Master's Thesis

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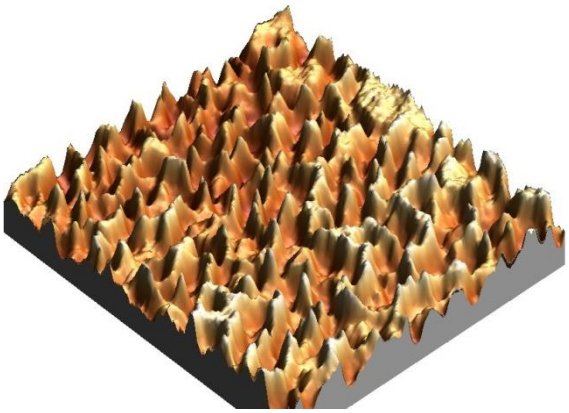
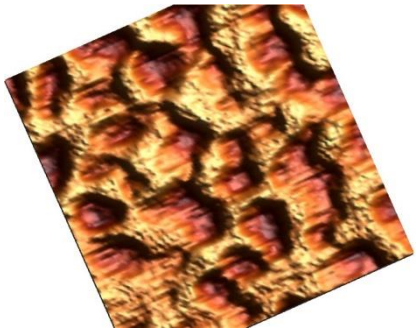
Self-Assembled Surface Layer Proteins from *Corynebacterium glutamicum* as a Matrix for Cutinase Immobilization





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ABSTRACT

One of the key challenges in nanoscience is the technological utilization of self-assembly systems; a process wherein molecules will spontaneously associate under equilibrium conditions into reproducible aggregates of supramolecules. Supramolecular structures and devices in the dimensions of a few to tens of nanometers have been developed using crystalline bacterial cell surface proteins (S-layer proteins). Treatment of whole bacterial cells with detergents and chaotropic agents enable the isolation of S-layer proteins of different molecular weights, having the intrinsic property to recrystallize into two dimensional arrays at a broad spectrum of surfaces and interfaces. The well-defined arrangement of functional groups of the S-layers on solid support permits the binding of molecules including enzymes in defined regular arrays. This can be applied in the nanofabrication of analytical based biosensors among other applications. The immobilization of enzymes facilitates the separation of the enzyme from the reaction mixture and can significantly lower the cost of the enzyme.

The aim of this project is to isolate and purify crystalline surface layer proteins from the bacteria cell envelope of a Gram positive bacteria; *Corynebacterium glutamicum* using a detergent (SDS) and high molar concentrations of a chaotropic agent (guanidine hydrochloride), characterize the isolated S-layer proteins by SDS PAGE in order to determine its molecular weight and do an AFM analysis of the self-assembled S-layers on a mica surface and finally immobilize a hydrolytic enzyme; cutinase on the self-assembled S-layer proteins. An esterase activity test for cutinase will be used to study the binding of cutinase to the self-assembled S-layers on the mica surface. The immobilized cutinase activity will be compared with the activity of the enzyme in solution.

Key Words: Self-assembly, Surface Layer Proteins, Enzyme Immobilization, Cutinase, Atomic Force Microscopy

Abbreviations

AFM	Atomic Force Microscopy
EDC	1-ethyl-3(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene Glycol Tetraacetic Acid
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
MES	4-Morpholineethanesulfonic acid sodium salt
NHS	N-hydroxysuccinimide
PBS	Phosphate Buffered Saline
SDS	Sodium Dodecyl Sulphate

PREFACE

This thesis is submitted in fulfilment of the requirements for a Master`s degree in Nanobiotechnology at the Institute of Physics and Nanotechnology at the Faculty of Engineering and Science of Aalborg University, Denmark. The experiments and assays were carried out within the period from October 17, 2013 to June 2, 2014.

The report is partitioned into parts, chapters and sections. Part one is the introduction and gives a global insight of the concepts considered in the report. The second part describes the experimental methods used in the investigations done in the project while the third part presents the results obtained from the experiments and assays carried out. Part four is the discussion and it analyses the results obtained relating them to the experiments performed and other previous knowledge. The conclusions arrived at in the project are presented in part five.

Figures in the report are sequentially numbered in each chapter.

Referencing is done using a number method, where a bracket contains a number relating to the source position in the reference section. The sources are listed with surnames and initials of the authors, title, journal, volume, number, year and page of the book/article.

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Part I

Introduction

CHAPTER
ONE

INTRODUCTION

Contents

1.1 Self- Assembly

Self–assembly is a fundamental principal that generates organized structures [1]. It involves reversible processes in which pre-existing components or disordered components of a pre-existing system form structures with a pattern, achieved through their global energy minimization [2, 3]. Self–assembly can either be static or dynamic. When the ordered state occurs with the system in equilibrium and does not dissipate energy, it is referred to static self-assemble. Dynamic self-assembly set in when the ordered state dissipates energy. Self-assembly can be observed at all level from macro to nano scale [3]. Some examples of natural self-assembly include the formation of lipid bilayer, micelle formation, protein folding, DNA double helix, self-assembled monolayers (SAMs) [2] etc.

Molecular self-assembly involves strategies for the nanofabrication and designing of molecules and supramolecular entities in a manner that shape-complementarity will lead to aggregation into desired structures. This enables nanobiotechnologists with the tremendous ability to fabricate controlled nanostructures. Self-assembly performs most of the difficult steps involved in nanofabrication; like those involving atomic level modification of structures [4]. This is achieved through utilization of highly developed techniques of synthetic chemistry. Self-assembly also help to incorporate structures directly as components in the final system. It is also an important tool used in biology for the development of complex functional structures [4]. Self-assembly produces structures that are relatively defect-free and self-healing since it requires that the target structures are thermodynamically the most stable ones open to the system. The main challenge of self-assembly is that we do not yet know how

to carry it out and cannot mimic processes that are known to occur in biological systems at other elementary levels [2, 4].

Self-Assembled Monolayers (SAMs)

The formation of monolayers by surfactant molecules at surfaces is one example of the general phenomena of self-assembly. SAMs are formed when surfactant molecules adsorb spontaneously on surfaces in a monomolecular layer. They offer a unique combination of physical properties that allow fundamental studies of interfacial chemistry, self-organization and solvent-molecule interactions. SAMs are very valuable substrates in organometallic, physical, organic, bioorganic, bioanalytical and electrochemistry because of their well-defined arrays and ease of functionalization [5]**.**

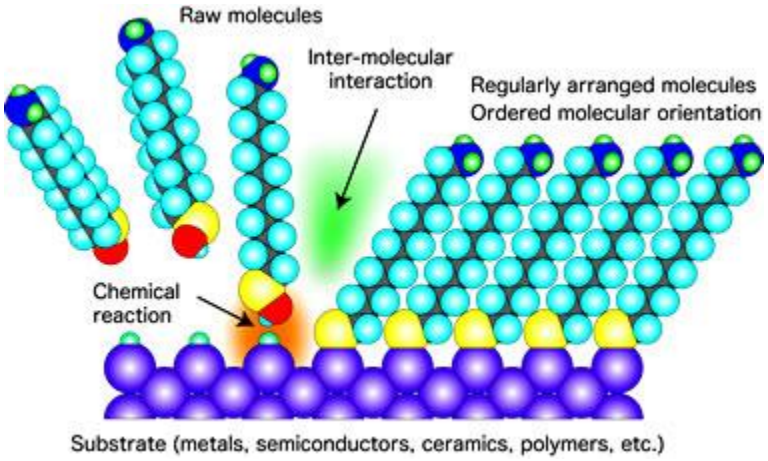


Figure 1.1: Schematic illustration of self-assembled monolayers

Micelle formation

Micelle formation is another example of natural self-assembly phenomena. Micelles are organized molecular assemblies of surfactants [6]. Amphiphilic molecules (molecules having both polar or charged groups and nonpolar regions) in aqueous solution form aggregates called micelles. In a micelle the nonpolar tails are sequestered in the interior while the polar heads form an outer shell in contact with water molecules .The kind of aggregates formed during micellization is determined by factors like the nature and size of the polar or ionic head, length of the nonpolar tail, the acidity of the solution, the presence of added salts and temperature. The shape and size of the micelle can be changed by changing these parameters. The size of the micelle is described by the aggregation number (the number of amphiphilic molecules forming the aggregate**).** The critical micelle concentration (CMC) is the concentration at which amphiphilic molecules (for example; surfactants) will spontaneously form micelles [6]. This is monitored by the sudden change of the physical and chemical properties of the solution. Thus, the CMC represents a phase separation between single molecules of surfactant and surfactant aggregates in dynamic equilibrium [7].

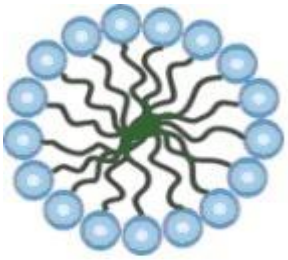


Figure 1.2: Schematic representation of a micelle in aqueous solution

Amphiphilic molecules can also form micelles in nonpolar organic solvents. In this case, micelle aggregates are called inverse micelles because the hydrocarbon tails are exposed to the solvent, while the polar heads point towards the interior of the aggregate to escape the contacts with the solvent**.** A "pocket" is formed which is particularly suited for the dissolution and transportation of polar solutes through a nonpolar solvent because the reverse micelles are able hold relatively large amounts of water in their interior [6, 7].

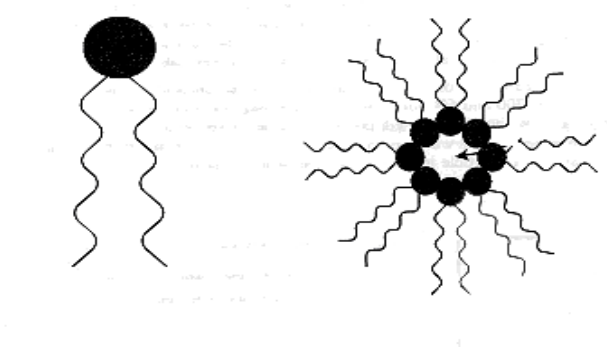


Figure 1.3: A reverse micelle. Polar heads point toward the interior, while the non-polar tails are exposed to the nonpolar solvent. The arrow indicates water "sequestered" in the interior

1.2 Surface layer proteins

S-layers are self-assembled crystalline protein lattices that form the outermost cell envelope component that cover many bacteria and almost all archaea. They have been optimized for years as constituent elements of one of the simplest systems of self-assembly. They are composed of numerous identical subunits forming a symmetric, porous, lattice-like layer that completely covers the cell surface. The subunits are held together and attached to cell wall carbohydrates by non-covalent interactions, and they spontaneously reassemble in vitro by an entropy-driven process [8]. The functional groups on S-layer lattices have a well define arrangement that permits molecules and particles to bind on it in well define regular arrays. The biological functions of S-layer proteins are poorly understood, but in some species S-layer proteins mediate bacterial adherence to host cells or extracellular matrix proteins or have protective or enzymatic functions, serving as molecular sieves [9]. S-layer proteins when isolated have the intrinsic property to recrystallize into two dimensional mono- or double layers in solution, and at interfaces forming arrays on a broad spectrum of surfaces like mica, silicon and interfaces such as planar lipid films [8].

S-layer proteins exhibit oblique, square or hexagonal lattice symmetry composed of a single protein or glycoprotein species (molecular weight of 40 to 200kDa) and represent the simplest biological membranes that have developed during evolution. S-layer proteins are generally 5 to 10 nm thick with identical pores of size 2 to 8 nm in diameter and identical morphology [10]. The inner and outer surface corrugation of S-layer proteins shows a remarkable difference. The inner face topography with respect to the bacterial cell is more corrugated than the outer one. Chemical modification of surface properties of S-layer proteins of most Bacillaceae revealed a net negatively charged inner face and neutral outer face [9]. The net negative surface charge on the inner face is attributed to an excess of carboxyl groups. These carboxyl groups are found in equimolar amounts on the outer face rendering it neutral.

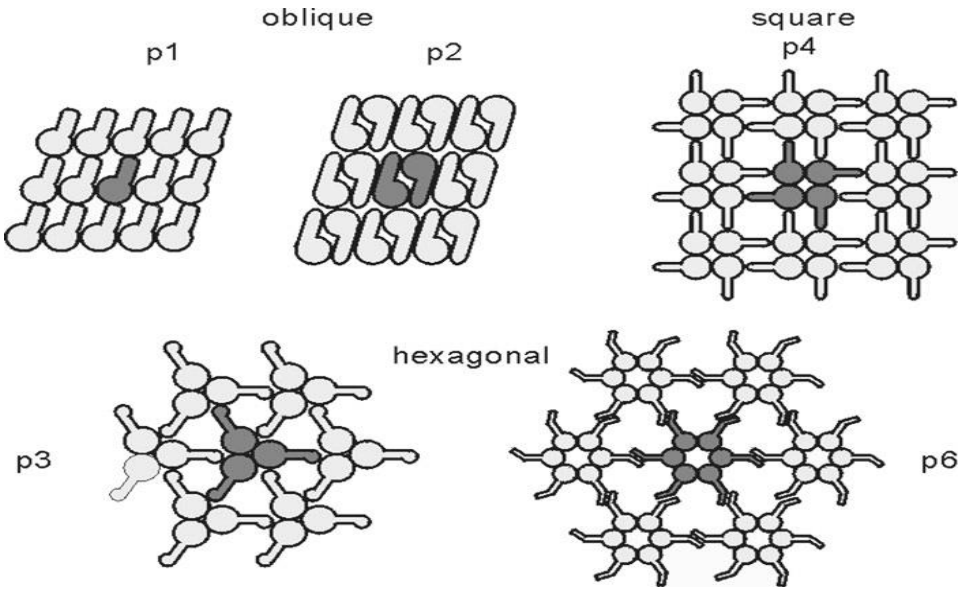


Figure 1.4: Schematic representation of S-layer lattice types grouped according to the possible two-dimensional space group symmetries. [11]

The constituent subunits in S-layer lattices interact with each other and with the supporting cell envelope layer such as the plasma membrane, outer membrane or peptidoglycan by a combination of non-covalent forces including hydrogen or ionic bonds, and hydrophobic or electrostatic interactions [12]. Hydrophobic interactions play a particularly important role in the assembly process of S-layer proteins because the S-layer proteins are composed of high proportions of non-polar amino acids. [13]. Studies have revealed that the intermolecular forces between the S-layer subunits are stronger than the forces binding the crystalline array to the supporting envelope layer [14]. The dynamic recrystallization and reorganization process of the S-layer lattice on the bacterial cell surface in the course of cell growth and cell division has been related to the existence of specific (lectine type) binding domains on the N-

terminal part of the S-layer proteins for secondary cell wall polymers that are covalently attached to the peptidoglycan matrix of the cell wall [15]. S-layer protein species and a host of other environmental parameters such as pH value, ion composition and/or ionic strength and temperature determine the structural size and form of the S-layer self-assembly products. Rapid nucleation of subunits into oligomeric precursors of several units determines the initial phase, followed by slow assembly of the aggregates into larger crystalline arrays [16]. The amino acid sequence of polypeptide chain and consequently the tertiary structure of the S-layer species determine the self-assembly properties. Double layers may also be formed during S-layer self-assembly process in which case the two monolayer constituents face each other either with their inner or outer sides and often stabilized by divalent cations interacting with polar groups [17].

S-layer proteins have the intrinsic property to form extended crystalline arrays on solid supports. This is an important feature for the functionalization of surfaces. Transmission electron microscopy (TEM) and atomic force microscopy (AFM) are techniques that have been employed to study the recrystallization of S-layer proteins on solid substrates. Measurements by AFM with chemically functionalized probes reveal repulsive surface forces between S-layer protein monolayers and both hydrophobic and hydrophilic probes. These repulsive forces could be interpreted to be steric forces that arise from the presence of solvated macromolecular chains protruding from the S-layer surface. The macromolecules on the S-layer proteins are possibly secondary cell wall polymers (SCWP) and are involved in specific binding of the S-layer proteins to the supporting layer containing peptidoglycan and remain attached to the solubilized S-layer protein [18]. The SCWPs are thought to support or facilitate the S-layer protein recrystallization leading to the formation of monocrystalline layers on solid substrates

The formation of monolayers at surfaces and interfaces and the ability to reassemble in suspension are some fundamental features of S-layers in nanobiotechnological applications [19]. The repetitive features of S-layers have led to their use as immobilization matrices for binding of monolayers of functional molecules (e.g., enzymes, antibodies, and immunogens) in a geometrically well-defined way. The controlled immobilization of biomolecules in an ordered fashion on solid substrates and their controlled confinement in definite areas of nanometer dimensions are key requirements for many applications including the development of bioanalytical sensors, biochips, molecular electronics, biocompatible surfaces, and signal processing between functional membranes, cells, and integrated circuits [19]. S-layer lattices recrystallized on solid supports (e.g., silicon wafers) can be patterned by microlithographic procedures as required for lab-on-a-chip technologies [20]. Another line of development is directed towards the use of S-layer self-assembly products in suspension as a combined carrier-adjuvant system against infection with pathogenic bacteria in the immunotherapy of cancers and in antiallergic immunotherapy. S-layers of pathogenic organisms on the other hand have been identified to be essential for virulence. Partially purified cell products or whole-cell preparations are currently used as attenuated vaccines against fish pathogens [21]. The ability of subunits of S-layer proteins to recrystallize into coherent lattices on functional lipid membranes including liposomes is another broad application of S-layer proteins. These S-layer-stabilized lipid membranes mimic the supramolecular structure of those archaeal

envelopes which possess S-layers as exclusive wall components or virus envelopes. They can be used in diagnostics, as vaccines, for drug targeting or delivery, and for gene therapy [22]

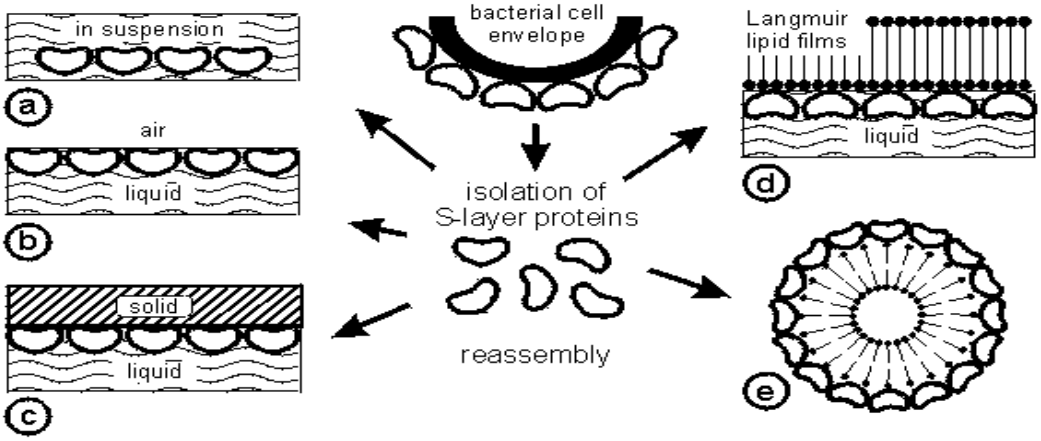


Figure 1.5: Schematic drawing of the isolation of S-layer proteins from bacterial cells and their reassembly into crystalline arrays in suspension (a), at solid supports (b), at the air-water interface (c), on lipid films (d) and on liposomes (e). The orientation of the recrystallized lattice is determined by the physicochemical properties of the surfaces.

1.3 Corynebacterium glutamicum

C. glutamicum is a non-pathogenic, rod shaped gram positive bacteria found in soil, vegetables, animal faeces and fruits. It is facultatively anaerobic and heterotrophic. It is widely known for its role in the production of monosodium glutamate (a flavor enhancer) and as a natural producer of glutamic acid. *C. glutamicum* has been developed for the production of most biogene amino acids, nucleotides and vitamins.

The cell envelope of *C. glutamicum* possess an unusual cell wall dominated by a heteropolysaccharide termed as arabinogalactan (AG), which is linked to both mycolic acids and peptidoglycan, forming the mycolyl arabinogalactan-peptidoglycan (mAGP) complex [23**].** Some strains have the cell enveloped covered by an S-layer. It is suggested that the S-layer is anchored to a hydrophobic layer of the bacterial wall via its C-terminal hydrophobic sequence. The S-layer may be detached from the cell as organized sheets by detergents at room temperature [24]. Treatment of whole *C. glutamicum* cells with detergents results in the isolation of S-layer proteins with different apparent molecular weights, ranging in size from 55 to 66 kDa. The S-layer of *C. glutamicum* is characterized by hexagonal lattice symmetry [25].

1.4 Enzymes

Enzymes are biochemical catalysts that speed up reactions by providing an alternative reaction pathway of lower activation energy. The enzyme usually takes part in the reaction but does not undergo permanent changes and thus remain chemically unchanged at the end of the reaction. This implies that the enzymes only alter the rate of the reaction and does not affect the position of the equilibrium. Most enzymes have a high degree of specificity, binding a specific molecule and converting it to a specific product. The specificity of the enzyme is as a result of the shape of the enzyme molecules. Enzymes are also highly efficient [26].

Enzymes are proteins; polymers of amino acids. The proteins in enzymes are usually globular. Some enzymes have extramolecules associated with them apart from the amino acids. These molecules assist in the reaction they catalyze. The protein portion of an enzyme is called the apoenzyme. The non protein part is referred to as the cofactor. Cofactors can be loosely bound (coenzyme) or tightly bound (prosthetic groups).The term holoenzyme describes the complete enzyme consisting of the apoprotein and the cofactor. Intra-and intermolecular bonds that hold proteins in their secondary and tertiary structures are disrupted by pH and temperature changes. This affects the shape and also the catalytic activity of an enzyme rendering enzymes pH and temperature sensitive [26, 27].

An enzyme active site is the part of the molecule that has just the right shape and functional group to bind to one of the reacting molecules. The substrate is the reacting molecule that binds to the enzyme. The enzyme and the substrate form a reaction intermediate with lower activation energy than the reaction between the reactants without the enzyme. Once the intermediate reacts with another reactant the enzyme will reform.

The Lock and key hypothesis is the simplest model to represent the functioning of an enzyme. The substrate simply fits into the active site to form a reaction intermediate [26].

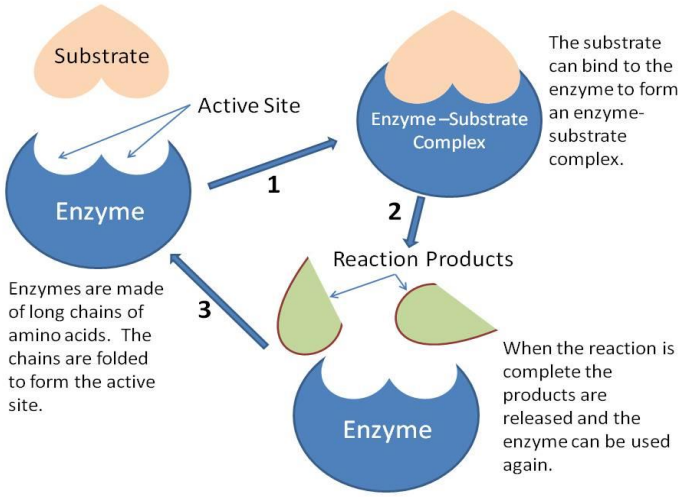


Figure 1.6: Illustration of the Lock and key hypothesis of enzyme function

In the induced fit hypothesis of enzyme function, the shape of the enzyme molecule changes as the substrate molecules approach it [27]. This model is more sophisticated and relies on the fact that the enzyme molecules are flexible due to the ability of single covalent bonds to rotate freely.

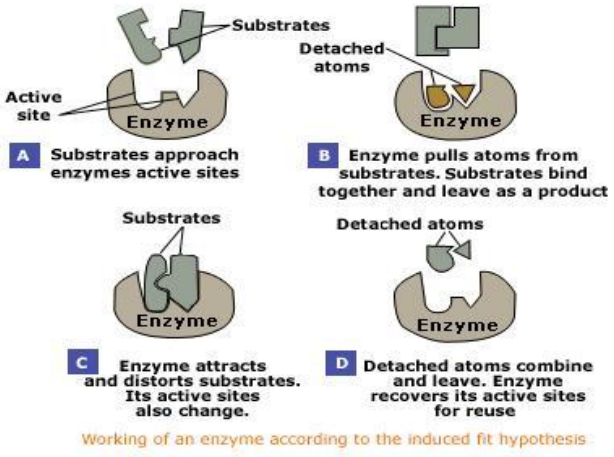


Figure 1.7: Illustration of enzyme action by the induced fit hypothesis

Inhibitors are substances that reduce or even stop the catalytic activity of enzymes in biochemical reactions by blocking or distorting the active site. Active inhibitors occupy the enzyme active site and prevent a substrate molecule from binding to the enzyme. Active inhibitors are also referred to as competitive inhibitors because they compete with the substrate for the enzyme active site. Non active site directed or noncompetitive inhibitors attach to the other parts of the enzyme molecule, distorting its shape [27].

Enzyme Immobilization

Immobilization of macromolecules can be generally defined as a procedure leading to their restricted mobility. Immobilized enzymes are enzymes which are either covalently bound or absorbed onto the surface of an insoluble support or enzymes that are physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously. The binding of enzymes onto solid material started far back in the 1950s when immobilized enzymes (with restricted mobility) were intentionally prepared [28]. Recently, the attachment of enzymes onto prefabricated artificial or natural carriers has been given special emphasis. Immobilization of enzymes helps to fully exploit the economical and technical advantages of biocatalysts in organic synthesis based on the isolated enzymes [28]. This enables the easy separation of the enzyme catalyst from the reaction mixture and will therefore drastically lower the cost of the enzyme.

Enzymes are immobilized in order to facilitate the separation of the enzyme from the product. This helps to simplify the enzyme application and support an efficient and reliable reaction

technology. The immobilization of enzymes also permits the reuse of the enzyme and provides cost advantages which are often essential prerequisite for the establishment of an enzyme catalyzed process. Above all, immobilized enzyme preparations are more convenient to handle [28].

The enzyme yield activity after immobilization depends not only on losses due to the binding procedure but also as a result of the diminished availability of the molecules within pores or from slowly diffusing substrate molecules. These limitations lead to lower catalytic efficiency of the immobilized enzyme. On the other hand, immobilized enzyme preparations provide a balanced overall performance based on low mass transfer limitations, reasonable immobilization yield and high operational stability. The improved stability under working conditions compensates for the drawbacks and hence an overall benefit [28].

For the immobilization of biologically active macromolecules (including enzymes), the carboxylic acid groups from S-layer proteins are mostly activated with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) so that they could subsequently react with the free amine groups from the enzyme. Immobilized enzymes retain 16 to 60% of the initial activity. To improve the activity preservation, flexible sparcers like 6-aminocapronic acid are used [29]. Immobilization through sparcer molecules is of particular advantage to enzymes that are small enough for being entrapped inside the pores or for penetrating the pore openings to some extent. For example, β-glucosidase with molecular mass of 66kDa retained only 16% of its activity after direct coupling to EDC-activated carboxylic acid groups of S-layer proteins [29], but a 10 fold increase in activity to 160% was achieved when the enzyme was immobilized through sparcers.

Methods of Enzyme immobilization

The immobilization of enzymes can induce novel characteristics to the enzyme [30]. The immobilization procedure should therefore be performed in a way that will allow the enzyme to maintain its active conformation and necessary catalytic flexibility and also avoid disturbance of the catalytically essential residues of the enzyme. In addition to their use in industrial processes, the immobilization techniques are the basis for making a number of biotechnology products with application in diagnostics, bioaffinity chromatography, and biosensors

There are four principal methods available for immobilizing enzymes; covalent binding, entrapment, crosslinking, adsorption and immobilization via disulfide bonds [30].

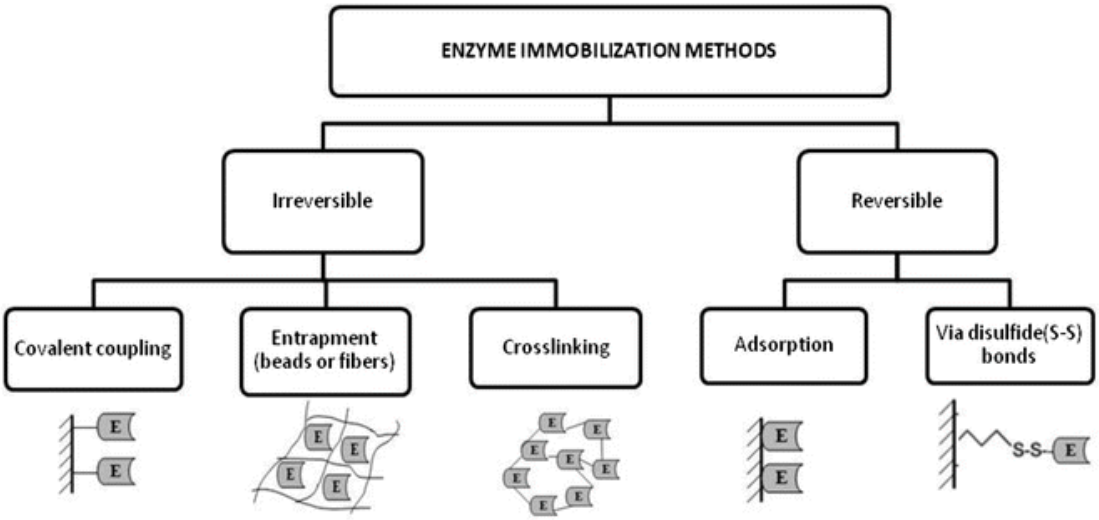


Figure 1.8: Schematic representation of the main different methods of enzyme immobilization

Each immobilization method has its advantages and drawbacks. The choice of the most appropriate technique depends on the nature of the enzyme (biochemical and kinetics properties) and the carrier (chemical characteristics, mechanical properties). So, the interaction between the enzyme and support provides an immobilized enzyme with particular biochemical and physicochemical properties that determine their applicability to specific processes [31, 32, 33].

The immobilization of enzymes by covalent coupling has a major advantage in that the enzyme is not released into the solution upon use due to the stable nature of the bonds formed between the enzyme and the matrix. A difficult requirement however is to exempt the amino acid residues essential for the catalytic activity from the covalent linkage to the support. To improve the activity yield, the coupling reaction is sometimes performed in the presence of analogues substrates [34].

Covalent methods of immobilizing enzymes are often used when there is a strict requirement for the absence of the enzyme in the product. The coupling methods are in two folds; (1) matrix activation by addition of a reactive function to a polymer; (2) modification of the polymer backbone to produce an activated group. These activation processes are designed to generate electrophilic groups on the support which in the coupling step will react with the strong nucleophiles on the enzyme. The enzyme is linked to the support through amide, ether, thio-ether, or carbamate bonds, strongly bound to the matrix and in many cases it is also stabilized. Usually, the S-layer lattice is crosslinked with glutardialdehyde before the free carboxyl groups are activated with 1-ethyl- 3, 3'(dimethylamino) propylcarbodiimide (EDC). The activated carboxyl groups can then react with free amino groups of the foreign macromolecules leading to stable peptide bonds between the S-layer matrix and the

immobilized protein molecules [35]. Once the enzymatic activity decays, the matrix is discarded together with the enzyme. To avoid this problem, enzymes are covalently attached to solid matrices through disulfide bonds. The great potential advantage of this approach is the reversibility of the bonds formed between the activated solid phase and the thiol-enzyme, because the bound enzyme can be released with an excess of a low-molecular-weight thiol (e.g., dithiothreitol [DTT]). This is of particular interest when the enzyme degrades much faster than the adsorbent, which can be reloaded afterwards [36].

Enzyme immobilization by entrapment differs from covalent bond formation (coupling methods) described above, in that the enzyme is not bound to the matrix or membrane. There are different approaches to entrapping enzymes such as gel or fiber entrapping [37**],** and micro-encapsulation [38]. The use of these methods is practically restricted by mass transfer limitations through membranes or gels.

Enzyme immobilization by crosslinking is based on multipoint attachment through intermolecular crosslinking between the enzyme molecules [39**].**

Adsorption is a non covalent interaction. Nonspecific adsorption is the simplest immobilization method and based mainly on physical adsorption or ionic binding [40]. By physical adsorption, the enzymes are attached to the matrix via van der Waals forces, hydrogen bonds or hydrophobic interactions. In ionic binding, the enzymes are attached through salt linkages. The nature of forces involved in non-covalent immobilization result in processes which can be reversed by changing the conditions that influence the strength of the interaction (pH, ionic strength, temperature, or polarity of the solvent). Immobilization by adsorption is a mild, easy to perform process, and usually preserves the catalytic activity of the enzyme and therefore economically attractive, but may suffer from problems such as enzyme leakage from matrix when the interactions are relatively weak [40].

Covalent coupling with EDC and NHS

For the immobilization of proteins to occur, the carboxyl groups on our S-layer proteins need to be activated. This activation is achieved through the use carbodiimide compounds with EDC being water soluble the most commonly used and readily available carbodiimide compound [35]. It is often used for aqueous crosslinking. The water insoluble DCC counterpart is often utilized for non-aqueous organic synthesis [41].

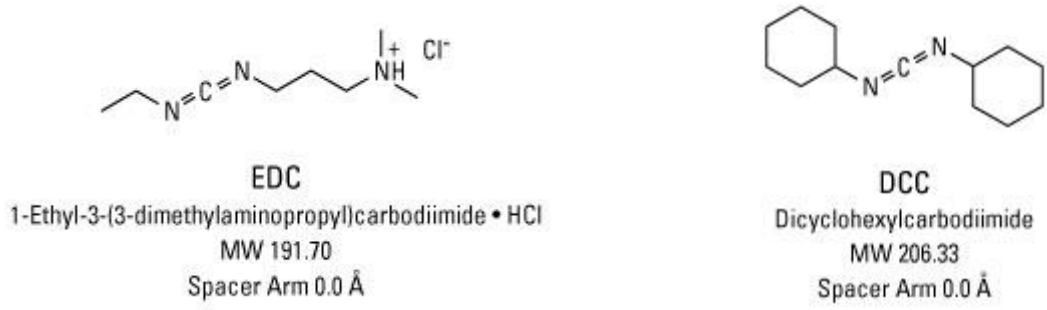


Figure 1.9: Chemical structures of carbodiimides EDC and DCC [41]

Carbodiimides are considered as zero-length carboxyl-to amine crosslinkers because no portion of their chemical structure results in part of the final bond between the coupled molecules [41]. The ability of EDC to crosslink primary amines to carboxylic acid groups is a very versatile and powerful tool for immobilizing macromolecules and preparing biomolecular probes [41].These are applications used in most protein and cell biology detection and analysis methods.

Initially, EDC reacts with carboxylic groups resulting in the formation of an active O-acylisourea intermediate. The primary amino groups subsequently displace this active intermediate leading to the formation of an amide bond with the original carboxyl group.

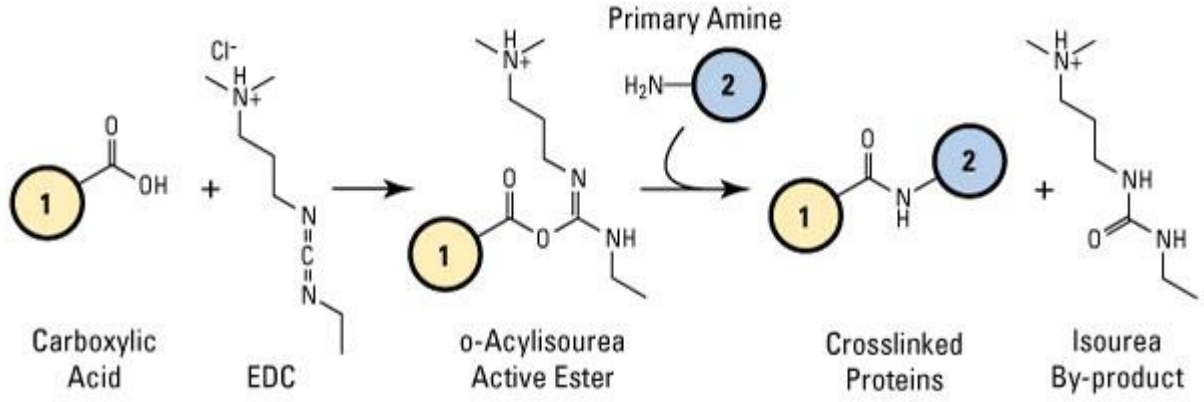


Figure 1.10: EDC crosslinking reaction scheme. Molecule 1(For example; S-layer protein) and molecule 2 can be an enzyme (cutinase).

N-hydroxysuccinimide (NHS) is usually used in combination with EDC coupling procedures. NHS helps to improve the efficiency by the formation of a dry-stable (amine reactive) intermediate [41]. The O-acylisourea intermediate that is formed by EDC is not stable in aqueous solution. If it does not react with an amine, the result will be the hydrolysis of the intermediate. NHS couples with EDC and results in the formation of an NHS ester which is more stable than the O-acylisourea intermediate and therefore permits an efficient conjugation at physiological pH to primary amines.

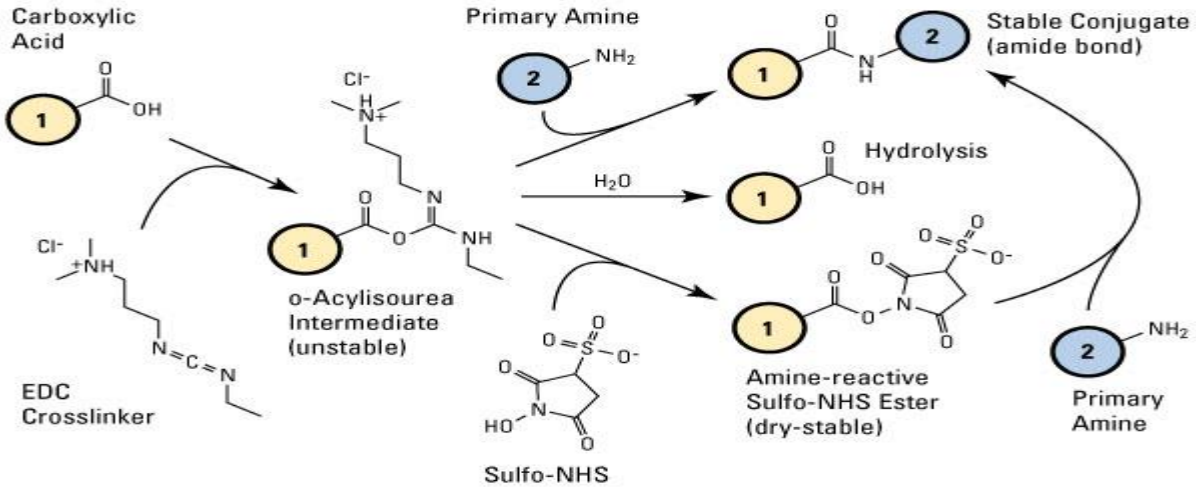


Figure 1.11: NHS / EDC crosslinking reaction scheme. Carboxyl-to-amine crosslinking using the carbodiimide EDC and sulfo-NHS. Addition of Sulfo-NHS to EDC reactions (bottom-most pathway) increases efficiency and enables molecule (1) to be activated for storage and later use.

1.5 Cutinase

Cutinase is a hydrolytic enzyme that is involved in the degradation of the cuticular polymer (cutin) in higher plants. Cutin is a polyester composed of hydroxyl and epoxy fatty acids [42]. Cutinase plays an important role in virulence, hence several studies have been performed to elucidate its biochemical and physiological properties [43]. Cutinase is important because of its applications in the dairy industry for the hydrolysis of milk fat and in the oleochemistry industry. It can be applied in the synthesis of structured triglycerides, polymers, surfactants, ingredients for personal care products, pharmaceutical and agrochemicals containing one or more chiral centers. It also has important applications in the degradation of plastics. For example, the synthetic polyester, Polycaprolactone has been hydrolyzed to water soluble products by cutinase [43].

Cutinase is a compact one domain molecule about $45\times30\times30Å^3$ in size. It is a 197-residue protein and has a molecular weight of around 22KDa [43, 44]. It contains highly conserved stretches with four invariant cysteines that form two disulfide bridges. The disulfide bridges play an important role in the structural integrity of the cutinase molecule. Studies have revealed that the disruption of the disulfide bridge affects the conformation and activity of the enzyme because the cleavage distorts the active site area in a manner that the catalytic triad would no longer be close enough for catalysis [44].The catalytic serine in cutinase is situated at the middle of a sharp turn between a β-strand and an α-helix. Cutinase is therefore a member of the α/β hydrolases, with the central β-sheet consisting of five parallel strands, covered by two and three helices on either side of the sheet. The catalytic triad Ser-120,Asp-175,His-188 is accessible to solvent and located at one extremity of the protein ellipsoid,

surrounded by the loop 80-87 and the more hydrophobic loop 180-188 [45].The active site is very flexible, explaining why cutinase is adapted to different substrates [46].

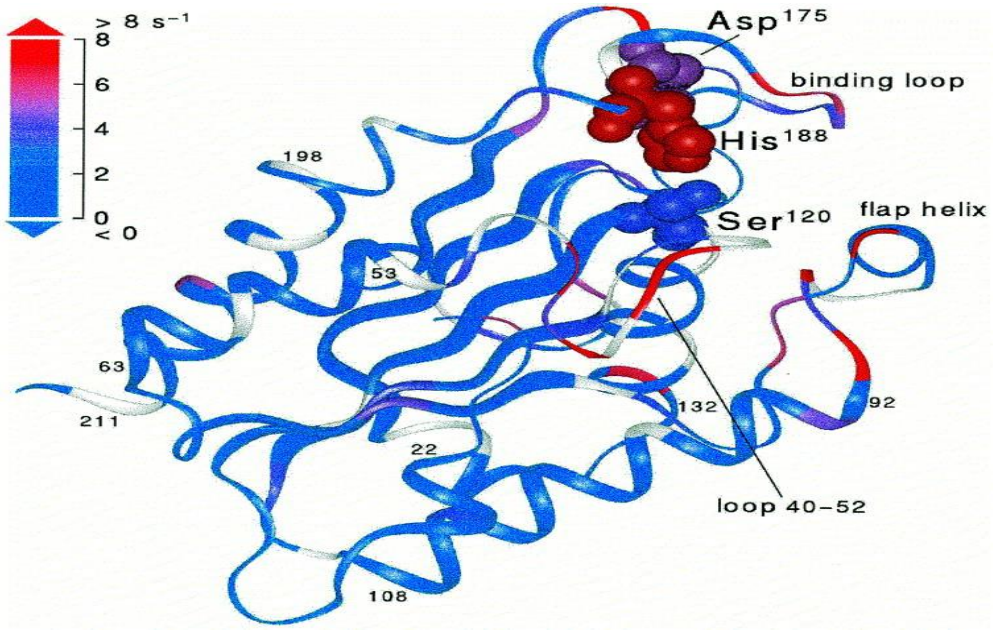


Figure 1.12: 3D backbone fold of cutinase showing accessibility of the active site region. The dynamics of the backbone is also indicated. Red, mobile; blue, rigid. The mobility data are derived from NMR relaxation experiments, where the difference is taken of the 15N R2 and R1 relaxation rates (colour coding level is indicated in inset). Residues for which relaxation data are lacking are indicated in white [47].

Cutinase differs from classical lipases even though they present lipolytic activity in that they do not exhibit interfacial activation. Interfacial activation is necessary for lipases to display their activity because the existence of the hydrophobic lid or flap covers the catalytic site. This conformational change of the lid is closely related to the interfacial activation process [48] and results in a large increase in the hydrophobic surface that is stabilized by interaction with the interface. The lid movement in lipases causes the formation of an oxyanion hole by a group of hydrogen donors that help to stabilize the catalytic carbonyl oxygen with a negative charge resulting from the intermediate complex formed during nucleophilic attack on the sessile bond of the substrate [48]. Although two side chain bridges of amino acid Leu-81 and Val-184 and Leu-182 and Asn-82 exist, the catalytic serine of cutinase is not buried under surface loops. The catalytic serine is accessible to solvent and substrate. Cutinase is not activated by the presence of interfaces due to the absence of the flap masking the active site serine as in other lipases. The flap (lid) is important for substrate docking and could also be related to substrate specificity. Cutinase having an exposed active site serine can behave like an interfacial activated lipase [49].

Activity test for cutinase

The activity of cutinase, being a hydrolytic lipase is often followed by the *p*-nitrophenyl butyrate hydrolyzing activity test. It is spectrophotometric esterase activity assay with p-NPB as substrate [50]. Cutinase hydrolyses *p*-NPB into butyric acid and *p*-nitrophenol. The protonation of *p*-nitrophenol in the presence of a base leads to the formation of *p*-nitrophenoxide anion with a change in absorbance at 420nm, the clear solution becomes yellow [51].

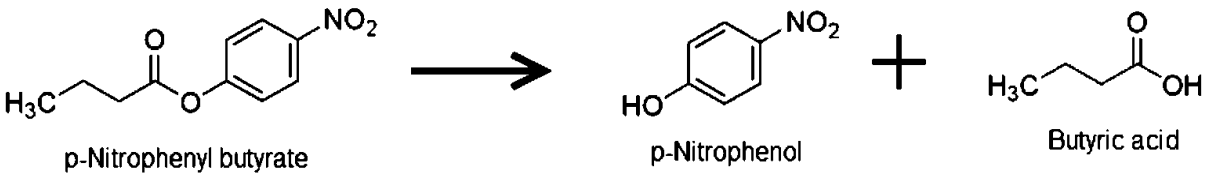


Figure 1.13: Hydrolysation of p-Nitrophenyl butyrate by Cutinase

Part II

Materials and Methods

CHAPTER
TWO

MATERIALS AND METHODS

Contents

This chapter describes the experiments for the growth and extraction of the S-layers from the bacteria cell, the characterization of the isolated S-layers, the protocol for the covalent coupling of cutinase to the S-layers and the assays for enzyme activity.

2.1 Bacterial growth

Corynebacterium glutamicum strain from the freezer was grown in minimal medium 1 (M1 medium) in a batch culture. 1 liter of M1 medium was composed of 5g casein peptone (Fluka) and 3g meat extract (Fluka). The medium was supplemented with 5g of $C_3H_5NaO_3$. Cells were cultivated on 500ml medium in a 2000ml shaking flask at a stirring speed of 250rpm and temperature of 30^0C until mid-exponential growth was attained (about 24 hours).

2.2 Isolation of S-layer proteins

Most of the techniques used for isolation and purification of S-layer proteins involve the mechanical disintegration of cells, and subsequent differential centrifugation to separate cell wall fragments [52]. In the case of most gram positive organisms, detergents (such Triton X-100) are usually used to treat the crude cell wall preparations in order to dissolve the plasma membrane contaminants. Chaotropic agents such as guanidine hydrochloride which loosen the bonds of the supporting polymer layer without dissociating the S-layer lattice are then used to obtain S-layer fragments by digestion of the peptidoglycan layer [52]. S-layers have

also been removed from the outer membrane of isolated cell envelope by treatment with low concentrations metal chelating agents (e.g., EDTA, EGTA), SDS, cation substitution, and detergents or combinations of them.

Isolation with Guanidine Hydrochloride

The protocol used for the isolation of the S-layer proteins is that described by Schuster and Sleytr (2005) with some modifications [52]. A 500ml cell culture grown to about OD_{600} of 0.72 was used. The cells were pelleted by centrifugation at 12,000rpm for 15 minutes at 4^0C.The cells were washed twice with 50ml of 25mM HEPES buffer and centrifuged at 12,000rpm for 15 minutes. The pellet was resuspended in 10ml HEPES buffer and ultrasonicated on ice at 40% amplitude for 50 minutes in 10 seconds cycle and 10 seconds break. The insoluble debris was removed by centrifugation at 11,000rpm for 5 minutes. The supernatant was centrifuged at 11,000rpm for 15 minutes. Contaminating plasma membrane fragments were extracted by suspending the crude cell wall preparation (pellet collected) in 10ml of 0.5% Triton X-100 and left at room temperature for 20 minutes with frequent shaking. The solution was centrifuged at 11,000rpm for 5 minutes and washed 3 times with 4ml of 25mM HEPES buffer. The solution was pelleted by centrifugation at 12,000rpm for 20 minutes. The pellet (cell wall fragments) was resuspended in 5ml of 5M guanidine hydrochloride (Flucka) and incubated at 60^0C for about 20 hours. It was centrifuged at 20,000rpm for 30 minutes. The supernatant was dialysed for about 24 hours against 3litres of milli Q water at 4^0C. The dialysed product (self-assembled product) was pelleted by centrifugation at 20,000rpm for 60 minutes and the pellet was resuspended in 1ml solution of $Cacl_2$ and stored at 4^0C.

Isolation with Sodium Dodecyl Sulfate

Extraction of the S-layer proteins using SDS was according to the protocol of Peyret et al. (1993) with minor modifications [53]. Cells from a 250ml culture were harvested by centrifugation at 5000g for 20 minutes. The cell pellet was washed 3 times with milli Q water and resuspended in 25ml of 2% SDS. The cell suspension was incubated with intensive stirring at a speed of 300 rpm at 60^0C for 2 hours and was then centrifuged again at 5000*g* for 10 minutes. The supernatant was centrifuged at 10,000g for 1 hour and the pellet suspended in 3ml of 2% SDS. The solution was dialysed against 3litres of milli Q water at 4^oC for 24 hours.

2.3 Characterization of Surface layer proteins

The S-layer proteins were characterized by SDS-PAGE according to Laemmli (1979) [54] with 10% separation gels in order to determine the molecular weight. Further characterization of the S-layers was done by AFM in semi contact mode. Single isolated S-layer subunits from many prokaryotic organisms have the ability to assemble into regular lattices identical to those observed on intact cells upon removal of the disrupting agents used for their isolation, e.g. by dialyzing [12,24]. Therefore the isolated S-layer proteins were dialysed against water for about 24 hours to remove the disrupting agent used for isolation before scanning by AFM.

SDS PAGE

A one-dimensional denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) with a 4% stacking gel and a 10 % resolving gel [54] was performed to determine the molecular weight of the S-layer proteins.
The resolving gel composed of 3.75ml milli Q water, 2.5ml of 1.5M Tris-Hcl pH 8.8,100µl of 10% SDS,50µl of 10%APS,3.6ML OF 30% Acrylamide Bis and 10µl TEMED. The stacking gel contained 3.05ml milli Q water,1.25ml of 0.5M Tris-Hcl pH 6.8,50µl of 10% SDS,30µl of 10% APS,0.65ml of 30% Acrylamide Bis and 10µl TEMED. The protein samples were denatured by heating to 95^0C for about 5 minutes.

10µl of sample and 10µl of sample buffer were mixed and placed on a heating block at 95^0C for 5 minutes and then placed immediately on ice for at least 1 minute. The samples were loaded onto the gel and electrophoresed for 90 minutes at 130V in 10x running buffer. The molecular weight was determined using 10µl of unstained low molecular weight marker. The stacking gel was eliminated and the separation gel was rinsed with milli Q water and stained with Coomassie blue stain for about 60 minutes. The gel was immersed into the destaining solution and kept overnight with shaking.

Atomic force microscopy of S-layer proteins

Mica surfaces were freshly cleaved (to render it clean and adhesive so that proteins will be able to adhere on them) and incubated with 10µl of sample solution for about 10 minutes at room temperature. The sample was carefully rinsed on the mica surface with 1–2 ml of milli Q water to remove weakly attached proteins and dried under a gentle flow of nitrogen gas. Imaging was performed at room temperature in semi contact mode using AC160TS cantilever. The apparatus used was a NTEGRA TS-150 table stable atomic force microscope. Images were constructed and analyzed using WSxM and Gwyddion freeware [55,56].

2.4 Covalent coupling of cutinase to S-layer proteins

The protocol used for coupling cutinase to the S-layer proteins is adapted from the protocol described by *Grabarek and Gergely* [30] with some modifications. EDC and NHS are used in the coupling procedure because they enable the sequential coupling of two proteins without affecting the carboxyls of the second protein [30].

1mg/ml of S-layer proteins were prepared in 1ml of 0.1M MES buffer pH 6.2 for 30minutes. 1mg/ml of cutinase was also prepared in 1ml of phosphate buffered saline (PBS) pH 7.1. EDC (0.4mg) and 0.6mg of NHS equilibrated at room temperature were added to the MES buffer prepared S-layer protein solution and left to react for 15minutes at room temperature. The cutinase prepared in PBS buffer was added to the EDC/NHS activated S-layer proteins and kept at room temperature for 2 hours.

2.5 Cutinase activity assay

A continuous spectrophotometric assay with p-nitrophenyl butyrate as substrate was used to determine the esterase activity of cutinase immobilized on S-layer proteins on a mica surface [57, 58]. 10µl of coupled S-layers and cutinase was deposited on a freshly cleaved mica surface and left for 30 minutes. Weakly bound proteins were gently washed off from the mica surface with milli Q water. The attached proteins on the mica surface were dried under a gentle flow of nitrogen gas. The p-nitrophenyl butyrate substrate was prepared by adding 10µl of p-nitrophenyl butyrate to 980µl of 50mM Tris-HCl buffer of pH 7.5. 10µl of the prepared p-nitrophenyl butyrate solution was deposited on the S-layer/cutinase immobilized proteins on the mica surface. A control experiment was done by depositing the prepared p-nitrophenyl butyrate on mica surface with pre-deposited, rinsed and dried S-layer proteins only and another with pre-deposited and then rinsed cutinase only.

The cutinase activity was determined by measuring the optical density of the S-layer/cutinase sample when reacted with p-nitrophenyl at different time intervals at a wavelength of 420nm and compared with the optical density of cutinase reacted with the p-nitrophenyl substrate. The optical density of S-layers only was also measured as a control assay. A plot of time against optical density at 420nm of the different samples was made and the S-layer immobilized cutinase activity was compared to the activity of the pure cutinase sample.

Part III

Results

CHAPTER
THREE

RESULTS

Contents

This chapter presents the results obtained from the various experimental procedures in isolating and characterizing S-layer proteins and the immobilization of cutinase to the S-layer proteins.

3.1 Growth of Bacterial and isolation of surface layer proteins

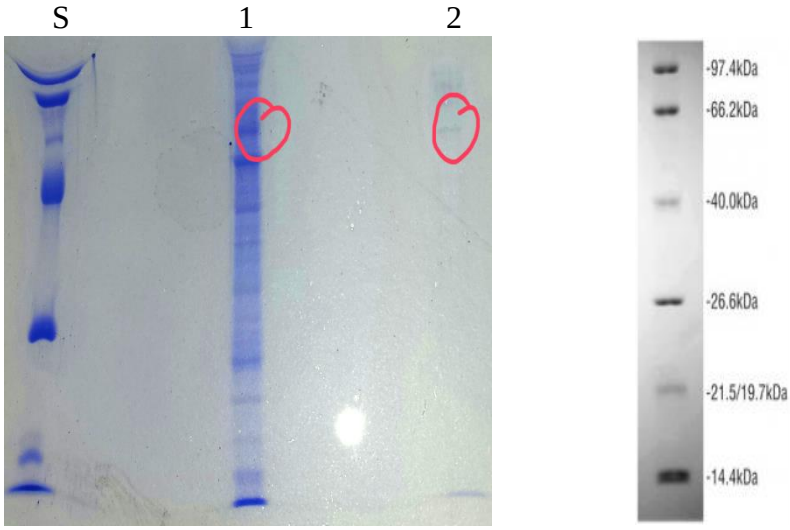
C. glutamicum was grown overnight at 30^{o}C in a batch culture medium until the growth entered the stationary phase. The cells were broken by ultrasonic treatment and the intact and broken cells were separated by centrifugation. Isolation of the S-layer proteins commenced immediately after the cells were harvested. The isolation of the S-layer proteins was done with guanidine hydrochloride and SDS.

3.2 Characterization of S-layer proteins

SDS-PAGE

The molecular weights of the isolated S-layer proteins were determined by SDS–PAGE and to compare the purity of the proteins isolated with the different protocols. Figure 3.1 shows the SDS-PAGE results of the S-layer proteins isolated. The molecular weight of the S-layer proteins from *C. glutamicum* is approximately 66kDa [19]. A band around this size is seen on the gel indicating that S-layers were successfully isolated. Several bands corresponding to different proteins are seen on the lane for S-layer proteins isolated with guanidine hydrochloride indicating that other proteins were isolated by this protocol. This indicates that

the S-layer proteins isolated using SDS are more pure than S-layer proteins isolated with guanidine hydrochloride.

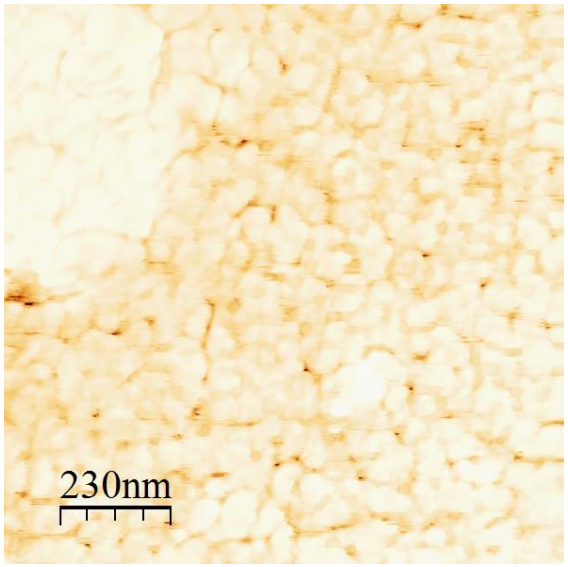


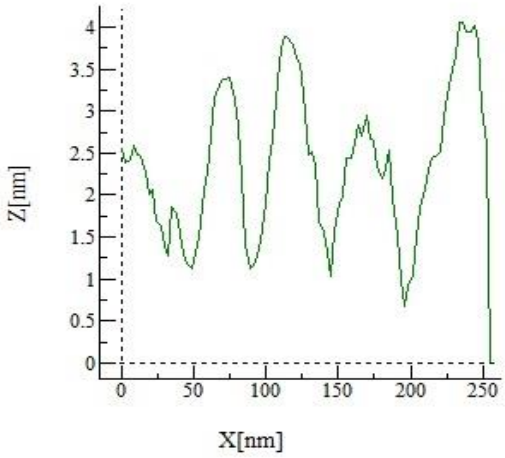
(a) (b)

Figure 3.1: (a) S-layer proteins isolated from C. glutamicum. Lane 1 represents S-layer proteins isolated with Guanidine hydrochloride while lane 2 represents S-layer proteins extracted with SDS. Lane S is the low molecular weight protein ladder with size range from 14.4 to 97.4 kDa. Lane S is also shown in (b).The molecular weight of S-layer proteins from C. glutamicum is between 55 to 66 kDa. A band in this range is shown on lanes 1 and 2.

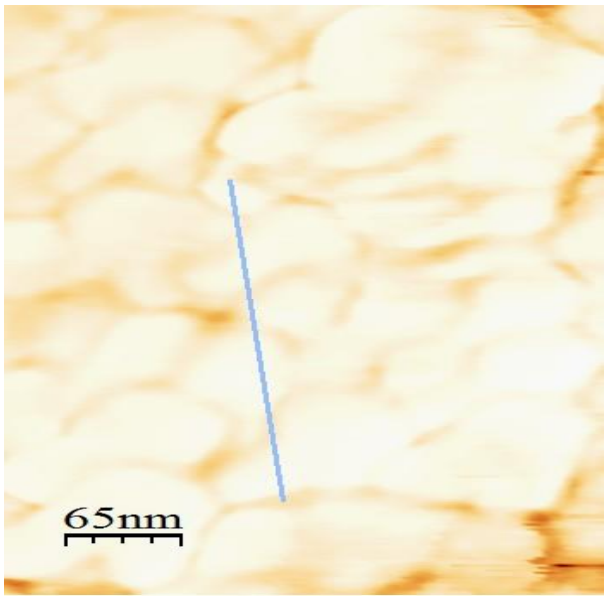
AFM of the isolated S-layer proteins

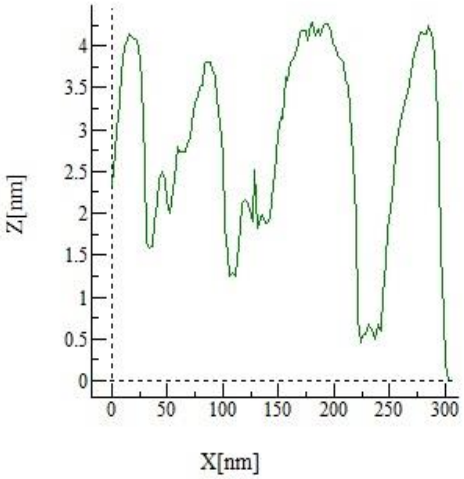
The isolated S-layers were further studied and characterized on a mica surface by AFM. Figure 3.2 shows high resolution images of S-layer proteins isolated with SDS on a mica surface. The height scan of the self-assembled S-layer proteins is shown in figure 3.2 (a) and the maximum estimated height is found to be around 4 nm. The higher resolution reconstruction image on Figure 3.2 (c) shows the S-layer lattice symmetry.





(a) (b)

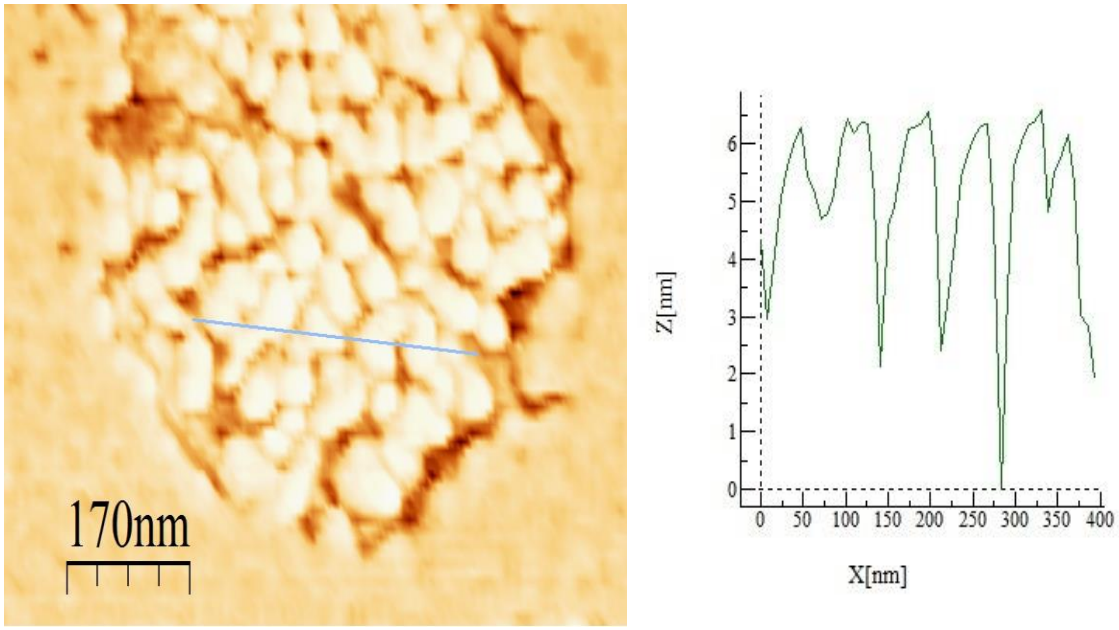




(c) (d)

Figure 3.2: S-layers isolated with SDS from C. glutamicum. (a) A height scan of the S-layers on mica surface. (b) A height profile of the line marked on (a). (c) A higher resolution of a section from (a). (d) A height profile of the line marked on (c).

Figure 3.3 shows high resolution images of S-layer proteins isolated with guanidine hydrochloride on a mica surface. The height scan of the self-assembled S-layer proteins is shown in figure 3.3 (a) and a maximum height is found to be 6 nm.



(a) (b)

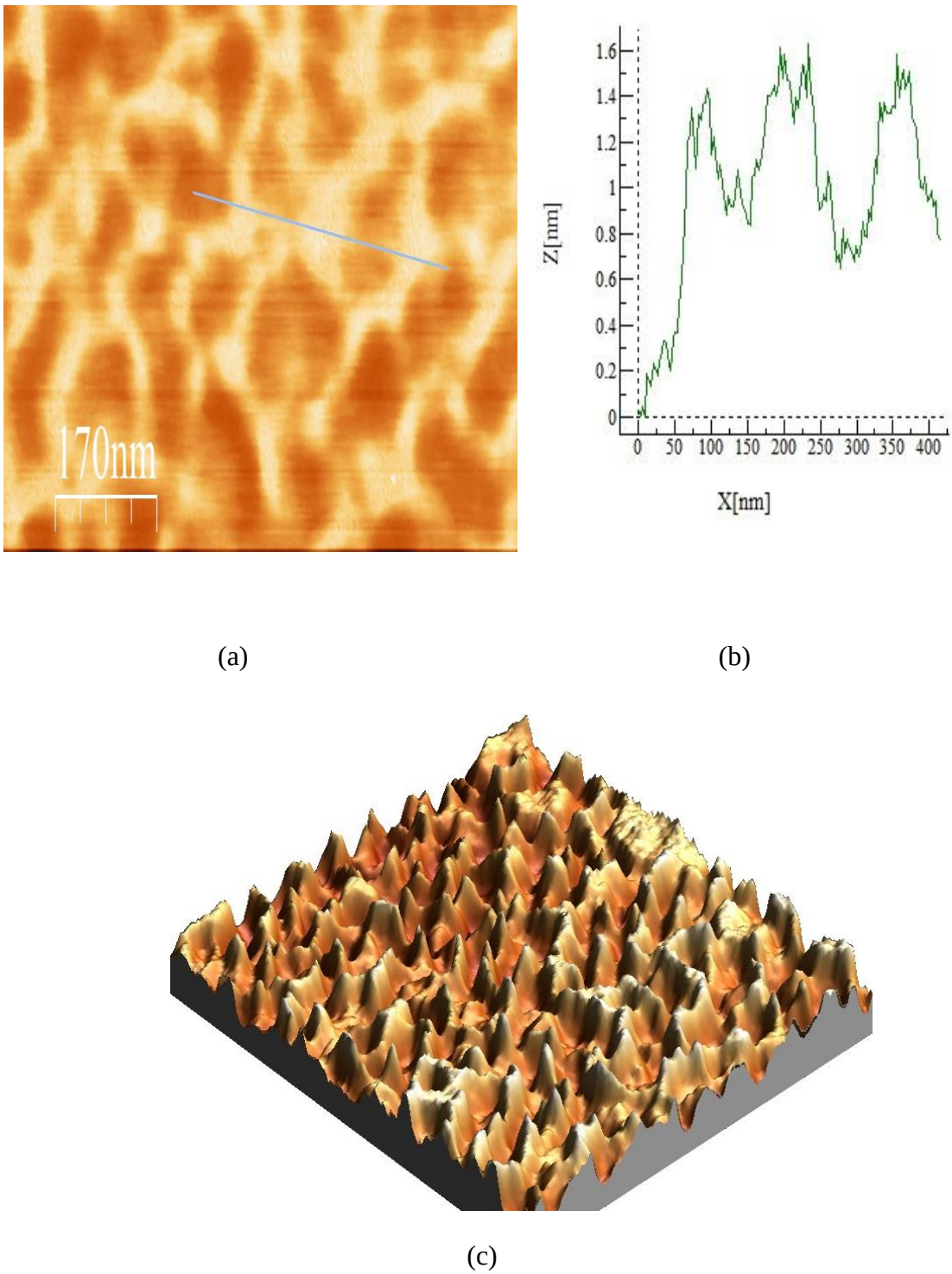
Figure 3.3: AFM images of S-layers isolated with guanidine hydrochloride from C. glutamicum. (a) Height scan of the S-layers on mica surface. (b) A height profile of the line marked on (a).

A very close view of the AFM images shows that a pattern of hexagonal S-layer lattice is formed but the pattern is not ordered and has an approximate height of 4 to 6nm.

3.3 Covalent coupling of Cutinase to S-layer proteins

The success of the covalent coupling of cutinase to S-layer proteins was determined using p-nitrophenyl butyrate assay for esterase activity. A colour change from clear solution to yellowish was visible on the mica surface on which the cutinase immobilized to S-layers was deposited (rinsed with water to remove weakly bound S-layers and dried by a gentle flow of nitrogen gas) upon the addition of the p-nitrophenyl butyrate. No yellow colour was visible following the addition of p-nitrophenyl butyrate on the mica surfaces on which only S-layers or cutinase were deposited, rinsed with water and dried.

After the covalent coupling of the cutinase to the self-assembled S-layer proteins, AFM was again used to characterize the S-layer-cutinase complex on the mica surface.

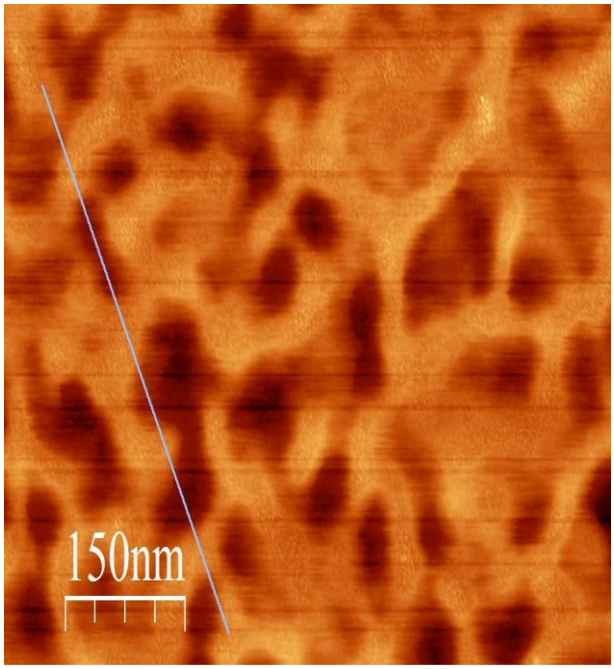


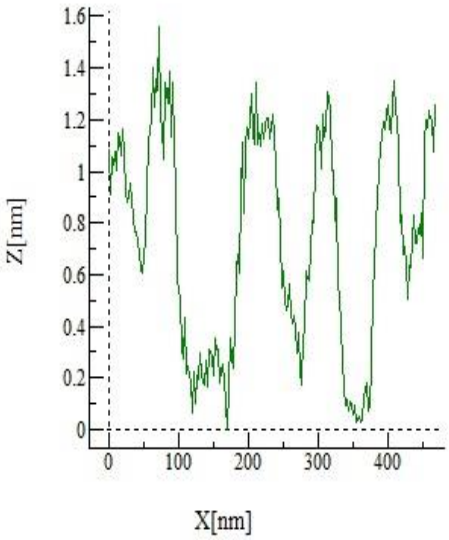
(a) (b)

(c)

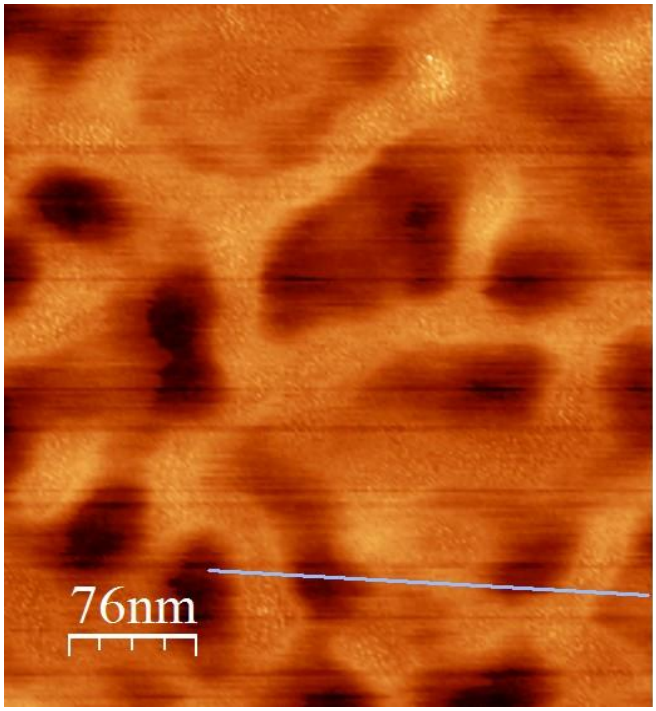
Figure 3.4: AFM image of Self-assembled S-layer proteins on mica surface isolated from C. glutamicum using SDS with cutinase immobilized on the self-assembled S-layers. (a) A height scan of the S-layer-cutinase complex. (b) A height profile of the line marked on (a). (c) A 3D reconstruction of (a)

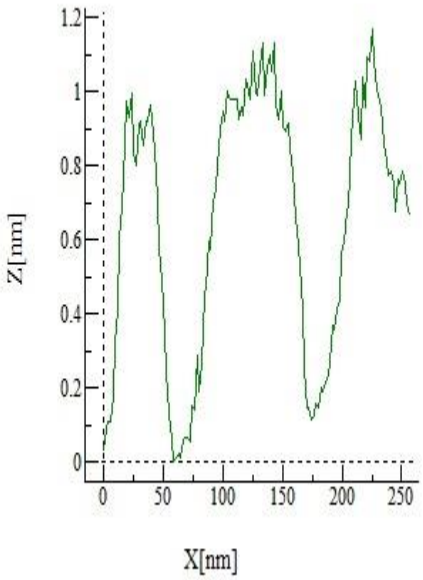
AFM images showed a decrease in the height of the self-assembled S-layer lattice after the immobilization of cutinase to the self-assembled S-layer proteins on mica surface. The maximum estimated height after cutinase immobilization was found to be approximately 1.6 nm.



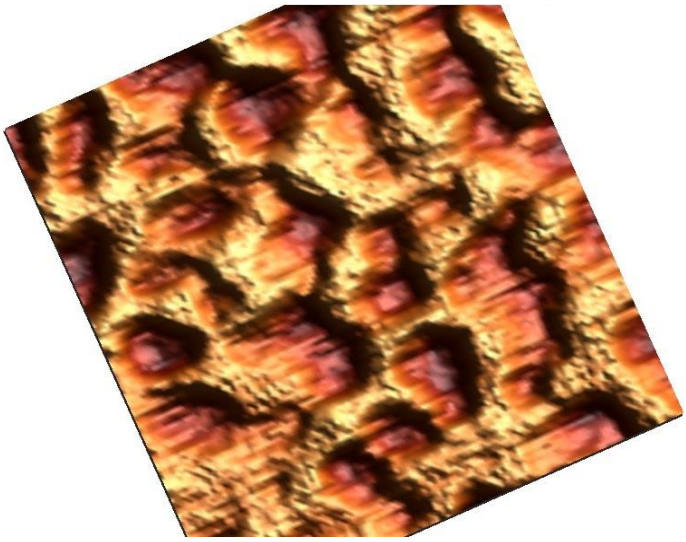


(a) (b)





(c) (d)



(e)

Figure 3.5: AFM image of Self-assembled S-layer proteins on mica surface isolated from C. glutamicum using guanidine hydrochloride with cutinase immobilized on the self-assembled S-layers. (a) A height scan of the S-layer-cutinase complex. (b) A height profile of the line marked on (a). (c) Height scan of (a) at higher resolution (d) Height profile of the line marked on (c). (e) A 3D reconstruction of (c)

Figure 3.6 is a graph of absorbance versus time for cutinase (T45P variant) immobilized on self-assembled S-layer proteins and pure T45P cutinase variant with p-nitrophenyl butyrate as substrate.

From the graph there is an almost linear increase in the absorbance with increasing time of reaction between the pure cutinase and the substrate. An almost similar linear increase is observed with the cutinase immobilized on the self-assembled S-layers reacting with the substrate. However, the pure cutinase sample attains its peak absorbance faster than the cutinase immobilized on the S-layers. The absorbance of the S-layer sample only remains almost constant.

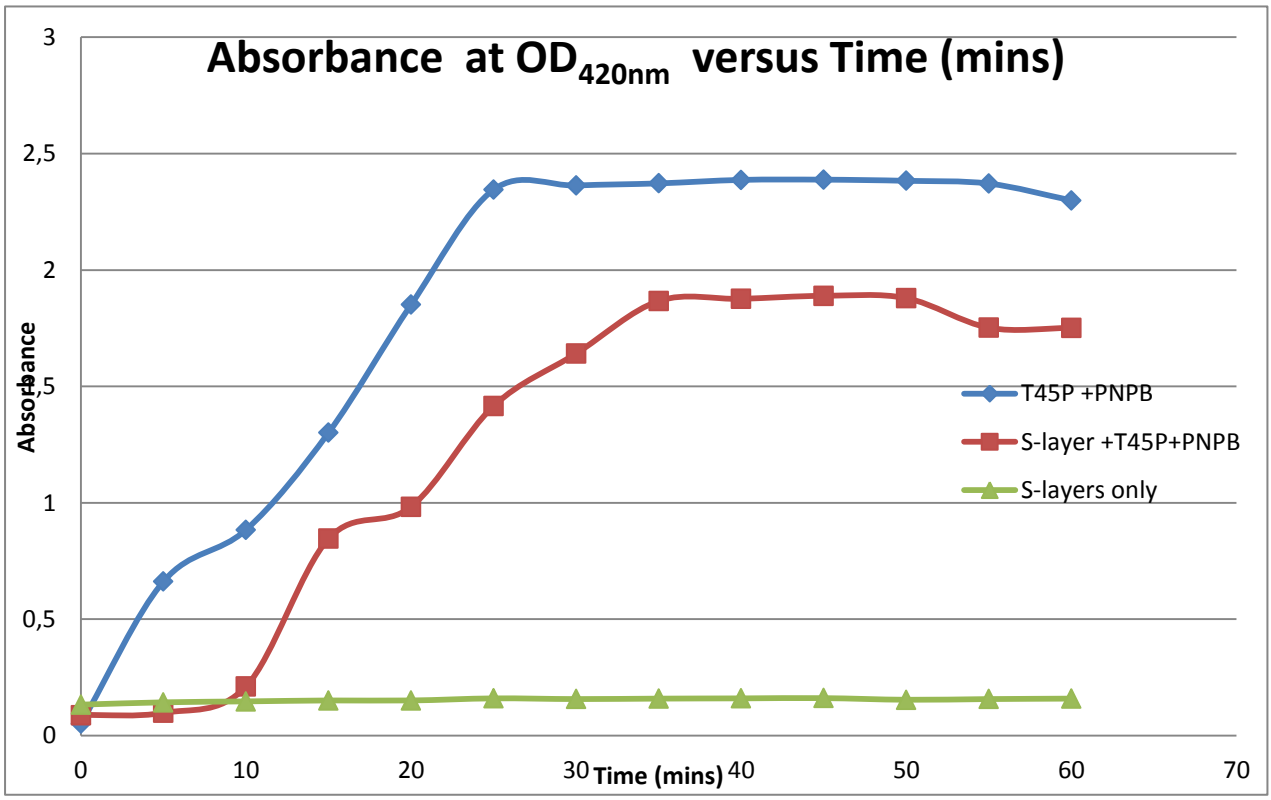


Figure 3.6: Plot of absorbance measured at 420nm against time (in minutes) for cutinase (T45P variant) immobilized on self-assembled S-layer proteins and pure T45P cutinase variant with p-nitrophenyl butyrate as substrate.

The activity of the pure cutinase and the cutinase immobilized on the S-layer proteins can be estimated from the absorbance-time graph based on the assumption that the cutinase activity is proportional to the absorbance per time. The gradient or slope of the most linear portion of the graph gives an approximation of the cutinase activity because the absorbance per time is proportional to the rate of cutinase activity in moles of the p-nitrophenol butyrate used.

To calculate an approximate value for the pure cutinase activity, the slope of the best straight line on the graph was determined preferably between the absorbance of 0,5nm to 1,5nm so as to have a high enough signal (good signal to noise ratio) because this region is still in the linear range of the spectrophotometer detector.

Slope of graph for pure cutinase at points (15, 1.301) and (10, 0.884) is 0.0834

Slope of graph for cutinase immobilized on S-layer at points (35, 1.867) and (25, 1.416) is 0.0451

Percentage of retained cutinase activity= (0.0451/0.0834)*100 = 54.07%

The figures calculated above are values of absorbance per time and are the values for the enzyme activity in disguise because absorbance per time is proportional to the rate of enzyme activity in moles of substrate used. To convert the absorbance measurements to product concentration we use Beer-Lambert law;

Absorbance per time = Absorptivity *concentration per time * Path length.

The concentration per time can then be determined from the absorptivity, path length and the absorbance per time. The concentration per time is the rate of the enzymatic reaction. The specific activity can be derived by dividing the total activity (rate of the enzymatic reaction) by the amount of enzyme used.

Part IV

Discussion

CHAPTER
FOUR

DISCUSSION

C. glutamicum was grown in M1 medium at 30^{o}C and at a stirring speed of 250rpm. This provided optimal requirements for the growth of the bacteria and the formation of crystalline surface proteins on the bacterial cell wall.

The isolation of S-layers using guanidine hydrochloride involved the use of 0.75% Triton X-100. Triton X-100 enabled the removal of contaminating plasma membrane fragments from the crude cell wall preparations. The ultrasonication step was performed at intervals and on ice in order to avoid autocatalytic processes and to maintain a stable temperature because during the ultrasonication energy is dissipated. Dialyzing after the extraction of the S-layers was done mainly to get rid of the disrupting agent used for isolation in order to enable the S-layers to self-assemble into regular lattices resembling those seen on the intact cells [59]. S-layers will only assemble into regular lattice if the disrupting agent used in their isolation is removed (for example; by dialysis).

The isolation with SDS is faster and easier and also results in more pure S-layer proteins than the isolation protocol using guanidine hydrochloride. However, it has a drawback being the difficulty to remove the disrupting agent (SDS) by dialysis in order to enable the isolated S-layers to self-assemble into regular lattices. The isolation protocol involving guanidine hydrochloride is more tedious and time consuming and also results in the isolation of other proteins because the experimental treatment of the cells with guanidine hydrochloride resulted in the solubilization of the cytoplasmic membrane or in cell lysis [53]. It however results in S-layers that have the intrinsic property to self-assemble into regular lattices more easily once the disrupting agent (guanidine hydrochloride) is removed by dialyzing against water.

The SDS-PAGE and AFM results indicated the presence and successful isolation of S-layers from the *C. glutamicum* cell membrane. SDS-PAGE results showed a band for the expected weight of 66kDa for *C. glutamicum* S-layer proteins found in literature [25], giving indication that S-layer proteins were isolated. Several other bands were seen on the SDS-PAGE results for the S-layers isolated with guanidine hydrochloride indicating a relative lower purity compared to the S-layers isolated with SDS. The SDS-PAGE results revealed that the isolation with SDS yielded a more pure S-layer protein sample than the isolation with

guanidine hydrochloride because the experimental treatment of the cells with SDS did not result in the solubilization of the cytoplasmic membrane or in cell lysis [53]. Treatment of the cell wall with guanidine hydrochloride being a chaotropic agent leads to solubilization of the cytoplasmic membrane. This implies that the protocol using guanidine hydrochloride resulted not only in isolation of S-layer proteins but also the isolation of membrane proteins and even lipids. The S-layers proteins were contaminated by membrane proteins and possibly lipids.

The AFM images showed a pattern of not well ordered (not very regular) S-layer lattices on the mica surface. The absence of a well ordered lattice could be because the isolated S-layer proteins failed to self- assemble properly. This could probably be because the disrupting agent used for the isolation (SDS or guanidine hydrochloride) was not completely removed from the S-layer solution. S-layer proteins will only self-assemble to regular lattices on surfaces if the disrupting agent used in their isolation is removed [12,24], for example, by dialysis. However, dialyzing results in an increase in volume and a more dilute S-layer protein sample. The diluted S-layer protein sample may not self-assemble properly unless it is slowly concentrated again possibly by centrifugal filtration. This can cause the S-layers to self-assemble into double or even multi layers rather than assembling in a monolayer. When double layers are formed during the self-assembly process, the two constituent monolayers face each other with either their inner or outer side. When double layers are formed, they can only by stabilized by divalent cations interacting with polar groups [17].

Previous studies on the self-assembly process revealed that the initial phase is determined by rapid nucleation of the subunits into oligomeric precursors made up of several unit cells. This is followed by the reassemble of the aggregates into larger crystalline arrays [16].

The structural form and size of the self-assembly products also depends on environmental parameters like pH value, temperature, ion composition and/or ionic strength and on the particular S-layer protein species.

High AFM image resolutions of the S-layer proteins isolated from *C. glutamicum* showed a 6-fold symmetry that corresponds to the p6 symmetry found in other reports [25, 60]. The height of the lattice was found to be between 4 to 6nm. This also corresponds to previous findings were the height of S-layers from *C. glutamicum* was found to be 4.6nm [25, 60]. Properties of the self-assembled S-layer proteins are determined by the amino acid sequence of the polypeptide chains and consequently their tertiary structure [14].

After the immobilization of cutinase to the self-assembled S-layer proteins, the height of the S-layer proteins was found to decease to about 1.6nm probably because the cutinase was entrapped within the pores of the S-layers.

In the immobilization of cutinase to the self-assembled S-layers, the carboxyl groups were activated with EDC. The activated carboxyl groups then reacted with the free amino groups from cutinase resulting in the formation of stable peptide bonds between the S-layer matrix and the cutinase [35]. No portion of the chemical structure of EDC results in part of the final bond formed between the S-layer and the cutinase. For this reason EDC is considered as a zero-length carboxyl-to amine crosslinker [41].

NHS was used in combination with EDC to improve the efficiency of the crosslinking through the formation of a dry stable (amine-reactive) intermediate. NHS coupled with the EDC and formed an NHS ester which was more stable than the O-acylisourea intermediate and led to an efficient conjugation to the primary amines of the cutinase.

The change in colour to yellow following the addition of p-nitrophenyl butyrate to the cutinase coupled to S-layer protein lattice on the mica surface was indication of the successful conjugation of the cutinase to the self-assembled S-layer proteins on the mica surface. The cutinase immobilized on the S-layers catalyzed the hydrolysis of p-nitrophenol butyrate that was added into butyric acid and p-nitrophenol. Protonation of the p-nitrophenol in the presence of a base led to a colour change from clear solution to yellow as a result of the formation of a p-nitrophenoxide anion. The absence of a colour change after addition of p-nitrophenyl butyrate on the mica surface on which only S-layers was deposited was because there was no cutinase to hydrolyse the substrate to the product. No colour change was also noticed on the mica surface on which only cutinase was deposited because the cutinase could not self-assemble on the mica surface and was washed off when rinsed with milli Q water.

The absorbance –time graph for the cutinase activity correlates to the characteristic graph for enzyme action. Upon the addition of the substrate (p-NPB), cutinase immediately starts to hydrolyze the p-NPB. Initially the absorbance was low and increased almost steadily. Once the substrate was introduced the enzyme started catalyzing the conversion of the substrate to the product. The substrate concentration was decreasing as the product concentration was increasing and the increase in product formation was reflected in the increase in absorbance. The increase in absorbance stopped at a certain point when the substrate was completely used up. At that point there was no longer substrate for the cutinase to hydrolyse and the product was in its maximum concentration as reflected by the high absorbance (point of highest colour intensity).

The pure T45P cutinase variant took a lesser time to completely hydrolyse the p-NPB substrate than the T45P cutinase variant covalently coupled to S-layer proteins. Also the amount of p-nitrophenoxide anion formed as product was higher with the pure cutinase than with the cutinase immobilized on the S-layer proteins as seen on the graph. These differences were also seen in the estimated enzyme activity values in terms of absorbance per time for the pure cutinase and the cutinase immobilized on the self-assembled S-layer proteins. The estimated cutinase activity in terms of absorbance per time for the pure cutinase catalyzed reaction was found to be 0.0834 and that catalyzed by cutinase coupled to self-assembled S-layers was estimated to be 0.0451. This implies that the cutinase coupled to self-assembled S-layer proteins retained about 54.07% of its original activity. It has been reported from previous experiments that immobilized enzymes usually retain 16 t0 60% of their initial activity [29].

The major advantage for chemically coupling enzymes is that the enzyme is not released into solution upon use because the bond formed between the enzyme and the matrix is very stable. It is however difficult to exempt the amino acid residues essential for the catalytic activity from the covalent linkage. The close to 50% loss in activity of the cutinase immobilized on S-

layer proteins can therefore be accounted for by maybe the involvement of some amino acid residues essential for cutinase catalytic activity in the covalent bond between the self-assembled S-layers and the cutinase. The activity yield can be improved by performing the reaction in the presence of analogues substrates [34]. Covalent coupling is often used when there is strict requirement for the absence of the enzyme in the product. Once the enzymatic activity decays, the matrix is discarded together with the enzyme.

The reduction in enzymatic yield for cutinase covalently coupled to self-assembled S-layers could also be caused by mass transfer effects including diminished availability of the cutinase molecules within the pores of the S-layers and also decrease in the mobility of the substrate molecules. To improve the activity preservation, flexible sparcers like 6-aminocapronic acid could be use [29]. Immobilization through sparcer molecules is of particular advantage to enzymes that are small enough (including cutinase with a size of about $45\times30\times30 Å^3$ and a molecular weight of about 22kDa [42]) for being entrapped inside the pores or for penetrating the pore openings to some extent. For example, previous experiments showed that β-glucosidase with molecular mass of 66kDa retained only 16% of its activity after direct coupling to EDC-activated carboxylic acid groups of S-layer proteins [29], but a 10 fold increase in activity to 160% was achieved when the enzyme was immobilized through sparcers.

CHAPTER
FIVE

CONCLUSION

From the experiments and assays carried out the follow conclusions were obtained

The rod shaped, gram positive bacteria; *C. glutamicum* grown in M1 medium has its outermost envelope covered with S-layer proteins.

The S-layer proteins on the outermost envelope of *C. glutamicum* can be isolated using high molar concentrations of chaotropic agents like guanidine hydrochloride and detergents like SDS with the isolation using SDS giving a more pure S-layer protein sample compared to isolation with guanidine hydrochloride.

Characterization of the isolated S-layer proteins by SDS-PAGE shows that S-layer proteins are successfully isolated and have a molecular weight of about 66kDa.

Further characterization of S-layer proteins on mica surface by AFM indicate that the S-layer proteins self-assemble on mica surface and form regular lattices with heights of about 4 to 6nm and a p6 symmetry.

A spectrophotometric assay with p-nitrophenyl butyrate as substrate and AFM shows that cutinase was covalently immobilized on the self-assembled S-layer proteins on the mica surface.

The cutinase immobilized on the self-assembled S-layer protein matrix retained about 54.07% of its original activity.

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