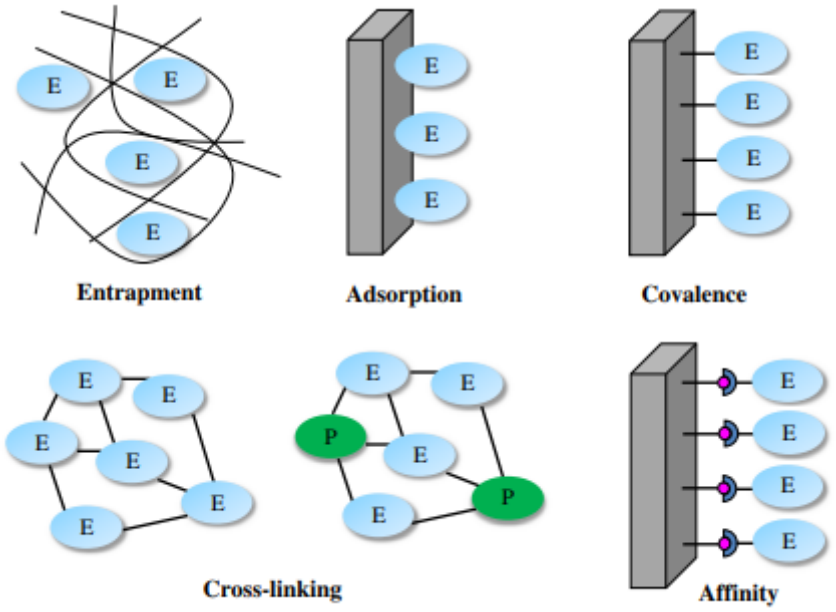


*Figure 7: Schematic representation of the main different methods of enzyme immobilization E: enzyme P: protein [9]*

## Entrapment

In three-dimensional matrices, such an electropolymerized film, an amphiphilic network composed of polydimethylsiloxane (PDMS), a photopolymer, a silica gel, a polysaccharide or a carbon paste, enzymes can be immobilized. It is simple to perform this immobilization. In the same sensing layer, enzyme, mediators, and additives can all be simultaneously deposited. The biological component is left unaltered in order to maintain the enzyme's activity throughout the immobilization procedure. Increased operational and storage stability is a common characteristic of biosensors based on physically entrapped enzymes. However, restrictions like biocomponent leaching and potential diffusion barriers may limit the performances of the systems [9].

## Adsorption

The simplest method of physical immobilization involves the adsorption of enzymes onto solid supports. The solid support is placed in contact with the enzyme solution for a predetermined amount of time after the enzyme has been dissolved in a solution. The unadsorbable enzyme is then eliminated by buffer washing. The adsorption mechanisms are based on weak bonds, such

as electrostatic and/or hydrophobic interactions and Van der Waal forces. This method generally doesn't damage enzyme activity and doesn't involve functionalizing the support in any way. The enzymes are only loosely bound to the support, and desorption of the enzyme due to changes in temperature, pH, and ionic strength appears to be the main issue despite the fact that this immobilization method results in little to no enzyme inactivation. Because of this, enzyme-based adsorbed biosensors have poor operational and storage stability. The non-specific adsorption of additional proteins or substances is also a disadvantage [9].

### Covalence

Covalent coupling of enzymes to polymeric supports is a popular chemical immobilization method used to develop enzymatic biosensors. For this purpose, biocatalysts are bound to the surface through functional groups that they contain and that are not essential for their catalytic activity. The binding of the enzymes to the solid support is generally carried out by initial activation of the surface using multifunctional reagents (e.g., glutaraldehyde or carbodiimide), followed by enzyme coupling to the activated support, then the removal of excess and unbound biomolecules. The carrier support can either be an inorganic material (e.g., controlled pore glass), a natural (e.g., cellulose) or synthetic polymer (e.g., nylon). Membranes (e.g., Immunodyne, Ultra bind) that are already pre-activated have also been used. Covalent immobilization can be performed directly onto the transducer surface or onto a thin membrane fixed onto the transducer.

### Cross-linking

Immobilization of enzymes by cross-linking with glutaraldehyde or other bifunctional agents such as glyoxal or hexamethylenediamine is another well-known approach to develop biosensors. The enzyme can be either cross-linked with each other or in the presence of a functionally inert protein such as bovine serum albumin. This method is attractive due to its simplicity and the strong chemical binding achieved between biomolecules. The main drawback is the possibility of activity losses due to the distortion of the active enzyme conformation and the chemical alterations of the active site during crosslinking. Several conductometric biosensors based on the immobilization of enzymes in a gel obtained by co-reticulation with glutaraldehyde in the presence of BSA have been reported for the detection of different molecules such as heavy metals, nitrite, or pollutants. The most common cross-linking agent in biosensor applications is glutaraldehyde, which couples with the lysine amino groups of enzymes. For example, a biosensor was developed for heavy metal ion determination by

cross-linking alkaline phosphatase with BSA in saturated glutaraldehyde vapor on the electrode surface. Storage stability in buffer solution at 4 °C was more than 1 month. A gravimetric glucose biosensor was also reported for selective detection of blood glucose levels. GOD was immobilized onto cantilever surface by cross-linking with glutaraldehyde and BSA. The enzyme-functionalized microcantilever underwent bending due to a change in surface stress induced by the reaction between glucose and immobilized GOD. No external physical parameters, such as potential or current, were applied during the measurements, so the selectivity only depended on the enzyme reaction. This biosensor was very selective and species such as catechol, ascorbic acid and 4- acetaminophen had no effect on the response of the cantilever biosensor [9], [12].

### Affinity

Efforts have been achieved in order to develop biosensors based on oriented and site-specific immobilization of enzymes. A strategy is to create (bio)affinity bonds between an activated support (e.g., with lectin, avidin, metal chelates) and a specific group (a tag) of the protein sequence (e.g., carbohydrate residue, biotin, histidine). This method allows to control the biomolecule orientation in order to avoid enzyme deactivation and/or active site blocking. Several affinity methods have been described to immobilize enzymes through (strept)avidin-biotin, lectin-carbohydrate, and metal cation–chelator interactions. An enzyme can contain affinity tags in its sequence (e.g. a sugar moiety) but, in some cases, the affinity tag (e.g. biotin) needs to be attached to the protein sequence by genetic engineering methods such as site-directed mutagenesis, protein fusion technology and post-transcriptional modification [9].

There are benefits and drawbacks to each immobilization technique. The type of enzyme, the transducer, and the associated detection mode all have an impact on which technique is most sensible and appropriate. Depending if a biosensor application is focused on stability or maximum sensitivity, different enzyme immobilization techniques are best. It is also necessary to take into account the process' difficulty, cost, and reproducibility. Sensitivity decrease if enzyme denaturation or conformational changes result from immobilization, or if the enzyme has been effected, particularly at its active site [9].

*Table 1: The main advantages and drawbacks*

| | Binding nature | Advantages | Drawbacks |
|---|---|---|---|
| Adsorption | Weak bonds | ◦ Simple and easy<br>◦ Limited loss of enzyme activity | ◦ Desorption<br>◦ Non-specific adsorption |
| Covalent coupling | Chemical binding between functional groups of the enzyme and those on the support | ◦ No diffusion barrier<br>◦ Stable<br>◦ Short response time<br>◦ High enzyme activity loss | ◦ Matrix not regenerable<br>◦ Coupling with toxic product |
| Entrapment | Incorporation of the enzyme within a gel or a polymer | ◦ No chemical reaction between the monomer and the enzyme that could affect the activity<br>◦ Several types of enzymes can be immobilized within the same polymer | ◦ Diffusion barrier<br>◦ Enzyme leakage<br>◦ High concentrations of monomer and enzyme needed for electropolymerization |
| Cross-linking | Bond between enzyme/cross-linker (e.g. glutaraldehyde)/inert molecule (e.g. BSA) | ◦ Simple | ◦ High enzyme activity loss |
| Affinity | Affinity bonds between a functional group (e.g. avidin) on a support and affinity tag (e.g. biotin) on a protein sequence | ◦ Controlled and oriented immobilization | ◦ Need of the presence of specific groups on enzyme (e.g. His, biotin) |

The main advantages and drawbacks of each immobilization method are presents in the table.

## 4. Biosensor performance parameters

In the research field of new sensing devices, the parameters listed below help to define the performance of the different biosensors, being relevant to improve existent technology and make possible the incorporation into industrial processes. These guidelines enable a reliable comparison among the different devices and facilitate the development of new prototypes.

### Sensitivity

The sensitivity of a sensor can be understood as the minimum amount of analyte that the device is able to detect. Is defined by the relationship (mostly linear) between the output electrical quantity (signal) and the analyte concentration. Mathematically is represented as $y = m * c$ where $y$ is the output signal, $m$ is the sensitivity of the biosensor and $c$ is the concentration of the analyte in the sample. Techniques such as platinum deposition or the use of films that adsorbed and concentrate the analytes, i.e. $Nafion^{TM}$ are used to enhance the electrode performance [21].

### Limit of detection (LOD)

The limit of detection is defined as the lowest analyte concentration in the sample that can be reliably distinguished from the analytical noise (signal produced in the absence of analyte) and at which detection is feasible but not necessarily quantitated as an exact value [22]. Acording the reported by the International Council of Harmonization, 2005 this parameter can be estimated by the calculation of the signal-to-noise ratio, visual evaluation, calculation from the

standard deviation of the blank or performing the calculation based on the calibration curve using samples containing an analyte in the range of the linear detection.

### Stability

Biosensor stability can be defined as the degree of suceptibility to ambient disturbances in and around the biosensing system. This parameter may vary considerably according the operational conditions, the type of biological receptor and the transducer used. According to Thevenot et al. (2001) [23] for the operational stability determination is recommended to consider the continuous or sequential contact of the biosensor with the analyte solution, the analyte concentration, temperature, pH, buffer composition, presence of organic solvents, and sample matrix composition. In general stability is one of the most relevant factors in real-life applications having special influence in the medical and food monitoring field.

### Reproducibility

Reproducibility is the ability of a biosensor to produce similar responses for a duplicated experimental set-up. This parameter is based on the the precision (ability of the sensor to provide similar results every time the sample is measured) and accuracy (capacity to provide a mean value close to the true value when the sample is measured more than once) of the transducer and electronics in the device. It is generally determined for the analyte concentrations within the usable range [24].

### Selectivity

The selectivity is the ability of a biosensor to detect a specific analyte present in a mixture. It varies depending on the type of biological receptor and transducer chosen for the sensor design. According to Thevenot et al. (2001) there are two methods for the determination of the biosensor selectivity. The first one consists in the measuring of the biosensor response to interfering substances by a calibration curve for each of those. Then this data is plotted and compared to the calibration curve of the analyte under identical conditions. The selectivity is expressed as the ratio of the signal output with the analyte alone to that of the interfering substance alone at the same concentration as that of the analyte. In the second method interfering substances at the expected concentration are added into the measuring cell containing the usual analyte concentration. In this case the selectivity is expressed as the percentage of variation of the biosensor response. In general selectivity is the main

consideration when choosing bioreceptors, being an important parameter to study the performance of this kind of devices.

# Chapter 2. Ammonium biosensor: State of Art

## 2.1 Detection methods of ammonium

Over the last years, the measurement of ammonium ($NH4^+$) for different purposes has become increasingly important. Various methods have been developed for quantification of ammonia concentration in different samples. Ammonia has been determined by a flow injection analysis (FIA) system constructed with a sequential immobilized enzyme reactor and chemiluminescence detector [25], HPLC coupled with fluorescent detector, and colorimetric methods (Indophenol-based reaction and Berthelot reaction) [6].

Ammonium is converted to glutamate by glutamate dehydrogenase (GLDH) in the presence of 2-oxoglutarate and NADH, followed by the oxidation of glutamate by glutamate oxidase (GXD) to produce hydrogen peroxide ($H_2O_2$) that is detected by a chemiluminometric method using luminol and hexacyanoferrrate (III). The measurement of urea in serum was then performed using this system in conjunction with immobilized urease. In addition, the indophenol blue (IPB) colorimetric method measures the concentration of ammonium through the reaction of blue indophenol dye with phenolic agent, which, under the right oxidizing conditions, absorbs light between 630 and 720 nm [26]. The immobilized GLDH reactor coupled with spectrofluorimetric detection was reported for measurement of ammonium. The enzymatic consumption of NADH in the presence of 2-oxoglutamate can be monitored at 340 nm, which is proportional to the concentration of ammonium [27].

However, all these conventional methods for ammonia measurement are time-consuming and tedious. Several methods involved highly toxic reagents and interference by other photoactive substances, resulting in quite large biases of the analysis [6]. Spectrophotometric monitoring of NADH is always interfered by other photoactive substances or suspended particles in samples. Chromatographic methods need pre-column derivatization to treat the samples for fluorescent detection [28]. Although ammonium can also be measured by simply using an ammonium-sensing electrode, it has suffered from interferences caused by the pH of sample or working solution, as well as low-molecular weight amines or cations [27].

The importance of enzyme-based amperometric biosensors result in the development of rapid, accurate and easy device for specific measurement of target analyte in complex matrices such as blood and food product samples. A flow-injection biosensor system employing GLDH and GXD and creatinine deiminase (CRDI) has been described for the determination of creatinine in urine, immobilized CRDI hydrolyzes creatinine with a generation of ammonium, which was subsequently detected by GlDH and GXD immobilized on an oxygen electrode. However, this

system suffers from low sensitivity and large sample volume for low-concentration determination. Recently, two serially connected reactors immobilized with GLDH and GXD individually have been applied for monitoring of 2-oxoglutarate produced in a fermentation process. The concentration of 2-oxoglutarate was detected electrochemically due to the generation of $H_2O_2$. Nevertheless, significant interference from electroactive substances has decreased the reliability (e.g. ascorbic acid, uric acid and glutathione). Few biosensors have been reported up to this point for quickly determining ammonium in aquatic environments [27], [29].

The rapid determination of ammonium with a bienzyme sensor is described as, the bienzyme sensor was constructed by immobilizing glutamate dehydrogenase and glutamate oxidase on a Clark-type oxygen electrode. GLDH consumes ammonium for specific amination of 2-oxoglutarate in the presence of NADH. GXD consumes dissolved oxygen (DO) for the oxidative deamination of glutamate produced by GLDH. DO acts as an essential material for the enzymatic activity of GXD and is consumed with a maximum rate that is proportional to the concentration of ammonium during the measurements. A detectable signal, measured as the first derivation of the current-time curve, was monitored at -600 mV versus Ag/AgCl by the Clark electrode. Both enzymes were entrapped by a poly(carbamoyl) sulfonate (PCS) hydrogel, that was sandwiched between a dialysis membrane and a Teflon membrane. Electroactive interferences were eliminated by the oxygen membrane. The schematic diagram of the bienzyme system is shown in Fig. 11. The practicality of this biosensor has been studied by measuring the ammonium content in wastewater collected from a wastewater treatment facility and comparing with the results obtained by using a commercial ammonium testing kit.

## 2.2 Biological enzyme detection

More recently, enzymatic biosensors have been developed for ammonium detection in different samples. Most biosensors related to ammonia nitrogen detection are based on glutamate dehydrogenase (GLDH) and alanine dehydrogenase (AlaDH). Relevant studies are listed in Table 2.

*Table 2: Overview of ammonia nitrogen detection methods based on biological enzyme detection*

| Chemistry | Type of transduce | Range | LOD | RSD | Response time | Type of sample | Ref. |
|---|---|---|---|---|---|---|---|
| GLDH | Ultraviolet spectrophotometry | 0.005–0.5 mM | 0.005 mM | 5.9% (1 mM, n = 8) | – | Artificial water sample | [115] |
| GLDH-Dph, MTT | Ultraviolet spectrophotometry | 16.8–70 μM | 11 μM | – | – | Fishpond water | [116] |
| GLDH, GXD | Amperometry | 10–300 μM | 2.06 μM | – | 2 s | Wastewater sample | [117] |
| GLDH-Dph | Amperometry | 2.5–500 μM | 2.5 μM | 4.17% (n = 5) | – | Artificial water sample | [118] |
| AlaDH | Amperometry | 10–100 mM | 0.18 mM | 1.4–4.9% (30 mM, n = 5) | – | Spiked river water | [119] |
| AlaDH, AuNPs, C8-DT | Amperometry | 0.1–0.5 mM | 0.01 mM | – | <1 min | Wastewater | [120] |
| AlaDH | Amperometry | 1.67–56.67 μM | 0.47 μM | – | – | River water | [121] |
| AlaDH, $Fe_3O_4$@Au nanoparticles | Amperometry | 5–250 mM | 2.1 mM | – | – | Latex samples | [122] |
| AlaDH, CNT | Amperometry | 0.05–500 mM | 0.001 mM | – | 30 s | Artificial water sample | [123] |
| AlaDH | Amperometry | 0.1–300 mM | 0.01 mM | – | 20 s | Real water sample | [124] |
| Palmitic acid, Nonactin, PVC membrane | Potentiometry | 1 μM–100 mM | 1 μM | <0.5–1.0% (n = 5) | 1–2 min | Artificial ammonia solution | [125] |
| L-GLDH, L-GXD, Prussian blue-carbon electrode | Potentiometry Amperometry | 0–100 μM (potentiometric) | 0.2 μM (potentiometric) 2100 μM (amperometric) | – | – | Artificial water sample | [126] |

GLDH: glutamate dehydrogenase; GLDH-Dph: glutamate dehydrogenase/diaphorase; MTT: thiazolyl blue tetrazolium bromide; GXD: glutamate oxidase; AlaDH: alanine dehydrogenase; AuNPs: gold nanoparticles; C8-DT: 1,8-octanedithiol.

Glutamate dehydrogenase (GLDH), alanine dehydrogenase (AlaDH), leucine dehydrogenase (LeuDH), phenylalanine dehydrogenase (PheDH) and valine dehydrogenase (ValDH) are the main members of the amino acid dehydrogenase family, all of which are $NAD(P)^+$ dependent, and their enzymatic reactions are accompanied by catalytic reduction ammonization. Amino acid dehydrogenase is derived from bacteria, fungi, plant cells and animal organs and can be purified in a laboratory. The activity of GLDH is higher than 200 U in 1 mg of solid powder, and the best activity is at pH 8.5. The optimum pH of other kinds of amino acid dehydrogenase is about 10.5, and the enzyme activity is less than 50 U/mg. The natural GLDH usually exists in the form of homologous hexamer, and its subunit structure includes substrate-binding domain and cofactor-binding domain. The Chrystal structure of glutamate dehydrogenase is shown in the fig 8. Lysine residues are the key active sites for catalytic reaction, and they are distributed at the crack junction between the two domains [30].

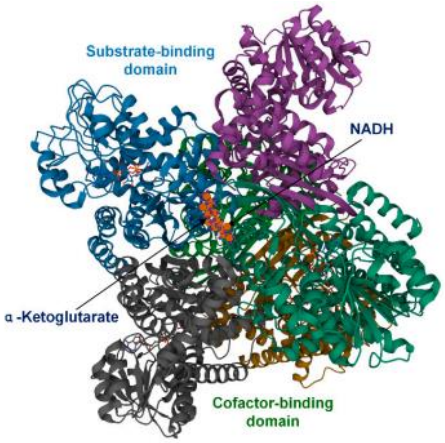


*Figure 8: Chrystal structure of glutamate dehydrogenase from Corynebacterium glutamicum*

The principle of a GLDH reaction and related spectroscopic detection can be summarized as follows: immobilized GLDH requires a coenzyme β-nicotinamide adenine dinucleotide (NADH) and ammonium for enzymatic conversion of α-ketoglutarate into ι-glutamic acid. As shown in fig. 9, during the reaction, NADH is oxidized to form $NAD^+$ and the ammonium concentration can be indirectly measured from the consumption of NADH monitored at a specific wavelength.

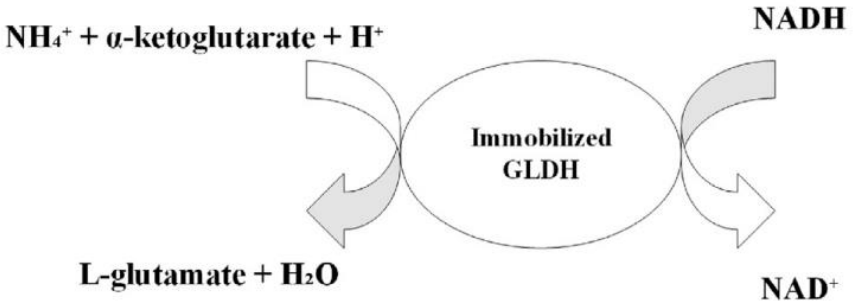


*Figure 9: Mechanism for ammonium ion catalyzed by immobilized GLDH in the presence of a-ketoglutaric acid and reduced NADH.*

Azmi et al. [1] performed enzyme detection with a linear response of 0.005-0.5 mM and an LOD of 0.005 mM, achieving good reproducibility and RSD of 5.9% (n =8). However, NADH is usually provided in excess and for the unreacted NADH, a double enzyme GLDH/diaphorase combined with MTT can be used to improve detection performance.

Excess unreacted NADH can be oxidized to form NAD via diaphorase (Dph) in the presence of MTT. When MTT receives electrons, it is reduced to the purple color. By measuring the reaction of the two enzymes, the detection range of the system can be improved to 16.8-70 mM and with an LOD of 11μM.

In the presence of ammonium, the enzymatic reaction produces an electron transfer with the reaction proportional to the ammonium concentration. Thus, measurement of the ammonium concentration can be achieved by detecting the current or voltage using various electrochemical methods. Immobilized GLDH and glutamate oxidase (GXD) can be used on Clark-type oxygen electrodes to determine the ammonium concentration and the principle of this reaction is shown in fig. 10. In the presence of NADH, GLDH consumes ammonium to perform a specific amination reaction of 2-oxoglutarate, and GXD consumes dissolved oxygen (DO) via oxidative deamination of glutamic acid produced by GLDH. Therefore, DO is a basic substrate that is required for GXD enzymatic activity and is consumed at the maximum rate proportional to the ammonium concentration, generating charge transfer at the electrode. In addition, bilayer

GLDH/diaphorase increased the linear range of ammonium detection from 10e300 mM to 2.5-500 mM with good repeatability [3].

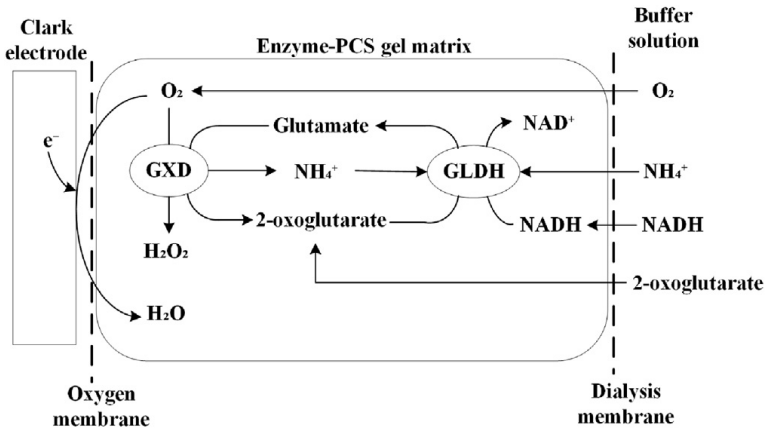


*Figure 10. Schematic diagram of the GXD/GLDH double ammonium biosensor*

However, due to the DO demand, the enzyme analysis method using GLDH and GXDwas gradually replaced by a method featuring alanine dehydrogenase (AlaDH). AlaDH is similar to GLDH and exhibits high specificity for ammonium. The principle of AlaDH-based detection methods is as follows: AlaDH reversibly azoxylates pyruvate to form L-alanine in the presence of NADH and ammonium.

Electron transfer can be monitored through examination of NADH oxidation to determine the current generated during the electrochemical processes. Simultaneously, the enzymatic redox reaction consumes ammonium and the generated current is directly proportional to the change in ammonium concentration.

In the AlaDH determination of ammonium, the pyruvate substrate and reduced NADH are necessary. However, Tan et al. demonstrated that ammonium detection can be realized using an accumulated methacrylate film. The enzymatic reaction between the AlaDH and pyruvate consumes ammonium and the accumulated methacrylate film responds linearly with the change in ammonium concentration. A screen-printed carbon electrode based on this design was prepared and detected ammonium in the range of 10e100mMwith an LOD of 0.18mM[119]. In addition,1,8-octanediol (C8-DT) and multilayer AuNPs were deposited directly on the surface of a gold electrode and subsequently modified with AlaDH to achieve a detection range of 0.1e0.5 mM with an improved LOD of 0.01 mM [120].

In addition to the enzymes mentioned above, other enzymatic detection systems have been developed. For example, incorporation of macromolecules containing active ends (palmitic acid) into a PVC membrane containing non-actin as an ammonium carrier was reported [125].

The PVC membrane selective electrode containing palmitic acid showed better performance than a similar carboxylated PVC membrane electrode. In addition, L-glutamate oxidase and Prussian blue carbon electrodes can be used to fabricate a sensor chip, and the enzyme electrode can detect ammonia from the potentiometric and amperometry measurements with detection limits of 0.2 and 2100 mM, respectively [126]. Active substances present in plant extracts, such as orchid extract, can also be used for ammonia nitrogen detection because orchid receptors produce a coloring phenomenon. By controlling the sampling process, high and low concentrations of ammonia nitrogen can be detected. For samples with high ammonia nitrogen content, the linear range of the sensor was determined to be 5e40 mM with an LOD of 2.12 mM at a flow rate of 1.0 mL/min and injection volume of 100 mL. For wastewater samples with low ammonia nitrogen contents, higher sensitivity is required. At a decreased flow rate of 0.3mL/min and increased injection volume. of 1000 mL, the obtained linear range was 1e5mMwith an LOD of 0.76 mM [127]. Similarly, Jaikang et al. [128] prepared the extract of butterfly pea flower into a natural indicator-immobilized paper and obtained the linear calibration results of ammonia nitrogen ~0.71e7.14 mM by gas pervaporation and diffusion method. These methods represent an improvement over traditional methods and are a kind of green chemical analysis, because the use of biological reagents can completely eliminate the influence and negative consequences of chemical reagents. However, thesemethods are notmature and have requirements on the extraction of substances.

Ammonia biological enzyme detection methods exhibit good selectivity and high sensitivity with the ability to be adapted for detection of low and high concentration ranges of ammonia nitrogen. Currently, biosensors based on biological enzymes, such as GLDH, GXD, AlaDH, for the detection of ammonia or ammonium concentration via enzymatic reactions are promising. The reaction between GLDH and GXD requires sufficient DO, while AlaDH does not require DO as an essential component, which is a significant advantage. In terms of detection performance, nano science can improve the sensitivity of enzyme analysis. In addition, electrochemical detection has many advantages in biosensor because of its rapid response time, small volume requirements, low power consumption, low manufacturing cost, and wide dynamic response range. Therefore, it is an attractive sensing platform for environmental water analysis.

# Chapter 3. Aim of the thesis

The aim of the presence study has been to develop and optimize an electrochemical bienzymatic biosensor for the control of ammonium in must grape during alcoholic fermentation.

The application of NADH-dependent dehydrogenase-based biosensor for ammonium detection using glutamate dehydrogenase and diaphorase enzymes has been evaluated. The electrochemical oxidation of NADH was used as indicator reaction for quantification of ammonium concentration.

The research activities have been focused on the identification of the optimal enzymes amount to immobilize on polyaniline – modified screen printed electrodes, as well as the cofactor involved in the enzymatic reaction.

The developed biosensor has been characterized through amperometric transduction technique and the main analytical parameters, such as sensitivity, linear range, limit of detection, reproducibility and operational stability have been investigated.

Finally, a hydroalcoholic model solution has been used to verify the efficacy of the developed biosensor for the on line monitoring of ammonium during winemaking process.

# Chapter 4. Materials and Methods

## 4.1 Reagents

Aniline ($C_6H_7N$), poly(2-acrylamido-2-methyl-1-propanesulfonic acid, PAAMPSA, Mw = kD), poly(ethylene glycol), diglycidyl ether (PEDGE, Mw = 500 Da) solution, β-nicotinamide-adenine dinucleotide phosphate (reduced form, NADPH, >90%), sodium phosphate dibasic ($Na_2HPO_4$), sodium phosphate monobasic monohydrate ($NaH_2PO_4$-$H_2O$), potassium chloride (KCl) and diaphorase (DP) have been purchased from Sigma Aldrich (St. Louis, MO, USA). Glutamate dehydrogenase (GLDH), Ammonium chloride and α-ketoglutarate has been purchased from Merck (Italy).

## 4.2 Synthesis of PANI - PAAMPSA

Screen-printed carbon electrodes (SPCEs), based on a three electrode (working/auxiliary/reference) layout, were produced in three steps by screen printing different consecutive ink layers on transparent polyester films. The first layer of a carbon/graphite ink (G-Went, Pontypool, UK) was deposited to define the conducting track and the working electrode, the second one was a silver/silver chloride ink (Acheson Colloiden B.V., city, The Netherlands) used as pseudo-reference electrode, while the third layer consisted in an insulating ink (G-Went). The diameter of the working electrode was 2.8 mm.

The electropolymerization of aniline was conducted on the SPCE surface after an electrochemical electrode treatment according to Albanese et al. [21]. Conductive PANI-PAAMPSA polymer was electrochemically synthesized on SPCEs by cyclic voltammetry (CV), according to the following method: SPCEs were soaked in an aqueous solution containing 0.5 M aniline, 1 M hydrochloric acid and 22 g/100 g PAAMPSA; deposition was started by sweeping the potential from −200 to +900 mV on the first scan, to initiate polymer growth; then a potential between −200 and +780 mV, at a scan rate of 50 mV·s−1 was cycled 10 times. The resulting film was washed in 1 M HCl solution to remove the reaction products from the film, then five CV scans were performed, from −500 to +500 mV at 50 mV·s−1 in 1 M HCl, to fully reduce the film.

## 4.3 Biosensor manufacturing

GLDH and DP enzymes were immobilized with PEDGE on PANI-PAAMPSA/SPCE. The immobilization of the enzymes were carried out by dropping consecutive volumes of PEDGE (5 $mg \cdot mL^{-1}$), GLDH and DP on PANI-PAAMPSA modified electrode and left overnight to get dried up. The biosensors were stored overnight at 4 °C when not in use.

## 4.4 Experimental procedures

All the electrochemical experiments were conducted using a PGSTAT 204 potentiostat/galvanostat (Autolab) interfaced with NOVA software. The amperometric measurements have been performed in a Flow Injection Analysis (FIA) apparatus at room temperature.

The biosensors have been placed in a homemade electrochemical wall-jet flow cell while a constant potential of 100 mV vs. Ag/AgCl has been applied. Carrier solution PB 0.1 M at pH 7 from reservoir has been pumped with a peristaltic pump (Minipuls 3, Gilson, Villiers Le Bel, France) at 0.5 $mL \cdot min^{-1}$ flow rate to the injection valve (Sample injection valve, Omnifit, Danbury, CT, USA) equipped with a 100 μL sample loop. Moreover, the bienzymatic scheme required the addition of NADH cofactor and α-ketoglutarate injected in the system with standard (ammonium chloride) or real sample solutions so their optimum concentrations have been evaluated.

## 4.5 Analysis of real sample

The capability of the biosensor developed in this work to measure ammonium content in grape must samples was investigated. A model solution with alcohol content 7.8%vol. was spiked with different amount of ammonium (10 μM, 50 μM, 100 μM) and analysed with the biosensor.

# Chapter 5. Results and discussion

## 5.1 Modification of SPCE with PANI/PAAMPSA and detection of NADH

The bienzymatic GLDH/DP biosensor for ammonium detection works on the basis of the following reactions:

$$NH_4^+ + \text{NADH} + \alpha - \text{ketoglutarate} \rightarrow \text{NAD}^+ + \text{NADH}_{\text{unreacted}} + \text{L} - \text{glutamate} + H_2O$$

$$\text{DP}_{\text{ox}} + \text{NADH}_{\text{unreacted}} \rightarrow \text{DP}_{\text{red}} + \text{NAD}^+ + H^+$$

$$\text{DP}_{\text{red}} + \text{PANI}_{\text{red}} \rightarrow \text{DP}_{\text{ox}} + \text{PANI}_{\text{ox}}$$

$$\text{PANI}_{\text{ox}} \rightarrow \text{PANI}_{\text{red}} + +2e^- + H^+$$

In the presence of ammonium, GLDH catalyzed the conversion of α-ketoglutaric acid to L-glutamate. During the enzymatic sequence reaction, NADH is oxidized to $NAD^+$. The consumption of NADH depends on the ammonium concentration present, which may result in excess of unreacted NADH. This excess of unreacted NADH is then oxidized to $NAD^+$ in the presence of diaphorase and mediator. The current produced corresponds to NADH concentration.

The drawback of this approach is represented by the high overpotential (1 V *vs*. SPCE) for the direct electrochemical oxidation of NADH, which is accompanied by electrode fouling. This overpotential lead to the oxidation of other electroactive species in the sample that can interfere with the determination of the ammonium [31].

To avoid this approach this problem recent approaches have used conducting polymers that led to NADH oxidation at lower potential [35, 37] . Among various conducting polymers polyaniline (PANI) [38–40], and its doped forms with poly(2-acrylamido-2-methyl-1-propane sulfonic acid) (PAAMPSA) [32], poly(acrylic acid) and poly(styrene sulfonate) have been extensively studied as important conducting materials able to increase the electrical, electrochemical, and optical properties of the sensors.

PANI polymer can exist in different forms. The fully oxidized and reduced states, referred to as pernigraniline base (PB) and leucoemeraldine base (LB), respectively, are electrochemically inactive. Emeraldine base (EB) is the intermediate oxidation state of PANI containing an equal number of repeated alternate oxidized and reduced forms (PB and LB). The conductive form of PANI is ”*emeraldine salt*“ (ES) and is the protonated form of EB.

PANI polymer used in biosensor applications is the soluble and electrical conductive form of emeraldine which normally exists in acidic media. Acid conditions are not favorable for both the activity of enzymes and the stability of NADH. The use of PAAMPSA during the polymerization of aniline gives to the resulting polymer proper levels of solubility and electrical conductivity at higher pH values in the neutral and alkaline range [32].

The electropolymerization of PANI-PAAMPSA film on SPCE is illustrated in fig. 11.

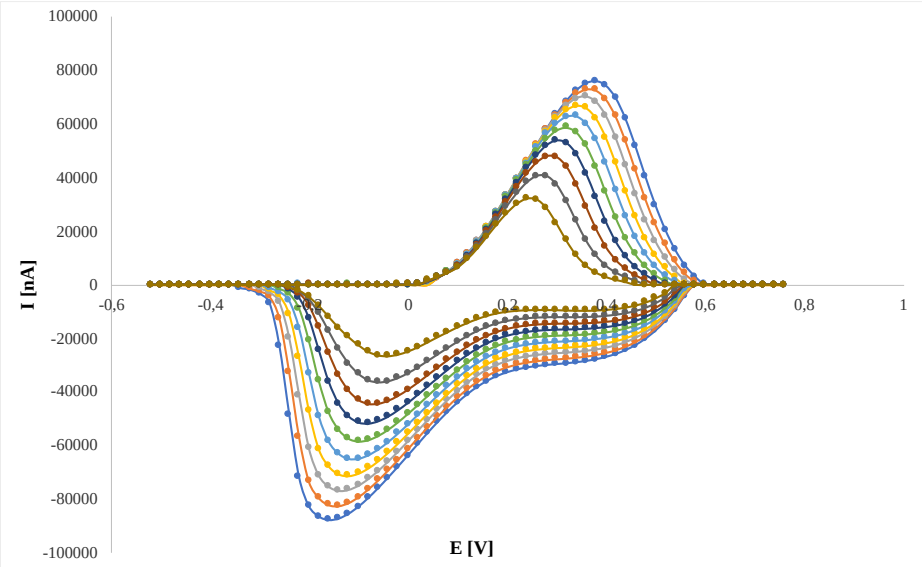


*Figure 11: Electrochemical synthesis of PANI-PAAMPSA on SPCE surface*

The results show an increase in the current during the scans of the CV which indicates the formation and growth of the conducting polymer PANI-PAAMPSA as a thin film on the working electrode. Moreover the increase of current with electropolymerization cycles means that the polymer thickness is increasing [7]. After 10 cycles of CV electropolymerization, the peak currents approached a nearly steady-state value.

The capability of PANI-PAAMPSA film as conductive polymer for NADH oxidation was investigated by CV of PANI-PAAMPSA/SPCE with and without 0.4 mM NADH (Fig. 12.).

After the electropolymerization on PANI the ability of electrode was checked to able to read the cofactor. The peak increased compared to the peak with the buffer. This has proven that the electrode is able to detect and analyze the amount of cofactor.

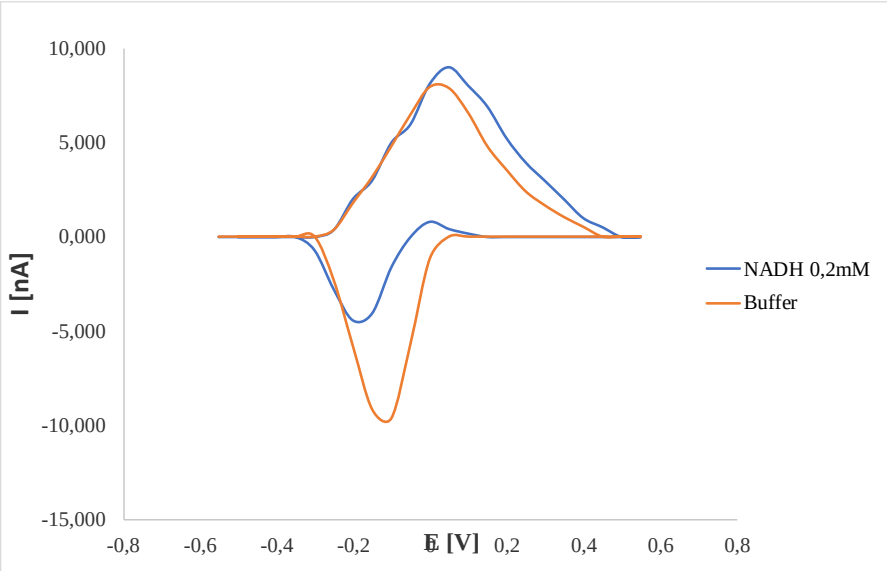


*Figure 12: CV of PANI-PAAMPSA/SPCE in PBS, pH 7 without NADH (a) and with 0.4 mM NADH (b).*

During the CV of PANI-PAAMPSA polymer oxidation and reduction peaks were observed between −100 and 50 mV, thus presenting the transitions between the LB/EB and EB/PB states of the PANI [38]. The CV recorded in the presence of NADH displayed an increase of the anodic peak (at 0.1 V) due to the electrocatalytic oxidation of the NADH by ES and a significant reduction of the cathodic one. For this reason, the working potential at 0.1 V was chosen during the optimization trials of bienzymatic ammonium biosensor.

The sensitivity of PANI-PAAMPSA modified electrode was evaluated by chronoamperometry at 0.1 V vs. Ag/AgCl injecting NADH at several concentrations into flow injection system.

The results (fig. 13) highlight the capability of PANI/PAAMPSA modified SPCE to detect NADH at 0.1 V in a linear range from 0.005 to 0.5 mM with a sensitivity of 1000.9 nA/mM and a limit of detection of 5 µM.

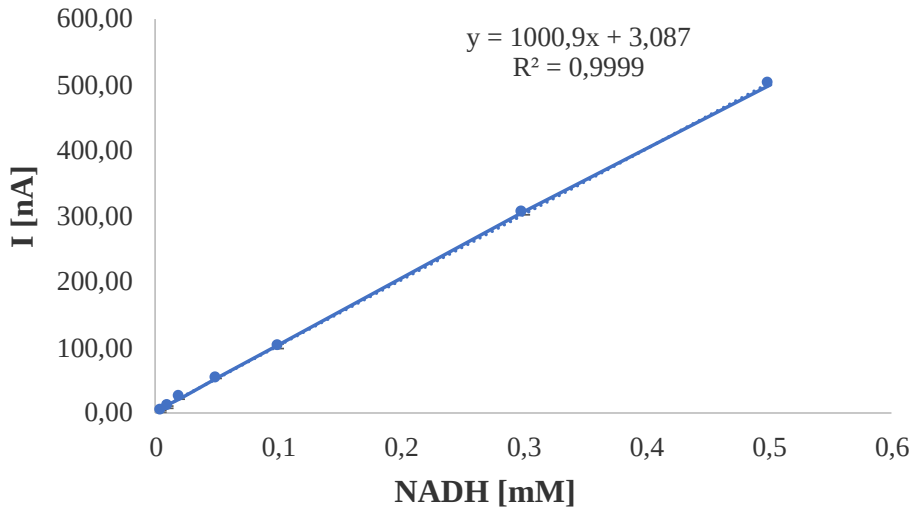


*Figure 13: Characterization of NADH oxidation in PBS pH 7.*

## 5.2 Optimization of bienzymatic ammonium biosensor

### 5.2.1 Enzymes optimization

The amount of enzymes loading employed can affect the sensitivity of the developed biosensor. In this work, bi-layer immobilization of GLDH and diaphorase in SPCE electorde was used. Upon increasing the enzymes loading, the sensitivity of the biosensor increased. Thus, the enzyme loading of 10mg/ml for both GLDH and diaphorase were used for the preparation of the biosensing films. The results of the different amount of enzymes shown in the Figure 16.-17.

Immobilized lower, 5 µl of both enzymes, we have got a close linear range (Fig.14). Then in order to increase the sensitivity and have larger linear range, higher amount of enzymes, 10 µl of both enzymes, were mmobilized (Fig.15).

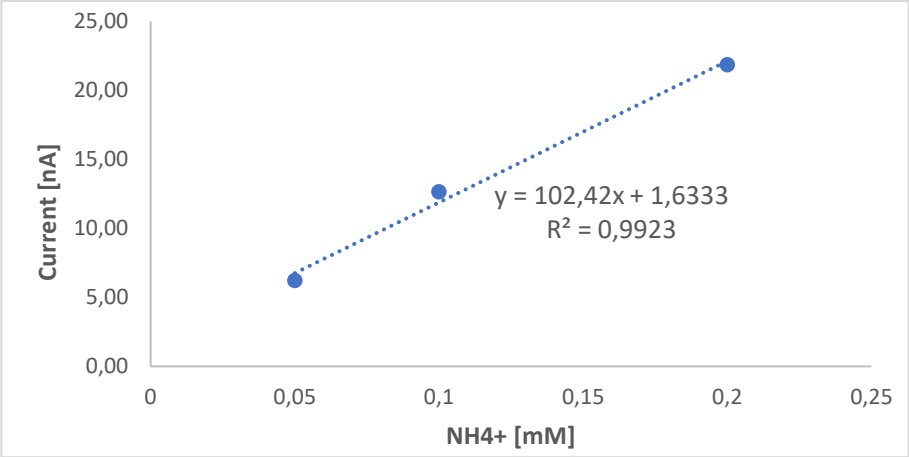


*Figure 14: Calibration curve of 5 mg/ml of Glutamate dehydrogenase and Diaphorase*

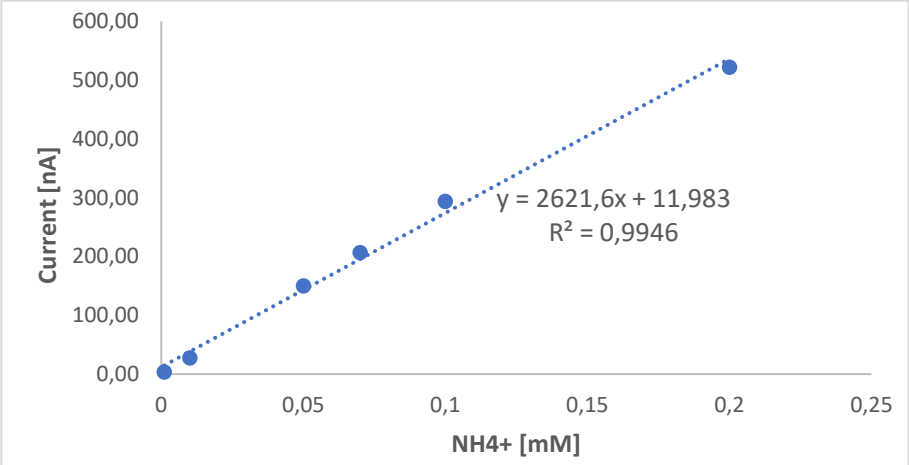


*Figure 15: Calibration curve of 10 mg/ml of Glutamate dehydrogenase and Diaphorase*

### 5.2.2 NADH Alpha-keto glutaric acid optimization

To further improve the performance of the developed biosensor, the effect of different NADH and α-ketoglutaric acid concentrations were also evaluated. The influence of the biosensor sensitivity at various NADH concentrations is shown in Fig. 16. The sensitivity increased with increasing NADH concentration and reaches an optimum value at a concentration of 0.18 mM. Above this concentration, a decrease in sensitivity was observed. This behavior may be due to an excess of NADH concentration which can competitively inhibit enzymes activity [39].

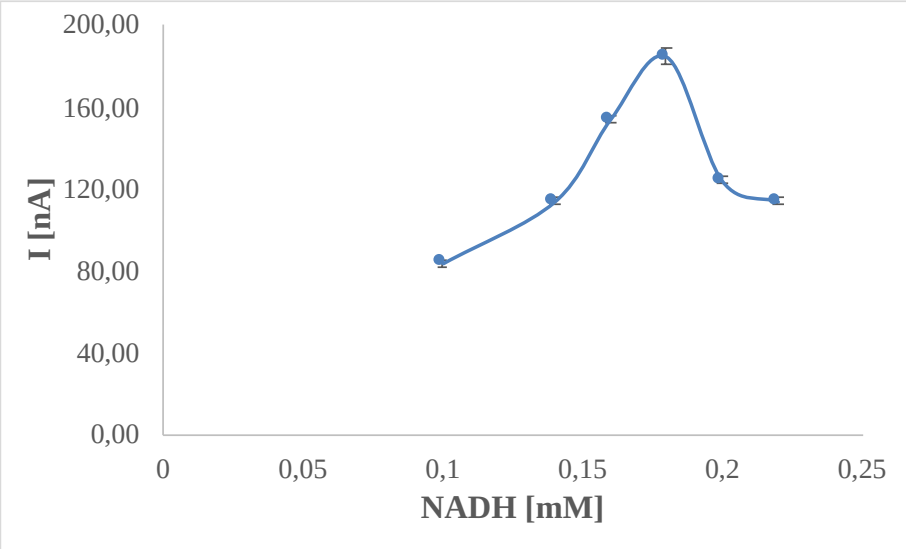


*Figure 16: Effect of NADH concetration ont he biosensor sensitivity.*

The effect of α-ketoglutaric acid concentration on biosensor performance was further evaluated at various concentrations ranging from 0.6–1.0 mM. As shown in Fig. 17, the optimum concentration of α-ketoglutaric acid was obtained at 1.8 mM. Further increase in the concentration of α-ketoglutaric acid results in reduction of the biosensor sensitivity, which may attribute to an inhibitory effect of α-ketoglutaric acid on the enzyme activity [39].

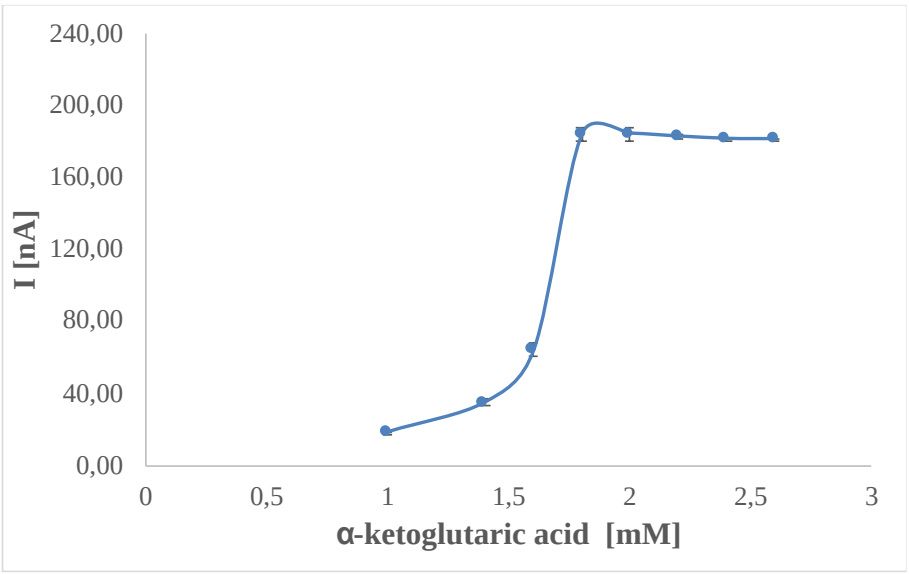


*Figure 17: Effect of α-ketoglutaric acid concentrations on the biosensor sensitivity. The concentration of NADH were fixed at 0.18 mM.. Concentration of NH4Cl ranging from 0 to 0.5 mM*

Hereinafter the injected solution was prepared by solving 250 µl NADH 0,18 mM and 339 µl α-ketoglutarate 1,8 mM.

## 5.3 Analytical performances of optimized ammonium biosensor

Under optimized experimental conditions calibration curve for ammonium chloride determination was evaluated (Fig. 18).

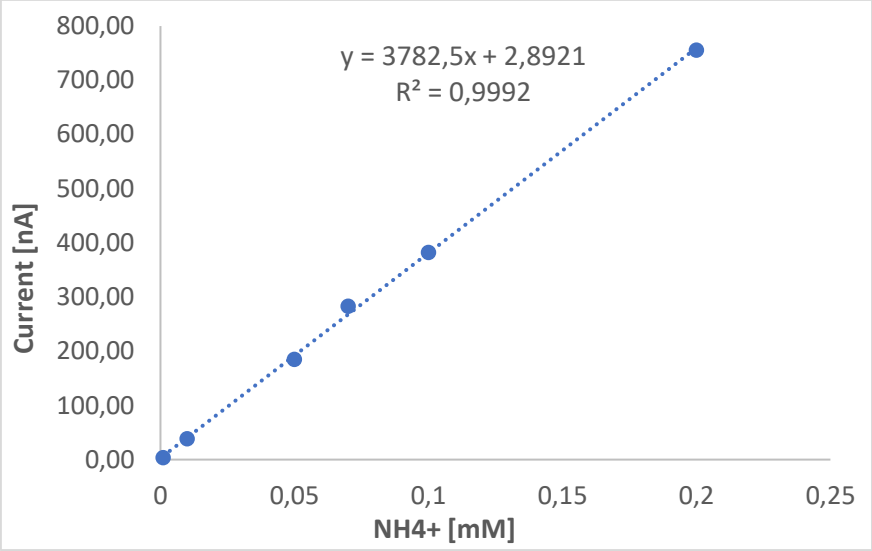


*Figure 18: Optimized parameters for ammonium chloride*

A linear response was obtained in the concentration range 1 – 200 µM ($R^2$=0.9992) with detection limit of 1 µM. The reproducibility of the developed biosensor was also evaluated, and it was found to be good with a relative standard deviation (RSD) of 4,66 % (n=6).

The comparison study between the developed biosensor with an optical biosensor used an optical biosensor based on GLDH immobilized in a chitosan film was conducted for the determination of ammonium in the concentration range of 0.005 to 0.5 mM (slope = 0.1619, $R^2$ = 0.9991). The reproducibility in the optical biosensor fabrication was investigated at ammonium ion, a-ketoglutaric acid, and NADH concentrations of 1.0, 0.15, and 0.15 mM, respectively. The reproducibility of the fabricated biosensor was found to be good with a relative standard deviation (RSD) of 5.9% (n = 8). The stability of the biosensor was also evaluated to be at least 1 month when stored dry at 4°C [1]. The results showed that for the optical biosensor similar amount of NADH concentrations was used and higher amount of a-

ketoglutaric acid, but in this case the minimum concentration was 0,005 mM and the developed biosensor was 0,001 mM and with better relative standard deviation.

Comparing to an other amperometric biosensor, where the ammonium concentration was determined with an Amperometric Biosensor Based on Alanine Dehydrogenase, with concentration in the range of 0.03–1.02 mg/L with a limit of detection (LOD) of 8.52 μg/L. (slope = 0.000524, $R^2$ = 0.974) [40]. In this case, compared to the developed biosensor, the developed biosensor showed better sensitivity in a higher linear range.

The developed biosensor compared to two different biosensor used for the $NH4^+$ determination, showed better results in terms of sensitivity and linear range.

A crucial parameter of the biosensor related to its applicability for the monitoring of food processes is the operational stability, defined as the retention of enzyme activity when it is in use. This parameter was evaluated by injecting 11 samples of ammonium chloride solution (0.01 mM) during an interval time of about 6 h. The measurements were taken in every half hour with the optimized parameters of NADH and α-ketoglutaric acid. The enzyme was immobilized on the electrode on the previous day and stored at 4°C at overnight. The data reported in fig.19 shows a high repeatability of the current during the experiment with only 3.9% sensitivity loss during this test period.

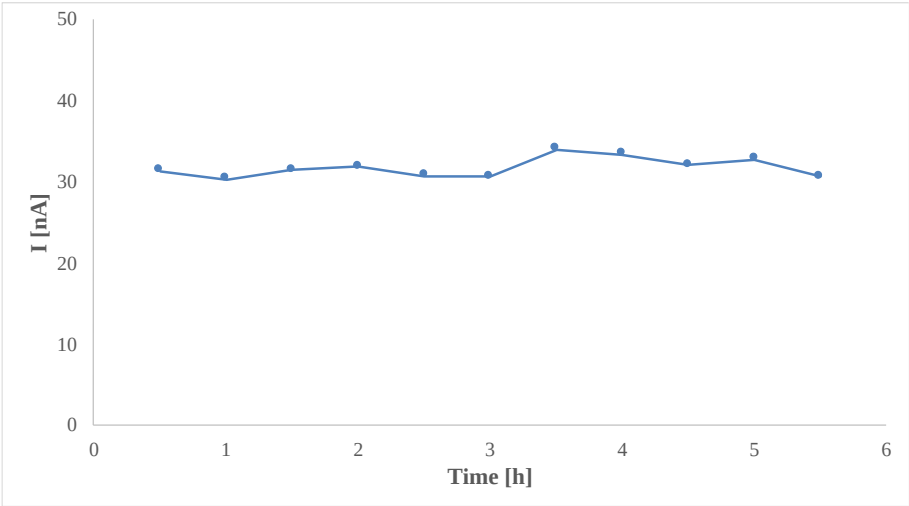


*Figure 19: Operational Stability of ammonium chloride during 6 h working.*

## 5.4 Analysis in real sample

The possibility to apply the developed bienzymatic biosensor for the detection of ammonium in real samples during alcoholic fermentation was studied. Alcoholic solutions were spiked with three different amounts of ammonium and analyzed by the developed immunosensors. The results, reported in Table 3, highlight that the developed biosensor exhibited a good recovery percentage.

*Table 3. Ammonium results in spiked alcoholic solutions obtained by developed biosensor*

| Spiked concentrations [μM] | Biosensor Result [μM] | Recovery [%] |
|---|---|---|
| 10 | 10.23±0.67 | 102.3±6.7 |
| 50 | 50.12±1.34 | 100.24±2.92 |
| 100 | 99.34±3.45 | 99.01±2.79 |

Based on these results, it is possible to assert that the biosensor can be considered a fast analytical technique for the control of ammonium in food samples.

# Conclusion

The bienzymatic biosensor to determinate of ammonium based on bi-layer GLDH/DP immobilized in a PANI-PAAMPSA/SPCE has been successfully developed. The use of PANI-PAAMPSA polymers allowed the direct electrochemical oxidation of NADPH at lower potential in opposition to the higher potential commonly required for carbon electrodes.

The linear range, low detection limit, high sensitivity, and operational stability, showed the potential of the proposed biosensor as a highly capable analytical device for a fast analytical technique for the control of ammonium in food samples.

As compared to the conventional methods, and other biosensors, the developed biosensor shows good analytical performance with a simple examination procedure.

# References


[1] N. E. Azmi, M. Ahmad, J. Abdullah, H. Sidek, L. Y. Heng, and N. Karuppiah, "Biosensor based on glutamate dehydrogenase immobilized in chitosan for the determination of ammonium in water samples," *Anal. Biochem.*, vol. 388, no. 1, pp. 28–32, May 2009, doi: 10.1016/j.ab.2009.02.005.

[2] B. Šljukić, C. E. Banks, A. Crossley, and R. G. Compton, "Lead(IV) oxide–graphite composite electrodes: Application to sensing of ammonia, nitrite and phenols," *Anal. Chim. Acta*, vol. 587, no. 2, pp. 240–246, Mar. 2007, doi: 10.1016/j.aca.2007.01.041.

[3] D. Li, X. Xu, Z. Li, T. Wang, and C. Wang, "Detection methods of ammonia nitrogen in water: A review," *TrAC Trends Anal. Chem.*, vol. 127, p. 115890, Jun. 2020, doi: 10.1016/j.trac.2020.115890.

[4] F. Zamora, "Biochemistry of Alcoholic Fermentation," in *Wine Chemistry and Biochemistry*, M. V. Moreno-Arribas and M. C. Polo, Eds. New York, NY: Springer New York, 2009, pp. 3–26. doi: 10.1007/978-0-387-74118-5_1.

[5] A. Gobert, R. Tourdot-Maréchal, C. Sparrow, C. Morge, and H. Alexandre, "Influence of nitrogen status in wine alcoholic fermentation," *Food Microbiol.*, vol. 83, pp. 71–85, Oct. 2019, doi: 10.1016/j.fm.2019.04.008.

[6] S. A. Rahman, J. Abdullah, H. Sidek, and N. E. Azmi, "Mediated Amperometric Biosensor for the Determination of Ammonium," vol. 4, no. 3, p. 15, 2012.

[7] P. N. Bartlett, P. R. Birkin, and E. N. K. Wallace, "Oxidation of b-nicotinamide adenine dinucleotide (NADH) at poly(aniline)-coated electrodes," p. 10.

[8] A. F. Collings and F. Caruso, "Biosensors: recent advances," *Rep. Prog. Phys.*, vol. 60, no. 11, pp. 1397–1445, Nov. 1997, doi: 10.1088/0034-4885/60/11/005.

[9] A. Sassolas, L. J. Blum, and B. D. Leca-Bouvier, "Immobilization strategies to develop enzymatic biosensors," *Biotechnol. Adv.*, vol. 30, no. 3, pp. 489–511, May 2012, doi: 10.1016/j.biotechadv.2011.09.003.

[10] S. N. Sawant, "Development of Biosensors From Biopolymer Composites," in *Biopolymer Composites in Electronics*, Elsevier, 2017, pp. 353–383. doi: 10.1016/B978-0-12-809261-3.00013-9.

[11] Y. H. Hui and F. Sherkat, Eds., "Biosensor Technology for FoodProcessing, Safety, and Packaging," in *Handbook of Food Science, Technology, and Engineering - 4 Volume Set*, 0 ed., CRC Press, 2005, pp. 2351–2370. doi: 10.1201/b15995-147.

[12] R. Monošík, M. Streďanský, and E. Šturdík, "Biosensors - classification, characterization and new trends," *Acta Chim. Slovaca*, vol. 5, no. 1, pp. 109–120, Apr. 2012, doi: 10.2478/v10188-012-0017-z.

[13] H. M. Pan, S. Gonuguntla, S. Li, and D. Trau, "3.33 Conjugated Polymers for Biosensor Devices ☆," in *Comprehensive Biomaterials II*, Elsevier, 2017, pp. 716–754. doi: 10.1016/B978-0-12-803581-8.10144-4.

[14] S. Dzyadevych and N. Jaffrezic-Renault, "Conductometric biosensors," in *Biological Identification*, Elsevier, 2014, pp. 153–193. doi: 10.1533/9780857099167.2.153.

[15] J. Updike and G. Hicks, "The Enzyme Electrode," *Nature International Journal of Science*, 1967.

[16] S. Patris, M. Vandeput, and J.-M. Kauffmann, "Antibodies as target for affinity biosensors," *TrAC Trends Anal. Chem.*, vol. 79, pp. 239–246, May 2016, doi: 10.1016/j.trac.2015.12.005.

[17] S. Sharma, H. Byrne, and R. J. O'Kennedy, "Antibodies and antibody-derived analytical biosensors," *Essays Biochem.*, vol. 60, no. 1, pp. 9–18, Jun. 2016, doi: 10.1042/EBC20150002.

[18] A. Kumar, S. P. Dash, and D. Sharma, "DNA Based Biosensors for Detection of Pathogens," *Plant Dis. Manag. Pp 31-35 Sci. Publ.*, 2001.

[19] Q. Liu, C. Wu, C. Hua, N. Hu, Zhou Jun, and P. Wang, “Cell-Based Biosensors and Their Application in Biomedicine”.
[20] F. Carvalho and P. Fernandes, “Recent developments in enzyme immobilization for food production,” in *Addition in Food Products and Processing Through Enzyme Technology*, 2022.
[21] “Lavín et al. - 2018 - On the Determination of Uncertainty and Limit of D.pdf.”
[22] D. Ambruster and T. Pry, “Limit of Blank, Limit of Detection and Limit of Quantitation. The Clinical Biochemist Reviews,” 2008.
[23] D. R. Thevenot, K. Toth, R. A. Durst, and G. S. Wilson, “Electrochemical biosensors: recommended definitions and classification_,” 2001.
[24] N. Bhalla, P. Jolly, N. Formisano, and P. Estrela, “Introduction to biosensors,” *Essays Biochem.*, vol. 60, no. 1, pp. 1–8, Jun. 2016, doi: 10.1042/EBC20150001.
[25] M. Tabata, T. Murachi, J. Endo, and M. Totani, “Use of various types of column reactors for flow-injection analysis,” *J. Chromatogr. A*, vol. 597, no. 1–2, pp. 435–442, Apr. 1992, doi: 10.1016/0021-9673(92)80142-H.
[26] H. S. Ruppersberg, M. R. Goebel, S. I. Kleinert, D. Wünsch, K. Trautwein, and R. Rabus, “Photometric Determination of Ammonium and Phosphate in Seawater Medium Using a Microplate Reader,” *Microb. Physiol.*, vol. 27, no. 2, pp. 73–80, 2017, doi: 10.1159/000454814.
[27] R. C. H. Kwan, P. Y. T. Hon, and R. Renneberg, “Amperometric determination of ammonium with bienzyme/poly(carbamoyl) sulfonate hydrogel-based biosensor,” *Sens. Actuators B Chem.*, vol. 107, no. 2, pp. 616–622, Jun. 2005, doi: 10.1016/j.snb.2004.11.028.
[28] L. J. Nagels, “Chapter 29 Enzyme reactors and enzyme electrodes for the liquid chromatographic determination of carbohydrates,” in *Journal of Chromatography Library*, vol. 66, Elsevier, 2002, pp. 1071–1100. doi: 10.1016/S0301-4770(02)80054-9.
[29] “flow injectionbiosensor system.pdf.”
[30] C. Wang, T. Wang, Z. Li, X. Xu, X. Zhang, and D. Li, “An Electrochemical Enzyme Biosensor for Ammonium Detection in Aquaculture Using Screen-Printed Electrode Modified by Gold Nanoparticle/Polymethylene Blue,” *Biosensors*, vol. 11, no. 9, p. 335, Sep. 2021, doi: 10.3390/bios11090335.
[31] B. Prieto-Simón and E. Fàbregas, “Comparative study of electron mediators used in the electrochemical oxidation of NADH,” *Biosens. Bioelectron.*, vol. 19, no. 10, pp. 1131–1138, May 2004, doi: 10.1016/j.bios.2003.10.010.
[32] J. Tarver, J. E. Yoo, T. J. Dennes, J. Schwartz, and Y.-L. Loo, “Polymer Acid Doped Polyaniline Is Electrochemically Stable Beyond pH 9,” *Chem. Mater.*, vol. 21, no. 2, pp. 280–286, Jan. 2009, doi: 10.1021/cm802314h.
[33] D.-W. Yang and H.-H. Liu, “Poly(brilliant cresyl blue)-carbonnanotube modified electrodes for determination of NADH and fabrication of ethanol dehydrogenase-based biosensor,” *Biosens. Bioelectron.*, vol. 25, no. 4, pp. 733–738, Dec. 2009, doi: 10.1016/j.bios.2009.08.016.
[34] I. Katakis and E. Domnguez, “Catalytic electrooxidation of NADH for dehydrogenase amperometric biosensors,” *Mikrochim. Acta*, vol. 126, no. 1–2, pp. 11–32, Mar. 1997, doi: 10.1007/BF01242656.
[35] X.-W. Hu, C.-J. Mao, J.-M. Song, H.-L. Niu, S.-Y. Zhang, and H. Huang, “Fabrication of GO/PANi/CdSe nanocomposites for sensitive electrochemiluminescence biosensor,” *Biosens. Bioelectron.*, vol. 41, pp. 372–378, Mar. 2013, doi: 10.1016/j.bios.2012.08.054.
[36] V. Mazeiko, A. Kausaite-Minkstimiene, A. Ramanaviciene, Z. Balevicius, and A. Ramanavicius, “Gold nanoparticle and conducting polymer-polyaniline-based

nanocomposites for glucose biosensor design," *Sens. Actuators B Chem.*, vol. 189, pp. 187–193, Dec. 2013, doi: 10.1016/j.snb.2013.03.140.
[37] S. Prakash, T. Chakrabarty, A. K. Singh, and V. K. Shahi, "Polymer thin films embedded with metal nanoparticles for electrochemical biosensors applications," *Biosens. Bioelectron.*, vol. 41, pp. 43–53, Mar. 2013, doi: 10.1016/j.bios.2012.09.031.
[38] S. Prasannakumar, R. Manjunatha, C. Nethravathi, G. S. Suresh, M. Rajamathi, and T. V. Venkatesha, "Non-enzymatic Reduction of Hydrogen Peroxide Sensor Based on (Polyaniline-polystyrene Sulphonate) - Carboxylated Graphene Modified Graphite Electrode:," *Port. Electrochimica Acta*, vol. 30, no. 6, pp. 371–383, 2012, doi: 10.4152/pea.201206371.
[39] P. Ödman, W. B. Wellborn, and A. S. Bommarius, "An enzymatic process to α-ketoglutarate from l-glutamate: the coupled system l-glutamate dehydrogenase/NADH oxidase," *Tetrahedron Asymmetry*, vol. 15, no. 18, pp. 2933–2937, Sep. 2004, doi: 10.1016/j.tetasy.2004.07.055.
[40] T. Ling Ling, M. Ahmad, and L. Yook Heng, "An Amperometric Biosensor Based on Alanine Dehydrogenase for the Determination of Low Level of Ammonium Ion in Water," *J. Sens.*, vol. 2011, pp. 1–10, 2011, doi: 10.1155/2011/980709.

**NYILATKOZAT**

**a diplomadolgozat nyilvános hozzáféréséről és eredetiségéről**

A hallgató neve: Hencz Anita

A Hallgató Neptun kódja: AGENOA

A dolgozat címe: Development of an enzymatic biosensor for nitrogen control for alcoholic fermentation

A megjelenés éve: 2023

A konzulens tanszék neve: Élelmiszeripari Méréstechnika és Automatizálás Tanszék

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Kelt: 2023.04.29

Belső konzulens

[1] A megfelelő aláhúzandó.
[2] A megfelelő aláhúzandó.

## A DIPLOMADOLGOZAT TARTALMI KIVONATA

Diplomadolgozat címe: **Development of an enzymatic biosensor for nitrogen control for alcoholic fermentation**

A dolgozatot készítő hallgató neve: **Hencz Anita**

Élelmiszermérnöki mesterképzés, nappali tagozat

*Belső témavezető:* Dr. Baranyai László (tanszékvezető, Magyar Agrár- és Élettudományi Egyetem)

*Külső témavezető:* Donatella Albanese (Full professor at the University of Salerno, Department of Industrial Engineering), Dr. Francesca Malvano (Ph.D. at University of Salerno, Department of Industrial Engineering)

### *Összefoglaló*

The aim of the present study has been to develop and optimize an electrochemical bienzymatic biosensor for the control of ammonium in must grape during alcoholic fermentation**.**

The biosensor has been developed with glutamate dehydrogenase (GLDH) and diaphorase (DP) enzymes immobilized on screen-printed carbon electrode (SPCE) modified with polyaniline/poly (2-acrylamido-2-methyl-1-propane sulfonic acid) (PAAMPSA).

The conductive polymer electrodeposited on the working electrode surface was able to increase the electrochemical properties of the biosensors and allowed the detection of NADH at low potential (0.1 V) in a linear range from 0.005 to 0.5 mM with a sensitivity of 1000.9 nA/mM and a limit of detection of 5 μM.

The electrochemical oxidation of NADH was used as an indicator reaction for the quantification of ammonium concentration. The bi-enzymatic biosensor has been optimized concerning GLDH/DP enzymatic unit ratio, as well as for NADH and alpha-ketoglutaric acid.

Under optimized conditions, ammonium was detected in a linear range from 1 μM to 200 μM (R2=0.9992), with a detection limit of 1 μM and a relative standard deviation (RSD) of 4,66 % (n=6 biosensors).

Finally, the biosensor has been applied to alcoholic solutions with three different amounts of ammonium. Based on these results, it is possible to assert that the biosensor can be considered a fast analytical technique for the control of ammonium in food samples.