CELLULOSE BINDING PROTEIN BASED GENERIC BIOSENSORS

Ali Sinan Kara
118815005

Dr. Öğr. Üyesi Yiğit Dağhan Gökdel

Institute of Graduate Programs
Electrical and Electronics Engineering Master's Degree Program

İstanbul Bilgi University
2021

Cellulose Binding Protein Based Generic Biosensors

Selüloz Bağlanma Modülü Bazlı Jenerik Biyosensörler

Ali Sinan Kara

118815005

Tez Danışmanı:	Dr. Öğr. Üyesi Yiğit Dağhan Gökdel İstanbul Bilgi Üniversitesi	(İmza): ……
Jüri Üyesi:	Doç. Dr. Emel Timuçin Acıbadem Üniversitesi	(İmza): ……
	Dr. Öğr. Üyesi Baykal Sarıoğlu İstanbul Bilgi Üniversitesi	(İmza): …….....

Tezin Onaylandığı Tarih : 13.08.2021
Toplam Sayfa Sayısı : 78

Anahtar Kelimeler:	Keywords:
1) Yüzey Baskılı Elektrotlar	1) Screen-printed Electrodes
2) Selüloz Bağlanma Modülü	2) Cellulose Binding Module
3) Amperometri	3) Amperometry
4) Biyosensör	4) Biosensor
5) Nanofibril Selüloz	5) Nanofibril Cellulose

Foreword

This thesis was created with my late advisor Dr. Özgür Gül. He was my mentor for 6 years and I owe him everything I know. I hope I can follow his high standards for a scientist and make him proud with my work in the future. I want to dedicate this thesis to Dr. Özgür. I can never pay my debt to you for making me the researcher I am today, but I will certainly endeavor to do so.

I want to express my gratitude to my advisor Dr. Yiğit Dağhan Gökdel for helping me to finish my thesis in one of the hardest times in my life. Also, I want to thank Dr. Emel Timuçin and Dr. Kıvanç Bilecen for helping me with my thesis. I want to thank Dr. Ayça Çeşmelioğlu Gül for helping me in even in a time that is darkest for her. Lastly, I want to thank my colleague Lara Naserikojasteh for supporting me through my thesis.

Abstract

CELLULOSE BINDING PROTEIN BASED GENERIC BIOSENSORS

Biosensors are used in various industries. They have great importance in medicine and food industry. Quick prototype production is important for new biosensor production. In this work, a novel method for enzyme immobilization is described to make way for quick biosensor prototype production. Cellulosomes are enzyme complexes that target and degrade cellulosic materials. They utilize a Cellulose binding protein/module (CBM) to attach themselves to the cellulosic material. CBM fused enzymes are used in this work to immobilize enzymes on nanofibril cellulose (NFC) covered screen-printed electrodes. Cellulosic materials are abundant and cheap, thus making them a suitable immobilization material. It is shown in this work that enzymes can be expressed as CBM-fused chimeric proteins to create quick and reliable biosensors.

Özet

SELÜLOZ BAĞLANMA MODÜLÜ BAZLI JENERIK BIYOSENSÖRLER

Biyosensörler farklı endüstrilerde sıklıkla kullanılmaktadırlar. Özellikle sağlık ve gıda endüstrisinde yaygın olarak kullanılırlar. Hızlı prototipleme, yeni biyosensörlerin üretimi için önemlidir. Bu çalışmada, hızlı biyosensör prototiplemesi için yeni bir immobilizasyon metodu denenmiştir. Selülozom, selülozik materyallere bağlanan ve parçalayan bir enzim topluluğudur. Yapısında selüloza bağlanmak için bir selüloz bağlanma modülü bulundur. Bu çalışmada selüloz bağlanma modülü ile birleştirilen enzimler elektrot yüzeyleri nanofibril selüloz kaplı yüzey baskılı elektrotların üzerine immobilize edilmiştir. Selülozik materyaller ucuz olmaları sebebiyle immobilizasyon için uygun materyallerdir. Bu çalışmanın sonucu olarak, enzimlerin selüloz bağlanma modülü ile birleştirilerek rekombinant üretimi ile hızlı ve güvenilir biyosensör üretiminin gerçekleştrilebilineceği gösterilmiştir.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

SPE	Screen-printed electrode
CBM	Cellulose binding module
NFC	Nanofibril cellulose
PASC	Phosphoric acid swollen cellulose
WE	Working electrode
RE	Reference electrode
DI	Dielectric
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PVC	Polyvinyl chloride
PP	Polypropylene
PMMA	Poly (methyl methacrylate)
NaOAc	Sodium acetate
LB Media	Lysogeny broth / Luria-Bertani
INT	Iodonitrotetrazolium chloride
NAD+	Nicotinamide adenine dinucleotide
MPMS	1-Methoxy-5-methylphenazinium methyl sulfate
TAE	Tris Acetic acid EDTA
TBS	Tris buffer saline

1. INTRODUCTION

1.1. WHAT IS A BIOSENSOR?

Biosensors can be defined as devices generating signals proportional to the desired analyte concentration in a reaction. Difference between a conventional sensor and a biosensor comes from the fact that biosensors incorporate a specific biorecognition element which is sourced from biological origins. Biosensors are used in various fields such as environmental monitoring, drug development, medicine, and research. In environmental monitoring, biosensors can be used as pollutant detectors. In drug design biosensors can be used as test devices for the newly developed drugs. For the medicine, biosensors can be used as diagnostic devices to detect biomarkers on bodily fluids (Sweat, urine, spinal fluid, blood). Application areas of biosensors are given in figure 1.1 below.

Figure 1.1. Application areas of biosensors[1].

First biosensor is developed by Leland C. Clark Jr. “The father of biosensors” in 1956. He created the “Clark electrode” for the detection of oxygen. Clark electrode led way to the development of the glucose biosensor by the immobilization of glucose oxidase (GOx) enzyme on to the Clark electrode. First commercial glucose sensor was produced by Yellow Spring Instruments in 1975[1]. Glucose sensors was a landmark for biosensors and diabetes management. Today, coupled with screen-printing and continues biosensor technologies, glucose biosensors revolutionized Type 1 and Type 2 Diabetes Melllitus treatment. Glucose biosensors are the most advanced and worked on biosensors to date and they make up the 31 per cent of the total biosensor market[2]. Biosensors become an important research area for point-of-care applications in medicine and environmental monitoring. A conventional biosensor assembly is given in figure 1.2, which consists of following components.

1.1.1. Analyte

The substance which the biosensor is built to detect. It could be anything from biological substances (DNA, RNA, Microorganisms, Proteins) to chemicals (pollutants, carcinogens, drugs, heavy metals, etc.)[1].

1.1.2. Biorecognition Element / Bioreceptor

A molecule with biological origin, which is used to specifically recognize the analyte. This could be enzymes, DNA, RNA, proteins, or whole cells. Resulting interaction between bioreceptor and the analyte will produce a signal (thermal change, pH change, color change, conformation change, mass change, light generation, etc.) as a result. This process is called biorecognition[1].

1.1.2.1. DNA

DNA is used can be used as bioreceptor in many ways. DNA Hybridization based biosensors take advantage of complementary DNA base pairing. Oligonucleotides with short 20-40 base long, single-stranded DNA molecules with fluorescent probes attached are immobilized on to electrode surface. Biorecognition event occurs when analyte binds to the DNA molecule thus creating a photometric or electrochemical signal. Synthetic DNA constructs called

Aptamers are also used frequently for biosensor purposes. They show great selectivity for their target molecules[3].

1.1.2.2. Enzymes

Enzymes are used as biorecognition elements in electrochemical biosensors. In amperometric biosensors, when enzymes interact with their target analytes, resulting biorecognition event can produce electrons or chemical substances such as H_2O_2, which can be measured as current when a stable potential is applied. Resulting current/time plot will be relative to the analyte concentration[4]. A good example for this is the glucose biosensor. Glucose oxidase (GOx) enzyme interacts with the glucose in the blood stream and produces H_2O_2. H_2O_2 molecules then reacts with either another enzyme such as Horseradish peroxidase (HRP) which generates 2 electrons from H_2O_2 or a mediator such as Prussian Blue, which also reacts with H_2O_2 to produce electrons. Produced electrons are then measured with amperometry, resulting in a glucose reading.

1.1.2.3. Antibodies

Antibodies are proteins produced by the plasma cells of the immune system to target pathogens and foreign agents invading the body. They are large Y-shaped proteins that are specific to a target which is called an antigen. Antibody fragments can be produced recombinantly to be used in biosensors. Biosensors that utilize antibodies are called immunosensors[5]. A good example to immunosensors is the generic lateral flow assay-based pregnancy test. Immobilized antibodies are used to target human chorionic gonadotropin (HCG) hormone in urine. HCG is a biomarker for pregnancy[6].

1.1.2.4. Whole Cell

Genetically engineered cells can be utilized as bioreceptors. By genetically engineering bacterial cells to produce reporter proteins in presence of an analyte, a biosensor can be built. Generally, biorecognition events produce a fluorescent or colorimetric signal. Biosensors that utilize whole-cells are used for environmental monitoring of heavy metals, pollutants and they have some use in medicine and drug development[6].

1.1.3. Transducer

Transducers in biosensors turns the interaction between the bioreceptor and the analyte into a measurable signal. This change of energy form is called signalization. End product is usually an optical or electrical signal. Following are some of the common transducers used in biosensors[1].

1.1.3.1. Electrochemical Transducers

Electrochemical transducers turn chemical events into electrical signals. Enzymes are the most common bioreceptors for electrochemical transducers. The reaction between bioreceptor and the analyte usually creates a detectable chemical species that can be measured. H_2O_2 and reactive oxygen species (ROS) are good examples of this. H_2O_2 will be processed further into releasing electrons, which can be detected with using electrochemical techniques. These techniques are amperometry, cyclic voltammetry and impedance spectroscopy. Amperometry is the process of detecting current change relative to the time. Generated current in biorecognition event will be linear to the analyte concentration[7]. In cyclic voltammetry resulting current is measured when a time dependent potential is applied to the electrode. A voltagram is produced as a result, which is a plot of applied potential versus current. Reactions with defined oxidation and reduction events are suitable for detection with cyclic voltammetry[8]. Impedance spectroscopy is used to detect the change in conduction after the occurrence of biorecognition event. Change in the conductance is relative to the analyte concentration.

1.1.3.2. Photometric Transducers

Photometric transducers rely on the light signal generated by the biorecognition event. This change could be a colorimetric one (Color change), a fluorometric one (fluorescence emission) or luminescent one. Generally an optical detector is used to pick up the signal[7]. Optical detectors can be a basic camera, a spectrophotometer, or a microplate reader. Microarray technology is a good example for biosensors that are utilizing photometric transducers.

1.1.3.3. Piezoelectric Transducers

Piezoelectric transducers depend on the principle of piezoelectric effect. Piezoelectric effect is when application of mechanical stress to piezoelectric material creates an electric potential. Also application of an electric potential to the piezoelectric material will cause a mechanical change on the piezoelectric material[9]. A piezoelectric sensor is constructed which can detect binding and mass changes when biorecognition event is occurred. Change in mass causes the piezoelectric material to bend (Mechanical change) which creates an electric potential because of the piezoelectric effect. Mechanical change and the resulting electric potential are relative to analyte concentration.

1.1.3.4. Calorimetric Transducers

Chemical reactions are either endothermic which requires heat or exothermic which releases heat. Released heat can be measured. In biosensors, chemical reaction that releases heat in the biorecognition event can be used for analyte detection. Released heat will be relative to the concentration amount[7].

1.1.4. Electronics

Electronics of a biosensor is where the transduced signal is processed and made ready for the display. Complex calculations or basic transformations can take part here. Signal amplification occurs at this part and the signal is turned into an digital one from an analog one[1]. Signal can be delivered to display via wired connection or wireless connection. Smartphones and computers can be utilized for complex calculations instead of using discrete electronics designs.

1.1.5. Display

Display is the final component of the biosensor. It could be in the form of a screen, print out or a digital message. It could give quantitative or qualitative result. Result can be given as graphs, numbers or images[1].

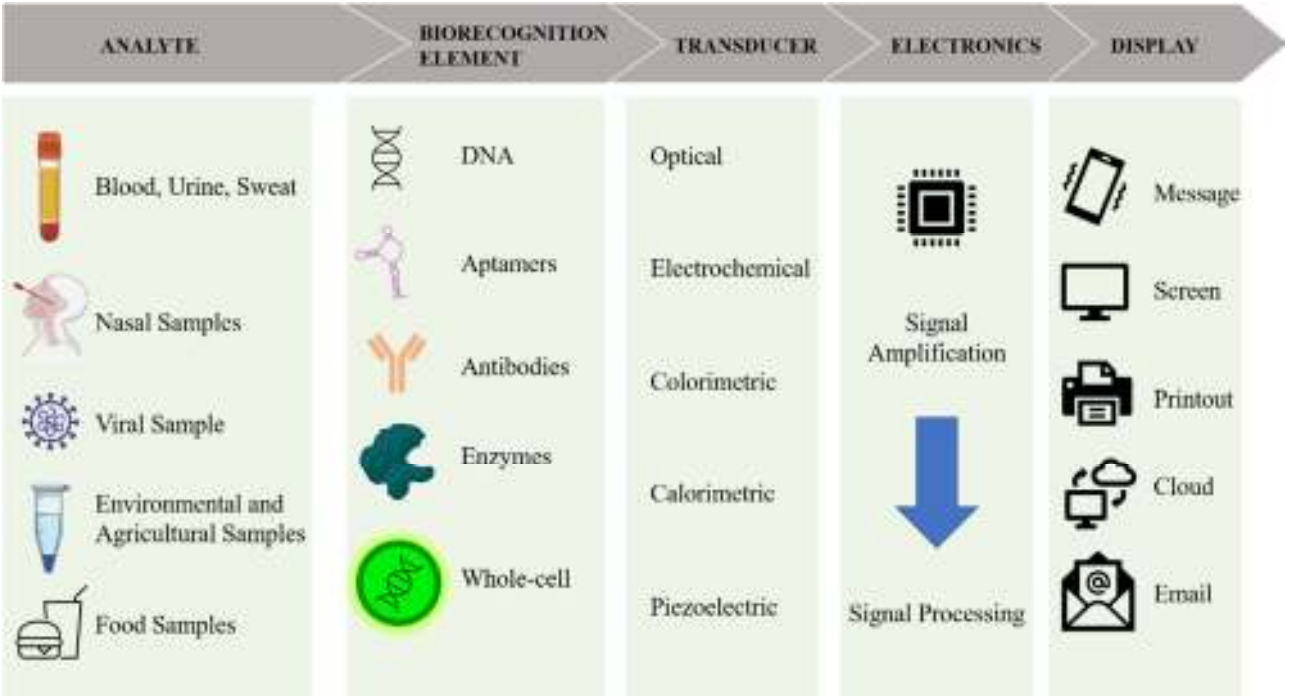

Figure 1.2: Components of a biosensor[10].

1.2. CHARACTERISTICS OF A BIOSENSOR

1.2.1. Selectivity

For a biosensor, selectivity is the most important characteristic. Selectivity is the ability of a bioreceptor to target specifically the target analyte and not the other elements in the testing solution. Without selectivity, a biosensor cannot function[1].

1.2.2. Stability

Stability can be defined as the resistance of a biosensor to time and environmental disturbances[1]. These disturbances can be temperature, humidity, altitude, degradation of the bioreceptor. Effectiveness of a biosensor can decrease with time. Thus, stability window and conditions of a biosensor is very important for its accuracy and precision.

1.2.3. Sensitivity

Sensitivity is the range that a biosensor is able to detect an analyte accurately and precisely. Detection range is called limit of detection (LOD)[1]. In medical and environmental monitoring application LOD can get low as ng/ml.

1.2.4. Linearity

Linearity can be defined as accuracy of a biosensors measured response. connection between the analyte amount and the measured response should be mathematically linear[1]. Biosensors are constructed to stay in a linear range for the application they are made for.

1.2.5. Reproducibility

Reproducibility can be defined as the ability of a biosensor to give identical measured responses in repeated experiments[1]. This is paramount for medical applications. Reproducibility can be characterized by accuracy and precision of biorecognition event, transducer, and electronics. Precision is the ability to produce similar results in every trial. Accuracy can be defined as the ability of a biosensor to give results that are as close as possible to the "true" value when multiple trials are performed[1].

1.3. LACTATE MONITORING

Lactate monitoring has uses in medicine, food production and sports science. Lactate is a product of carbohydrate metabolism thus making it a clinical biomarker in medicine and sports science. Also, lactate monitoring is used for fermentation process control in food industry. Lactate is levels in a person's blood, depends on the lactate production of their muscles, erythrocytes, and rate of metabolism in liver. Low lactate levels are a biomarker for low oxidation rate in tissues, cancer, diabetes, organ failure and sepsis. Being able to detect sepsis makes point-of-care monitoring of lactate a very important research area. High levels of lactate also a biomarker for organ failure, drug overdose and sepsis. Normal lactate level in blood of a healthy person is between 2mmol L^{-1} and 4 mmol L^{-1}. Higher levels are

shown to be a sign of acute sepsis and extreme levels such as 7-8 mmol L^{-1} are shown to indicate lethal Anastomotic bowel leak, which requires early diagnosis for a successful treatment. In food industry, lactate monitoring is commonly used in food production facilities. Fermentation processes are controlled by lactate monitoring and foods that are marketed as "low lactate containing foods" are checked for their lactate content. In sports medicine, lactate monitoring is used to establish lactate threshold of athletes. Lactate threshold is a biomarker for fatigue. Training programs are design to improve lactate thresholds of athletes. Electrochemical biosensors utilizing enzymes are commonly used for lactate monitoring. Most common enzymes used in electrochemical lactate biosensors are lactate oxidase (LOx) and lactate dehydrogenase (LDH)[11].

1.3.1. Lactate Oxidase (LOx)

Lactate oxidase is an enzyme that can be obtained from various bacterial sources such as *Aeroccoccus viridans*, *Pediococcus* and *Mycobacterium smegmatis*. It is a globular flavoprotein with a size of 5 nm. Lactate oxidase catalyzes the oxidation of L-lactate into pyruvate and H_2O_2 when in presence of O_2. This reaction can be used in an electrochemical biosensor set-up for lactate monitoring. Resulting H_2O_2 is electrochemically active and can be either reduced or oxidized to create a current that is relative to the analyte (lactate) concentration[12]. Chemical oxidation of H_2O_2 can be accomplished via a mediator chemicals such as Prussian blue or another enzyme such as horseradish peroxidase (HRP)[13]. A typical enzymatic reaction of a lactate oxidase biosensor is given below[12].

$$L - Lactate + O_2 \xrightarrow{Lactate\ oxidase\ (LOx)} pyruvate + H_2O_2 \quad (1)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (2)$$

1.3.2. Lactate Dehydrogenase (LDH)

LDH is a protein found in the blood cells and hearth muscles and various other tissues. It has a quaternary structure. LDH has medical importance for the detection of tissue damage. LDH is released when a particular tissue is damaged, thus making LDH a biomarker for

tissue damage. L-LDH converts L-lactate into pyruvate and NADH. LDH is classified as an oxidoreductase, which catalyzes oxidation of α-hydroxyacids. As a dehydrogenase, L-LDH transfers a hydride between molecules. L-LDH is used in biosensor development with its coenzyme NAD. In presence of NAD, L-LDH converts L-lactate into pyruvate and NAD shuttles electrons while being converted in to NADH. When a potential applied to the electrode surface, NADH is oxidized. Reaction generates electrons which creates a current proportional to the lactate concentration. Chemical process of electron generation in a generic L-LDH biosensor is given below[14].

$$L - lactate + NAD^+ \xrightarrow{L-LDH} Pyruvate + NADH + H^+ \quad (3)$$

$$NADH \xrightarrow{Electrode} NAD^+ + H^+ + 2e^- \quad (4)$$

1.4. CARBOHYDRATE BINDING MODULE / CELLULOSE BINDING DOMAIN (CBM/CBD)

Cellulosome is a protein complex, which is found in cell surface of some bacteria for degradation of plant cell wall polysaccharides. Cellulosome is made up of scaffolding containing large cohesin subunit and a catalytic subunit containing dockerins. The interaction between dockerins and cohesins are specific to each other depended on the type of dockerins and cohesisins. Same types will bind to each other with an affinity constant higher than most protein interactions. Carbohydrate binding module (CBM) is found on the end of the scaffolding of the cellulosome complex. It has no catalytic activity. Purpose of CBM is to recognize and tightly bind to polysaccharides for bringing celluloseome into closer contact with the polysaccharide for increasing catalytic activity of the cellulosome complex[15]. *Clostridium thermocellum* has a cellulosome complex containing enzyme called cellobiohydrolase 9A (Cbh9A), which its structure is widely researched and available in literature. Mutations are used on the CBM part of the enzyme to increase its affinity to cellulose making it a great option for enzyme immobilization on cellulosic materials such as nanofibril cellulose (NFC), nanocrystalline cellulose (NCC) and paper[16]. Cellulosome complex of *Clostridium thermocellum* ‘s cellobiohydrolase 9A is given in figure 1.3.

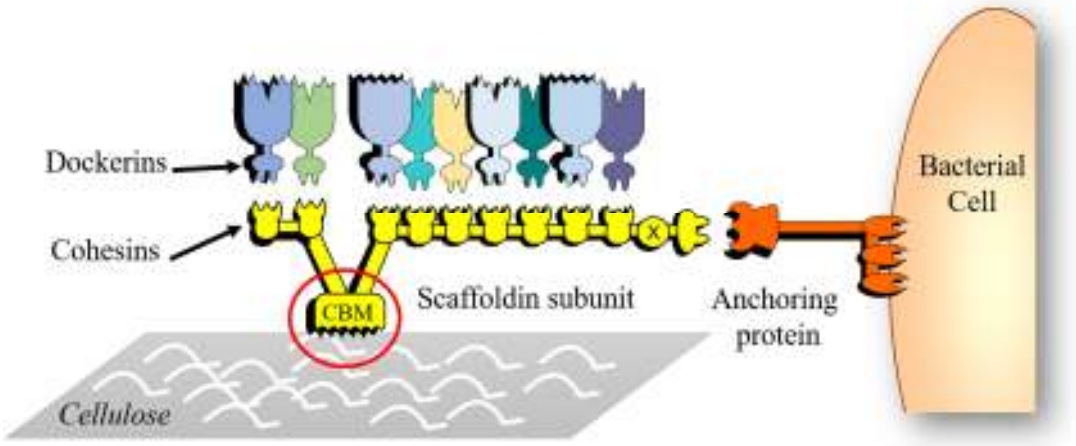

Figure 1.3. Drawing of the proposed cell-surface interaction of the cellulosomal components of *C. thermocellum*. CBM is shown with red circle[16].

1.5. SCREEN-PRINTED ELECTRODE BASED BIOSENSORS

Electrochemical experiments always dependent on an electrochemical cell set-up, using metallic electrodes. Recently, research is focused on reducing the size and manufacturing cost of electrochemical cells for point of care diagnostic applications. Carbon based materials became an interesting choice for electrochemical cell development. Usage of carbon pastes made development of cheap, small, and affordable electrochemical cells. Different printing approaches are used for electrochemical electrode fabrication such as roll-to-roll, pad-printing, and screen-printing. All these techniques have advantages and disadvantages. Pad-printing for example can be used for thin-film transfer to produce electrochemical electrodes, but they it is not ideal for mass production. On the other hand, Screen-printing has the ability for the mass production of an electrochemical electrode. Because of mass production ability and cost advantages, screen-printing revolutionized the electrochemistry field by combining power of laboratories with point of care applications. A good example to the screen-printed electrode biosensor would be the glucose sensors used today. It became a billion-dollar industry featuring screen-printed glucose electrodes for point of care patient testing. These electrodes improved diabetes management immensely and helped millions of patients. Other than commercial glucose sensors, there are many other biosensor designs utilizing screen-printed electrodes. Examples can be found in literature about such sensors used for detection of narcotics and drugs in a subject's urine or lactate biosensors utilized in medicine and sport science for various lactate determination purposes.

To keep in mind, other than enzymes DNA is also utilized with screen-printed electrodes to detect radiation damaged DNA. Another biorecognition element other than enzymes are proteins that target certain cancer biomarkers. This process allows for early detection of tumor cell formation within the body. Screen-printed electrode surface can be modified with various processes and chemicals for enzyme immobilization and reaction mediation. Easiest method for modification of the surface is the drop-cast method which a small volume of liquid is dropped on the electrode. This could be a polymer, which becomes a gel after a while or can be a chemical that needs to be dried. Screen-printing allows precise control over electrode geometry. This allows the production of compact electrode systems that can work with microliter volumes of samples. The most common electrochemical techniques used with screen-printed electrodes are cyclic voltammetry and amperometry[17]. A typical voltametric and amperometric electrode consists of 3 main parts. A working electrode, which the biorecognition event happens on. It could be made from platinum, carbon, or gold for easier protein immobilization. Second component is a counter/auxiliary electrode which is used to complete the electrode circuit and let the current flow. Third component is a reference electrode, which uses mostly a mixture of Ag/AgCl. Purpose of the reference electrode is to have a fixed potential that is comparable against the potential applied to the working electrode which is than measured and controlled[18]. These 3 main parts are printed on a substrate such as plastic (PVC, PP, PE) or ceramic and covered with a dielectric layer to prevent short circuits[19]. A screen-printed electrode designs is given in figure 1.4.

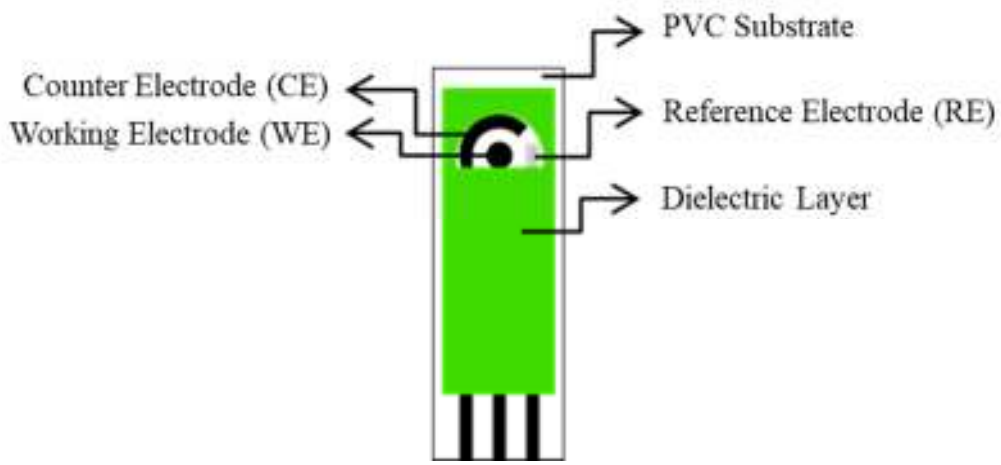

Figure 1.4. Design of a generic screen-printed electrode made for amperometry applications.

1.5.1. Screen-printing Process

Screen-printing is a process used in various fields such as textile, electronics, research, and media. Process consists of a very simple yet effected manufacturing method. First, a silk, nylon or stainless-steel mesh is stretched on an aluminum or wood frame. This is called a screen. A stencil design is imprinted on the screen. The substrate is placed under the screen and printing ink is deposited on the screen. With the help of a squeegee, ink is smeared on the screen and transferred to the substrate. Last step is the curing of the ink[20]. Simple description of the screen-printing process is given in figure 1.5.

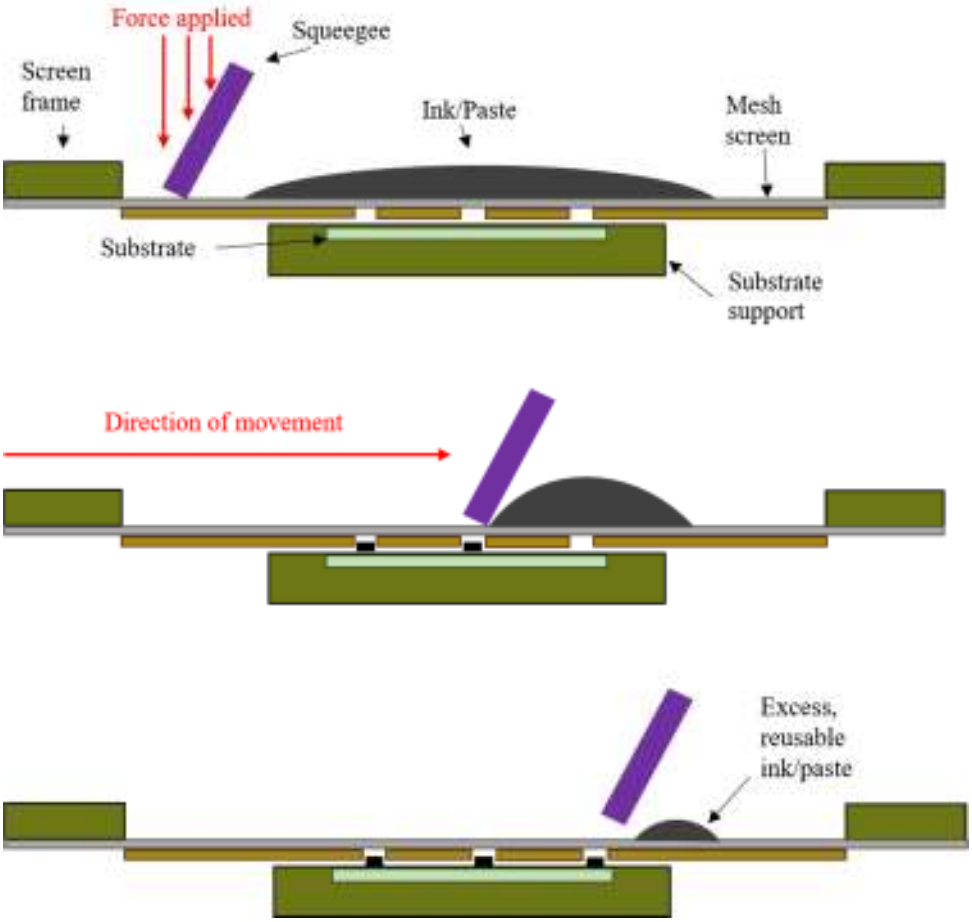

Figure 1.5. Simple description of screen-printing[17].

1.5.1.1. Screen-printing Machinery

Screen-printing machinery can be divided into 3 groups. Manual machines are operated via hand and usually consists of a screen holder and a table with vacuum capabilities connected

to it. Substrate is placed over the table and secured in place via vacuum. Shown in figure 1.6. Semi-Automatic machines operate the printing process automatically. Screen change, ink deposition and substrate feeding are done manually. Shown in figure 1.6. Fully automatic machines are belt-fed, computer aided screen-printing machines. They accomplish the whole process automatically and can be attached to a curing oven with a belt for further automation. Shown in figure 1.6.

Figure 1.6. A) Manual screen-printer, B) Semi-automatic screen-printer, C) Fully automatic screen-printer.

1.5.1.2. Screen

Screen is the most important part in screen-printing workflow. For designs that include different materials or overlapping shapes, more than one screen can be used in screen-printing process. For screen-printed electrodes, electrodes that require different materials are usually printed with different screen after each print is cured. There are 3 main components to consider in a screen. First one is the screen frame. It could be wood or metal. Metal is considered expensive but more robust and durable. Most of the time aluminum is the metal of choice. Wood is a cheaper option, but it is more prone to damage. Second component is the stencil-design. Desired design is imprinted on the stretched mesh via UV emulsion. Design is printed on an opaque film. Emulsion is applied on the stretched mesh (figure 1.7 A) and then the film is placed between the screen and a UV light source (figure 1.7 B). Emulsion thickness is determined by the viscosity of the emulsion. Thin emulsion will cause disfigured prints and will be more prone to damage. Too thick emulsion will block some of the ink from releasing causing in complete prints. Emulsion proceeds to polymerase in locations that are exposed to the UV light. Opaque film blocks UV access according to the

desired stencil design. After the procedure, screen is washed with water and un-polymerized emulsion, where UV light is blocked by the opaque film, is removed. Product is then cheeked for openings on the polymerized emulsion[20].

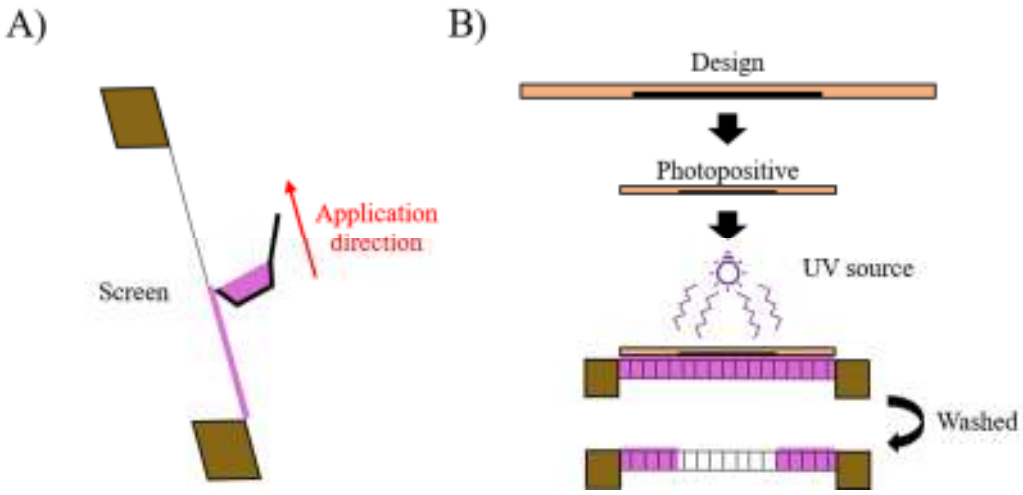

Figure 1.7. A) Emulsion application, B) UV curing of the emulsion[20].

Last part of the screen is the mesh. For the mesh, nylon, polyester, or stainless steel is used. It should be noted that, stainless steel has one distinct feature, which ink should be chosen specially for it to avoid oxidation damage. All these materials have different properties, which is discussed in table 1.1.

Table 1.1. Comparison of screen mesh materials from best to worst[17].

Screen Requirements	Polyester	Stainless Steel	Nylon
Flexibility	Best	Average	Worst
Resilience	Best	Average	Worst
Percentage of open area	Average	Best	Average
Stability of print size	Average	Best	Worst
Damage from squeegee	Average	Worst	Best
Accidental damage	Average	Worst	Best
Cost	Best	Worst	Best
Minimal snap-off from large areas	Average	Best	Worst

Mesh is stretched and glued to the screen-frame. Mesh holds the stencil design even after a pressure is applied upon it by the squeegee and the printer. While designing the frame and

the mesh, opening area is calculated. Passage of the paste though the screen is determined by the percentage of the open area. For better prints and more ink transfer, type of the mesh should be chosen allowing higher percentage of open area. Open area is calculation is given below[17]:

$$Open\ Area = \frac{(mesh\ area)^2}{(wire\ diameter + mesh\ opening)^2}\ x\ 100\% \qquad (5)$$

Mesh count (*M*) is the area between crossing threads of the mesh. All 3 mesh materials have various versions with different mesh counts. Mesh count should be considered according to the specifications of the ink manufacturer and the design requirements of the print. Another measurement unit is the wire diameter, which describes a single thread's diameter[17]. Description of the mesh count and wire diameter is given in figure 1.8.

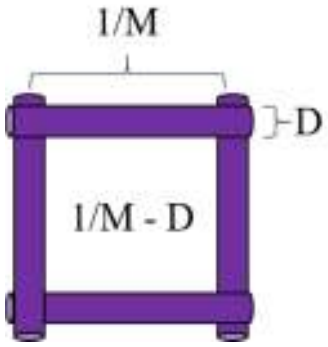

Figure 1.8. Illustration of mesh count[17].

With mesh count size and wire diameter one can calculate the mesh opening, which is a crucial calculation for knowing what size of ink particles can pass through the screen and suitable with the material at hand. General formula for the calculation of the mesh opening is given below:

$$O = \frac{1}{M} - D \qquad (6)$$

An example set of design imprinted ready to use screens for printing screen-printed electrodes are given in figure 1.9.

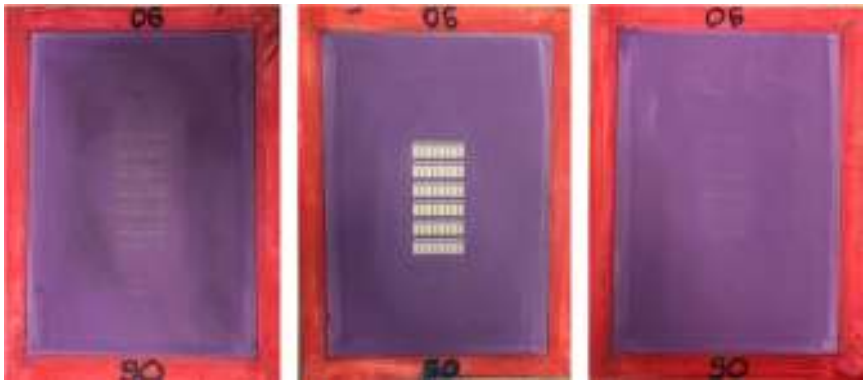

Figure 1.9. Example set of screen-printing screens with designs imprinted.

1.5.1.3. Squeegee

Squeegee is a vital part of the screen-printing processes. It is used to push printing ink through the screen, on to the substrate. Squeegee must be resilient and flexible, thus making material choice an important decision. Most used squeegee material is polyurethane. Polyurethane squeegees are very durable, lasting about 20.000 prints. Squeegees are made with different toughness levels. Softer squeegees bent more easily and cover more surface. Squeegees are generally made 10 mm wider than the print area[17]. Pressure applied with the squeegees must be constant and application angle must be precise. In literature, best application angle for the squeegee is given as 60 degrees and with the application of pressure tip of the squeegee must bent to 45 degrees against the screen[20]. Effects of squeegee angles are given in figure 1.9. Squeegee usage and application angle is done manually by a person in manual screen-printing process. In Semi-automatic and full-automatic screen printers, squeegee angle is fixed by the operator and the pressure is entered to the machinery.

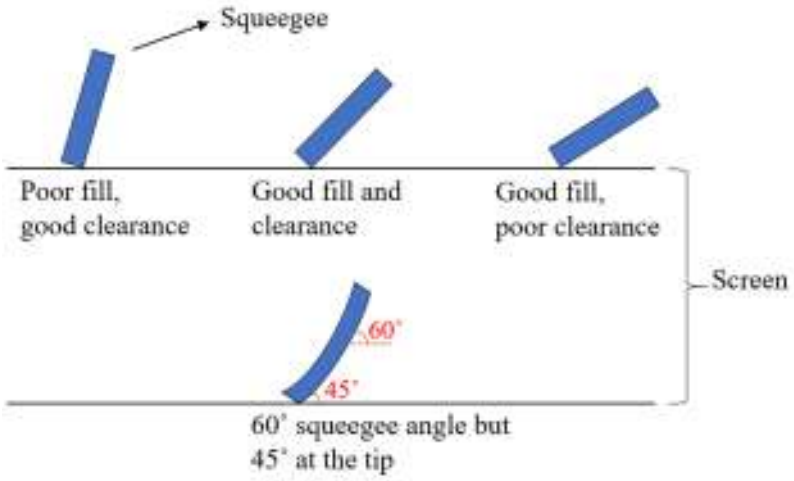

Figure 1.10. Comparison of squeegee application angles[20].

1.5.1.4. Ink

Choice of ink is an important decision in screen-printed electrode design. Ink should be chosen according to the biorecognition element, the biorecognition reaction that will occur and the substrate. Carbon materials are popular choices for screen-printed electrodes. Most of the commercial screen-printed electrodes utilize carbon materials to some extent. These materials include graphite, graphene, carbon nanotubes (CNTs), and many materials derived from them. Carbon materials show great conductivity, and they could be easily utilized as inks when mixed with a resin[21]. Carbon inks are usually modified or covered with some mediator for helping or directly taking part in the biorecognition event. Materials like Prussian Blue are mixed or deposited electrochemically to the surface of the carbon inks. Prussian Blue is a mediator for the reduction of H_2O_2 when O_2 is present, which makes it a great choice for biorecognition elements that are part of oxidase class enzymes[22]. Other additives such as CNTs or multiwalled CNTs (MCNTs) are utilized for both enzyme immobilization and electron transfer capabilities[21]. Silver is another material utilized in screen-printed electrodes. Plain silver is an excellent material for printing conductive paths for connecting WE, RE, CE and outside connectors. Modified silver, such as silver with silver chloride (Ag/AgCl) is utilized for RE production because of its fixed stable potential[23]. Platinum and gold electrodes are also utilized as WE. Platinum is an alternative to carbon inks. Gold is used in immunosensors for its ability to bind antibodies, which is well described in literature[24]. Dielectric inks are usually plastics based and used for insulating conductive paths and electrodes. When an ink is chosen for application, its viscosity is an important factor. Solvents are used to dilute inks for screen-printing.

Viscosity should be decided according to the screen and thinning must be performed via the instructions given by the ink manufacturer. Figure 1.10. shows the right amount of thinning required for printing.

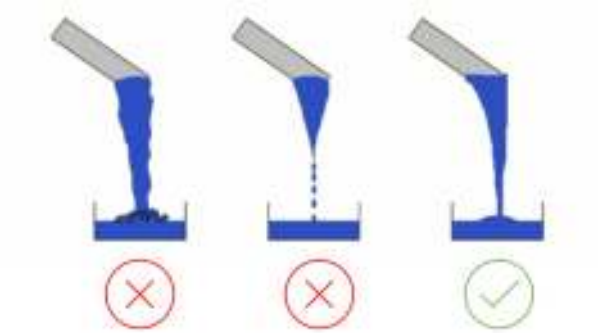

Figure 1.11. Viscosity example for screen printing (Right amount is shown with green check mark)[20].

Screen-printing inks require curing when the whole printing process is complete. Curing is used to evaporate solvent used in the ink. Removal of solvent causes ink molecules to "stack" on each other and create a conductive stack on the substrate[17]. Whole process is described in figure 1.11.

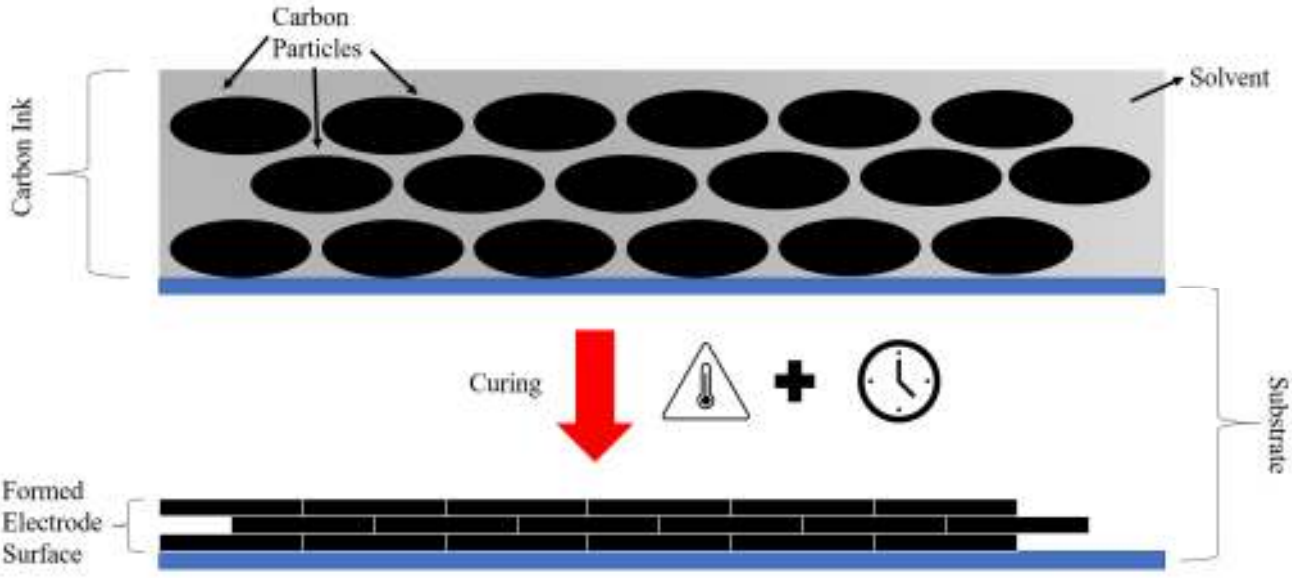

Figure 1.12. "Stacking" of carbon ink after evaporation of the solvent via curing (Heat applied in a fixed time)[17].

Curing process should be applied according to ink manufacturer's instructions and durability of the substrate. Temperature and curing time are usually given by the ink manufacturer. In

some cases, temperature can be lowered, and time can be increased to preserve substrate integrity.

1.5.2. Enzyme Immobilization Methods

Biosensors require stable enzymes for accurate measurements. One of the best ways of increasing enzyme stability is to immobilize enzymes on to the electrode surface. Most enzymes are naturally unstable and costly to produce. Thus, in biosensors, immobilization is important to reduce enzyme waste in manufacturing, increase stability for end product and increase durability of the biosensor[25]. Immobilized enzymes can be defined as enzymes that are "physically attached or confined to a solid matrix or surface while also keeping their catalytic activity intact in continuous and repeated usage"[26]. Basic requirements for immobilization techniques are protection from microbial contamination, providing adequate support, ability to increase enzyme specificity/activity, inertness and showing no inhibitory effect on the biorecognition event. Immobilization can be used to create multi-enzyme biosensors. There are many immobilization techniques utilized in biosensor production. The most important ones are adsorption, covalent bonding, entrapment, encapsulation, and cross linking. Figure 1.12. gives examples of each method.

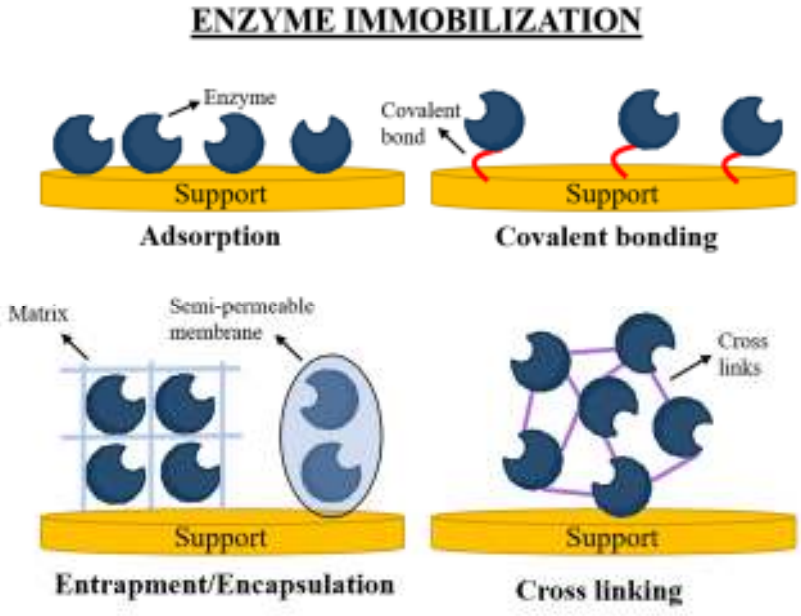

Figure 1.13. Most common enzyme immobilization methods[26].

1.5.2.1. Adsorption

Adsorption is the most basic immobilization technique for enzymes. It results from week interactions such as Van der Waal's forces, hydrophobic interactions, and electrostatic interactions. Adsorption protects the enzyme from proteolysis, aggregation, and hydrophobic interactions. Disadvantages of adsorption are poor operational and storage stability and nonspecific protein contamination of the immobilization surface. Adsorption can be divided into 3 subcategories called, physical adsorption, electrostatic adsorption, and hydrophobic adsorption. Physical adsorption is simple, either enzyme is dropped on the support matrix and dried out or support matrix is dipped in an enzyme solution and kept there for some time. Both methods are followed by a wash step to get rid of excess unbound enzyme. Disadvantages of physical adsorption are enzyme leakage, susceptibility to environmental changes (pH, temperature, salinity, humidity, etc.) and lack of homogeneity of immobilized enzymes[26]. Electrostatic adsorption is dependent on the charge of the surface and the charge of the enzyme. With using opposite charges one can immobilize enzymes on to a charged surface or matrix. Last adsorption method is using hydrophobic interactions. Displacement of water molecules in a support matrix or on the enzyme surface. This creates a binding because of the resulting entropy gain[26].

1.5.2.2. Covalent Bonding

Covalent binding utilizes functional groups on enzyme molecules to create stable complexes with support matrices via usage of covalent bonds. Important factor is that the functional group that is used for immobilization should not have a functional role on the enzymatic activity. This is important for preventing inhibition of the enzyme. Most common targets for covalent bonding are side chains of lysine (ϵ-amino group), cysteine (thiol group) and glutamic acid and aspartic acid (carboxylic group). Most common functional groups involved in covalent bonding are the following: Carboxylic group, sulfhydryl group, amino group, phenolic group, thiol group, indole group, imidazole group and hydroxyl group. To create covalent bonds, first the surface is activated via using linker molecules such as glutaraldehyde or carbodiimide. Second step is to introduce the enzyme and let the linker molecules (glutaraldehyde or carbodiimide) create a bridge between the enzyme and the activated surface via covalent bonding. Covalent bonding is great for creating strong bonds between the support matrix/surface and the enzyme, thus limiting enzyme leakage to

minimum. Disadvantage of the covalent bonding is the fact that enzymes are immobilized from their functional group which in turn can cause denaturation of the enzyme[26].

1.5.2.3. Entrapment / Encapsulation

Entrapment is the processes of trapping enzymes in a support matrix. Enzymes are not attached to the matrix directly instead; they are trapped by a matrix which allows substrate and product transfer but keeps the enzyme in place. Entrapment is a two-step process. First, enzyme is mixed with a monomer solution. Secondly, monomer solution is polymerized via a chemical reaction or environmental change (Heat, electricity, pH, etc.). Resulting polymer matrix will have enzymes trapped in it. Because enzymes are not part of the polymerization reaction, they are trapped inside without being directly bonded to the support matrix. Because of this, entrapment can improve enzyme stability while reducing the enzyme leakage and denaturation. Encapsulation is a similar process to entrapment. Difference comes from the fact that enzymes are encapsulated by a semi-permeable membrane instead of getting trapped in a support matrix. This semi-permeable membrane allows transport of selected molecules and also helps to modify the enzymes solubility, pH stability, amphilicity. Disadvantages of entrapment and encapsulation comes from the fact that that they are product of polymerization. Polymerized matrix can be too thick for enzymes substrates to reach the active site of enzymes, thus reducing the activity. Also, polymerized matrix can have large pores, which can cause enzyme leakage instead of achieving enzyme entrapment. Entrapment immobilization can be achieved via lots of different methods such as, photopolymerization, sol-gel process, electropolymerization. For encapsulation, micro encapsulation is sued to create microcapsules. Electrochemical polymerization is achieved via application of a fixed potential or current to an enzyme containing monomer solution. Polymerization is facilitated by the reactive radical species released from oxidation or reduction of monomers in the solution. Polymerization starts from the electrode surface and continuous from there. To produce electropolymerized films, electronically conducting polymers such as, pyrroles, thiophenes, polyindoles and polypyrroles are used. The most important advantage of electropolymerization is the ability to control polymerization via electricity, thus allowing controlled thickness and formation of the entrapment matrix[26]. Sol-gel process is creating polymer matrices made up of metal-oxide, silica and organosiloxane. Sol-gel process is a low temperature process, meaning the chemical reaction for polymerization does not require heat, which in turn makes sol-gels attractive

immobilization matrices for enzyme entrapment purposes[27]. To produce a sol-gel, there are 2 methods available. First one is called colloidal method which is not preferred for enzyme immobilization. Second method, which is widely utilized for enzyme immobilization is the polymeric method. In polymeric method, a metal alkoxide precursor such as tetramethoxysilane (TEOS) is dissolved in a liquid. When pH is acidic, hydrolyzation will occur and formation of silanol (Si-OH) groups will begin. Then, a base such as potassium hydroxide will be added to the mixture to initiate condensation and creation of siloxane (si-O-Si) polymers which is a great matrix for enzyme entrapment. TEOS based sol-gel is widely used in literature for biosensor production[28][27]. Benefits of sol-gel methods are their stability and their ability to increase biosensor sensitivity. Disadvantages of the sol-gel method is cost of materials and fragility of produced the sol-gel polymers when mechanical pressure is applied. Photopolymerization is the method of using radiation to polymerase photopolymers. Photopolymerization is a chain-growth polymerization which when UV or visible light is applied to the photopolymer (resin), polymerization occurs. When exposed to light, cross-linking of monomers start creating a polymer. It is a good method for enzyme immobilization because it generates no heat. Enzymes are mixed with the resin before application of the light source. Microencapsulation is when enzyme is encapsulated by a spherical semi-permeable membrane. This membrane can be composed of polymers, lipoproteins, lipids, or nonionic materials. Two methods are utilized for microencapsulation. First method is using coacervation, which separation of enzyme micropelets are carried out in a water immiscible solvent. Second method is called interfacial polymerization in which polymerization of monomers are occur in the interface of two immiscible substances. With encapsulation, resulting membrane will let small particles like substrate and products through while blocking enzymes from getting out. Most obvious advantage of this method is the fact that it encapsulates the enzyme thus making enzyme's charge and polarity as same as the membrane. Disadvantages of this method is the importance and difficulty of getting the pore size right. Large pores can lead to enzyme leakage[26].

1.5.2.4. Cross-Linking

Cross-linking is an irreversible enzyme immobilization method. It depends on the creation of intermolecular cross-linkages between the enzymes. Usually, a multifunctional reagent is used to link the enzymes into three-dimensional cross-linked aggregates. Enzymes are not

bound to any support. They are cross-linked to each other. Two methods are existing for cross-linking enzymes. First one is called cross-linking enzyme aggregate (CLEA) and the second one is called cross-linking enzyme crystals (CLEC). A cross-linking enzyme such as glutaraldehyde is utilized in both methods. With glutaraldehyde, free amino groups of lysine residues on near enzymes are cross-linked to each other. CLEC method utilizes crystalized enzymes with glutaraldehyde. Because enzymes are crystallized, they tend to be very stable and work with higher efficiency. CLEA is an improvement over CLEC. CLEA method uses salts, organic solvents, and non-ionic polymers to form enzyme aggregates. In this form, enzymes retain their catalytic activities. Cross-linking is a simple but effective method. Leakage is minimized by the strong bonds created between enzyme molecules. One of the advantages of cross-linking is that microenvironment of the enzyme can adjusted to improve enzyme stability. Disadvantage of this method comes from the use of glutaraldehyde. It could cause severe enzyme modifications thus causing conformational change and loss of activity. To overcome this, inert proteins such as bovine serum albumin (BSA) and gelatin is added to the immobilization step[26].

1.5.3. Potentiostat

Electrochemical transducers are the method of detection in screen-printed electrode-based biosensors. Analytes are detected via amperometry or potentiometry. Electronic circuitry that performs amperometry and potentiometry is called a potentiostat[29]. Potentiostat creates a specific potential drop between the working electrode and the analyte solution. Also, when current is generated in the biorecognition event, it is amplified by the potentiostat. A potentiostat can use different electrochemical techniques such as amperometry, cyclic voltammetry, linear voltammetry, etc. Potentiostat instruments can be produced as laboratory level expensive machines or open source do it yourself (DIY) devices[30][31]. Basic requirement for a potentiostat is the ability to control and measure electrical current with a corresponding software. Software can be a complex computer program or just a basic calculation script which will display the resulting value. Portable potentiostats are becoming revolutionary tools for point of care diagnosis. They are utilized in in situ environmental monitoring, disease management and diagnosis, sports science. A good example to portable consumer grade potentiostat is the glucose meter. Glucose meter is a basic potentiostat, which is calibrated to the screen-printed electrode-based glucose strips of the producing company. Millions of diabetics use portable glucose meters in their diabetes

management to check their blood glucose levels. Modern potentiostat are designed specifically for their application areas. There are potentiostat systems able to use Bluetooth and wi-fi for wireless data transfer[32][33]. Some examples of potentiostat systems are given in figure 1.14.

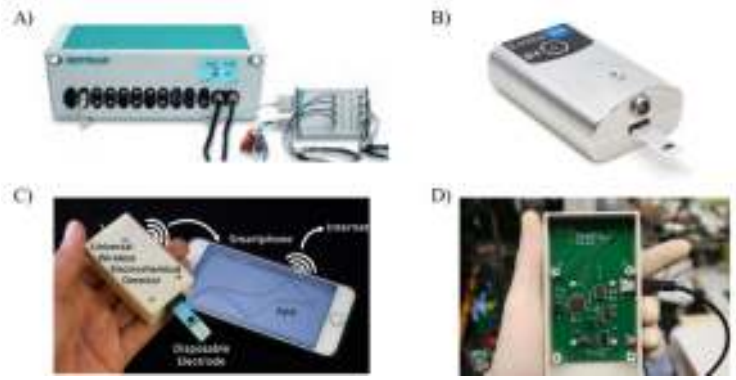

Figure 1.14. Potentiostat examples. A) Metrohm Autolab laboratory scale potentiostat. B) PalmSnese EmStat Blue portable potentiostat. C) Open-Source Potentiostat for Wireless Electrochemical Detection with Smartphones. D) CheapStat: An Open-Source, ‘‘Do-It-Yourself’’ Potentiostat for Analytical and Educational Applications[33][31].

1.6. RECOMBINANT ENZYME PRODUCTION

Recombinant protein production is a widely used protein engineering technique. A desired protein gene from an organism is cloned into a plasmid then transformed to a different organism which will perform protein expression[34]. Recombinant enzyme production is a subcategory of protein production which interest itself with only enzyme production. Enzymes are very important for various industries. Enzymes are utilized in various industries such as, cleaning products, medicine, animal feed, food, drug, and alcohol. The ability to express same enzyme exponentially more or a better modified version of it is very important need for all these industries. The solution to this need is to produce enzymes recombinantly. To call an enzyme “recombinant” there are some rules to follow. First, amino acid sequence of an enzyme should be same as its original source but must be expressed in a nonnative organism. Secondly, expression organism should be genetically modified. Lastly, enzyme can be modified in its sequence to improve its properties. Recombinant DNA technology is the basis of these procedures. İsolation of a target gene and then transformation of another organism via a carrier such as a plasmid containing the isolated gene are called “cloning”[35]. Steps for cloning a gene is given below:

1. Selection of gene of interest from a suitable donor organism. A screening process or a DNA sequencing can be performed to decide on the organism[35].
2. Choosing of a host organism. Choosing the right host organism is important for expression of the gene product. It should allow our target gene to be replicated, transcribed, and translated. In some cases, must perform some post-translational modifications[35].
3. A suitable vector for the host organism. Most common vector is double-helix circler DNA fragments called "Plasmids". Plasmids can be found in cytoplasm's of bacteria. Plasmid containing an origin of replication and an antibiotic resistance gene which can be utilized for selection. Gene of interest is cloned into multiple cloning site (MCS)[35].
4. Target gene can be isolated from the donor organism via PCR reaction using suitable primers. Shotgun cloning can be utilized. Also, gene can be synthesized synthetically[35].
5. Enzymatic reactions are performed on the insert(gene) and the vector (Plasmid) to bring them together. First, restriction enzymes are used which, can cut a DNA fragment from a specific palindromic sequence. Restriction digestion allows the creation of compatible "sticky ends" between the insert and the vector. After the restriction digestion ligation procedure is applied. DNA ligase ligases insert into the vector via the "sticky ends"[36].
6. Vector with the recombinant gene is introduced into the host cells. Most popular method is to use an *E. coli* strain made competent via electroporation, chemically or using sonication. This step is called transformation[35].
7. Recombinant clone isolation. Cells are grown on agar plates with a selective marker (antibiotic). Cells with the recombinant insert will have the necessary antibiotic resistance genes to survive in the antibiotic containing agar plate. A single colony is picked from the plate[35].
8. Fermentation, cultivation, and bioreactor process. Cells are grown in large scale fermenters or flasks with best media required for expression. In this media, ingredients are chosen for regulatory DNA elements such as promotors, regulators, and inhibitors. Inducers are chemicals that promote gene expression on a specific promotor. Most used promoter system is Lac operon. Lac operon is induced with the

usage of Isopropyl β-D-1-thiogalactopyranoside (IPTG). IPTG is a chemical mimic of allolactose[34].

9. Enzyme purification. Enzyme purification can be done via following methods: (1) Affinity chromatography which depends on an affinity tag expressed with a protein such as Histidine tags. (2) Size exclusion chromatography is used to separate proteins by size. (3) Ion-exchange chromatography is used to separate proteins using charge. (4) Salting-out is used to precipitate proteins using salt concentration. (5) Dialysis is used to separate a specific size of protein via a dialysis membrane[37].
10. Quantification and analysis. SDS-Page and Native-Page electrophoresis are used to analyze the resulting proteins. Size of the protein is compared to the wild type one to make sure right protein is purified. Nanodrop spectrophotometry is utilized to quantify the amount of protein expressed and purified[38].

2. Materials

2.1. SCREEN PRINTING PROCESS

2.1.1. Hardware

- 3x 70x40cm Aluminum Screen – Ceren Serigrafi
- Manuel PCB Screenprinter – NeoDen PM3040
- Guillotine Cutter - Ideal
- Polyurethane Squeegee – Ceren Serigrafi
- Potentiostat - BlueSense EmStat3 Blue
- Vacuum Compressor
- Incu-line Incubator - VWR
- Pipette Set – AXYGEN
- Digital USB Microscope - Jiusion

2.1.2. Dyes, Chemicals and Substrate Materials

- Carbon Paste - Gwent ink C2030519P4
- 60:40 Silver/Silver Chloride Paste – Gwent ink C2130809D5
- 120x100 PVC and PP sheets
- 200x200, 2.7mm PMMA
- Grey Dielectric Paste – Gwent ink D2070423P5
- Paint Retardant – Ceren Serigrafi
- Potassium hexacyanoferrate (III) – AlfaAesar A16946
- Nanofibril Cellulose (non-modified) – Akkim
- 0.1M NaOAc

2.1.3. Buffers

- 0.1M Potassium phosphate buffer
- 0.1M Potassium chloride buffer

2.1.4. Software

- PsTrace Software (Potentiostat) – BlueSense
- Office 365 Excel – Microsoft
- AutoCAD 2019 – Autodesk
- Inkscape – Opensource
- Amcap – USB Microscope Driver / Software

2.2. RECOMBINANT ENZYME EXPRESSION

2.2.1. Hardware

- Sorwall Lynx 4000 Centrifuge – Thermo Fisher SCIENTIFIC
- F12-6 x 500 LEX Rotor for Centrifuge – Thermo Fisher SCIENTIFIC

- A27-8x50 Rotor for Centrifuge – Thermo Fisher SCIENTIFIC
- MicroCL 17 Microcentrifuge - Thermo Fisher SCIENTIFIC
- JSSI – 300C Incubator - JSR
- Pipette Set – AXYGEN
- 300V Power Supply – VWR
- Mini-PROTEAN® Tetra System – BIO-RAD
- AKTAprime plus – GE Healthcare
- PHMT Thermo-shaker – Grant-bio
- DB-45 Dry Block for Strips – Grant-bio
- Vortex Genie 2 – Scientific Instruments
- Probe Sonicator – BANDELIN SONOPULS HD 2200
- 4˚C Deli Fridge - Uğur
- -21 Freezer – Uğur
- -81 Ultra Low Temperature Freezer – VWR
- QUANTUM Gel documentation system – VILBER LOURMAT
- Mini Gel II DNA Gel Runner – VWR
- Autoclave
- ProFlex™ 3 x 32-well PCR System – Applied Biosystems

2.2.2. Chemicals, Kits and Labware

- Culture Tubes
- 2.5L Ultra-yield Flasks
- 500ml Culture Bottle
- 50ml Centrifuge Tubes
- 1.5ml Eppendorf Tubes
- Aluminum Disposable Takeout Pans
- Polyacrylamide Gel (Appendix Table A10, A11)
- Tetramethylethylenediamine (TEMED)
- 10 percent Ammonium persulphate solution APS
- GeneJET® Plasmid Miniprep kit – Thermo Fisher SCIENTIFIC

- E.Z.N.A. Gel Extraction kit – OMEGA bio-tek
- Bradford Reagent – VWR
- Ni+ Beads
- Gravity Column
- 96-well plate
- 30kDa VIVASPIN Filter – Sartorius stedim biotech
- 1000x Kanamycin 50mg/ml stock solution
- 200x Protease Inhibitor (Appendix Table A13)
- LB Media
- T4 ligase and Buffer - NEB
- Kanamycin LB Agar Plate
- 1.5 percent Agarose Gel
- DNA/Protein Gel forming plates, glasses, and combs
- Ethidium bromide solution 0.025 percent (dropper) – ROTH
- PageRuler™ Plus Prestained Protein Ladder - Thermo Fisher SCIENTIFIC
- GeneRuler 1 kb Plus DNA Ladder - Thermo Fisher SCIENTIFIC
- 10X Tango Buffer – Thermo Fisher SCIENTIFIC
- 2x Phusion PCR MM
- DNA Clean Up Kit - Qiagen

2.2.3. Plasmids and Primers and Cells

- BL21 component cells
- XL1B component cells
- Genomic DNA from *L. paracasei*
- Primer Sets F-R for LDH and CBM fragment (Appendix Table A14)
- pDe2/BglA/Ct/CBM/CT plasmid (CBM) (Appendix Figure A3)
- pET28a-ybbR-HIS-CBM-CohI[39] (Appendix Figure A2)
- pET-28a (+)-6xH_DocCt_LoX (Appendix Figure A1)

2.2.4. Buffers

- 1x SDS Gel Running Buffer (Appendix Table A7)
- ZYM-5052 Auto-Induction Medium[40] (Appendix Table A12)
- Binding Buffer (Appendix Table A1)
- Wash Buffer (Appendix Table A2)
- Elution Buffer (Appendix Table A3)
- Sample Buffer (Appendix 1)
- SDS-PAGE Stain (Appendix Table A8)
- SDS-PAGE Destain (Appendix Table A9)
- 1x SDS-PAGE Sample Buffer (Appendix Table A6)
- 1x TBS Buffer
- 1x TAE Buffer

2.2.5. Software

- Geneious – Biomatters Ltd.
- VisionCapt - VILBER LOURMAT
- Benchling

2.3. ENZYME ACTIVITY TESTS

2.3.1. Hardware

- Incu-line Incubator - VWR
- Pipette Set – AXYGEN
- Varioskan® Flash Spectrophotometer - Thermo Fisher SCIENTIFIC
- PHMT Thermo-shaker – Grant-bio
- Vortex Genie 2 – Scientific Instruments

2.3.2. Chemicals and Substrates, Labware

- 1.5ml Eppendorf Tubes
- 96-well Plate
- 2mM INT
- Lactic Acid
- NAD +
- 150mM MPMS

2.3.3. Buffers

- 0.1M TRIS-HCl pH 8.2

2.3.4. Software

- ScanIt Varioscan - Thermo Fisher SCIENTIFIC
- Excel 365 – Microsoft
- IrfanView - Opensource

2.4. BINDING TESTS

2.4.1. Hardware

- MicroCL 17 Microcentrifuge - Thermo Fisher SCIENTIFIC
- Pipette Set – AXYGEN
- 300V Power Supply – VWR
- Mini-PROTEAN® Tetra System – BIO-RAD
- DB-45 Dry Block for Strips – Grant-bio
- Vortex Genie 2 – Scientific Instruments
- QUANTUM Gel documentation system – VILBER LOURMAT

2.4.2. Chemicals, Proteins and Labware

- Nanofibril Cellulose (non-modified) – Akkim
- Avicel
- Phosphoric Acid Swollen Cellulose (PASC)
- OGW 7 (A protein with CBM)
- OGW 15 (A protein without CBM)
- Protein Gel forming plates, glasses, and combs
- PageRuler™ Plus Prestained Protein Ladder - Thermo Fisher SCIENTIFIC
- Polyacrylamide Gel (Appendix Table A10, A11)

2.4.3. Buffers

- 1x SDS Gel Running Buffer (Appendix Table A7)
- SDS-Page Stain (Appendix Table A8)
- SDS-Page Destain (Appendix Table A9)
- 1x SDS-PAGE Sample Buffer (Appendix Table A6)

2.4.4. Software

- VisionCapt - VILBER LOURMAT

2.5. BIOSENSOR MEASUREMENTS

2.5.1. Hardware

- Potentiostat - BlueSense EmStat3 Blue
- Pipette Set – AXYGEN
- Laptop

2.5.2. Chemicals

- Lactic Acid
- 3M Potassium Chloride

2.5.3. Buffers

- 0.1M Phosphate Buffer pH:6.8

2.6. DOCUMENTATION AND FIGURES

2.6.1. Software

- Office 365 PowerPoint – Microsoft
- Office 365 Word – Microsoft
- Inkscape – Opensource
- Biorender
- Mendeley Desktop – Elsevier
- Google Drive

3. PROCEDURES

3.1. SCREEN PRINTING PROCESS

All screen-printing procedures are completed in Istanbul Bilgi University Protein Engineering Laboratory. AutoCAD and Inkscape have been used to design the finished electrode. AutoCAD drawing and the dimensions of the full electrode system is given in figure 3.15.

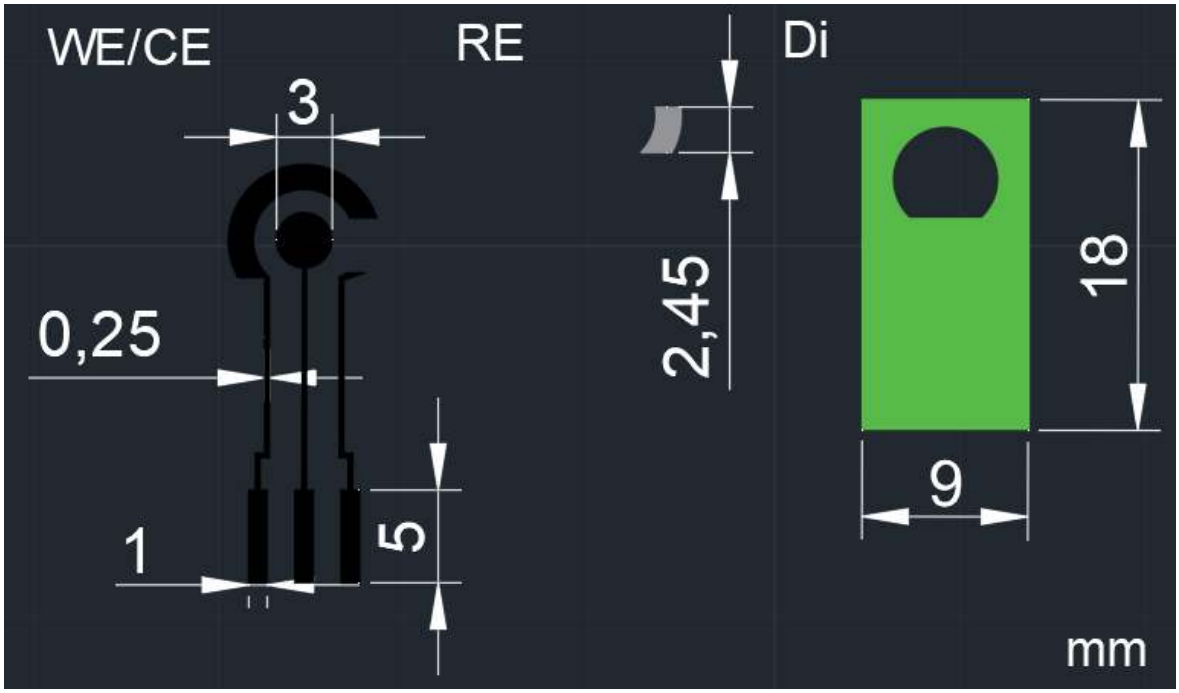

Figure 3.15. AutoCAD design of the full electrode system. WE = Working electrode, CE = Counter electrode, RE = Reference electrode, Di = Dielectric layer.

PDF files are prepared for each print layer. These layers were WE, RE, CE, Dielectric. PDF files are given to Ceren serigraphy company for production of printing screens with the required specifications provided with the order. Aluminum is chosen as the material for the screen frame. According to dye manuals provide by Gwent Ink, silk number is chosen as 100 and nylon is used for all the screens. Printing process is illustrated in figure 2.15.

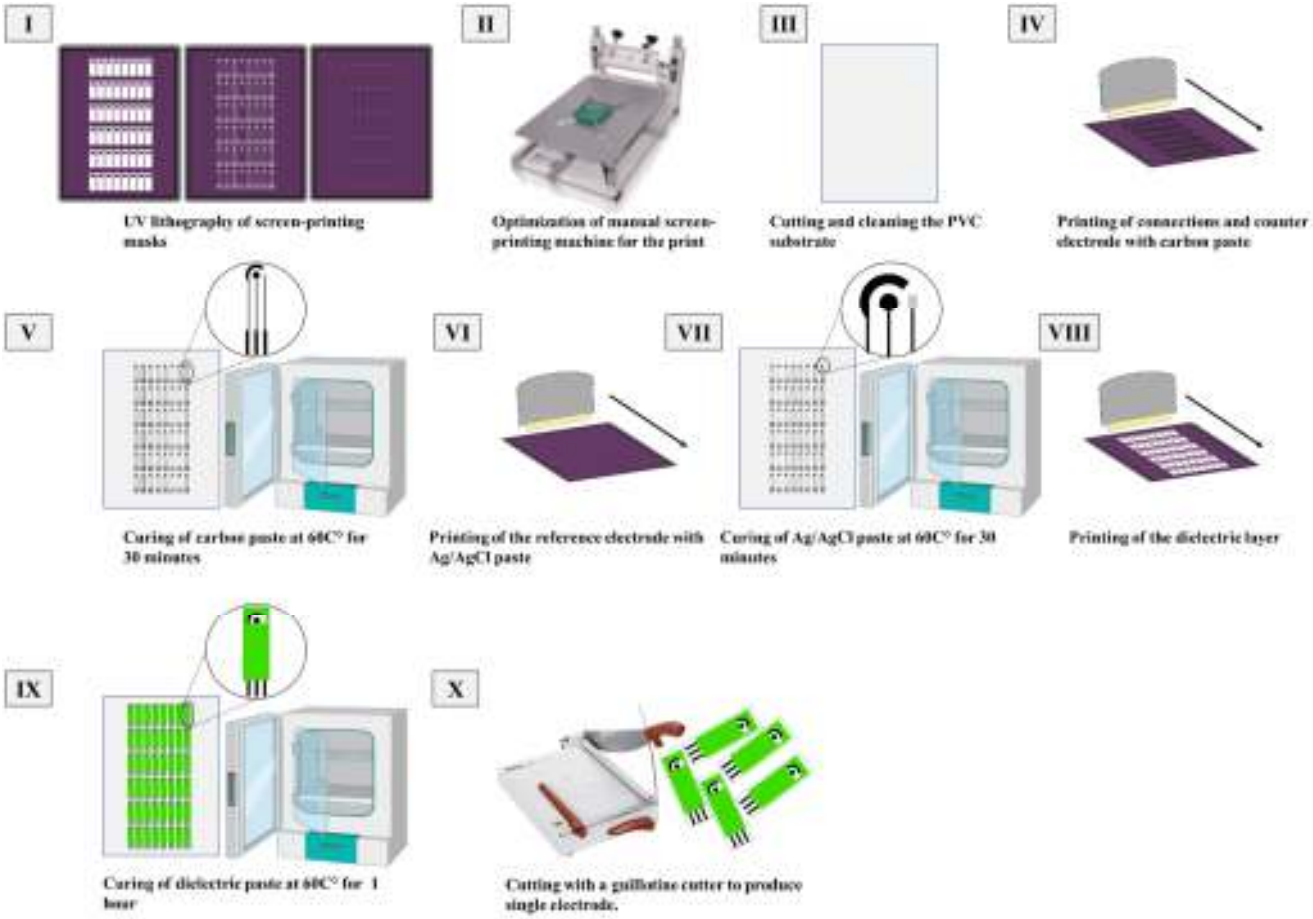

Figure 2.16. Illustration of screen-printing process of the electrodes.

Manual screen printer used in this thesis (NeoDen PM3040) lacks vacuum capabilities. To overcome this, a makeshift vacuum table has been created from 2.7mm PMMA. Alignment of screens are done via alignment crosses on 4 edges of the design. Alignment crosses are covered with masking band after application of the first layer to protect it from the dye. All the screen frames are built with dimension of 43x58cm. Design is made to produce 60 strips per 20x10cm PVC substrate. Printing distance between the substrate and the screen is calculated according to the table 2.2.

Table 2.2. Screen gap calculations. G is the ideal screen gap for printing. W is the inner area of the screen frame[20].

	Stainless Steel	**Polyester**	**Nylon**
G	W x .004	W x .006	W x .010

Since choice of screen material was Nylon, screen gap is calculated as following:

$$50cm\ x\ 0.010\ = 0.5cm = 5mm \qquad (7)$$

Screen-printer is carefully fixed to 5mm gap space with tightly screwed load bearing rods. This gap is very important for the release of the screen form the substrate after dye is applied on the screen and transferred to the substrate. If too little gap is provided, screen will not snap-off and cause misprints. If too much gap space is left, dye transfer will be reduced causing missing prints. After printing, visual inspection is done by a digital USB microscope. Electrodes are stored in a dehumidifier silica bag containing nitrogen purged box. Quality control is performed with potassium hexacyanoferrate (III) solutions. Potassium ferricyanide derivatives are commonly utilized for quality control of screen-printed electrodes[41][42]. Quality control is performed in two steps. First, 4 electrodes are randomly chosen and tested with 5mM potassium hexacyanoferrate in 0.1M KCl solution and the resulting voltagrams are compared for differences. Alternating voltage between -0.1V and 0.5V is used for the cyclic voltammetry with a scan rate of 0.01 V/s. Secondly, 0mM, 1.25mM, 2.5mM and 5mM potassium hexacyanoferrate solutions are prepared in 0.1M KCl. Electrodes are tested with each solution using cyclic voltammetry to observe linear peak current values. Alternating voltage between -0.1V and 0.5V is used for the cyclic voltammetry with a scan rate of 0.01 V/s. Surface activation of the WE are done to observe effects of surface activation on NFC coating. There were several surface application methods in literature. Amperometry with 0.1M PBS + 0.1M KCl solution at 1.8V for 10 minutes is chosen for surface activation trials[43]. NFC dilutions are prepared via serial dilution in 0, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256 concentrations. 9 activated and 9 non-activated electrodes are used. 2µl of NFC dilution is dropped on the electrodes and dried in 50˚C incubator. Dried electrodes are analyzed via USB microscope and results are documented. After the documentation electrodes are washed with 0.1M NaOAc to observe electrode's ability to retain NFC. Washed electrodes are analyzed again with USB microscope and compared with unwashed versions of them. To find the right amount of NFC concentration for enzyme immobilization without disrupting current flow on WE surface, different concentrations of NFC is dropped on to both electrodes and cyclic voltammetry is performed with 2.5mM potassium hexacyanoferrate in 0.1M KCl solution. Alternating voltage between -0.5V and 0.5V is used for the cyclic voltammetry with a scan rate of 0.01 V/s. Disruption of the current peaks are checked at the voltagram. All voltagram results are edited and analyzed in MS Excel 365 software.

3.2. RECOMBINANT ENZYME PRODUCTION

For enzyme production, our laboratory's optimized production protocol is used. Full protocol is given below.

3.2.1. Cloning

3.2.1.1. PCR

PCR reaction is run with appropriate program for the primers given in table 3.5. According to table 3.3. and 3.4., the PCR reaction is set up. The forward and reverse primers are designed with Geneious software. (Appendix Table A14) and tm calculations are made with NEB tm calculator. After PCR reaction, 3µl of PCR product loaded into 1.5% agarose gel with 1µl loading dye and 2µl DDW.

Table 3.3. PCR reaction for LDH gene cloning from *L. paracasei* plasmid DNA.

Materials	**Wild Type**	**CBM**
Plasmid DNA from *L. paracasei*	2µl	2µl
2x Phusion PCR MM	25µl	25µl
DDW	22µl	22µl
F_NcoI_LDH_L_paracasei_XhoI	0.5µl	-
R_NcoI_LDH_L_paracasei_XhoI	0.5µl	-
F_NcoI_LDH_L_paracasei_Kpnl	-	0.5µl
R_NcoI_LDH_L_paracasei_Kpnl	-	0.5µl
Total Reaction Volume	**50µl**	**50µl**

Table 3.4. PCR reaction for CBM gene fragment cloning.

pET28a-ybbR-HIS-CBM-CohI	2µl
2x Phusion PCR MM	25µl
DDW	22µl
F_CBM_LOx	0.5µl
R_CBM_LOx	0.5µl
Total Reaction Volume	50µl

Table 3.5. PCR reaction set-up for both LDH and CBM fragment cloning PCR reactions.

LDH	**CBM**	**Time**
95 □	95 □	10 min
95 □	95 □	20 sec
68 □	50 □	20 sec
72 □	72 □	2 min 15 sec
72 □	72 □	7 min
4 □	4 □	Hold

3.2.1.2. Gel Extraction

1. Gel extraction procedure is applied for both LOx and LDH fragments with E.Z.N.A Gel Extraction kit.
2. 1:1 volume Binding Buffer (Kit, XP2) is added into the Eppendorf tubes (gel piece containing) and incubated at 60□ until gels are completely melted. The tubes are inverted every 2-3 minutes.

3. Columns are placed to a new 2 ml Eppendorf tubes and 700µl solution added into the column for each and centrifuged at maximum rpm for 1 minute. Filtrates are discarded and procedure repeated until all samples are transferred to the column.
4. 300 ul Binding Buffer (Kit, XP2) is added and centrifuged at maximum rpm for 1 minute. Filtrates are discarded and the collection tubes are reused.
5. 700µl SPW Wash Buffer (Kit, ethanol added) is added and centrifuged at maximum rpm for 1 minute. Filtrates are discarded and the collection tubes are reused.
6. Empty columns are centrifuged at maximum rpm for 2 minutes to remove the ethanol completely.
7. Columns are transferred into clean tubes.
8. 30 ul elution buffer is added to the center of column for each. Incubate at room temperature for 5 minutes. Centrifuged at maximum rpm for 1 minute.

3.2.1.3. Restriction

Plasmids are restriction digested for cloning. Double digestion procedure is applied for pDE_2_BglA_Ct_CBM_Ct plasmid using NcoI and KpnI/XhoI restriction enzymes and for pET-28a(+)-6xH_DocCt_LoX using HindIII and NotI. The components on the tables 3.6., 3.7. and 3.8. are added to 1.5 ml Eppendorf tubes and incubated at 37□ for 1 hour. Gel extraction is performed aftermath to purify and check DNA constructs.

Table 3.6. Restriction digestion of pDe2/BglA/Ct/CBM/CT for Both wild type and CBM fused enzyme.

Material	**Wild Type**	**CBM**
pDe2/BglA/Ct/CBM/CT	25µl	25µl
NcoI	1µl	1µl
KpnI	0µl	3µl
XhoI	1µl	0µl
10X Tango Buffer	7µl	3.5µl
DDW	1µl	2.5µl

Total Reaction Volume	**35µl**	**35µl**

Table 3.7. Restriction digestion of LDH Fragments for cloning wild type and CBM fused enzyme from *L. paracasei* plasmid DNA.

Material	**Wild Type**	**CBM**
LDH Fragment	25µl	25µl
NcoI	1 µl	1µl
KpnI	0µl	3µl
XhoI	1µl	0µl
10X Tango Buffer	7µl	3.5µl
DDW	1µl	2.5µl
Total Reaction Volume	**35µl**	**35µl**

Table 3.8. Restriction digestion of pET-28a(+)-6xH_Docct_LoX.

pET-28a(+)-6xH_Docct_LoX	25µl
HindIII	1µl
NotI	1µl
10X Tango Buffer	7µl
DDW	1µl
Total Reaction Volume	35µl

3.2.1.4. Ligation

The components on the table 3.9. are added to PCR tubes and incubated at 16□ overnight. Same procedure is used for every construct created up to this step.

Table 3.9. Ligation procedure for all constructs. A, B, C are different plasmid concentrations to prevent mistakes. D is for control.

Ingredients	**A**	**B**	**C**	**D**
10x T4 ligase B.	1µl	1µl	1µl	1µl
T4 ligase	1µl	1µl	1 µl	1µl
Plasmid	1µl	2 µl	3 µl	2µl
Fragment	1µl	1µl	1 µl	0µl
DDW	6µl	5µl	4µl	6µl

3.2.1.5. Transformation to XL1B

1. All 10 ligation products and 200 ul XL1B cells are incubated on ice for 25 minutes.
2. Tubes are incubated at 42□ for 1 minute.
3. 800µl LB is added to tubes and incubated at 37□ for 45 minutes at incubator with shaker.
4. Centrifuged at 7500 rpm for 1 minute. The supernatant is discarded until 100µl remained.
5. Cells are plated into kanamycin resistant LB plates and incubated at 37□ overnight.

3.2.1.6. Colony PCR

PCR reaction set with 5µl of 2X Phusion MM, 0.1 ul of forward primer, 0.1 ul of reverse primer (Both primers for LDH and CBM are same as previous steps) and 4.8µl of nuclease free water is prepared. PCR program shown in the table 3.10.

Table 3.10. Colony PCR set-up.

LDH	CBM	Time
95 □	97 □	10 min
95 □	97 □	30 sec
68 □	53 □	30 sec
72 □	72 □	1 min 15 sec
72 □	72 □	7 min
4 □	4 □	Hold

3.2.1.7. Transformation to BL21

1. Add 1.5µl desired plasmid into 200µl BL21.
2. Incubate on ice for 25 min.
3. Incubate at 42°C for 60 sec.
4. Incubate on ice for 1min.
5. Add 800µl of LB Media.
6. Incubate for 45min at 37°C shaking (220 RPM) incubator.
7. Centrifuge for 1 min at 7500 RPM, discard to supernatant via pouring and a slight tap to the tube.
8. Use remaining liquid in tube to resuspend the cells.
9. Do a spread plate with the corresponding antibiotic.

3.2.2. Protein Expression

3.2.2.1. Pregrowth

1. Prepare 5ml of LB media in culture tube.
2. Add corresponding antibiotic to the media.
3. Take your transformation plate and get as much as cells as possible via using a small pipette tip.
4. Throw the pipet tip into the culture tube.

5. Incubate for 3 hours in 37°C shaking (220 RPM) incubator (Leave tubes half-open while incubating).

3.2.2.2. Protein Expression with ZYM5052 auto-induction medium

1. Prepare 1 L of ZYM-5052 auto-induction medium in 2.5L Ultra yield flask and close the lid via aluminum disposable takeout pan. Autoclave media.
2. Add corresponding antibiotic (Kan).
3. Add 20ml of 50x M buffer.
4. Add 20ml of 50x 5052 buffer.
5. Add 2ml of 500x $MgSO_4$
6. Add your 5ml pregrowth bacterial culture.
7. Put in 37°C shaking (220 RPM) incubator overnight.

3.2.2.3. Pre-Protein Purification

1. Transfer bacterial culture from 1L ZYM-5052 auto-induction medium in Ultra-yield flask to 500ml culture flask (Fill about 60% of the flask).
2. Centrifuge for at 6000RPM for 10 min at 15°C using F12-6 x 500 LEX rotor. Remove the supernatant. Repeat till 1L flask becomes empty.
3. Add 20ml Binding Buffer to resuspend cells, use shaker if necessary.
4. Transfer to a falcon tube. Top it up till 35ml with binding buffer.
5. Prepare for sonication. Put falcon buffers into a beaker full of ice (squirt some ethanol on the ice) to prevent heating while sonicating.
6. Put 175µl from 200xProtease Inhibitor (PI) just before sonicating.
7. Sonicate for 8 min 3 cycles.
8. Repeat for every tube.
9. Transfer contents of falcon tube into a 50ml centrifuge tube.
10. Centrifuge for 30 min at 6°C using A27-8x50 rotor.
11. Transfer the supernatant into a 50ml falcon tube.

3.2.2.4. Protein Purification

1. Prepare columns via adding 2ml Ni2+ beads and letting them settle.
2. Wash the column with ddH2O repeat this for 2 times.

3. Fill the column with binding buffer. Let it drip.
4. Fill the column with the protein supernatant. Let it drip.
5. Fill the column with washing buffer and check the protein content of droplets via Bradford assay (See: Bradford Assay).
6. Keep washing until Bradford assay stops turning blue.
7. Take 7 Eppendorf tubes and label them.
8. Put 500µl elution buffer to the column and collect the droplets in an Eppendorf tube. Repeat this 7 times for all the tubes.

3.2.2.5. Bradford Assay

1. Fill the wells of a 96-well plate with 200µl Bradford solution.
2. Take 20µl from droplets of the column and add it on to the wells while washing.
3. Observe color change, keep washing until little or no color change occurs.

3.2.2.6. SDS-PAGE analysis

20µl of each fraction of the purified enzyme are mixed with 10µl of 3x Sample buffer. Polyacrylamide gel is prepared (see materials) and gel electrophoresis is performed at 120V for 120 minutes. Gel is than stained for 40 minutes and destained overnight. Gel pictures are taken with QUANTUM Gel documentation system.

3.3. ENYZME ACTIVITY TESTS

Enzyme activity tests for LDH-CBM is done according to the literature. Master mix reaction buffers are prepared including 2mM INT, 0.1M TRIS-HCL at pH 8.2, 3mM NAD+ and various concentrations of lactic acid. Reactions are performed in a 96-well plate each containing various concentrations of LDH-LOX. Reaction volume was 50µl and plate is incubated at room temperature for 1 hour. Spectrophotometry is performed at 490 nm absorbance. To stop the reaction, 50µl 150mM MPMS is added to wells. Spectrophotometry results are then documented using MS Excel 365 and proceeded when enzyme activity is confirmed.

3.4. BINDING TESTS

Binding tests are performed to determine CBM ability to bind NFC and other cellulose substrates. Experiments are listed below.

3.4.1. PASC/AVICEL/NFC CBM Binding

For binding tests recombinant proteins already present in the laboratory are used. OGW_07 is a protein containing a CBM site. OGW 15 is a protein without a CBM site. 50ng/ul concentration is used from each cellulose derivative. 10µl of protein and 10µl of cellulose derivatives are incubated in a 1.5ml Eppendorf for 30 minutes on ice. For control, TBS is used. Figure 3.16 describes the incubation set up for the experiment.

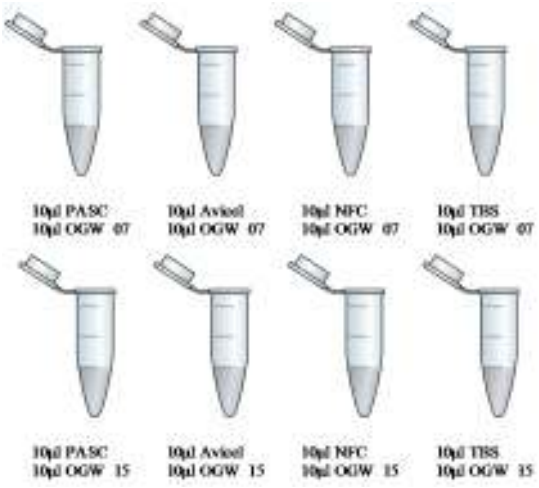

Figure 3.17. Incubation set up for PASC/AVICEL/NFC CBM binding.

After incubation tubes with cellulose derivatives are centrifuged at max speed for 15 minutes. 20µl of the supernatant is taken as sample I (Unbound) and remaining cellulose derivative pellets are resuspended with 20µl 1xTBS. 20µl are again taken from each tube as sample S (Bound). 20µl are also taken from each TBS only tubes as sample T(Total/control). Every sample is mixed with 10µl of 3xSample loading buffer and boiled for 10minutes at 98˚C. Polyacrylamide gel is prepared (see materials) and SDS-PAGE electrophoresis is performed at 120V for 120 minutes. Gel loading set up is given in table 3.3.

Table 3.11. Gel loading set-up for PASC/AVICEL/NFC CBM binding.

Marker PageRuler Plus Prestained #26619	OGW_07								OGW_15							
4µl	TBS	PASC		AVICEL		NFC			TBS	PASC		AVICEL		NFC		
	10µl	S	I	S	I	S	I		10µl	S	I	S	I	S	I	
		10µl	10µl	10µl	10µl	10µl	10µl			10µl	10µl	10µl	10µl	10µl	10µl	

Gel is than stained for 40 minutes and destained overnight. Gel pictures are taken with QUANTUM Gel documentation system.

3.4.2. Binding Test with Cohesin-Dockerin CBM interactions

For this test, proteins containing Cohesins and Dockerines already present at the laboratory is used. OGW_07 and OGW_09 proteins are CBM containing Cohesin proteins. OGW_56, OGW_58, sfGFP and GFP is used. OGW_07 interacts with OGW_58, OGW_09 interacts with sfGFP and OGW_56. GFP interacts with nothing. Preparation of samples are given in table 3.4.

Table 3.12. Sample preparation table for binding test with Cohesin-Dockerin CBM interactions.

Added	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
NFC	-	-	-	-	-	-	10µl	10µl	10µl	10µl	10µl	10µl	10µl	10µl
OGW_07	5µl	-	-	-	-	-	5µl	5µl	5µl	5µl	-	-	-	-
OGW_09	-	5µl	-	-	-	-	-	-	-	-	5µl	5µl	5µl	5µl
OGW_58	-	-	5µl	-	-	-	5µl	-	-	-	-	5µl	-	-
OGW_56	-	-	-	5µl	-	-	-	5µl	-	-	5µl	-	-	-
GFP	-	-	-	-	5µl	-	-	-	5µl	-	-	-	5µl	-
sfGFP	-	-	-	-	-	10µl	-	-	-	10µl	-	-	-	10µl

3.4.2.1. Experiment procedure

1. Incubate tubes for 30 min on ice.
2. Centrifuge at max speed for 2 min.
3. Remove supernatant.
4. Wash with 100µl 1xTBS. x3
5. Add 10µl of 3xSample buffer with DTT to the tubes.
6. Boil for 10 min at 98°C.
7. Load to SDS-PAGE.
8. Run at 120V for 120 minutes.
9. Stain the gel for 40 minutes.

10. Destain overnight.
11. Document gel with QUANTUM Gel documentation system.

3.5. BIOSENSOR MEASUREMENT

For biosensor measurement will be performed via dropcasting 2µl of purified enzyme (LDH-CBM) on to the NFC coated WE surface. Reaction will be performed in deli fridge at 4˚C for 30 minutes. Electrodes are then will be washed with DDW to get rid of the unbound LDH-CBM. Electrodes will be connected to the EmStat3 Blue potentiostat. 50µl of differing concentrations of lactic acid and 3mM NAD+ containing 0.1M KCl solutions will be dropped on the electrodes covering all three electrodes (WE, RE, CE). Immediately amperometry will be performed at 0.3V fixed potential for 60 seconds. Potentiostat's own computer software PSTrace 5.8 will be used for this experiment. Resulting voltagram will be analyzed via MS Excel 365.

4. RESULTS

4.1. SCREEN PRINTING PROCESS

An example of the resulting electrodes is given in figure 4.18. 60 electrode strips are made in 1 substrate sheet.

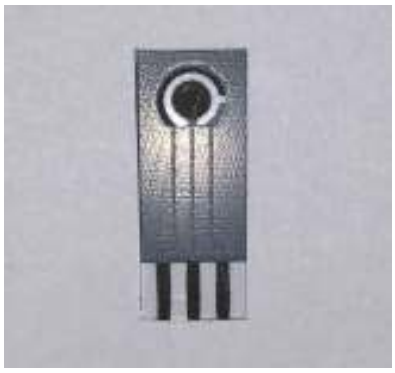

Figure 4.18. Screen-printed electrode sample.

Quality control is performed as described in the procedures. As can be seen in figure 4.19., between the 4 randomly selected electrodes there were little variation with the ferricyanide standard curve. This result was acceptable for manual printing.

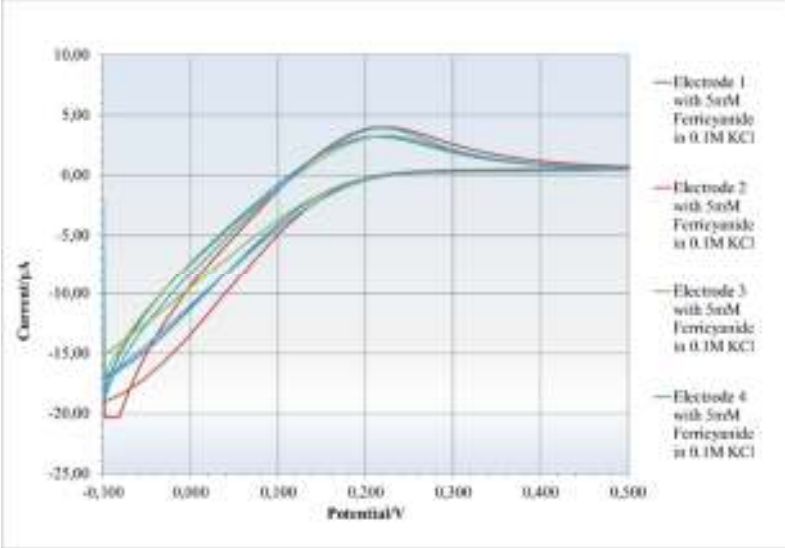

Figure 4.19. Ferricyanide (Potassium hexacyanoferrate) standard testing of 4 random electrodes. Small difference between peak currents are observed.

Second quality control test is performed to see the linearity of the electrode measurement. As seen in figure 4.20. and 4.21. electrodes showed linear measurement to increasing ferricyanide (Potassium hexacyanoferrate) concentrations.

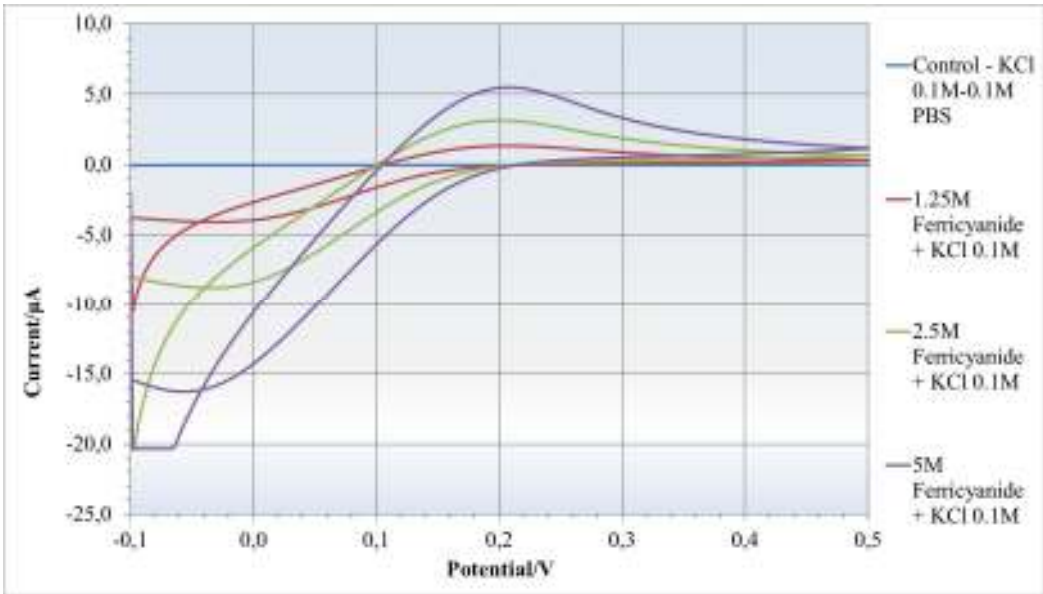

Figure 4.20. Cyclic voltammetry curves of increasing concentrations of ferricyanide (Potassium hexacyanoferrate).

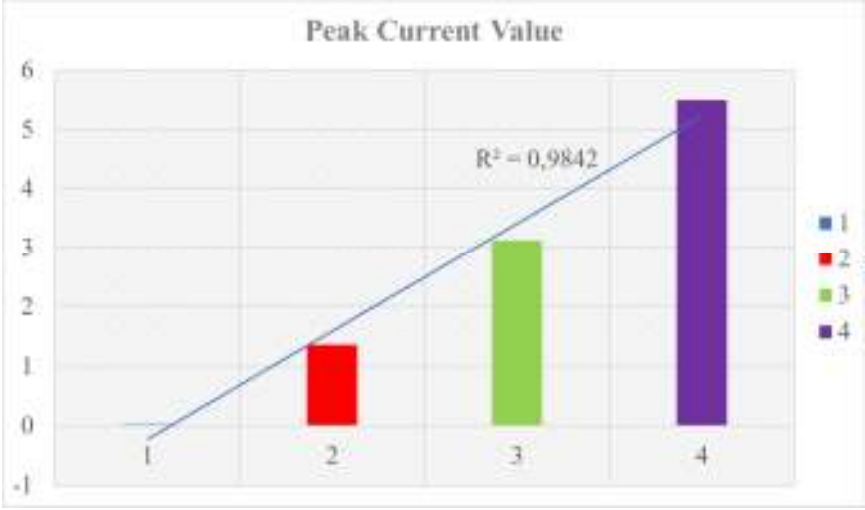

Figure 4.21. Peak current values taken from figure 4.20. to show linearity of the measurement. R^2 value is given to show linearity.

Electrodes are covered with NFC to observe their ability to retain NFC after liquid contact is made. Electrode activation with amperometry is tested to see if activation has any effect

on the ability of retaining NFC on, WE. As can be seen in figure 4.22., activated electrodes retain more NFC after getting washed.

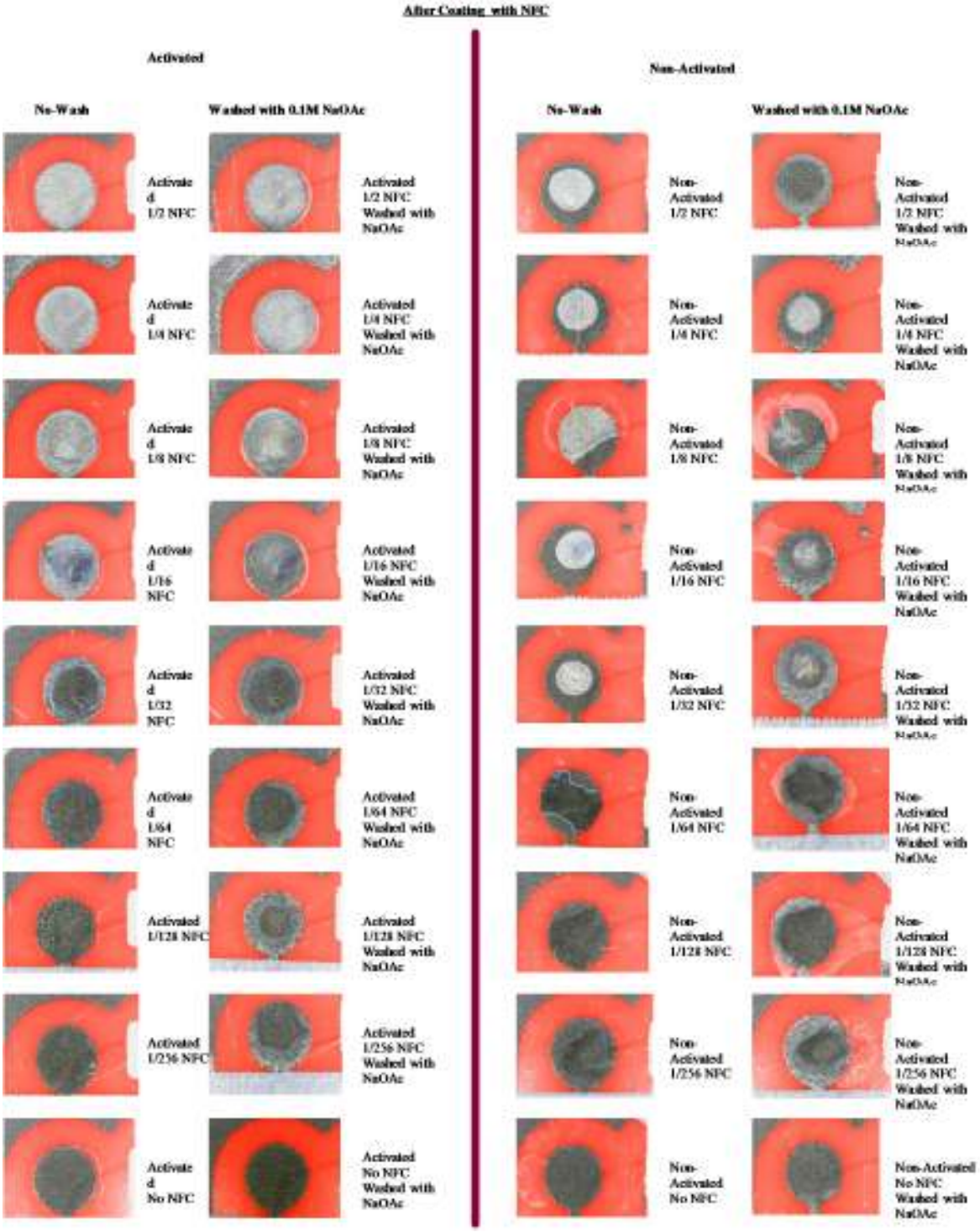

Figure 4.22. NFC retention tests performed on both amperometrically activated and non-activated WE surface.

Electrochemical testing is also performed on NFC covered electrodes to observe the effects of NFC on the ability of electrode to perform cyclic voltammetry. As can be seen in figure

4.23., ½ dilution of NFC has a high effect on the ability of electrode surface to pass current. Other dilutions showed dismissible inhibition on the electrodes ability to perform cyclic voltammetry.

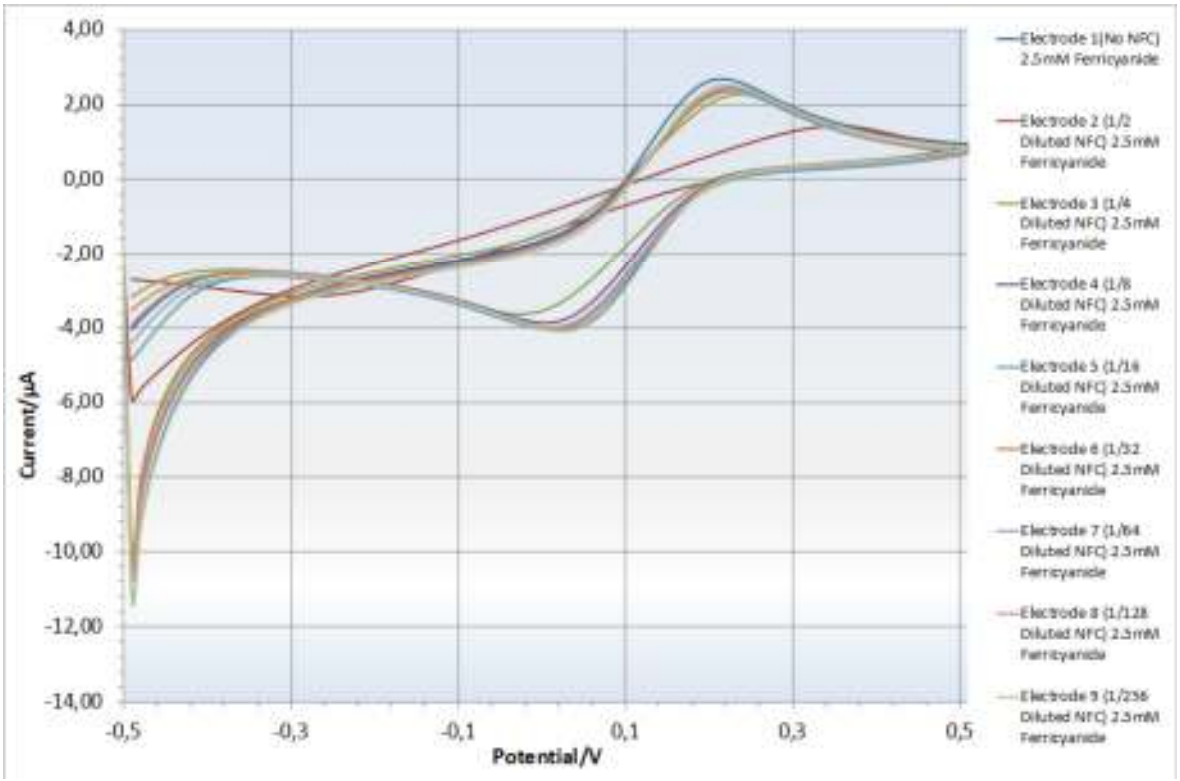

Figure 4.23. Effects of NFC on the ability of WE to perform electrochemistry. Tested with a standard of 2.5mM ferricyanide (Potassium hexacyanoferrate).

4.2. RECOMBINANT ENZYME PRODUCTION

4.2.1. LOx-CBM Production

CBM fused and non-fused LOx constructs are cloned and expressed. Resulting SDS-PAGE is given in figure 4.24 and it shows that CBM fused LOx is not expressed but non-fused LOx is expressed. This is the reason why LDH is chosen as the biorecognition element for this biosensor. Repeated trials and other protein purification methods also failed to express CBM fused lactate oxidase enzyme. Those trials are not included here because there was no change on the solubility or expressibility of the enzyme.

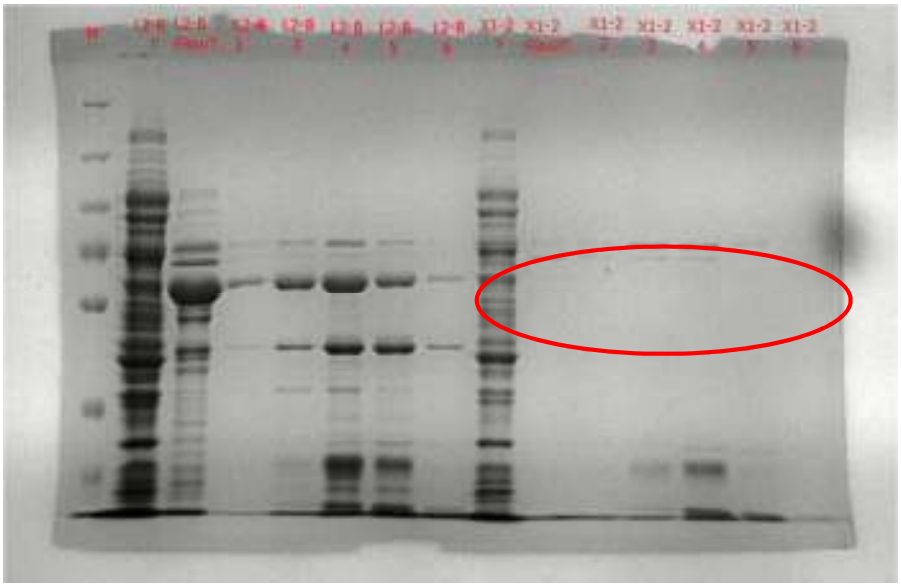

Figure 4.24. SDS-PAGE result of LOx recombinant enzyme expression process. Red circle show was CBM-fused lactate oxidase enzyme should be if it were expressed.

4.2.2. LDH-CBM Production

LDH gene is cloned from *Lactobacillus paracasei* plasmid DNA via PCR. Primers are given in Appendix Table A.14. 2 fragments are amplified. NcoI and KpnI is added to the fragment that will be inserted to the side of CBM gene found in pDE2/Bgla/Ct/CBM plasmid (Appendix Figure A.3). NcoI and XhoI is added to the second fragment for cloning only wild type LDH enzyme to the pDE2/Bgla/Ct/CBM plasmid. PCR results are given in Figure 4.25.

Figure 4.25. Gel electrophoresis of PCR reaction. Amplified fragments are shown with red circle.

pDE2/Bgla/Ct/CBM plasmid and 2 PCR amplified fragments are subjected to restriction digestion as given in table 3.7 and 3.6. Reaction is described in the procedures. After reaction is completed, gel electrophoresis is performed on the plasmids and fragments. Results are given in.

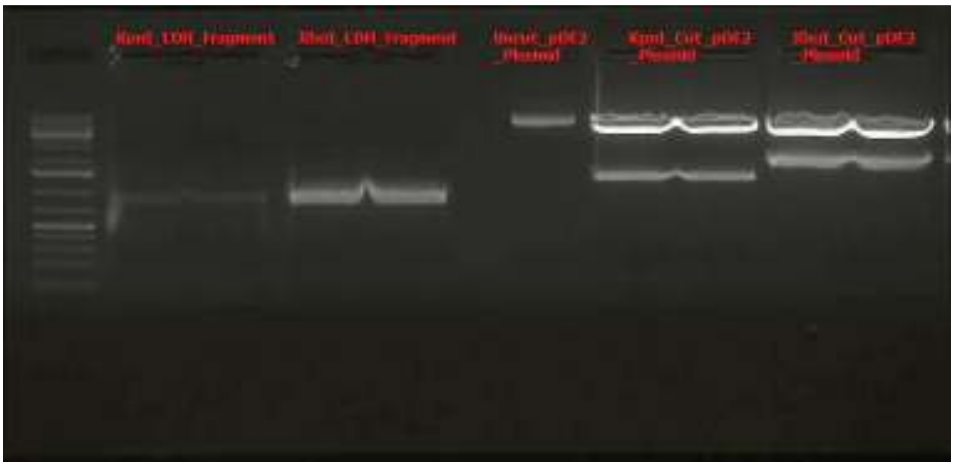

Figure 4.26. Gel result of restriction digested plasmids and fragments.

Ligation is performed as given in procedures. Extra step is added with DNA Clean Up kit from Quiagen to get rid of excess T4 Ligase. Protein expression is performed as given in procedures. SDS-PAGE is performed and given in Figure 4.27.

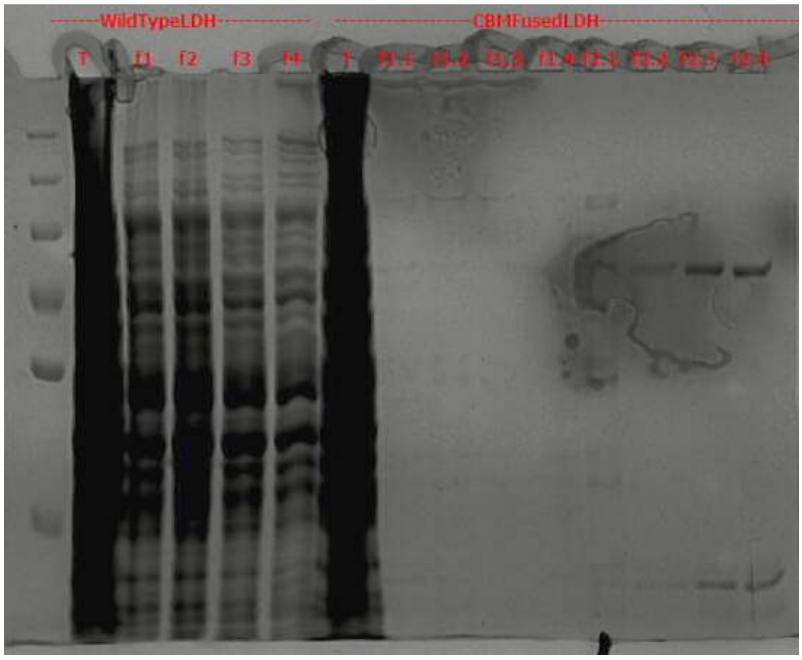

Figure 27. SDS-PAGE results of protein expression.

4.3. BINDING TESTS

First binding test results are given in figure 4.25 in which CBM containing OGW_07 protein clearly binds to cellulosic materials. Bound proteins are showed with symbol I. There is some remaining OGW_15 on S side but that is due to washing been not enough.

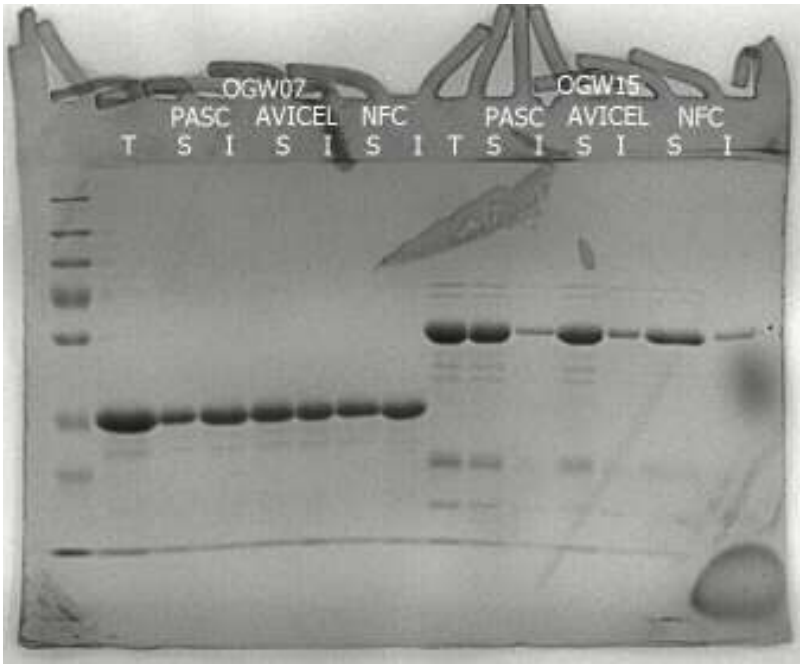

Figure 4.28. Binding test for CBM containing OGW_07 protein and non-CBM containing OGW_15 protein. T is total protein without NFC, S is unbound proteins and I denote bound proteins.

Second binding test incorporates cellulosome proteins cohesins and dockerins. It can be seen in figure 4.26 that OGW_56 and OGW_58 is bound to their respective proteins OGW_7 and OGW_9. OGW_56, OGW_58 and sfGFP are present in the gel after washing. This shows that OGW_7 and OGW_9 is bound to NFC and because they are also bound to OGW_56, OGW_58 and sfGFP they became remained on NFC after washing.

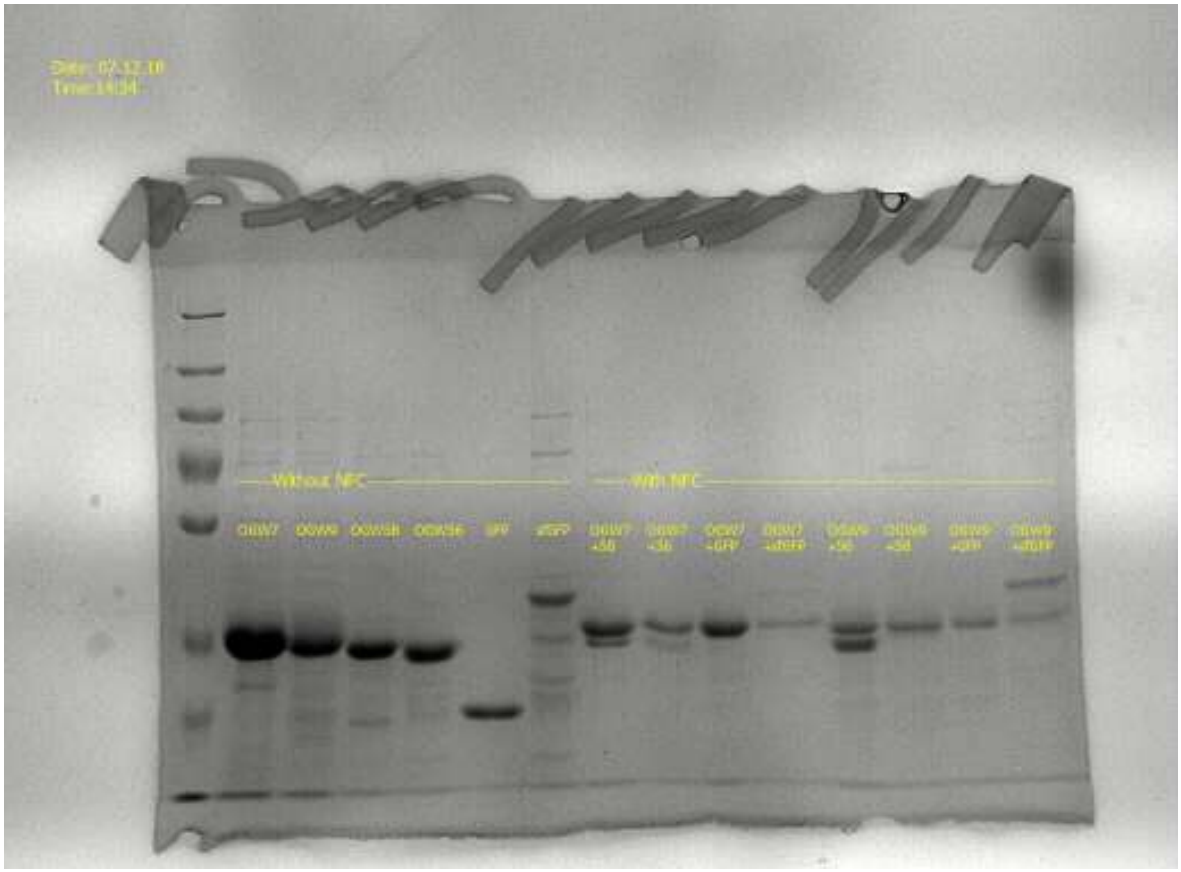

Figure 4.29. Binding test with CBM, cohesion and dockerin containing proteins. 2 bands can be observed with corresponding proteins which shows both NFC binding and binding between cohesins and dockerines.

5. Discussion

Screen printing with a PCB screen printer was a smart idea because of the screw holes present on the surface of the printer. Substrate immobilization is very important in screen printing. In this work, a makeshift vacuum table is screwed in PCB screw holes of the printer and substrate is immobilized on to the vacuum plate. As it can be seen in results, there some variation between electrodes. Manual printing is the cause of this variation and can be reduced with using an automatic or semi-automatic screen-printer. It should be noted that variation of electrodes in a batch can be dismissible small, but variations between batches require calibration for each batch. Recombinant enzyme production is a complicated and optimization dependent procedure. Unfortunately, in this work, CBM-fused LOx enzyme cannot be expressed in a recombinant manner. It is possible that even a small fusion such as a CBM site, effects protein folding of LOx enzyme adversely. LDH is already shown to be expressible with CBM fusion in literature[44]. Also, LDH does not need oxygen to work properly. Thus, LDH is chosen as the biorecognition element for this work. NFC is chosen as the cellulosic material because of its cheapness and storability. NFC retention is tested with activated WE and it was observed that activated WE can retain more NFC. This is not an important issue for disposable electrode production but for reusable sensors, this is important to note. Large amount of NFC also inhibits current flow to WE surface. ¼ dilution is shown to be enough for enzyme binding while retaining the ability to perform electrochemical measurements. This works shows that CBM and cellulosic materials such as NFC can be utilized as novel immobilization methods for biosensors. Final measurements cannot be performed because of COVID-19 pandemic. NAD+ chemical that was required got stuck in a shipping hell for 6 mounts and it did not arrive. Also, with CBM fusing enzymes, fast prototype biosensors can be created to fasten biomarker and drug testing. Future work continuing from this work could be the usage of other cellulosome proteins cohesines and dockerins to specifically control the location and the amount of enzyme immobilization on the WE surface of a biosensor. Unfortunately, because of the global COVID-19 pandemic and loss of my supervisor, this work could not include cohesion and dockerin utilizing biosensors.

6. Appendix

Table A 1. Binding buffer preparation.

Binding Buffer (25mM Imidazole)	Ingredient Amount
1M Imidazole	5ml
10X TBS	20ml
DDW	Up to 200ml

Table A 2. Wash buffer preparation

Wash Buffer (40mM Imidazole)	Ingredient Amount
1M Imidazole	8ml
10X TBS	20ml
DDW	Up to 200ml

Table A 3. Elution buffer preparation

Elution Buffer (200mM Imidazole)	Ingredient Amount
1M Imidazole	10ml
10X TBS	5ml
DDW	Up to 50ml

Table A 4. SDS-PAGE buffer for the lower gel.

Lower SDS-PAGE gel buffer (pH 8.8 + SDS)	Ingredient Amount
Tris	90.75g
SDS	1.75g
DDW	Up to 500ml

Table A 5. SDS-PAGE buffer for the upper gel.

Upper SDS-PAGE gel buffer (pH 6.8 + SDS)	Ingredient Amount
Tris	60.5g

SDS	3.7g
DDW	Up to 500ml

Table A 6 Sample buffer for protein samples. 1x used.

3x SDS-PAGE Sample Buffer	Ingredient Amount
Beta-mercaptoethanol	5ml
Bromophenol Blue	10mg
SDS	3g
Glycerol	10-24ml
Upper Tris Buffer (Appendix Table A5)	6.25ml
DDW	Up to 100ml

Table A 7. SDS-PAGE gel running buffer for electrophoresis.

10x Running Buffer	Ingredient Amount
Tris	151.43g
SDS	50g
Glycine	720.7g
DDW	Up to 5L

Table A 8. SDS-PAGE gel stain solution (Reusable).

SDS-PAGE Stain	Ingredient Amount
Coomassie Blue BB R-250	2g
Methanol	500ml
↓ Filter Though 3MM Filter Paper ↓	
Acetic Acid	100ml
DDW	400ml

Table A 9. SDS-PAGE gel detain solution.

SDS-PAGE Destain	Ingredient Amount
Methanol	400ml
Acetic Acid	140ml

DDW	1460ml

Table A 10. Lower gel preparation for polyacrylamide SDS-PAGE gel. Ingredients are added in sequence.

10 percent Lower Gel	**Ingredient Amount**
DDW	6.4ml
Acrylamide	3.3ml
1.5M Tris pH 8.8	3.25ml
10 percent APS	0.130ml
TEMED	0.005ml

Table A 11. Upper gel preparation for polyacrylamide SDS-PAGE gel. Ingredients are added in sequence.

10 percent Upper Gel	**Ingredient Amount**
DDW	2.5ml
Acrylamide	0.425ml
1.5M Tris pH 8.8	0.430ml
10 percent APS	0.034ml
TEMED	0.004ml

Table A 12. ZYM-5052 Medium preparation. All solutions must be autoclaved before mixing. 50x M must be prepared via sequentially dissolving the ingredients[40].

ZYM-5052 Medium Stock Solution	**Ingredients**	**Amount**
ZY	Tryptone	10g
	Yeast Extract	5g
	DDW	958ml
50x M	$Na_2HPO_4 – 7H_2O$	335g
	KH_2PO_4	170g
	NH_4Cl	134g
	Na_2SO_4	35.5g
	DDW	Up to 700ml
50x 5052	Glycerol	250g
	Glucose	25g

	Lactose	100g
	DDW	730ml
500x $MgSO_4$	$MgSO_4 – 7H_2O$	24.65g
	DDW	87ml

Table A 13. 200x Protease Inhibitor stock solution preparation.

200x Protease Inhibitor	**Ingredient Amount**
PMSF	350mg
Benzamidine	120mg
Benzamide	15mg
Ethanol	Up to 10ml

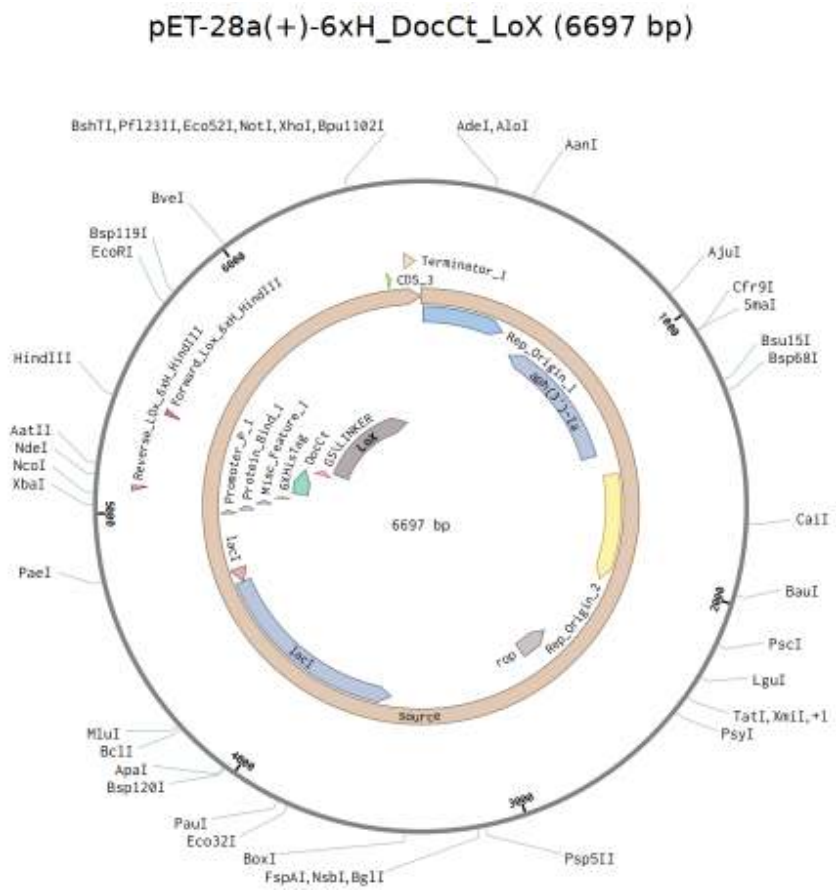

Figure A 1. Plasmid map of pET-28a(+)-6xH_DocCt_LoX.

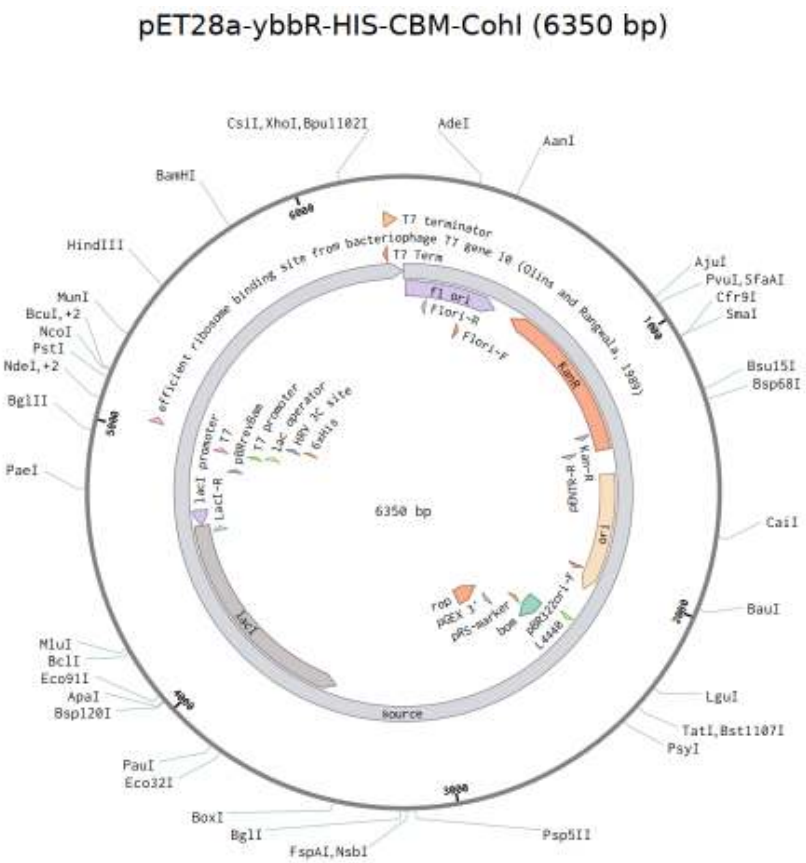

Figure A 2. Plasmid map of pET28a-ybbR-HIS-CBM-CohI[39].

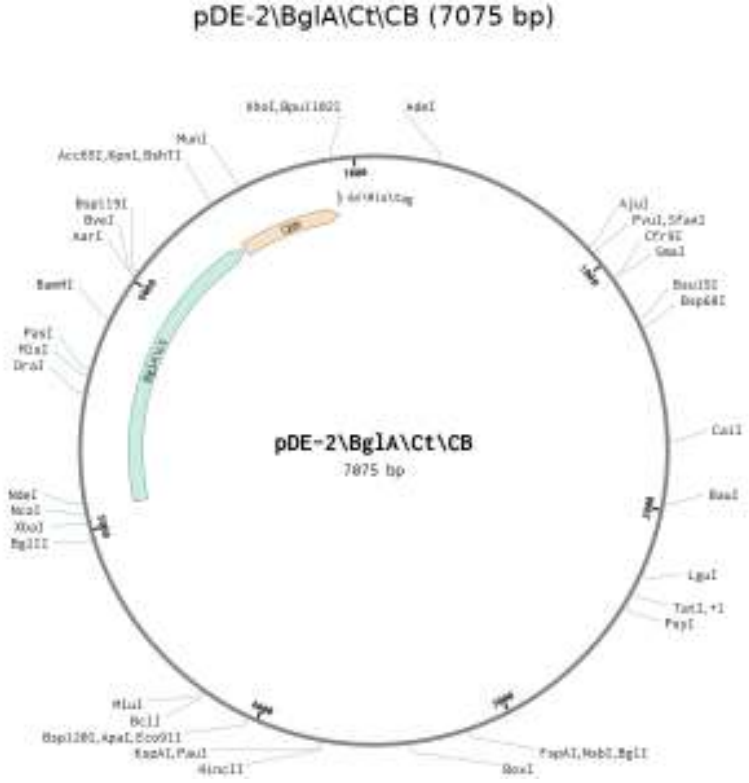

Figure A 3. Plasmid map of pDE-2\BglA\Ct\CB.

Table A 14. Primer Table.

PRIMER TABLE	
F_NcoI_LDH_L_paracasei_KpnI	5’ AATCCATGGATGATCTTCTGGGTTCAAATCC 3’
R_NcoI_LDH_L_paracasei_KpnI	5’ AATGGTACCTTAATTGGTGATGGCGC 3’
F_NcoI_LDH_L_paracasei_XhoI	5’ AATCCATGGATGATCTTCTGGGTTCAAATCC 3’
R_NcoI_LDH_L_paracasei_XhoI	5’ AATCTCGAGTTAATTGGTGATGGCGC 3’
F_CBM_to_LoX	5’ GGGCCATGGCAAATACACCGGTATCAGGC 3’
R_CBM_to_LoX	5’ CCCGGTCTCAAGCTTACTGCCACCGGGTTCTTAC 3’

7. References

[1] N. Bhalla, P. Jolly, N. Formisano, and P. Estrela, "Introduction to biosensors," *Essays Biochem.*, vol. 60, no. June, pp. 1–8, 2016, doi: 10.1042/EBC20150001.

[2] R. Thusu, "Strong Growth Predicted for Biosensors Market," 2010. [Online]. Available: https://www.fierceelectronics.com/components/strong-growth-predicted-for-biosensors-market.

[3] K. V, "DNA Biosensors-A Review," *J. Bioeng. Biomed. Sci.*, vol. 07, no. 02, 2017, doi: 10.4172/2155-9538.1000222.

[4] G. Rocchitta *et al.*, "Enzyme biosensors for biomedical applications: Strategies for safeguarding analytical performances in biological fluids," *Sensors (Switzerland)*, vol. 16, no. 6, 2016, doi: 10.3390/s16060780.

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

[6] S. Johnson, *The home pregnancy test*. Elsevier Inc., 2020.

[7] Y. H. Lee and R. Mutharasan, "Biosensors," *Sens. Technol. Handb.*, pp. 161–180, 2005, doi: 10.1016/B978-075067729-5/50046-X.

[8] C. S. Pundir, V. Narwal, and B. Batra, "Determination of lactic acid with special emphasis on biosensing methods: A review," *Biosens. Bioelectron.*, vol. 86, pp. 777–790, 2016, doi: 10.1016/j.bios.2016.07.076.

[9] Z. Ounaies, *Piezoelectric Materials*. 2008.

[10] G. Luka *et al.*, "Microfluidics integrated biosensors: A leading technology towards lab-on-A-chip and sensing applications," *Sensors (Switzerland)*, vol. 15, no. 12, pp. 30011–30031, 2015, doi: 10.3390/s151229783.

[11] A. Surleva, A. Marinova, V. Ivanova, R. Gradinaru, and S. Georgieva, "Enzyme Based Methods for Lactate Determination," vol. 52, no. April, pp. 2017–2513, 2017.

[12] L. Rassaei, W. Olthuis, S. Tsujimura, E. J. R. Sudhölter, and A. Van Den Berg, "Lactate biosensors: Current status and outlook," *Anal. Bioanal. Chem.*, vol. 406, no. 1, pp. 123–137, 2014, doi: 10.1007/s00216-013-7307-1.

[13] F. Ricci *et al.*, "Prussian Blue and enzyme bulk-modified screen-printed electrodes for hydrogen peroxide and glucose determination with improved storage and

operational stability," *Anal. Chim. Acta*, vol. 485, no. 1, pp. 111–120, 2003, doi: 10.1016/S0003-2670(03)00403-3.

[14] K. Rathee, V. Dhull, R. Dhull, and S. Singh, "Biosensors based on electrochemical lactate detection: A comprehensive review," *Biochem. Biophys. Reports*, vol. 5, pp. 35–54, 2016, doi: 10.1016/j.bbrep.2015.11.010.

[15] M. Li, Y. Yue, Z. J. Zhang, Z. Y. Wang, T. W. Tan, and L. H. Fan, "Site-Specific and High-Loading Immobilization of Proteins by Using Cohesin-Dockerin and CBM-Cellulose Interactions," *Bioconjug. Chem.*, vol. 27, no. 7, pp. 1579–1583, 2016, doi: 10.1021/acs.bioconjchem.6b00282.

[16] O. Yaniv, S. Petkun, L. J. W. Shimon, E. A. Bayer, R. Lamed, and F. Frolow, "A single mutation reforms the binding activity of an adhesion-deficient family 3 carbohydrate-binding module," *Acta Crystallogr. Sect. D Biol. Crystallogr.*, vol. 68, no. 7, pp. 819–828, 2012, doi: 10.1107/S0907444912013133.

[17] C. W. Foster, R. O. Kadara, and C. E. Banks, *Fundamentals of screen-printing electrochemical architectures*, no. 9783319251912. 2016.

[18] Y. Wang, H. Xu, J. Zhang, and G. Li, "Electrochemical sensors for clinic analysis," *Sensors*, vol. 8, no. 4, pp. 2043–2081, 2008, doi: 10.3390/s8042043.

[19] Z. Taleat, A. Khoshroo, and M. Mazloum-Ardakani, "Screen-printed electrodes for biosensing: A review (2008-2013)," *Microchimica Acta*. 2014, doi: 10.1007/s00604-014-1181-1.

[20] A. Hobby, "SCREEN PRINTING FOR THE INDUSTRIAL USER," *DEK Printing Machines Ltd.*, 1997. [Online]. Available: http://www.gwent.org/gem_screen_printing.html.

[21] Z. Chu, J. Peng, and W. Jin, "Advanced nanomaterial inks for screen-printed chemical sensors," *Sensors Actuators, B Chem.*, vol. 243, pp. 919–926, 2017, doi: 10.1016/j.snb.2016.12.022.

[22] F. Ricci and G. Palleschi, "Sensor and biosensor preparation, optimisation and applications of Prussian Blue modified electrodes," *Biosens. Bioelectron.*, vol. 21, no. 3, pp. 389–407, 2005, doi: 10.1016/j.bios.2004.12.001.

[23] W. Li, S. Yang, and A. Shamim, "Screen printing of silver nanowires: balancing conductivity with transparency while maintaining flexibility and stretchability," *npj Flex. Electron.*, vol. 3, no. 1, 2019, doi: 10.1038/s41528-019-0057-1.

[24] C. S. Huertas, M. Soler, M. C. Estevez, and L. M. Lechuga, "One-Step

Immobilization of Antibodies and DNA on Gold Sensor Surfaces via a Poly-Adenine Oligonucleotide Approach," *Anal. Chem.*, vol. 92, no. 18, pp. 12596–12604, 2020, doi: 10.1021/acs.analchem.0c02619.

[25] S. Datta, L. R. Christena, and Y. R. S. Rajaram, "Enzyme immobilization: an overview on techniques and support materials," *3 Biotech*, vol. 3, no. 1, pp. 1–9, 2013, doi: 10.1007/s13205-012-0071-7.

[26] H. H. Nguyen and M. Kim, "An Overview of Techniques in Enzyme Immobilization," *Appl. Sci. Converg. Technol.*, vol. 26, no. 6, pp. 157–163, 2017, doi: 10.5757/asct.2017.26.6.157.

[27] J. Wang, P. V. A. Pamidi, and D. S. Park, "Screen-printable sol-gel enzyme-containing carbon inks," *Anal. Chem.*, vol. 68, no. 15, pp. 2705–2708, 1996, doi: 10.1021/ac960159n.

[28] B. Lillis, C. Grogan, H. Berney, and W. A. Lane, "Investigation into immobilisation of lactate oxidase to improve stability," *Sensors Actuators, B Chem.*, vol. 68, no. 1, pp. 109–114, 2000, doi: 10.1016/S0925-4005(00)00469-X.

[29] S. M. Martin, F. H. Gebara, T. D. Strong, and R. B. Brown, "A fully differential potentiostat," *IEEE Sens. J.*, vol. 9, no. 2, pp. 135–142, 2009, doi: 10.1109/JSEN.2008.2011085.

[30] A. F. D. Cruz, N. Norena, A. Kaushik, and S. Bhansali, "A low-cost miniaturized potentiostat for point-of-care diagnosis," *Biosens. Bioelectron.*, vol. 62, pp. 249–254, 2014, doi: 10.1016/j.bios.2014.06.053.

[31] A. A. Rowe *et al.*, "Cheapstat: An open-source, 'do-it-yourself' potentiostat for analytical and educational applications," *PLoS One*, vol. 6, no. 9, 2011, doi: 10.1371/journal.pone.0023783.

[32] K. Krorakai, S. Klangphukhiew, S. Kulchat, and R. Patramanon, "Smartphone-based nfc potentiostat for wireless electrochemical sensing," *Appl. Sci.*, vol. 11, no. 1, pp. 1–13, 2021, doi: 10.3390/app11010392.

[33] A. Ainla *et al.*, "Open-Source Potentiostat for Wireless Electrochemical Detection with Smartphones," *Anal. Chem.*, vol. 90, no. 10, pp. 6240–6246, 2018, doi: 10.1021/acs.analchem.8b00850.

[34] T. W. Overton, "Recombinant protein production in bacterial hosts," *Drug Discov. Today*, vol. 19, no. 5, pp. 590–601, 2014, doi: 10.1016/j.drudis.2013.11.008.

[35] A. L. Demain and P. Vaishnav, *Production of Recombinant Enzymes*. Elsevier, 2016.

[36] C. Engler, R. Gruetzner, R. Kandzia, and S. Marillonnet, “Golden Gate Shuffling: A One-Pot DNA Shuffling Method Based on Type IIs Restriction Enzymes,” *PLoS One*, vol. 4, no. 5, p. 5553, 2009, doi: 10.1371/journal.pone.0005553.

[37] J. M. Berg, J. L. Tymoczko, and L. Stryer, *Bıochemistry, 5th Edition*, 5th ed., no. Chapter 4. New York: W H Freeman, 2002.

[38] European Commission EUROSTAT, “Protein production and purification,” *Nat. Methods*, vol. 5, no. 2, pp. 135–146, 2008, doi: 10.1038/nmeth.f.202.Protein.

[39] M. Otten *et al.*, “From genes to protein mechanics on a chip,” *Nat. Methods*, no. september, pp. 1–6, 2014, doi: 10.1038/nmeth.3099.

[40] F. W. Studier, “Protein production by auto-induction in high density shaking cultures.,” *Protein Expr. Purif.*, vol. 41, no. 1, pp. 207–234, 2005, doi: 10.1016/j.pep.2005.01.016.

[41] J. Wilson, “Cyclic Voltammetric Study of ferrocyanide/ferricyanide Redox Couple,” vol. 4, no. 3, pp. 4–6, 2000, doi: 10.1016/j.bios.2010.06.047.

[42] U. Kavčič and T. Pleša, “THE INFLUENCE OF PRINTING PROPERTIES OF SCREEN PRINTED ELECTRODES ON SENSITIVITY MEASURED WITH CYCLIC VOLTAMMETRY,” in *Proceedings of 9th International Symposium on Graphic Engineering and Design*, 2018, pp. 253–259, doi: 10.24867/GRID-2018-p31.

[43] S. Zuo, Y. Teng, H. Yuan, and M. Lan, “Direct electrochemistry of glucose oxidase on screen-printed electrodes through one-step enzyme immobilization process with silica sol-gel/polyvinyl alcohol hybrid film,” *Sensors Actuators, B Chem.*, vol. 133, no. 2, pp. 555–560, 2008, doi: 10.1016/j.snb.2008.03.024.

[44] G. Dai, J. Hu, X. Zhao, and P. Wang, “A colorimetric paper sensor for lactate assay using a cellulose-Binding recombinant enzyme,” *Sensors Actuators, B Chem.*, vol. 238, pp. 138–144, 2017, doi: 10.1016/j.snb.2016.07.008.