DOCTOR OF PHILOSOPHY

# The development of genetically engineered bacterial enzymes as sensor biological recognition elements for the detection of drugs and explosives

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# The development of genetically engineered bacterial enzymes as sensor biological recognition elements for the detection of drugs and explosives

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## Abstract

Recent security and environmental concerns have resulted in a plethora of research into the development of novel sensors for explosives and drugs. The high specificity of enzymes which interact with one or a range of specific substrates and catalyse only one type of chemical reaction, plus the possibility to use such enzymes to catalyse a specific biochemical reaction outside the cell has opened up a multitude of possibilities for the use of enzymes in analytical applications.

This work demonstrates biological recognition elements for both an explosive and drug biosensor based on the isolation and characterization of bacterial enzymes namely nitroreductases, due to their ability to degrade many commonly used explosives and carboxylesterases, because they have the potential to hydrolyse cocaine into its main metabolites. The enzymes have been genetically modified to incorporate the cys-tag (6 cysteine amino acids) at the enzyme N-terminus to enable the enzyme to adhere to the biosensor electrode surface (gold surface), subsequently the effect of cys-tag insertion on enzymes molecular (gene isolation, cloning, expression, and purification) and biochemical characterization (protein concentration, specific activity, $K_m$, $V_{max}$, pH and temperature), and observe the reaction pathways of the enzymes.

All the bacterial Nitroreductases (NfnB, YdjA, Dde_0086, and Dde_2199) showed considerable activity with different nitroaromatic compounds and displayed different rates of reaction for each substrate.

The optimum pH and temperature were evaluated along with their $K_m$ and $V_{max}$ values. Additionally bacterial carboxylesterases (PnbA1 and PnbA2) were found to be active with cocaine, and hydrolysed cocaine into benzoylecgonine and methanol rather than ecgonine methyl ester and benzoic acid following human liver carboxylesterase 1(hCE-1) in its pathway to hydrolyse cocaine.

The cys-tag insertion revealed its effect through decrease enzyme specific activity, protein concentration, $K_m$ and $V_{max}$ values, optimum temperature range for enzyme stability, however it does not restrict substrate entrance into the active site and all the bacterial nitroreductases and carboxylesterases remain active, furthermore it does not affect the optimum pH for enzyme activity.

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# List of Abbreviations

| | |
|---|---|
| **NTRs** | **Nitroreductases** |
| **CEs** | **Carboxylesterases** |
| **NADH** | **Nicotinamide adenine dinucleotide** |
| **FMN** | **Flavin mononucleotide** |
| **S** | **Substrate** |
| **E** | **Enzyme** |
| **ES** | **Enzyme-substrate complex** |
| **P** | **Product** |
| $[E]_{total}$ | **Total enzyme concentration** |
| $K_m$ | **Michaelis' constant** |
| $V_{max}$ | **Maximum velocity** |
| $k_1$ | **The rate of association S with E** |
| $k_{-1}$ | **The rate of dissociation S from ES** |
| $k_2$ | **The rate of formation product from ES** |
| **v** | **Velocity** |
| **Ar** | **Aromatic ring** |
| **NACs** | **Nitroaromatic compounds** |
| **ADNT** | **Aminodinitrotoluene** |
| **DNAT** | **Dinitroaminotoluene** |
| **TAT** | **Triaminotoluene** |
| **TNT** | **2,4,6-Trinitrotoluene** |
| **NB** | **Nitrobenzene** |
| **3-NBA** | **3-Nitrobenzoate** |
| **4-NT** | **4-nitrotoluene** |
| **DNEB** | **2,4-Dinitroethylbenzene** |
| **1,2-DNB** | **1,2-Dinitrobenzene** |
| **EC_YdjA** | ***E. coli* YdjA** |
| **GDEPT** | **Gene directed enzyme prodrug therapy** |
| **ADEPT** | **Antibody directed enzyme prodrug therapy** |

| | |
|---|---|
| VDEPT | Virus directed enzyme prodrug therapy |
| 3,5-DNBTF | 3,5-dinitro-trifluoromethylbenzene |
| 3,5-DABTF | 3,5-Diamino-benzene-trifluoride |
| 3,5-DABA | 3,5-Diaminobenzoic acid |
| CPT-11 | Irrinotecan |
| SN-38 | Potent topoisomerase I poison |
| 4-pp | 4-Piperidino-piperidine |
| rCE | Rabbit carboxylesterase |
| hCE-1 | Human liver carboxylesterase -1 |
| hCE-2 | Human liver carboxylesterase -2 |
| BEC | Benzoylecgonine |
| CEh | Cholesteryl ester hydrolase |
| ACAT | Acyl-CoA:cholesterol acyl-transferase |
| oxLDL | oxidized low-density lipoprotein |
| HDL | High-density lipoprotein |
| ChE | Cholesteryl ester |
| FC | Free cholesterol |
| ER | Endoplasmic reticulum |
| CRP | C-creative protein |
| Coc | Cocaine |
| 6-MAM | 6-mono acetylmorphine |
| IPTG | Isopropyl-beta-D-thiogalactopyranoside |
| IMAC | Immobilised metal ion a ffinity chromatography |
| SDS-PAGE | Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis |
| EDTA | Ethylenediaminetetra acetic acid |
| dNTP | Deoxynucleotide-tri-phosphate |
| TBE | Tris-borate- ethylenediaminetetra acetic acid |
| ATP | Adenosine triphosphate |
| LB | Luria-Bertani broth |
| O.D. | Optical Density |

| | |
|---|---|
| **MOPS** | **3-(N-morpholino)propanesulfonic acid** |
| **IM** | **Imidazole** |
| **PB** | **Phosphate buffer** |
| **BME** | **Beta-mercaptoethanol** |
| **IMS** | **Industrial methylated spirits** |
| **DDI** | **Doubly de-ionised** |
| **APS** | **Ammonium persulfate** |
| **TEMED** | ***N-N-N`-N`*-tetramethylethylenediamine** |
| ***P*-NPA** | **para-nitrophenylacetate** |
| **BSA** | **Bovine Serum Albumin** |
| **AZTs** | **Tetranitroazoxytoluene** |
| **HADNTs** | **Hydroxylamino-dinitrotoluenes** |
| **CocE** | **Cocaine** |
| **$A^{\circ}$** | **Angstrom** |
| **kb** | **Kilobase** |
| **kDa** | **Kilo Dalton** |
| **LB** | **Luria Bertani broth** |
| **NCBI** | **National centre for biotechnology information** |
| **PCR** | **Polymerase chain reaction** |
| **pET** | **Plasmid for expression by T7 RNA polymerase** |
| **$T_m$** | **Melting temperature** |
| **UV** | **Ultra violate** |
| **Vis** | **Visible** |
| **CAPS** | ***N*-cyclohexyl-3-aminoprpanesulfonic acid** |

# Acknowledgments

I am heartily thankful to my supervisors, Professor Maher Kalaji and Dr. Chris Gwenin, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject. Without their support and optimism, the accomplishment of this thesis would not have been possible. It is a pleasure to thank Professor Peter Williams for his advice on molecular biology and for his permission to work in the laboratory in the School of Biological Sciences during the course of this study. I owe my deepest gratitude to Dr. Catherin Kay, without her support I was still far from understanding advances in molecular biology. Special thanks go to Mr. Dennis Williams; the chief technician and all other technicians at both schools of chemistry and biological sciences.

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# Chapter 1- Introduction

## 1.1 Introduction

The field of biotechnology is gaining interest world-wide, and enzyme research has become an important area of biotechnology. Enzymes can be extracted from living tissues, and purified. Under controlled conditions of isolation, they retain their original level of activity; therefore, a purified enzyme can be used to carry on a specific biochemical reaction outside the cell. Enzymes can also be modified through gene manipulations for a variety of purposes including biosensors.

The use of enzymes in biosensor development has achieved enormous attention; a biosensor is an analytical device which incorporates a biological material (*recognition element*) with a physio-chemical transducer.[1] Molecular recognition elements are central for biosensing, and as such are considered a major player in future biosensor development. Enzyme recognition element based biosensors are very attractive for biosensor application due to a variety of measurable reaction products arising from a catalytic process.

The aim of this project is the development of biosensor recognition elements *via* engineer bacterial enzymes (*nitroreductases (NTRs) and carboxylesterases (CEs)*) which allow them to bind into a transducer surface of a biosensor. The active component of the proposed biosensors is an enzyme (recognition element) which reacts with the monitored substance and triggers a catalytic reaction, and the reaction product is then monitored by the detection system.

The main part of this study concentrates on bacterial NTRs as a biosensor recognition element for explosives detection since they are attracting researcher's attention due to their critical role in nitroaromatic-compounds (explosives) metabolism. Biorecognition element as a main part of biosensor need to be genetically modified to aid them to immobilise into a transducer surface that sense and translate the biological events into a measurable signal.

In the second part of this study bacterial carboxylesterases have been chosen as a biosensor recognition element which will used for narcotic detection because of their role in narcotics metabolism. The enzymes have to be genetically modified to immobilise into a transducer which will translate the reaction product into the detection system.

## 1.2 Biosensors

Explosives utilisation in terrorist and war activities cause great concern to public security of the whole world.[2] As an important pollution, explosives also cause health problems in both animals and human.[3] Even after degradation, the by-products of the common explosives are still toxic and carcinogenic.[4,5]

Narcotic abuse has become a critical health and social problem during the last few decades. One of the most dangerous illegal drugs available today is cocaine. Therefore, it has been necessary to develop sensitive methods for monitoring narcotics and explosives. During the past two decades biosensors have proved advantageous for detecting different analytes such as explosives,[6,7] and narcotics.[8,9] Biosensors can be defined as analytical devices translating biological event into an easily processor electronic signal through join a biologically active material with a physicochemical transducer for the detection of an analyte.[10,11] They consist of three major components (Fig 1.1), namely a sensitive biological elements (*in biosensors, the most common recognition element is an enzyme; others include antibodies, nucleic acids and receptors*) that recognises the target analyte which can be created by biological engineering; the transducer element (works in a physicochemical way) that translates the signal resulting from the interaction of the analyte with the biological element into another signal; and the signal processor that is responsible for the display of the results.

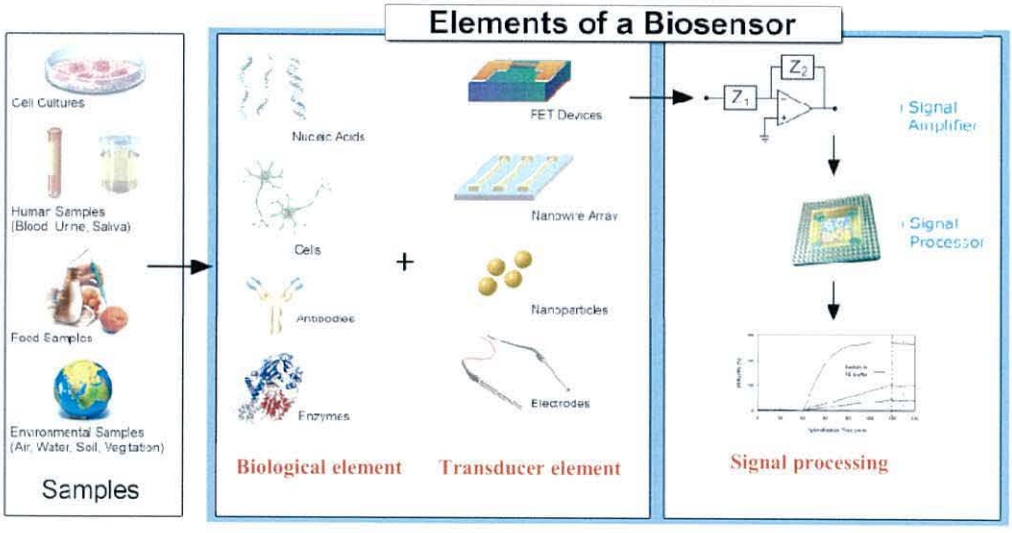



**Figure 1.1: Selected components of typical biosensors.**[12]

Electrochemical sensing usually needs three different electrodes; a reference electrode, a counter (auxiliary electrode) and a working electrode, also known as the sensing or redox electrode. The reference electrode, commonly made from Ag/AgCl, is kept away from the reaction site to keep a known and stable potential. The working electrode operates as the transduction element in the biochemical reaction, while the counter electrode creates a connection to the electrolytic solution so that a current can be applied to the working electrode. These electrodes should be both conductive and chemically stable. Therefore, the most commonly used compounds as electrodes are silicon, gold, carbon (graphite), and platinum.

The general performance of the biosensor primarily depends on the immobilisation of biomolecules in a suitable matrix and diffusional limitation of the analyte within this matrix and to a lesser extent on the instrumentation system used to acquire the signal generated by biochemical reaction at the biosensing membrane.[13,14,15]

## 1.3 Enzyme immobilisation

Immobilisation is the attachment of an enzyme to a solid matrix so that it cannot escape but can still react freely with its substrate. The rationale behind immobilisation is the easy separation of product from the biocatalyst. Enzymes may be immobilised by adsorption, covalent binding, entrapment, and membrane confinement, each method having its pros and cons.

The main advantages of immobilised enzyme are; easy separation from the reaction mixture, providing the ability to control reaction times and minimize the enzymes lost in the product, re-use of enzymes for many reaction cycles, and lowering the total cost Immobilisation of enzymes may have a considerable effect on their kinetics. This may be due to structural changes to the enzyme and the creation of a distinct microenvironment around it. The activity of an immobilised enzyme is governed by the physical conditions within this microenvironment not those prevalent in the bulk phase. The immobilisation matrix affects the partition of material between the product phase and the enzyme phase and imposes restriction on the rate of diffusion of material. Some effects of enzyme immobilisation are seen to be beneficial whilst others are detrimental with the economics of their use.

In order to develop a viable biosensor, the biological component has to be properly integrated to the transducer. There are four main methods for enzyme immobilisation:

### 1.3.1. Adsorption

It's probably the simplest way of preparing immobilised enzymes and can achieve high loadings on a suitable matrix which include ion-exchange matrices, porous carbon, clays, hydrous metal oxides, glasses polymeric resins. Simply, the procedure involves mixing a concentrated solution of enzyme with a suitable adsorbent, under appropriate conditions of ionic strength and pH, after a sufficient incubation period, by washing off loosely bound and unbound enzyme the immobilised enzyme will be produced in a directly usable form. Hydrophobic interactions, ionic bonds, hydrogen bonds, and *van der Waals* forces usually facilitate this binding. These weak interactions allow easy enzyme desorption from the matrix through changes in ionic strength, pH, or temperature.[16,17,18]

### 1.3.2 Covalent binding

This method of immobilisation involves covalent bond formation between the enzyme and the support material. It is an extensively researched technique and the most irreversible immobilisation method, and therefore potentially the most stable. The strength of binding is very strong and very little leakage of enzyme from the support occurs. The bond is normally formed between functional groups of the support and functional groups of the amino acid residues on the surface of the enzyme. A number of amino acid functional groups are suitable for participation in covalent bond formation such as amino group ($NH_2$) of arginine or lysine, the carboxyl group (COOH) of glutamic acid or aspartic acid, the hydroxyl group (OH) of threonine or serine, and the sulfydryl group (SH) of cysteine.[19,20,21]

### 1.3.3 Entrapment

Immobilisation by entrapment differs from adsorption and covalent binding in that the biomaterial is mixed with a monomer solution, which is then polymerized to a gel, thus trapping the enzymes in the pores of gels or fibres. Numerous matrices have been employed but the most favoured have been alginate, cross linked with linear chains of $Ca^{2+}$ ions, gelatine, and polyacrylamide. Unfortunately, this process suffers from three

major drawbacks; large diffusional barriers to the transport of substrate or product resulting in reaction retardation and long response times, continuous loss of enzyme activity since these materials generally do not have a narrow pore size distribution, and shrinkage or swelling of the polymer depending upon the ionic strength of the environment.[22,23,24]

### 1.3.4 Membrane confinement

Membrane confinement of enzymes may be achieved by a number of different methods, all of which depend for their utility on the semi-permeable nature of the membrane. It is possible to restrict the enzyme in a semi-permeable membrane which allows free passage of low molecular weight substrates and products but keeps the high molecular weight enzyme. The simplest of these methods is achieved by placing the enzyme on one side of the semi-permeable membrane whilst the reactant and product stream is present on the other side. Membrane confinement is a flexible method but expensive to set up.[25]

## 1.4 Enzymes

Enzymes are proteins that catalyze, or speed up, a chemical reaction and are not consumed during the reaction. In the 19$^{th}$ century, enzymes were first characterized as the agents that drive chemical reactions in cells.[26] They contain a special pocket or cleft called the active site which contains amino acid side chains that create a three-dimensional surface complementary to the substrate. Some enzymes require no chemical groups other than their amino acid residues for activity, others require an additional chemical component called cofactor which may be either one or more inorganic ions, such as $Fe^{2+}$, $Mg^{2+}$, $Mn^{2+}$, or $Zn^{2+}$, or complex organic molecules called coenzymes, that are derivatives of vitamins such as nicotinamide adenine dinucleotide ($NAD^{+}$), a coenzyme or metal ion that is covalently bound to the enzyme is called prosthetic group.[27,28]

In many enzymes, particularly those used in biosensors, the mode of action involves oxidation or reduction, which can be detected electrochemically. The detailed mode of action of enzymes can be described as follows:

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E+P \qquad \text{Equation 1.1}$$

Where **S** is the substrate, **E** is the enzyme, **ES** is the enzyme-substrate complex, and **P** is the product. The rate constant $k_1$ and $k_{-1}$ in reaction (1.1) govern the rates of association S with E and dissociation of S from ES, respectively. The rate constant for the second step is $k_2$, the rate of formation of product from ES. The formation and dissociation of ES complexes are usually very rapid reactions because only noncovalent bonds are formed and broken. In contrast, the conversion of substrate to product is usually rate-limiting. It is during this step that the substrate is chemically altered.

The steady-state approximation of the kinetic theory can be applied to the reaction system shown in equation (1.2). This approximation simply assumes that, during most of the time of the reaction the concentration of the ES complex is steady, i.e. constant; therefore the rate of formation of the complex from its components is balanced by the rate of its breakdown back to enzyme and forward to its products. Thus:

$$\mathbf{k_1\,[S][E] - k_{-1}\,[ES] - k_2[ES] = 0} \qquad \text{Equation 1.2}$$

Where:

$\mathbf{k_1\,[S][E] - k_{-1}\,[ES]}$ is the rate of formation of complex

and $\mathbf{k_2[ES]}$ is the rate of breakdown of complex

These rates are equal and opposite because of the steady-state approximation.

The enzyme concentration can be described in terms of the total $\mathbf{[E]_{total}}$, rather than the unknown, **[E],** so that $\mathbf{[E]_{total} = [E] + [ES]}$. Therefore equation 1.2 can be represented as:

$$\mathbf{k_1[S][E]_{total} - k_1[S][ES] - k_{-1}[ES] - k_2[ES] = 0} \qquad \text{Equation 1.3}$$

To calculate the concentration of the bound enzyme [ES], equation (1.3) can be re-written as:

$$\mathbf{[ES] = \frac{[E]_{total}\,[S]}{K_m + [S]}} \qquad \text{Equation 1.4}$$

$\mathbf{K_m = (k_{-1} + k_2)/k_1}$, where $\mathbf{K_m}$ is the Michaelis' constant.

When the concentration of S is very high, the enzyme is saturated, and essentially all the molecules of E are represent as ES. Under this condition, the velocity is at its maximum rate ($V_{max}$) and this velocity is determined by the total enzyme concentration and the rate constant $k_2$. Thus

$$\mathbf{V_{max} = k_2\,[E]_{total}}$$

Then, the overall rate of the reaction (rate of formation of products) is given by the Michaelis'-Menton equation, as follows:

$$\mathbf{v = \frac{d[P]}{dt} = \frac{-d[S]}{dt} = k_2\,[ES] = \frac{k_2\,[E]_{total}\,[S]}{K_m + [S]}} \qquad \text{Equation 1.5}$$

When [ S ] >> $K_m$, a maximum value of the rate constant, $\mathbf{V_{max}}$, is reached, so that

$\mathbf{V_{max} = k_2[E]_{total}}$, and when $\mathbf{[S] = K_m}$ , $\mathbf{v = V_{max}/2}$.

It is experimentally more convenient to plot the data in a straight-line form by inverting the Michaelis-Menton equation:[29]

$$\mathbf{1/v = \frac{K_m + [S]}{k_2[E]_{total}[S]} = \frac{K_m}{k_2[E_0][S]} + \frac{1}{V_{max}}} \qquad \text{Equation 1.6}$$

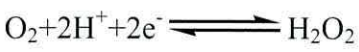
An important feature of enzymes is that they show highly specific interaction with one or few specific substrates and catalyse only one type of chemical reaction thus resulting in frequent use of enzymes in analytical applications (sensors). A typical application of a glucose sensor which was first described by Clark and Lyons in 1962,[30] is the determination of glucose concentrations by the use of an immobilised glucose oxidase membrane.

The glucose biosensor is the most successful amperometric-based chemical sensor and has been developed to a commercially available portable unit. A number of approaches to glucose sensing using amperometry has been reported but most are based on the glucose oxidase (GOX) catalysed oxidation of glucose to gluconic acid.

The basic reaction is the oxidation of an aldehyde group to a carboxylic acid group with the production of two electrons.

Equation 1.7

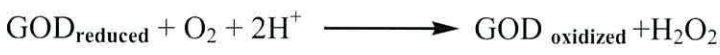
The electron acceptor in this reaction is normally molecular oxygen.



Equation 1.8

The overall reaction can be written as follows.

Equation 1.9



Equation 1.10

There are two ways of making use of this reaction for the quantitative determination of glucose. Firstly, the amount of hydrogen peroxide produced can be determined by anodic oxidation, and measuring the amount of current flowing which is related to the amount of glucose in the original sample. This has been done by chemically attaching the enzyme to the surface of a nylon mesh which is stretched over the surface of a platinum electrode which acts as the anode. The sensor is then placed in a small volume cell along with a Ag/AgCl reference electrode and an auxiliary electrode. Three electrodes are common in voltammetry, the current flows between the platinum and auxiliary electrodes, and the reference electrode monitors the potential.

A second way to determine glucose makes use of the oxygen probe to determine the decrease in the amount of oxygen in the sample solution after the oxidation of the glucose. This can be carried out by spreading a thin layer of glucose oxidase on the surface of the gas permeable membrane of the probe. The amount of dissolved oxygen remaining in the sample solution which diffuses across the membrane is determined, and is related to the concentration of glucose.

This study will focus on the use of genetically modified bacterial carboxylesterases and nitroreductases for use in the detection of narcotics and explosives.

## 1.5 Bacterial nitroreductases

Nitroreductases are NAD(P)H dependent flavoenzymes that mediate the reduction of a broad range of nitro-compounds. They were first identified in bacteria able to reduce chloramphenicol and nitrobenzoic acids.[31,32,33] Almost all bacterial nitroreductases occur as a homodimer (20-30 kDa subunits), contain FMN as a cofactor (Fig. 1.2a), and use NAD(P)H as electron donor (Fig. 1.2b) and catalyze the reduction of nitro-compounds using a ping-pong bi-bi mechanism. Nitroreductase genes are widely found within bacterial genome, but are also found in archaea and eukaryotic organisms.[34]

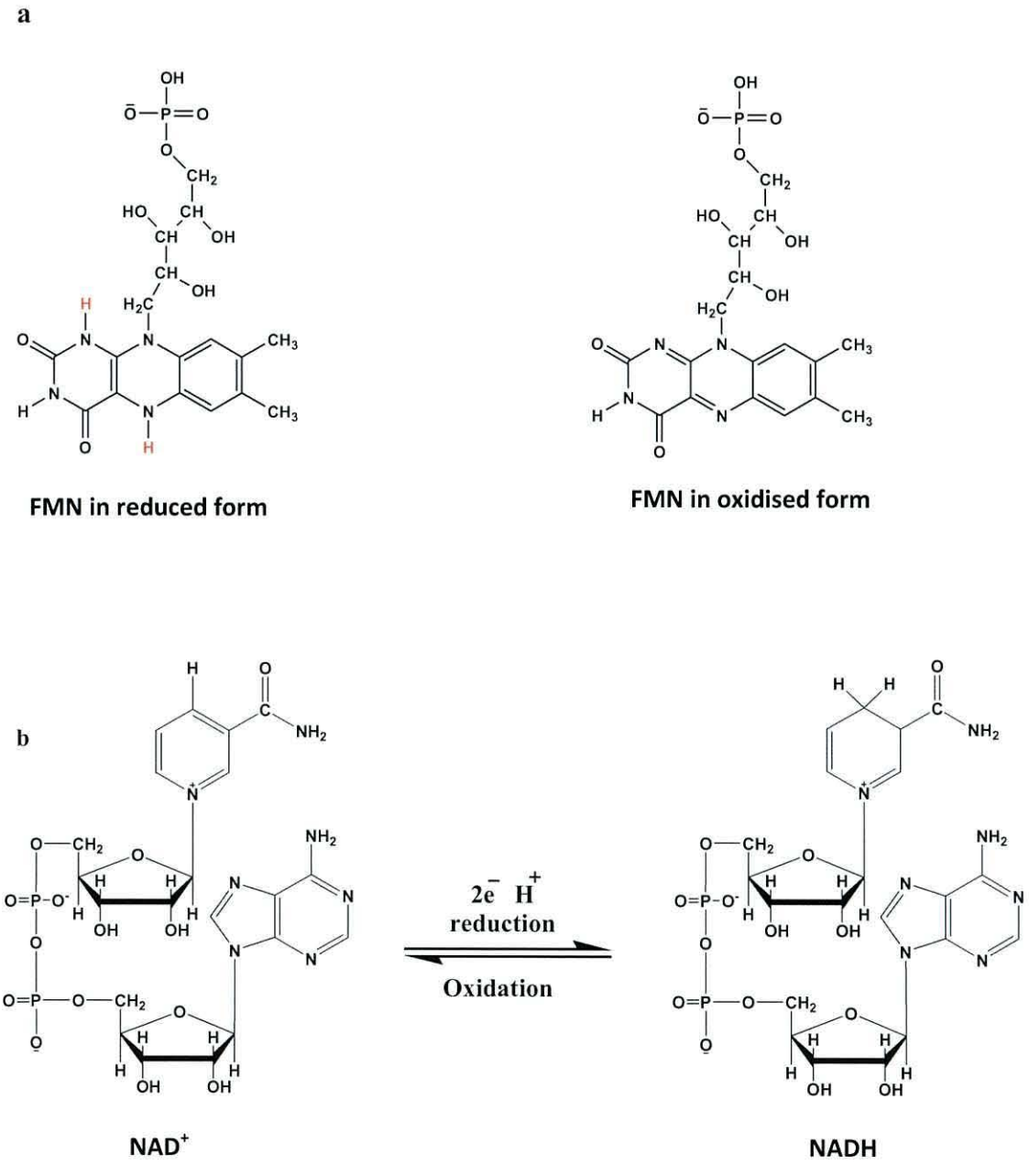



**Figure 1.2: Illustrating the reduction and oxidation of (a) FMN and (b) NADH.**

Nitroreductases have been classified into two main categories according to their response to oxygen (Fig.1.3): oxygen insensitive nitroreductases (type I) which catalyze the reduction of nitro-groups by transfer of two electrons from NAD(P)H to produce nitroso, hydroxylamino and amino derivatives,[35,36] while oxygen sensitive nitroreductases (type II) catalyze the reduction of nitro-groups through the addition of one electron to produce a nitro anion radical, which in the presence of oxygen can be reoxidised to the parent compounds with the concomitant formation of the superoxide anion in a futile cycle. [35,37]

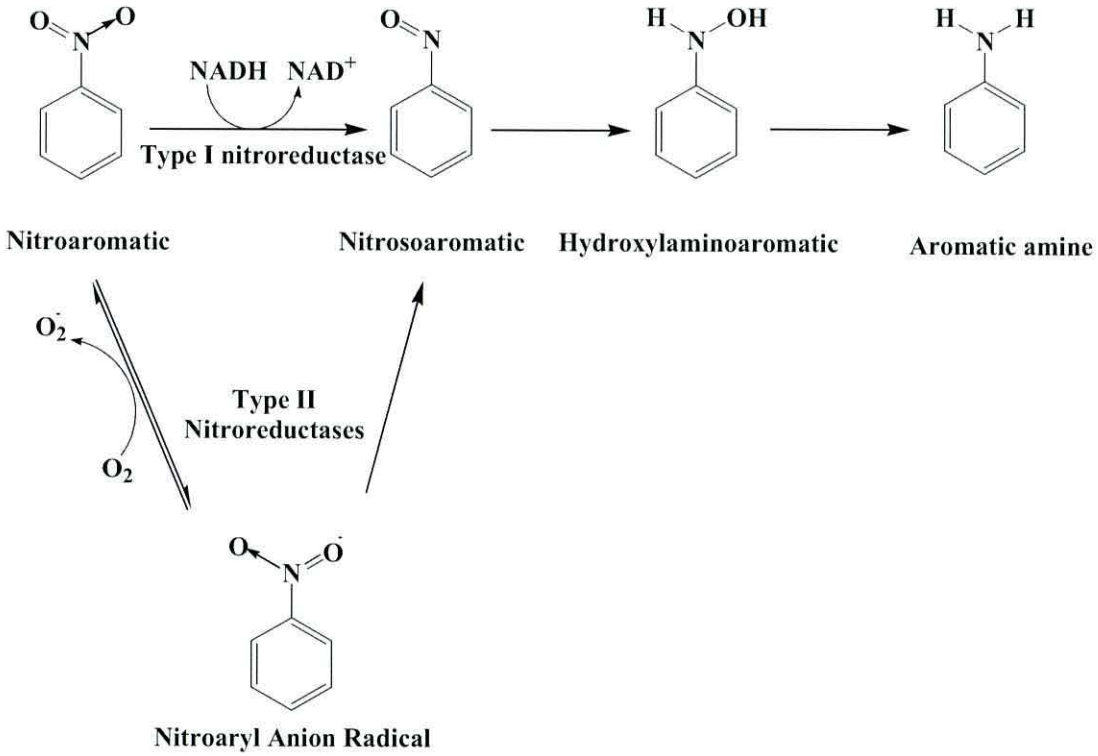



**Figure 1.3: Summary of Reductive Metabolism of Nitroaromatics Catalyzed by Nitroreductases.**

Enzymes with oxygen insensitive nitroreductase activity fall into two main groups: major oxygen-insensitive nitroreductases (group A) are usually NADPH-dependent which includes NfsA of *Escherichia coli,*[38] FRP (NADPH-flavin oxidoreductase) of *Vibrio harveyi,*[39] SnrA of *Salmonella typhimurium,*[40] and PnrA of *Pseudomonas putida JLR11.*[41] The minor oxygen-insensitive nitroreductases (group B) which can use both NADH and NADPH as electron donors, includes two subgroups: the B1 with the NfnB of *Escherichia coli,*[42] NR and RNR proteins of *Enterobacter cloacae,*[43,44] Cnr of *Salmonella typhimurium,*[45] PnrB nitroreductase of *Pseudomonas putida JLR11,*[41] NADPH-flavin oxidoreductase FRaseI of *Vibrio fischeri,*[46] DrgA

(quinine reductase) of *Synechocystis*,[47,48] and NPrA (major nitroreductase) of *Rhodobacter capsulatus*.[49] The YdjA proteins (putative nitroreductases) of *Escherichia coli*, and *Salmonella enterica*, and the NprB (minor nitroreductase) of *Rhodobacter capsulatus* [49] are included in the subgroup B2.

Recently bacterial nitroreductases have generated enormous interest due to their critical involvement in mediating nitroaromatic toxicity,[50,51,52] their potential use in nitroaromatic bioremediation,[53] and their use as a prodrug activator in directed anticancer therapies.[54,55]

### 1.5.1 Nitro-compounds

A wide variety of nitro-compounds are introduced into the environment as a consequence of their application in different aspects (Fig. 1.4). Some compounds are used as explosives, pesticides, herbicides, plastic, dyes, pharmaceuticals, and petroleum products. Others are produced as by-products of combustion processes.[56,57]

Natural nitro-compounds (Fig.1.4 top) are biodegradable by microorganisms and can serve as a source of carbon and energy. In contrast, xenobiotic compounds (Fig. 1.4 bottom) are recalcitrant and their biodegradability is limited because they contain structures or substituents that are not normally present. As revealed by different studies these compounds and their conversion products have toxic, mutagenic, and carcinogenic effect on various organisms ranging from bacteria to mammals,[58,59,60] and they are identified as a major environmental pollutants, therefore their removal is of significant interest.

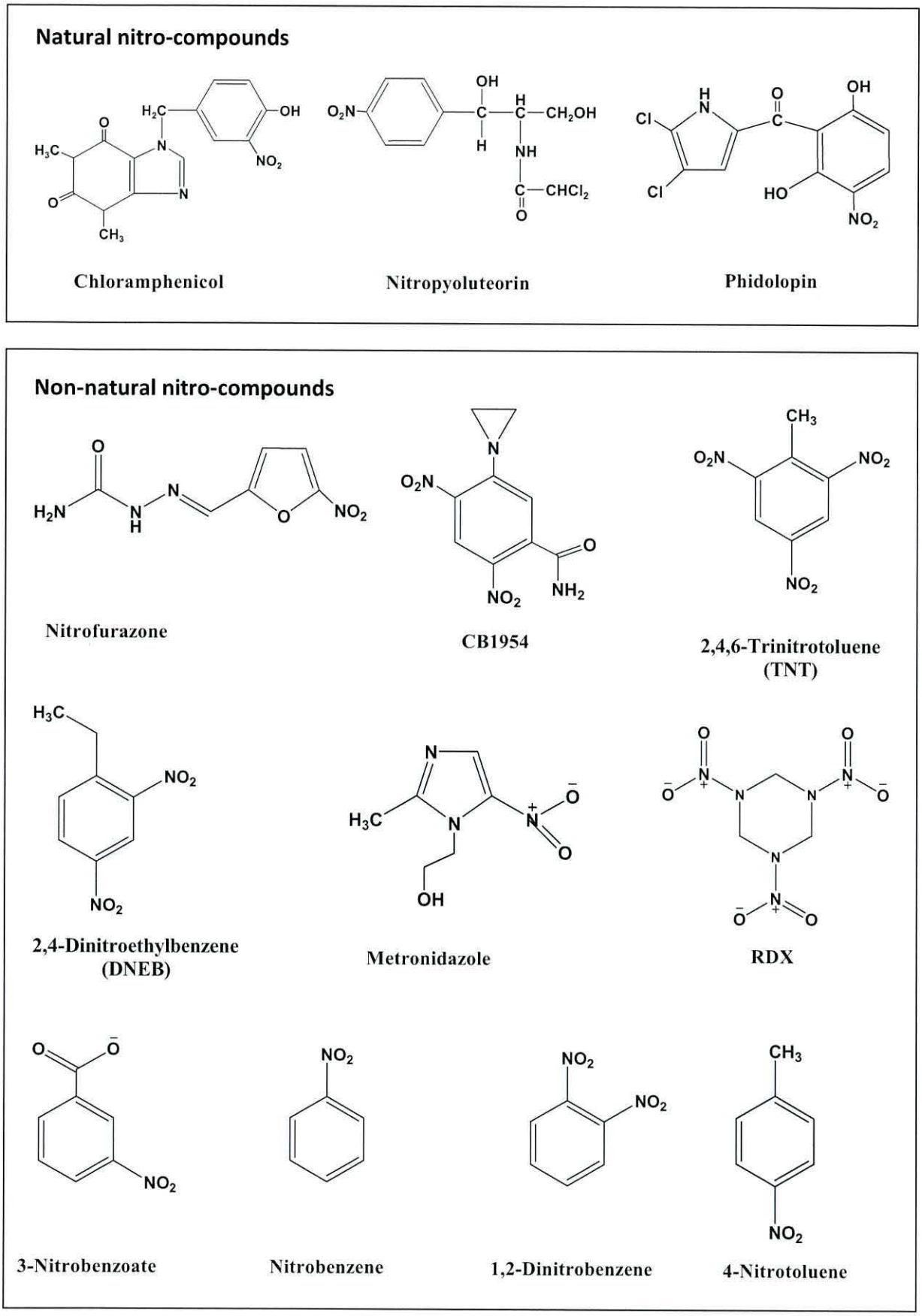



**Figure 1.4: Chemical structures of nitro-compounds including natural (top) and nonnatural or xenobiotic compounds (bottom).**

### 1.5.2 Bacterial degradation of nitro-compounds

Microorganisms have found the way to degrade nitro-compounds, to produce intermediates of central metabolic pathways. The oxidative attack of nitro-compounds (Fig.1.5) catalyzed by mono- and di-oxygenases under aerobic conditions, the hydroxyl derivatives generation through the aromatic ring oxidation followed by aromatic ring opening, is the expected pathway for aerobic degradation for mononitroaromatic compounds and some dinitroaromatics[61,62,63] whereas reductive metabolism (Fig.1.5) may occur both aerobically and anaerobically, through either the reduction of nitro groups to hydroxylamino or amino groups which is catalyzed by nitroreductases, or the reduction of the aromatic ring by the addition of hydride ions through hydride transferases to form hydride-Meisenheimer complexes, which may be further metabolized with the release of nitrite.[64]

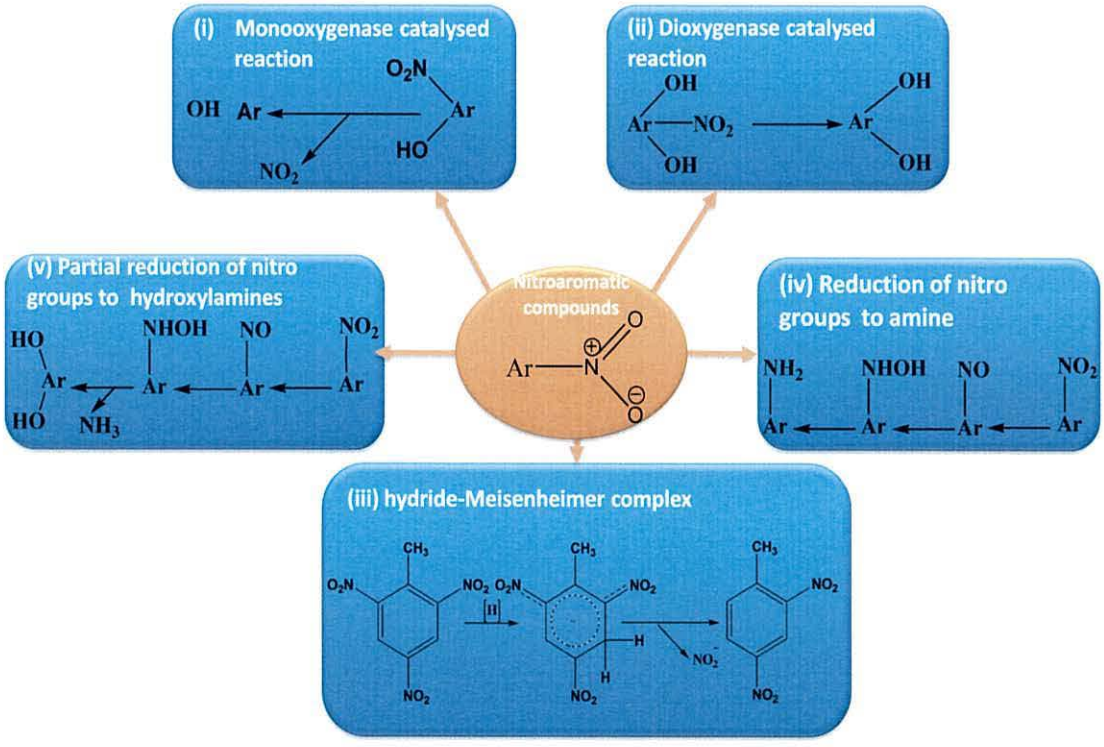



**Figure 1.5: Microbial strategies for degradation of nitro-aromatic compounds by aerobes (i, ii, iii and v ) and anaerobes (iv).[65]**

Polynitro-aromatic compounds favour reductive reactions due to the nitro groups electron withdrawing characters that causes an electron deficiency in the aromatic ring and impairs the oxidative degradation catalyzed by oxygenises. The reduction of the first nitro group is more rapid when compared with others, as the nitro

group reduction to amino group decreases the electron deficiency of the aromatic ring. Aminodinitrotoluenes (ADNT) and diaminonitrotoluenes (DANT) have been identified as the main intermediates in the reduction of TNT, and the complete TNT reduction to triaminotoluenes (TAT) identified only under anaerobic conditions due to the lower redox potential required to that found only in an anoxic environment.[66,67] The rate of nitro-aromatic reduction depends upon the number of nitro-groups and the chemical properties of the substituents in the aromatic ring.[68]

### 1.5.3 Bacterial nitroreductases crystal structure

The crystal structure studies of *Escherichia coli* NfsA, and NfsB; *Vibrio harveyi* FRP; *Enterobacter cloacae* NR; and *Vibrio fischeri* FRaseI revealed that nitroreductases are globular proteins with conserved domains for FMN binding and for the interactions with the nitroaromatic substrates and NAD(P)H electron donor.[69,70,71]

In spite of significant differences between proteins belonging to bacterial nitroreductases, they all share a tightly associated homodimeric structure with the FMN bound within a crevice formed by the dimer interface, and a characteristic $\alpha + \beta$ fold of the subunits. The central hydrophobic core consists of five-stranded $\beta$-sheets surrounded by $\alpha$-helices (Fig. 1.6). The FMN prosthetic groups interact with residues of both monomers, forming hydrophobic contacts to both subunits and hydrogen bonds to one of them. This set of interactions is well conserved in all nitroreductases and involves identical or similar residues.[72,73,74] The nicotinamide ring of NAD(P)H is located between the flavin isoalloxazine ring and the conserved Phe124 residue, as deduced from the position in the NfsB protein of the analogous ligand nicotinic acid.[69,73]

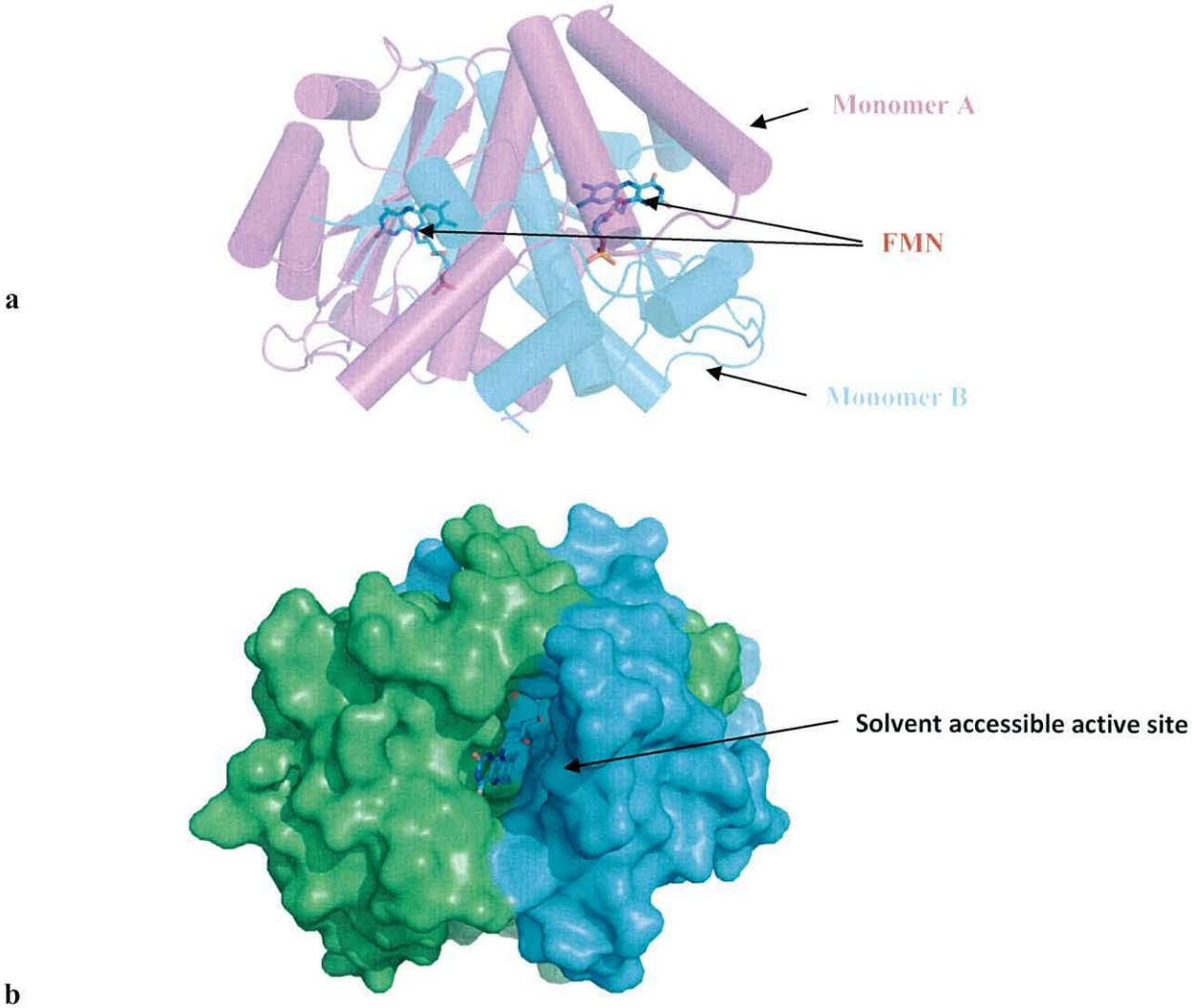



**Figure 1.6: (A) Ribbon diagram of the two monomers from the structure of *E. coli* NTR B with attached cofactor FMN, drawn with MOLSCRIPT (B) Surface diagram illustrating the solvent accessible active site represented by PYMOL Viewer.[69]**

## YdjA family protein (putative nitroreductases)

YdjA is the smallest NTRs reported to date, which is composed of less than 190 amino acids, whereas most NTRs are composed of at least 210 amino acids. [73,75,76] Although YdjA shows low sequence similarity to the other NTRs at the amino acid level, the crystal structure of *Escherichia coli* YdjA (EC_ydjA) revealed that its overall structure closely resembles those of other NTRs (Fig 1.7), specifically, the two monomers comprise an $\alpha + \beta$ fold domain and form the dimeric structure, and FMN molecules are bound at the dimeric interface.[77,78]

Comparison of the amino acid sequence of YdjA with those of other NTRs shows that about 30 amino acids are lacking in the middle of YdjA sequence, which comprises two helices in the other NTRs and restricts the cofactor and substrate binding and they are inserted between β2-strands and α5-helix in the EC_ydjA. The absence of the 30 internal amino acids in Ec_ydjA creates a wider and more flexible active site and this is considered as a fundamental difference which might distinguish the YdjA NTR family from other NTR family members.[75,76]

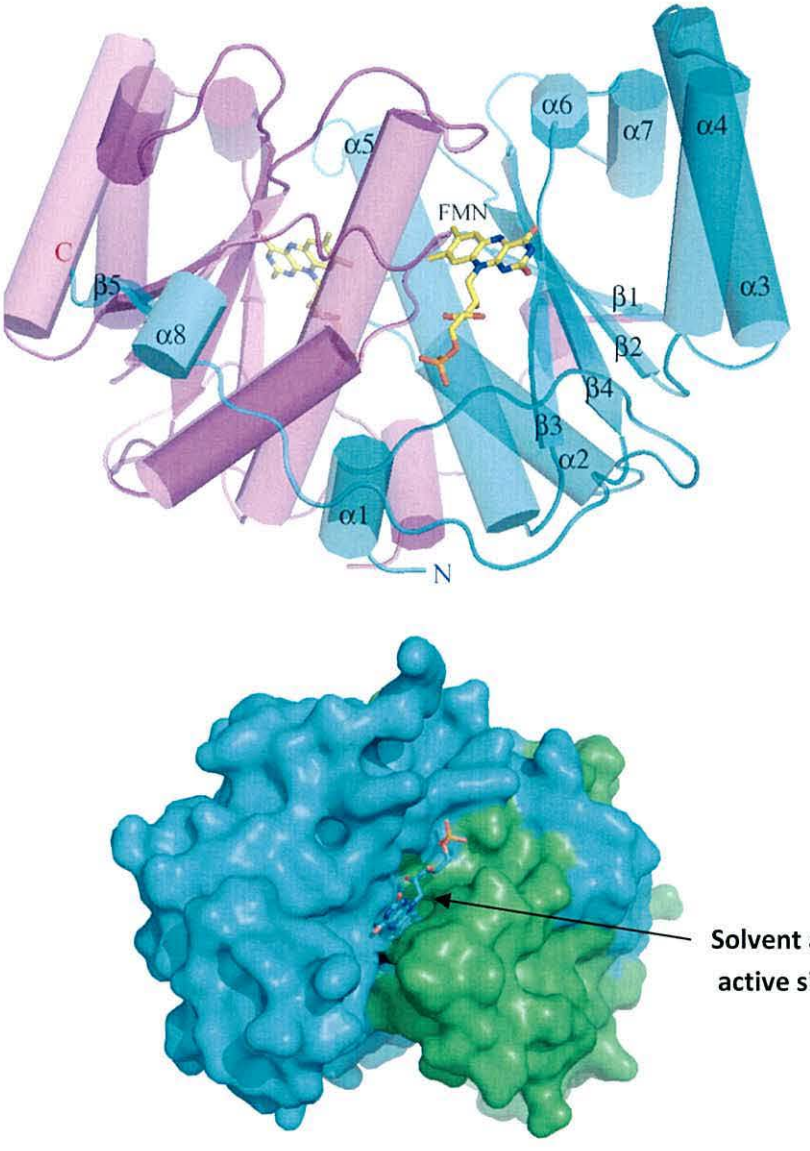



**Figure 1.7: (a) The dimeric structure of Ec_ydjA. Each monomer is differentiated by colour and labelled, and the bound cofactor molecules at the dimeric interface are drawn as stick models. (b) Surface diagram illustrating the solvent accessible active site drawn using PyMOL Viewer.[77]**

### 1.5.4 Physiological function of bacterial nitroreductases

Although the microbial ability for xenobiotic chemicals degradation may develop under the selective pressure of environmental pollution, xenobiotic chemicals cannot be regarded as physiological substrates of nitroreductases due to their release into the environment very recently by human activities.

As bacterial nitroreductases have broad substrate specificity and reduce different nitro-compounds (nitro-aromatic and nitro-heterocyclic), flavins, and quinones, different physiological functions have been suggested for these enzymes[79] involving Lawsone-dependent azo reductase,[80] oxidative stress,[81,82,83] bioluminescence,[39] cobalamin synthesis,[84,85,86] chromate or ferric ion reduction,[48,87,88] specific degradation pathways,[89,90] quinone reduction for detoxification.[80]

### 1.5.5 Nitro-aromatic compounds involved in this study

**Nitrobenzene (NB)**

Nitrobenzene is the simplest nitroaromatic compound and widely used in the manufacture of aniline, dyes, lubricating oils, pesticides, drugs and synthetic rubber. However, it has been listed as a priority pollutant due to its mutagenicity, recalcitrance and tendency to accumulate in the environment.[56,91] Bacterial degradation of nitrobenzene under anaerobic condition includes nitrobenzene reduction to aniline, and complete mineralization of aniline achieved by subsequent aerobic processes.[92] Nitrobenzene biodegradation has been described in different bacteria, such as *Streptomyces*,[93] *Bacillus*, *Pseudomonas*, *Klebsiella*,[94] *Comamonas*,[95] *Corynebacterium*, *Stapylococcus*, *Streptococcus*,[96] *Alcaligenes*, *Acinetobacter*, and *Flavobacterium,*[97] and *Micrococcus luteus*.[98] White rot fungi[99,100] and *Rhodotorula mucilaginosa* strain Z1[101] were found to degrade nitrobenzene, and recently nitrobenzene biodegradation *via* competing pathways in contaminated environments has been also reported.[102]

**3-Nitrobenzoates (3-NBA)**

Nitrobenzoate (NBA) can be considered as an important nitro-aromatic compound due to its participation as an intermediate in nitrotoluene degradation and its potential use as a substrate for biocatalytic transformation.[103,104] NBA degradation pathways remain unclear although all three NBA isomers (2-nitrobenzoate, 3-nitrobenzoate, 4-

nitrobenzoate) are degradable. NBA degradation to methane in a fully anaerobic system has been reported.[105] Although 3-NBA degradation by dioxygenase attack in *Pseudomonas strain JS51* and *Comamonas strain JS46,*[106] and by two monoxygenation steps in *Nocardia*[107] has been reported, very little is known regarding 3-nitrobenzoate degradation mechanism in comparison with the 2-nitrobenzoate, and 4-nitrobenzoate. A putative nitrobenzoate reductase was recently determined in the lactic acid bacterium *Lactobacillus plantarum WCFS1* strain. The enzyme was able to reduce 2,4-dinitrobenzoate and 4-nitrobenzoate, being unable to reduce other nitroaromatics. Therefore this enzyme might be more specific than other microbial nitroreductases that reduce a wider range of nitroaromatic compounds.[108]

**4-Nitrotoluene (4-NT)**

4-Nitrotoluene (4-NTs) is a common pollutant, precursor of TNT, and important derivative of the explosive industry. The degradation of 4-NT and generation of various metabolites has been studied by many researchers, including 4-NT degradation *via* partial reductive pathway in *Pseudomonas* strains TW3 and 4-NT. [109] Reduction of the nitro group to an amino group with simultaneous hydroxylation of the benzene ring of 4-NT in *Pseudomonas putida* OU83 has been previously demonstrated.[110] Oxidation of the methyl group of 4-NT to 4-nitrobenzyl alcohol has been demonstrated in *Pseudomonas sp.*[111] Biotransformation of 4-NT to 4-hydroxylaminotoluene in *Mycobacterium strain* HL 4-NT-1.[112]

**2,4,6-Trinitritoluene (TNT)**

This will be discussed in detail in chapter 4.

**2,4-Dinitroethylbenzene (DNEB)**

DNEB is a relatively rare explosive which was obtained from a previous project with the atomic weapons establishment.

**Bacterial oxygen insensitive nitroreductases**

In this study NfsB oxygen insensitive nitroreductase from *Salmonella* typhimurium LT2 (NCBI AE008722) which shares 88% amino acid identity with *Escherichia coli* NfsB nitroreductase, YdjA protein from *Salmonella paratyphi A* (NCBI CP000026) that shares 16% and 95% amino acids identity with *Escherichia coli* NfsB and Ec_ydjA respectively and two different nitroreductase family proteins (Dde_0086, and Dde_2199) from *Desulfovibrio desulfuricans* G20 (NCBI NC_007519) that share 51% and 17% identity respectively with *Escherichia coli* NfsB and 22% identity with each other, have been isolated, cloned, expressed in *Escherichia coli*, purified, and characterised using different nitro-compounds, and finally engineered through insertion of $cys_{12}$ tag at the enzyme N-terminus in order to use them as a biosensor for explosive detection.

### 1.5.6 Biotechnological applications of bacterial nitroreductases

The flexibility of this group of enzymes contributes the basis of their use in different biotechnological applications:

***Bacterial nitroreductases in cancer therapies***

The pharmaceutical application of nitroreductases in cancer therapy as a prodrug activator is associated with the ability of these enzymes to reduce nitro groups to the corresponding hydroxylamines which are further metabolized to form cytotoxic DNA cross-linking agents and subsequently cause cell death. There are essentially three strategies being used to introduce enzymes into the body. The first strategy is called gene directed enzyme prodrug therapy (GDEPT), which involves deliver of an enzyme-encoding gene to a tumour cell by use of a selective vector. The second is called antibody directed enzyme prodrug therapy (ADEPT). Here monoclonal antibodies are conjugated to a drug-activating enzyme to direct the enzyme to the tumour cell. Virus directed enzyme prodrug therapy (VDEPT) is very similar to (GDEPT), but in this case a virus as a carrier is used to deliver the enzyme. Recently recombinant anaerobic Clostridial spores have been used as a delivery vector for the nitroreductase gene.[113] The role of the NTRs in this approach is to enable the activation of prodrugs. The approach operates in two distinct steps. Firstly, a prodrug-activating enzyme is targeted and expressed in the tumour. In the second step, a non-toxic prodrug is administered

systemically where the systemically administered prodrug reaches the tumour and is converted to an effective anticancer drug, localized in the tumour tissue.[114,115]

Due to the role of the *Escherichia coli* nitroreductase NfsB in cancer therapy it has been well studied at structural and kinetic level,[116,117,118] and recently *Bacillus licheniformis* and *Bacillus amyloliquefaciens* nitroreductases have been used for the same purpose.[119,120]

The use of nitro-compounds as antibiotics such as nitrofuran-derivatives[121]**Error! Bookmark not defined.**and metronidazole[122, 123, 124] results from its activation as a toxic agent by nitroreductase.

***Bacterial nitroreductases in bioremediation***

Bioremediation can be defined as a biotechnological application of microorganisms, or their enzymes to degrade pollutants from the environment into a less toxic form. Microorganisms degrade contaminant nitro-compounds through reactions that occur as a part of their metabolic processes. Different aerobic and anaerobic mechanisms have been used for remediation of polynitroaromatic compounds.[125,126,127] The development of transgenic plants expressing bacterial nitroreductase to remove toxic pollutants from sites that have been polluted through industrial activities and military has been reported.[128, 129, 130] In addition, nitro-aromatic degradation by hybrid pathway through genetically modified bacterial strains,[131,132] microorganism or enzyme based biosensors and immunosensors to detect materials and sites polluted with nitro-aromatic compounds may be developed.[133]

***Bacterial nitroreductases as a biosensor for the detection of explosives and pollutants***

An amperometric biosensor for the nitro-containing explosives detection has been developed through using engineered minor oxygen insensitive nitroreductase NfsB from *E coli* k12 that enables the enzyme to form strong thiolate bonds on a biosensor gold electrode.[134]

The oxygen insensitive nitroreductase NfrA from *Bacillus subtilis* strain LAM, isolated from industrial waste contaminated with nitroaromatic compounds, has been used as a specific enzymatic biosensor for detecting target pollutant 3,5-dinitro-trifluoromethylbenzene (3,5-DNBTF). The enzyme catalyzes reduction of (3,5-

DNBTF) to its corresponding diamine 3,5-diamino-benzene-trifluoride (3,5-DABTF), irradiation at 310nm achieves total defluorination of (3,5-DABTF) to 3,5-diaminobenzoic acid (3,5-DABA). The defluorination approach offers a panel of detection signals that could be useful for the development of the specific 3,5-DNBTF biosensor.[135]

### 1.6 Bacterial Carboxylesterases

Carboxylesterases (CES) are ubiquitous enzymes that have been identified from different sources ranging from mammals[136] to bacteria,[137] and even in plants.[138] These enzymes cleave carboxylic esters into the corresponding alcohol and carboxylic acid, and they are members of α/β hydrolase family that share a common α/β hydrolase fold, a regulatory domain, and the catalytic triad of Ser, His, and Asp/Glu. [139, 140] They exhibit broad substrate specificities, catalyse hydrolytic and transesterification reactions of xenobiotics (carboxyl esters, thioesters and aromatic amides), narcotics (heroin and cocaine),[141] anticancer drugs such as irinotecan (CPT-11),[142] and detoxify numerous organophosphate and carbamate compounds used as insecticides (malathion) or chemical weapons (Sarin, Tabun and Soman),[143,144] and catalyze several reactions in cholesterol and fatty acid metabolism.[145,146,147]

#### 1.6.1 Carboxylesterase reaction mechanism

Carboxylesterases catalyse ester hydrolysis and substrate transesterification. Ester hydrolysis is base-mediated and requires water as co-reactant; the reaction is achieved in a two-stage reaction *via* a catalytic triad of Ser, His, and Asp/Glu (Fig.1.8). The active site glutamic acid exists as the charged form at neutral pH that facilitates removal of a proton from histidine, resulting in transfer of a proton from the adjacent serine to the opposing nitrogen of histidine; subsequently the generated oxygen nucleophile attacks the carbonyl carbon of the substrate. After the formation of tetrahedral intermediate of an acyl group (*a covalent acyl-enzyme intermediate is formed with the catalytic serine residue*), the alcohol product is released from the enzyme. The acyl-enzyme intermediate is attacked by water acting as the nucleophile to produce acyl product and the catalytic residues return to their original state.[148,149] In transesterification reactions alcohol can attack the acyl-enzyme intermediate generating an ester product. The transesterification of cocaine with ethanol to generate cocaethylene is a well-researched example.[150]

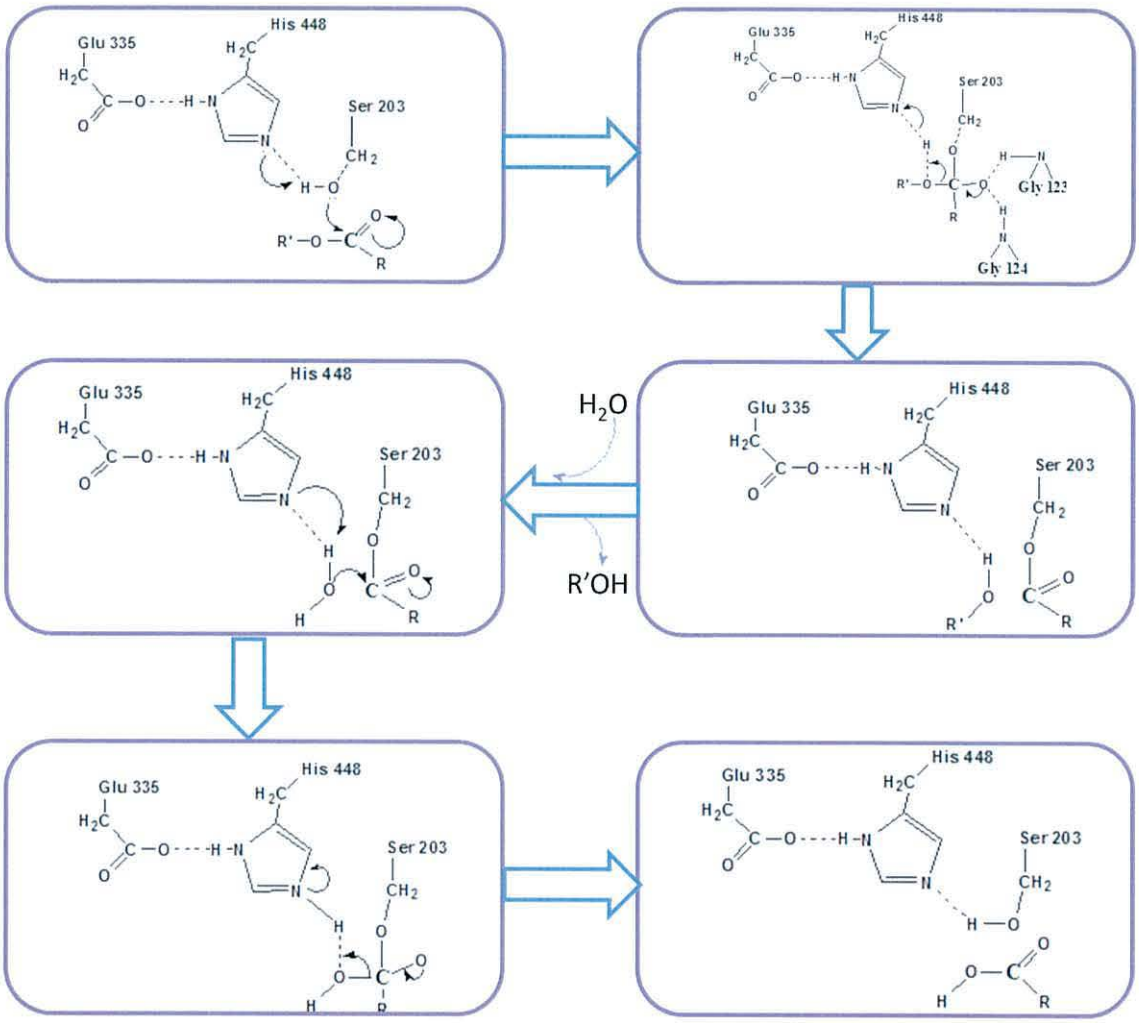



**Figure 1.8: A proposal mechanism for the action of carboxylesterase.**[151]

### 1.6.2 Carboxylesterases crystal structure

Recently carboxylesterase crystal structures from different organisms have been solved,[152,153,154,155] Despite sequence differences they share a typical α/β domain, and a catalytic domain of Ser, His, and Asp/Glu. The first crystal structure of a bacterial CE, that of *Pseudomonas fluorescens*, was reported in 1997,[153] and the first mammalian crystal structure was reported in 2002,[156] followed by that of hCE1.[154,155]

*Bacillus subtilis* pNB esterase (Fig. 1.9) which has been involved in this study is a α/β protein composed of a central 13-stranded β-sheet surrounded by 15 α-helices. The catalytic triad is formed by Ser-189, His-399, and Glu-310; this enzyme shares the rare use of Glu instead of Asp like acetylcholinesterase. The active site is placed at the base of a cavity with dimensions 20 Å by 13 Å by 18 Å. The active site entrance is formed by four loops. Residues 64–71 and 413–417 (unstructured WT loops) form one

side of the entrance. The rest of the entrance is formed by residues 316–320 (poorly structured electron density in WT) and 260–268. The residues 105–108, 193, 215–216, 268–275, 310–314, 362–363, and 399–400 comprise the active site cavity.[157]

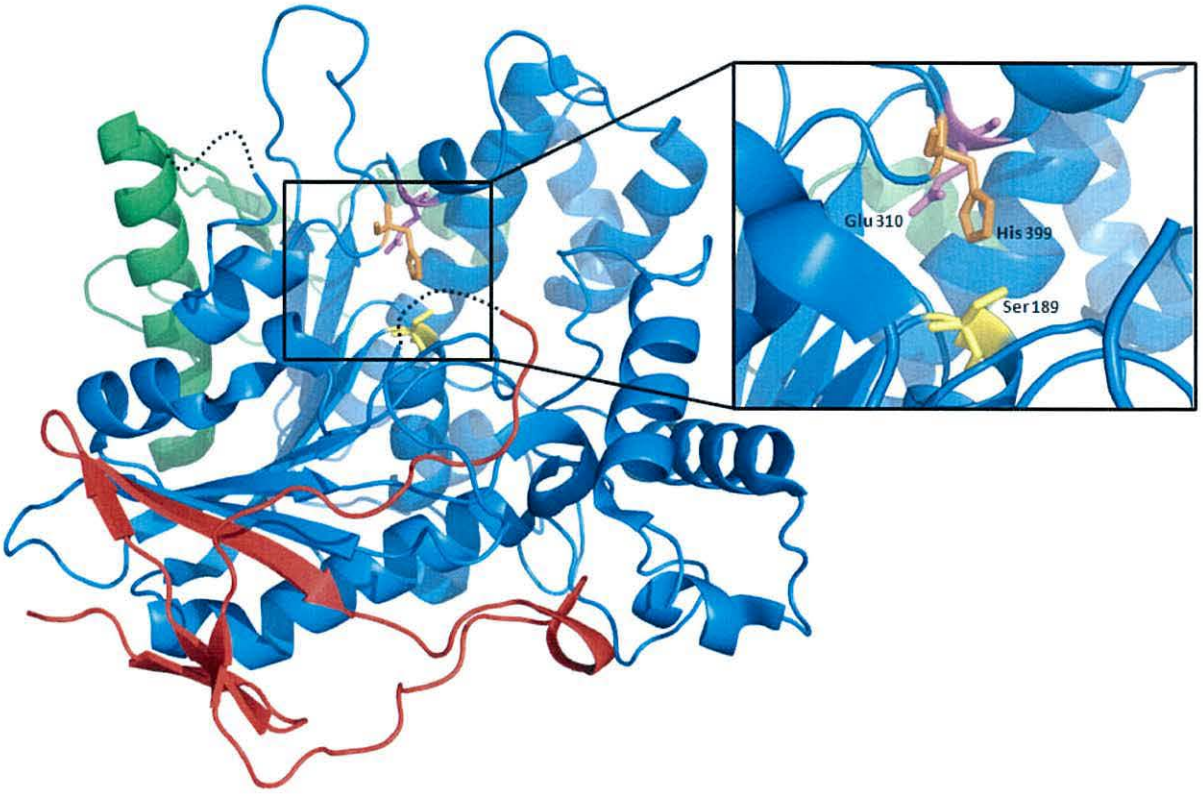



**Figure 1.9: The crystal structure of Pnb CE with secondary structural elements (PDB code 1EQ3).[157] The active site residues (Ser 189, Glu 310 and His 399) are enlarged. The figure was re-drawn using the PyMOL.**

### 1.6.3 Carboxylesterases biological role

Many environmental toxicants (parathions insecticides, and phthalate), narcotics (cocaine, heroin), and chemotherapeutic prodrugs (irrinotecan) contain ester moieties and, hence, are subject to catalysis by CEs. In the majority of cases, esterase-mediated hydrolysis results in inactivation of the drug, however, with the prodrugs capecitabine and CPT-11, the ester groups are removed to improve their water solubility.[158,159,160]

#### 1.6.3.1 *Xenobiotic metabolism*

Varying levels of anti-cancer prodrug CPT-11 activation have been noticed across different species, and rabbit liver carboxylesterase (rCE) was observed to be the most efficient enzyme in the activation of CPT-11.

The pro-drug CPT-11 requires enzymatic hydrolysis to generate the active metabolite SN-38, a potent topoisomerase I poison, and 4-piperidino-piperidine (4PP) (Fig 1.10). SN-38 affects covalent DNA-protein complex formation and eventually leads to cell death through inhibition of DNA transcription.[161]

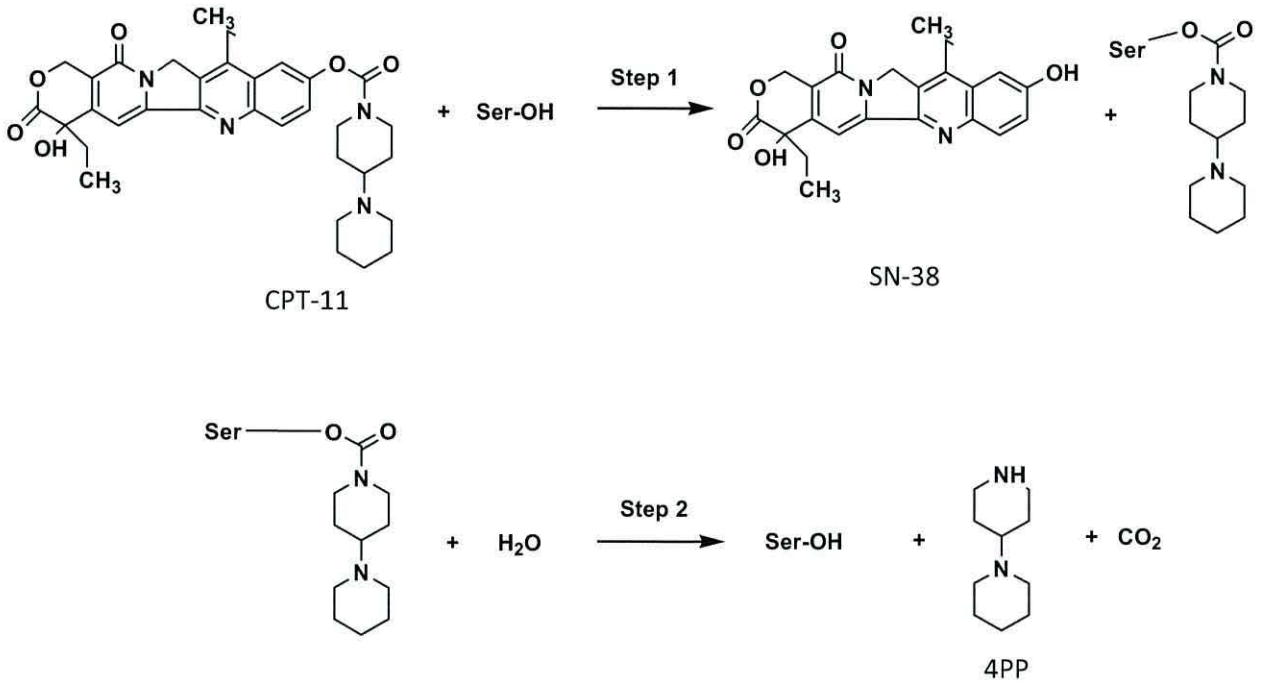



**Figure 1.10: Two steps of prodrug CPT-11 activation to topoisomerase poison SN-38 and 4-piperidino-piperidine (4pp) by carboxylesterase.[162]**

hCE-1 catalyzes the hydrolysis of cocaine methyl ester generating benzoylecgonine, a non toxic urinary metabolite of cocaine (Fig 1.11).[163,164,165] When cocaine and alcohol are abused together hCE-1 has the ability to generate the toxic cocaine metabolite cocaethylene (Fig 1.11).[166] Human liver carboxylesterases (hCE-1 and hCE-2) cleave the 3-acetyl group of heroin generating 6-monoacetylmorphine. However, 6-monoacetylmorphine hydrolysis to morphine is only achieved by hCE-2 with high catalytic efficiency.[164]

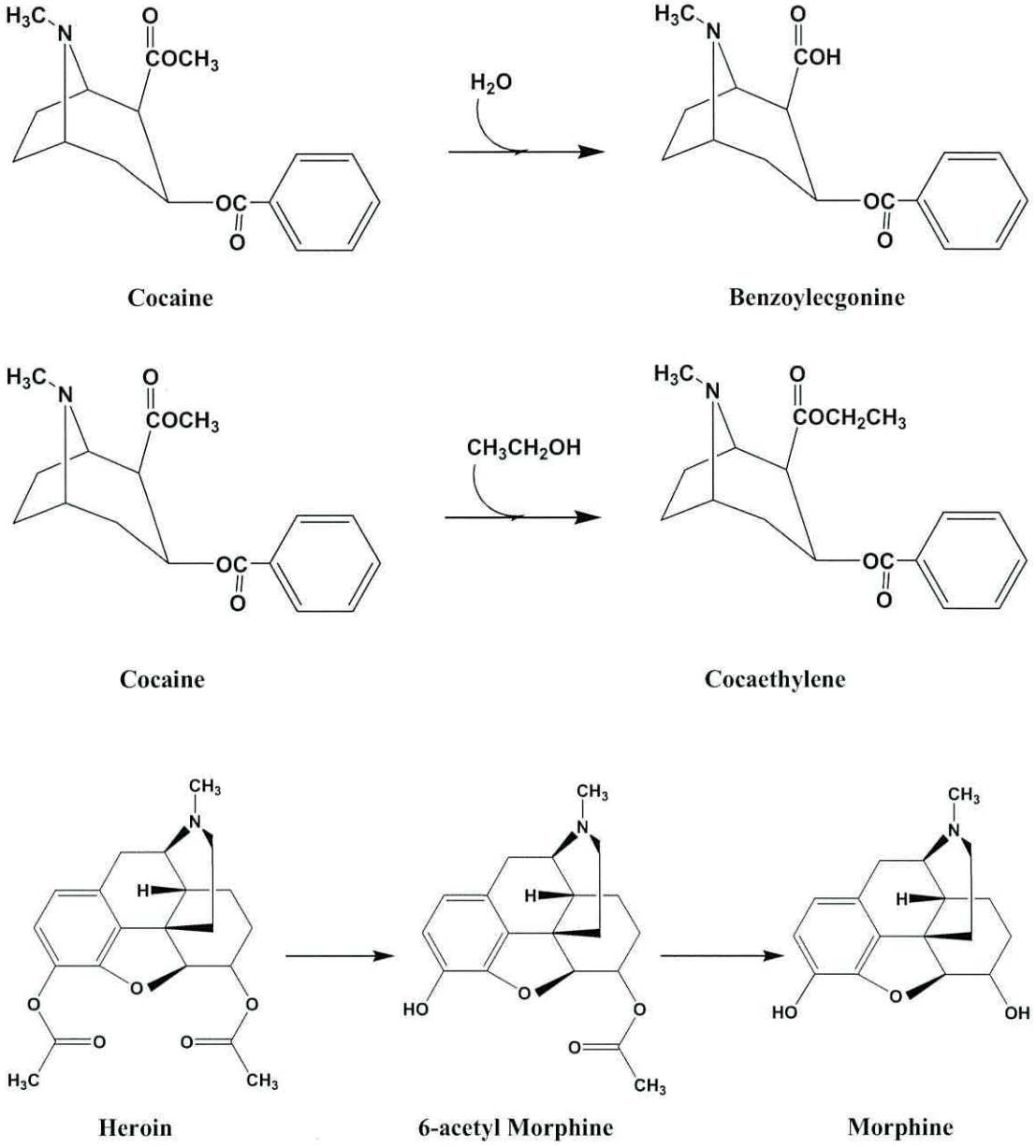



**Figure 1.11: Narcotics (cocaine & heroin) metabolism by hCE.**[167]

Detoxification of pesticides and chemical weapons (Tabun, Sarin, and Soman) is accomplished by CEs. Although these chemicals cause inactivation of the enzyme, it has been proposed that single-site mutations in the hCE-1 active site can generate an efficient organophosphate hydrolase enzyme capable of effectively detoxifying nerve agents.[168,169]

### 1.6.3.2 Endobiotic metabolism (Cholesterol and fatty acids metabolism)

CEs have been found to play a role in processing of cholesterol and fatty acids in the liver and peripheral tissues. CEs (hCE1) was shown to have cholesteryl ester hydrolase (CEH),[170] fatty acyl-CoA hydrolase,[171] and acyl-CoA:cholesterol acyl-transferase

(ACAT) activities.[172] In macrophages and monocytes CEs mediate metabolism of cholesterol from phagocytosed cell to cholesteryl esters. Conversely, CEs eliminate excess cholesteryl esters generated during lipid droplet and high density lipoprotein packaging. Thus CEs appear to play a role as a back-up system to reduce cellular levels of free cholesterol and cholesteryl esters through both the creation and the elimination of cholesteryl esters using transesterification and hydrolysis reactions respectively.

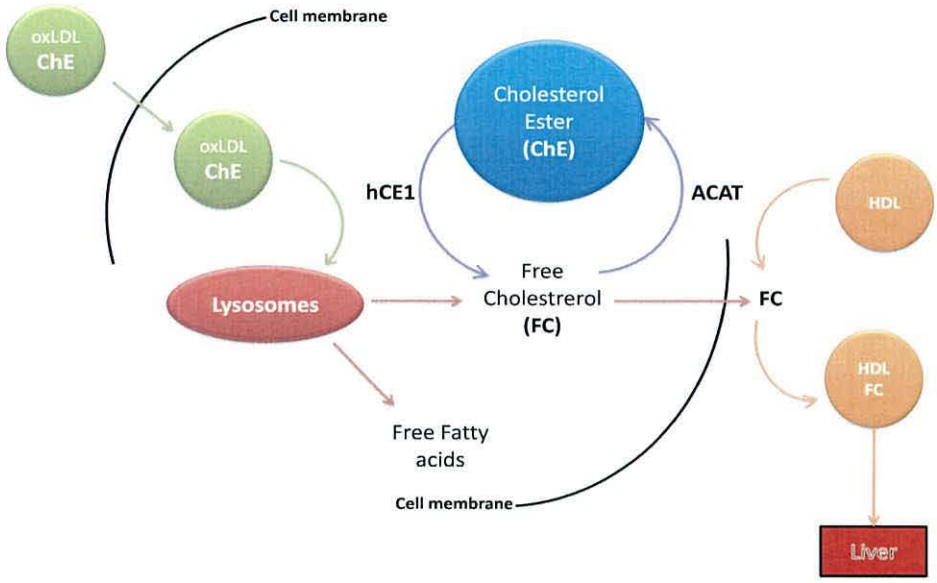



**Figure 1.12: Cholesteryl ester metabolism in macrophages. Abbreviations: oxLDL, oxidized low-density lipoprotein; HDL, high-density lipoprotein; ChE, cholesteryl ester; FC, free cholesterol; ACAT, acylCoA:acyltransferase; hCE1, human carboxylesterase 1.**[173]

### 1.6.3.3 Protein trafficking and retention in the endoplasmic reticulum

CEs seem to regulate protein trafficking and retention through binding to proteins within endoplasmic reticulum (ER). CEs bind directly to C-creative protein (CRP), the most sensitive serum marker for coronary heart disease, and then release it on the detection of tissue injury.[174] CEs have an N-terminal hydrophobic signal peptide that marks them for trafficking through ER; additionally a Lys-Asp-Glu-Leu (KDEL) sequence present at the C-terminus of the protein ensures retention within the cell.[175]

### 1.6.4 Carboxylesterases and narcotics (heroin and cocaine)

**Cocaine** (COC), an alkaloid obtained from *Erythroxylum coca* plant has become one of the most notably abused drugs, and remains one of the most common health problems.[176] Human CEs catalyze the rapid hydrolysis of ester linkages in COC to the

inactive benzoylecgonine (BZE), the main metabolite in both blood and urine (Fig 1.11)[177]. In association with alcohol, cocaine is converted into cocaethylene (Fig 1.11), an active compound responsible for an enhanced euphoric feeling.[178]

**Heroin** (3,6-diacetyl morphine) is a semi-synthetic opioid drug synthesized from morphine, a derivative of the opium poppy. It is rapidly metabolized by liver carboxylesterases *via* sequential deacetylation of ester bonds to 6-monoacetylmorphine (6-MAM), which is further hydrolysed to morphine (Fig 1.11). [179,180] Heroin ingestion is usually proved by the detection of 6-monoacetylmorphine not morphine, as codeine, which is used as a cough medicine, can also be metabolised to morphine.[181]

The role of CEs in narcotics metabolism and the illegal use of narcotics have encouraged considerable interest in the development of methods for their detection.

### 1.6.5 Microbial (*Bacillus*) carboxylesterases

There has been a great increase in the use of microbial carboxylesterases as biocatalysts in biomedical applications because of their tremendous abilities to achieve regio-, stereo-, and enantiospecific reactions.[182,183,184] p-Nitrobenzyl esters act as protecting groups on intermediates in the production of clinically important oral β-lactam antibiotics[185]. A *Bacillus* carboxylesterase has been used for stereo specific resolution of *R,S*-naproxen esters, which is an important anti-inflammatory drug,[186] and a *p*-nitrobenzyl esterase was genetically engineered in order to synthesize cephalosporin-derived antibiotics.[187]

The crystal structure of *Bacillus subtilis* Pnb CE has been solved and it is homologous to rCE and showed activity with the prodrug CPT-11. So far studies concerning the role of *Bacillus* or other microbial carboxylesterases in narcotic metabolism are scarce.

## 1.7 Genetic engineering, biomolecule immobilisation, and the aim of this study

The most significant scientific advance in recent years has been the development of technology which allows genes to be manipulated, altered, and transferred from organism to organism even to transform DNA itself. This has enabled us to use rapidly reproducing organisms such as bacteria as chemical factories producing useful, often life-saving, substances.

Since the first explanation by Clark and Lyons, that an enzyme could be integrated into an electrode surface to form a biosensor, the development of such electrochemical devices has made substantial progress. The advantages associated with these devices are their high selectivity and simple use in complex media as well as the possibility of developing compact and portable analyzers.

Some bacteria contain enzymes (Nitroreductases) which are able to degrade many commonly used explosives to less toxic products. In this study we attempted to isolate these enzymes from bacteria and subject them to genetic modification. This modification will enable the enzyme to adhere to the surface of an electrode sensor, where they remain active. There, they will generate an electrical signal when activated by the presence of a minute amount of an explosive molecule.

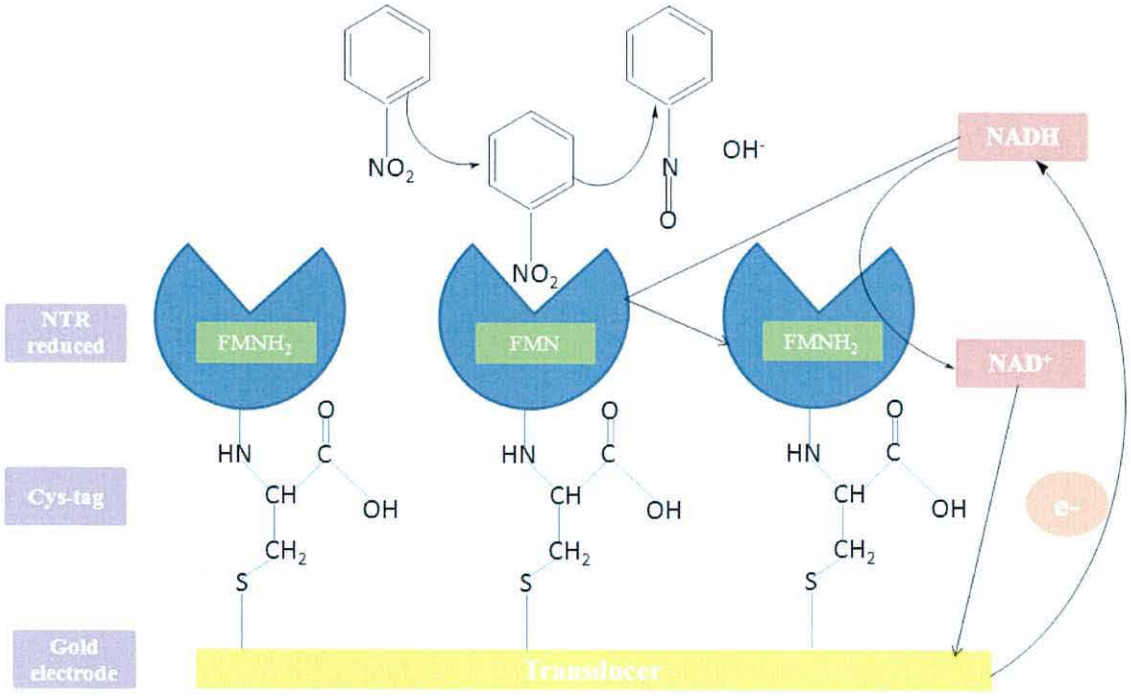



**Figure 1.13: The electron transfer from the electrode**

Human liver carboxylesterase (hCE1) have a key role in cocaine overdose treatment through catalysis of the cleavage of cocaine methyl ester bond to generate benzoylecgonine, the non-toxic cocaine metabolite. In the second part of this study we have made an effort to isolate *Bacillus* carboxylesterases as a model of mammalian carboxylesterases, and subject them to genetic modification. When the enzyme is able to adhere to an electrode surface with complete retention of its biological recognition properties, it will be used as a biosensor for narcotics detection.

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# Chapter 2-Material and methods

## 2.1 An introduction to cloning

Gene cloning is a process by which large quantities of a specific DNA are cloned or replicated, once the desired DNA has been isolated.[1] To clone a gene, a DNA fragment containing the gene of interest is isolated from chromosomal DNA using restriction enzymes and then inserted into a plasmid (small, circular self-replicating, extra-chromosomal DNA molecule and frequently carries genes of antibiotic resistance)[2] that has been cut with the same restriction enzyme. When the fragment of chromosomal DNA is joined with its cloning vector, it is called a "recombinant DNA molecule". Before introducing the vector containing the foreign DNA into host cells to express the protein, it must be cloned. Cloning is necessary to produce numerous copies of the DNA since the initial supply is inadequate to insert into host cells. Once the vector is isolated in large quantities, it can be introduced into the host cells (special bacterial cells); the host cells will then synthesize the foreign protein from the recombinant DNA. When the cells are grown in vast quantities, the foreign or recombinant protein can be isolated and purified in large amounts.[3]

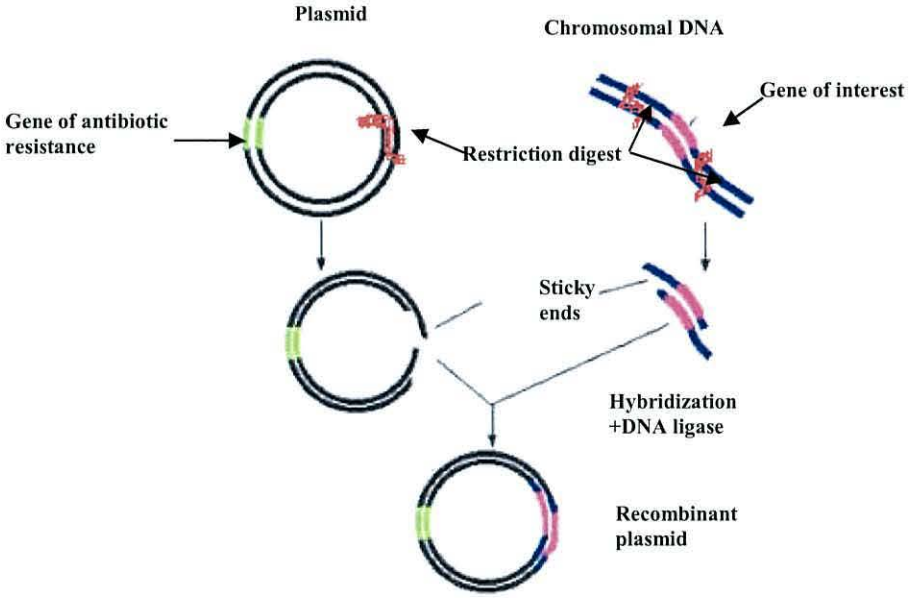


**Figure 2.1: Construction of a recombinant plasmid.**

The major tools of recombinant DNA technology are bacterial enzymes called restriction enzymes (also called restriction endonucleases), which were first discovered in the late 1960s. These enzymes naturally occur in bacteria as a defence mechanism against foreign DNA (bacteriophages). They have the ability to cut DNA molecules at very precise sequences of 4-8 base pairs called recognition sequences; these sequences are always symmetrical (One strand is identical to the other strand when read in the opposite direction).[4,5] Some of the enzymes create a staggered cut (sticky ends), where one strand of DNA is longer than the complimentary strand. Because of the staggered cuts, treatment of DNA from different sources with the restriction enzymes will generate regions, which complement one another. These regions will anneal together. These behaviours allow foreign DNA to be inserted into target DNA.[5,6] This is the basis of recombinant DNA technology. The first production of recombinant DNA molecule, using restriction enzymes, occurred in the early 1970s.[7]

### 2.1.1 Polymerase chain reaction (In vitro DNA cloning)

The polymerase chain reaction is a technique for quickly cloning a particular piece of DNA in the test tube rather than in living cells. The development of this technique resulted in an explosion of new techniques in molecular biology and a Nobel Prize for Kary Mullis in 1993.[8] The technique was made possible by the discovery of *Taq* polymerase, the DNA polymerase that is used by the bacterium *Thermophilus auquaticus* that was discovered in hot springs. This DNA polymerase is stable at the high temperatures need to perform the amplification, whereas other DNA polymerases become denatured.[8,9]

PCR reaction requires several basic components; DNA template, which contains the region of the DNA fragment to be amplified, two primers (short segment of nucleotides), which determine the beginning and end of the region to be amplified, *Taq polymerase*, which copies the region to be amplified, deoxynucleoside triphosphates, from which the DNA polymerase builds the new DNA, and buffer, which provides a suitable chemical environment for the DNA polymerase. This reaction is carried out in a thermal cycler, which heats and cools the reaction tubes within it to the precise temperature required for each step of the reaction.[10]

The PCR process usually consists of a series of twenty to thirty-five cycles. Each cycle consists of three steps. First the double stranded DNA has to be heated to ≈ 94°C in order to separate the strands. This step is called denaturing; it breaks apart the hydrogen bonds that connect the two DNA strands. Prior to the first cycle, the DNA is often denatured for an extended time to ensure that both the template DNA and the primers have completely separated and are single strand only. Next the temperature is lowered so the primers can attach themselves to the single DNA strands. This step is called annealing; the temperature of this stage depends on the primers and is usually 5°C below their melting temperature. Finally, the DNA polymerase has to copy the DNA strands; it starts at the annealed primer and works its way along the DNA strand. This step is called extension, and 72°C is the ideal working temperature for the polymerase. A final extension step is frequently used after the last cycle to ensure that any remaining single stranded DNA is completely copied.[9]

While a very powerful technique, PCR can also be very tricky, the selection of the primers is very important to the efficiency of the reaction. The primers must be very specific for the template to be amplified, and must not be capable of annealing to themselves. A wrong temperature during the annealing step can result in primers not binding to the template DNA at all, or binding at random.[8] The specificity of PCR depends on the primers used, while the size of the PCR products depends on the positioning of the primers on the target DNA.

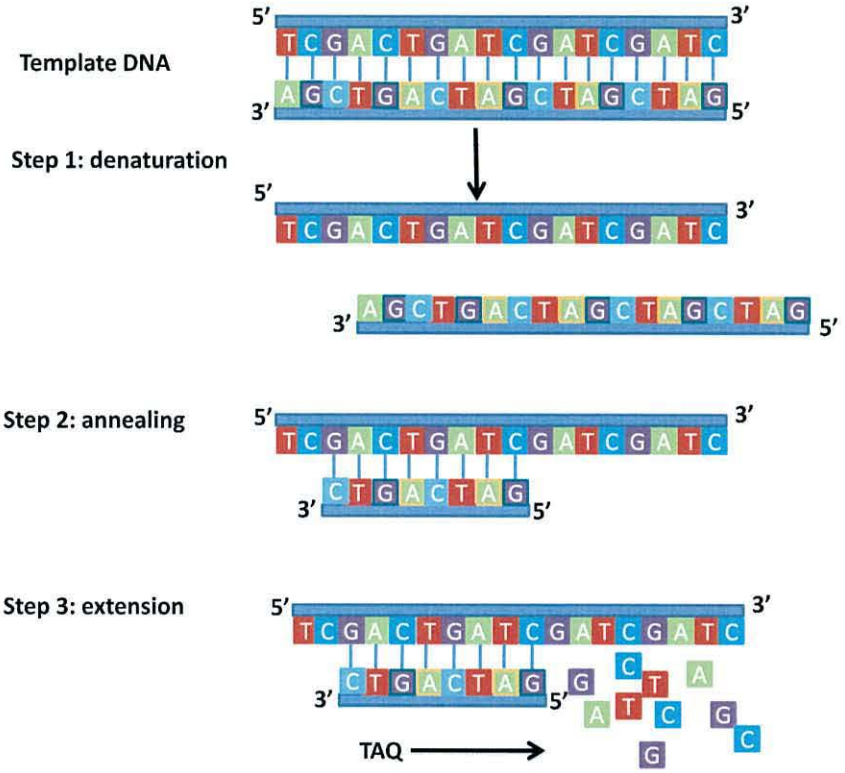


**Figure 2.2: A pictorial representation of polymerase chain reaction (In vitro DNA cloning).**

### 2.1.2 Detection and analysis of the PCR reaction product

Not every PCR is successful; therefore, before the PCR product is used in further applications, it has to be checked. Agarose gel electrophoresis is the easiest and commonest way of separating and analyzing DNA. The PCR product should be fragments of DNA of defined length. The technique of electrophoresis is based on the fact that DNA is negatively charged due to its phosphate backbone. For this reason, when an electrical potential is applied the DNA will move toward the positive pole. The gel matrix acts as a sieve for DNA molecules. Large molecules have difficulty getting through the holes in the matrix. Small molecules move easily through the holes. Because of this large fragments will lag behind small fragments as DNAs migrate through the gel. As the separation process continues the separation between the larger and smaller fragments increases. DNAs are visualized by staining the gel with ethidium bromide which binds strongly to DNA by intercalating between the bases and is fluorescent, meaning that it absorbs UV light and transmits the energy as visible orange light. Molecular weight markers (mixture of DNAs

with known molecular weight) are often electrophoresed with DNAs and used to estimate the sizes of unknown fragments.[3,11]

### 2.1.3 Cloning vector and bacterial transformation

A cloning vector is a small DNA vehicle (plasmid) that carries a foreign DNA fragment (gene of interest). The insertion of the foreign DNA fragments (PCR product) into the cloning vector is carried out by treating the vehicle and the gene of interest with the same restriction enzyme to generate complementary ends, then ligating the fragment together with the enzyme DNA ligase which joins 5`-phosphate and 3`-hydroxyl ends of the DNA.[12] The main features of cloning vectors are: sequences that permit the propagation of itself in a host cell (origin of replication), a multiple cloning site (polylinker region) to insert foreign DNA, the most versatile vectors contain a site that can be cut by many restriction enzymes, and a method of selecting for bacteria containing a vector with foreign DNA, usually accomplished by selectable markers for drug resistance.[13,14,15]

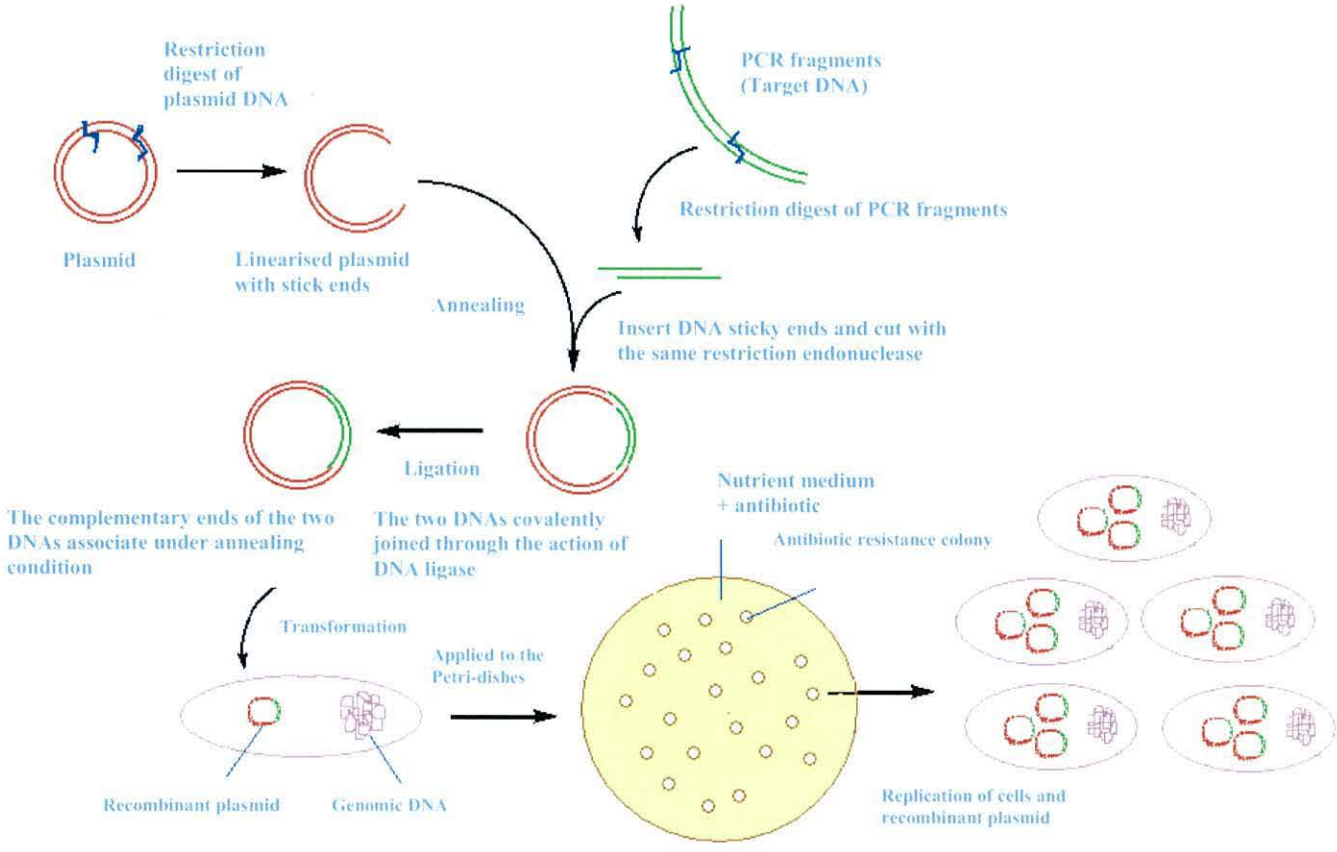


**Figure 2.3: A pictorial representation for cloning the vector**

Bacterial transformation is the process by which bacterial cells take up foreign DNA molecules (constructed plasmid). Since DNA is a very hydrophilic molecule, it will not normally pass through a bacterial cell's membrane. In order to make bacteria take in the plasmid, they must first be made "competent" to take up DNA. This is done by chilling cells and the constructed plasmid in the presence of divalent cations such as $CaCl_2$; this prepares the cell walls to become more permeable to plasmid. Placing them briefly at 42°C (heat shock), and then putting them back on ice, causes the bacteria to take in the plasmid.[2,4,6] To help the bacterial cells recover from the heat shock, the cells are briefly incubated with non selective growth media. Cells are then plated out on antibiotic containing media. Only bacteria that have acquired the plasmid can grow and form colonies on the plate.[11,16]

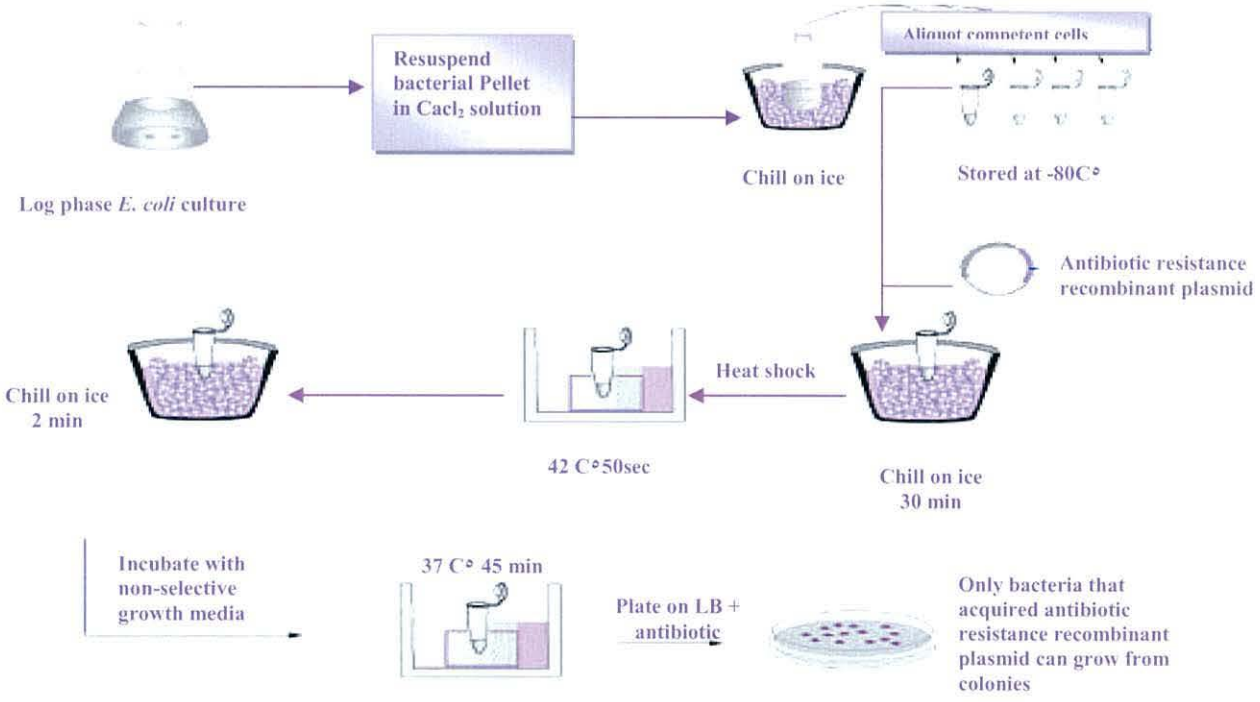


**Figure 2.4: Competent cell preparation and bacterial transformation.**

### 2.1.4 High- level expression of cloned gene (pET expression system)

Many proteins are expressed at low level in vivo, expression systems are designed to produce many copies of a desired protein within the host cell. The pET expression system (Novagen) was constructed to contain all of the genetic coding necessary to produce the protein, including a promoter (T7 promoter) appropriate to the host cell, *lac* operator region 3` to the T7 promoter, polylinker region downstream of the T7 promoter, *lacI* which codes for the lac repressor protein, a sequence which codes for ribosome binding, and a sequence which terminates transcription. The pET expression system is highly selective for bacteriophage T7 RNA polymerase. The specificity lies in the T7 promoter which only binds T7 RNA polymerase, the pET expression system involves not only an expression vector, but also a genetically engineered host bacteria *E. coli* strain Rosetta *(*Rosetta is a specific host strain designed to express proteins containing rare codons*)*. The gene for T7 RNA polymerase (gene 1) is inserted into the chromosome of *E. coli* and transcribed from the *lac* promoter; therefore, it will be expressed only if the inducer isopropyl-beta-D-thiogalactopyranoside (IPTG) is added. The T7 RNA polymerase will then transcribe the gene cloned into the pET vector.[6,17]

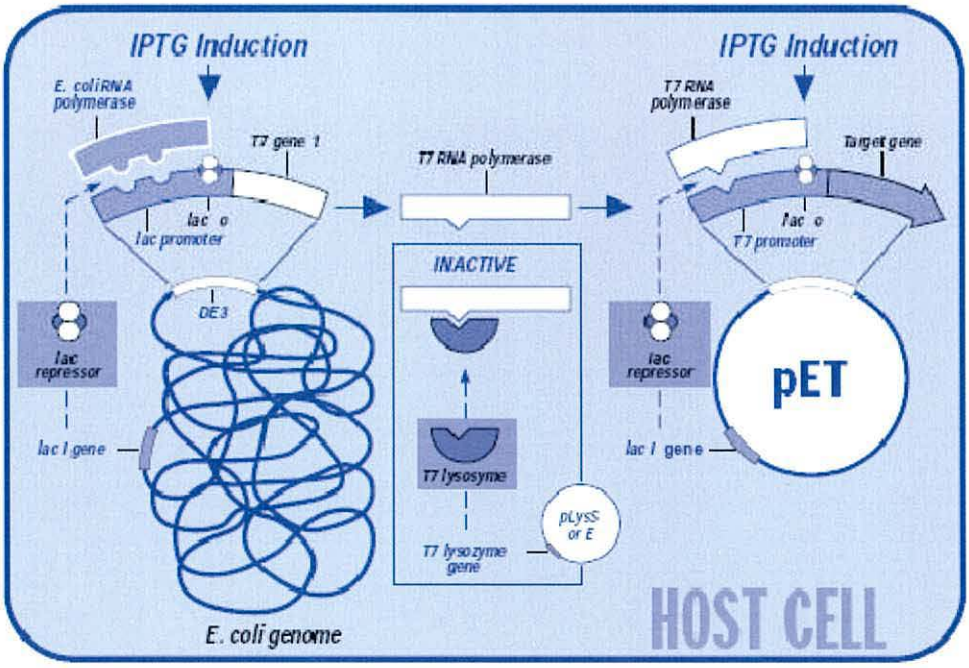


**Figure 2.5: Control elements of the pET expression system.** [6]

### 2.1.5 Purification of recombinant protein

Immobilised metal ion affinity chromatography (IMAC) is a popular and powerful way to purify proteins. It is based on the specific coordinate covalent binding between histidine or other amino acids (either naturally present on the surface of the protein or grafted with recombinant DNA techniques) and various immobilized metal ions, such as nickel.[6,18]

His-tags have affinity toward nickel ions, and thus can be immobilised on nickel ions in a resin which is packed in a column. Since the protein is the only component with a His-tag, the majority of proteins will pass through the column, and leave the His-tagged protein bound to the resin. The protein is competitively released from the column by eluting it with increasing amounts of imidazole, which competes with the His-tags for nickel binding.[4] The purity and the amount of protein can be assessed by SDS-PAGE (Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis).

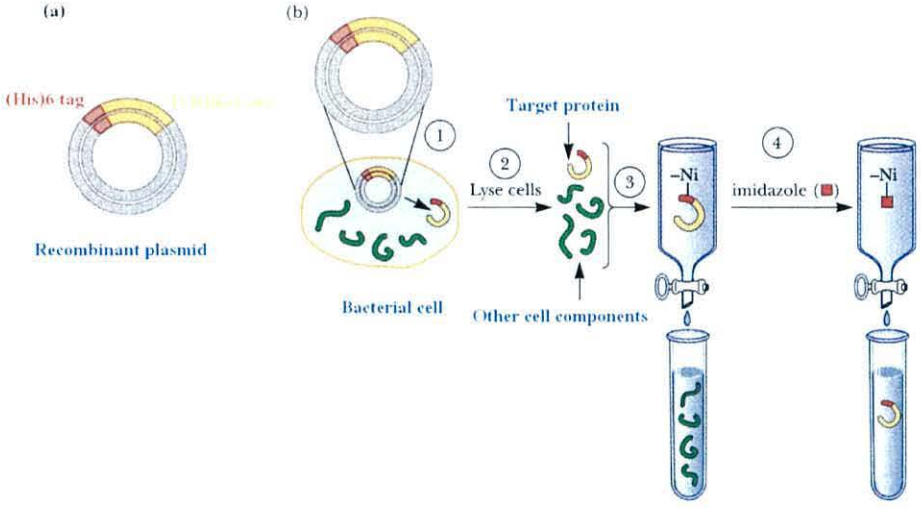


**Figure 2.6: Affinity chromatography and fusion proteins combine to efficiently purify a protein. (a) An expression vector that inserts six histidine codons at the N-terminus, following the histidines is a sequence of target recombinant protein. (b) (1) The vector is expressed to form target proteins. (2) The cell is lysed. (3) The lysate run over a nickel column, nickel attracts the histidine tag on the fusion protein, and the other cell components are washed out. (4) High concentrations of imidazole are used to elute the fusion protein.** [4]

## 2.2 Bacterial strains and plasmids

*E coli* DH5α competent cells were used for amplification of recombinant plasmids, while *E. coli* Rosetta strain was used as the expression host. The vector pET 28a(+) was used for expression. *Salmonella typhimurium* $LT_2$; *Salmonella paratyphi A; Desulfovibrio desulfuricans* G20 *Bacillus subtilis* strain 168 *and Bacillus licheniformis* ATCC 14580 were obtained from Chemistry department culture collection.

## 2.3 Genomic (Template) DNA extraction

### 2.3.1 *Gram positive bacteria (Bacillus subtilis and Bacillus licheniformis)*

The genomic DNA was extracted using Wizard® genomic DNA purification kit[19] by transferring 1 ml of overnight culture to a 1.5 ml microcentrifuge tube. Cells were pelleted by centrifugation at 13000-16000 xg for 2 min, and resuspended thoroughly in 480 µl of 50 mM EDTA. Appropriate lytic enzymes (lysozyme) were added to resuspend cell pellet in a total volume of 120 µl, gently mixed then incubated at 37°C for 30-60 min, and centrifuged for 2 min at 13000-16000 xg. The cell pellet was resuspended through adding 600 µl of nuclei lysis solution, and incubated at 80°C for 5 min to lyse the cells, then cooled to room temperature followed by addition of 3 µl of RNase solution to the cell lysate. The tube was inverted 2-5 times to mix, incubated at 37°C for 15-60 min then cooled to room temperature. RNase-treated cell lysate was vigorously vortexed at high speed for 20 min following addition of protein precipitation solution (200 µl), the sample was incubated on ice for 5 min, and centrifuged at 13000-16000 xg for 3 min. The supernatant containing DNA was transferred to a clean 1.5 ml microcentrifuge tube containing 600 µl of room temperature isopropanol, gently mixed by inversion until the thread-like strands of DNA formed a visible mass, then centrifuged at 13000-16000 xg for 2 min. The supernatant was carefully poured off, and the tube drained on clean absorbent paper, 600 µl of room temperature 70% ethanol added, and the tube gently inverted several times to wash the DNA pellet. The sample was centrifuged at 13000-16000 xg for 2 min, and the ethanol carefully aspirated. The tube was drained on clean absorbent paper and the pellet allowed to air-dry for 10-15 min. Finally 100 µl of DNA rehydration solution was added to the tube and DNA was rehydrated by incubating the solution overnight at 4°C, then the DNA was stored at 2-8°C.

### 2.3.2 *Gram negative bacteria (Salmonella typhimurium $LT_2$; Salmonella paratyphi A, and Desulfovibrio desulfuricans G20)*

The same protocol was used as for Gram negative bacteria except the steps using EDTA, and lytic enzyme addition were excluded.[19]

## 2.4 PCR protocol for isolation of nitroreductases and carboxylesterases

### 2.4.1Nitroreductase genes isolation

Nitroreductase genes; *nfnB* (*Salmonella typhimurium* $LT_2$), *ydjA* (*Salmonella enterica* paratyphi A, ATCC 9150), *Dde_0086* (*Desulfovibrio desulfuricans* G20), and *Dde_2199* (*Desulfovibrio desulfuricans* G20) were isolated using two different PCR kits; (proofstart polymerase[20], and KOD Hotstart polymerase[21]) and pre-designed primers listed in Table 1.

The PCR protocol using proofstart polymerase for *nfnB* isolation, was compiled by putting together 1 µl template DNA, 5 µl ProofStart PCR Buffer, (Owing to a uniquely balanced combination of KCl and $(NH_4)_2SO_4$ proofstart PCR buffer provides stringent primer-annealing conditions over a wider range of annealing temperatures and $Mg^{2+}$ concentrations), 1.5 µl dNTP (deoxynucleoside triphosphates ), 3 µl forward primer, 3 µl reverse primer, 1 µl ProofStart DNA polymerase, 1 µl MgSO4 , and 34.5 µl distilled water.

The PCR thermal cycler Fig. 2.7 was programmed to run in the following manner; initial activation step at 95°C for 5 min followed by 15 cycles of 94°C for 1 min (denaturation), -55-66°C for 1 min (annealing), and 72°C for 2 min (extension), after which the temperature was held at 72°C for 5 min to ensure that any remaining single stranded DNA was completely copied. Twenty cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, were followed with a final extension at 72°C for 10 min, finally the temperature was held at 4°C overnight (The DNA will not be damaged at (2-8°C) after just one night).

**Table 1: Oligonucleotide PCR primers. Underlined and bold letters refer to alteration to create a restriction site; the sequence in red is the restriction site for BamH1 and the sequence in green is the restriction site for HindIII, while blue sequence refers to the restriction site *SacI*.**

| | Bacteria | Gene / locus tag | Primers | Sequence | Restriction |
|---|---|---|---|---|---|
| Bacterial Nitroreductases | *Salmonella typhimurium* $LT_2$ | *nfnb* | Forward | 5`-CCAGGATCCTTTATGGATATCGTTTCTGTCGC-3` | *BamH1* |
| | | | Reverse | 5`-GGGCGTCAAGCTTAGGCAGGGGA-3` | *HindIII* |
| | | | Forward | 5`-CCAGGATCCTGTTGCTGTTGCTGTTGCTTTATGGATATCGTTTCTGTCGC-3` | *BamH1* |
| | *Salmonella enterica* Paratyphi A | *ydjA* | Forward | 5`-GCTGGATCCAATCAAATGGATGCACTA-3` | *BamH1* |
| | | | Reverse | 5`-CAGAAGCTTGACAGTTTTATCCCTCAGCG -3` | *HindIII* |
| | | | Forward | 5`-GCTGGATCCTGTTGCTGTTGCTGTTGCAATCAAATGGATGCACTA-3` | *BamH1* |
| | *Desulfovibrio desulfuricans* G20 | *Dde_0086* | Forward | 5`-AGGGGATCCGTCATGCATAGC-3` | *BamH1* |
| | | | Reverse | 5`-GGAAAGCTTCATTGCTGCCGCACG-3` | *HindIII* |
| | | | Forward | 5`- AGGGGATCCTGTTGCTGTTGCTGTTGCGTCATGCATAGC -3` | *BamH1* |
| | *Desulfovibrio desulfuricans* G20 | *Dde_2199* | Forward | 5`-ACGGGATCCGCCATGAATGAGAC -3` | *BamH1* |
| | | | Reverse | 5`-CGTCATTGAAGCTTGTCTTTGCGGCAGA -3` | *HindIII* |
| | | | Forward | 5`- ACGGGATCCTGTTGCTGTTGCTGTTGCGCCATGAATGAGAC -3` | *BamH1* |
| Bacterial Carboxylesterases | *Bacillus subtilis* strain 168 | *pnbA* | Forward | 5`-AAGGGATCCAACACAATGACTCATCAAATAG -3` | *BamH1* |
| | | | Reverse | 5`-CAGCACAAGCTTATCCTGTTTTCCCCA -3` | *HindIII* |
| | | | Forward | 5`- AAGGGATCCTGTTGCTGTTGCTGTTGCAACACAATGACTCATCAAATAG -3` | *BamH1* |
| | *Bacillus licheniformis* ATCC 14580 | *pnbA* | Forward | 5`-AAGGGATCCAATAAAATGTATGATACAACTGTCGAA -3` | *BamH1* |
| | | | Reverse | 5`- GGCGAGCTCCACAGGTCAGGCCCG-3` | *SacI* |
| | | | Forward | 5`- AAGGGATCCTGTTGCTGTTGCTGTTGCAATAAAATGTAT GATACAACTGTCGAA -3` | *BamH1* |

**Figure 2.7: PCR thermal cycler**

KOD Hotstart polymerase was used for *ydjA*, *Dde_0086*, and *Dde_2199* isolation, the 50 µl reaction mixture contained 5 µl, 10x KOD Hotstart buffer, 2 µl template DNA, 2 µl dNTP (deoxynucleoside triphosphates), 2 µl forward primer, 2 µl reverse primer, 1 µl KOD Hotstart DNA polymerase, 2 µl MgSO4, and 34 µl distilled water.

Amplification was 95°C for 3 min (initial activation) followed by 15 cycles of 95°C for 30s (denaturation), (66-56°C for *ydjA*, and 68-58°C for *Dde_0086*, and *Dde_2199*) for 30s (annealing), 75°C for 2 min (extension), after which the temperature was held at 75°C for 2 min, and 20 cycles of 95°C for 30s, (56°C for *ydjA*, and 58°C for *Dde_0086*, and *Dde_2199*) for 30s, 75°C for 2 min were followed with a final extension at 75°C for 2 min, finally the temperature was held at 4°C overnight.

### 2.4.2 Carboxylesterase genes isolation

Carboxylesterase genes *pnbA* from *Bacillus subtilis*, and *pnbA* from *Bacillus licheniformis* were amplified by Phusion™ High-Fidelty DNA Polymerase.[22] The PCR solution comprised of 10 µl 5x Phusion HF buffer, 1 µl template DNA, 1.5 µl forward primer, 1.5 µl reverse primer, 1 µl from 10mM dNTP, 0.5 µl Phusion DNA polymerase, and 34.5 µl distilled water.

The PCR program consisted of two steps; 15 cycles of 98°C for 30s (initial denaturation), 98°C for 10s (denaturation), 64-54°C for 30s (annealing), 72°C for 30s

(extension) followed by 72°C for 10 min (final extension) were used for the first step, while the second step included 20 cycles of 98°C for 10s, 54°C for 30s, 72°C for 30s followed by 72°C for 10 min, finally the temperature was lowered to 4°C overnight.

The success of the experiments was confirmed by 0.7% agarose gel electrophoresis, which consists of 0.7 g agarose and 100 ml of 1x Tris-borate-ethylenediaminetetra-acetic acid (TBE) in buffer pH 8.2, 2 µl Ethidium bromide was added to assist with analysis of the gel, and 200 ml of 1x (TBE) was used as a running buffer.

## 2.5 Purification of PCR products

The PCR products *(nitroreductases and carboxylesterases)* were purified from primers, nucleotides, polymerase, and salt using a QIAquick® PCR purification kit protocol.[23] This was achieved by adding 5 volume of buffer PB to 1 volume of PCR sample, the resulting sample was applied to the QIAquick spin column with a provided 2 ml collecting tube and centrifuged for 1 min, the flow through discarded and the spin column placed back into the collecting tube. Buffer PB allows the efficient binding of single or double stranded PCR product and the quantitative 99.5% removal of primers. Unbound molecules were washed away by adding 0.75 ml the ethanol-containing buffer PE to the spin column and centrifuged for 1 min, any residual ethanol from buffer PE, which may interfere with subsequent enzymatic reactions, was removed by an additional centrifugation for 1 min. DNA was eluted with 30 µl of purified water added to the centre of spin column, leave stand for 1 min, and centrifuged for 1 min.

## 2.6 Ligation of the gene of interest into the plasmid pET 28a (+)

### 2.6.1 Nitroreductase ligation

The purified PCR product (14 µl) was mixed with the appropriate restriction enzymes (2 µl of each), and 2 µl, 10x buffer solution. A separate mixture was made up of 2 µl of an over-expression plasmid pET-28a(+) (Appendix 1) (The pET-28a(+)carries a kanamycin (antibiotic) resistant gene and 6-histidine tag ($His_6$) at the N-terminal configuration, which are used for purification), the appropriate restriction enzymes (1 µl of each), buffer solution (1 µl, 10x), and purified water (5 µl), the two solutions were allowed to digest at 37°C for

45 min, later mixed, purified in accordance with the purification protocol (section 2.4), and vacuum dried for 40 min. The product was re-dissolved in 8 µl purified water, 1 µl ligase, and 1 µl ligase 10x buffer solution, which contains adenosine triphosphate (ATP) and $Mg^{2+}$, to promote the joining of the DNA, then allowed to ligate at 16°C overnight.

### 2.6.2 Carboxylesterase ligation

The purified PCR product (30 µl) which included restriction enzyme sites was vacuum dried for 40 min, then the resultant pellet mixed with the suitable restriction enzymes (1 µl of each), 1 µl ,10x buffer solution, and 7 µl purified water . In a separate 1.5 ml microcentrifuge tube, 2 µl of the plasmid vector pET-28a(+), appropriate restriction enzymes (1 µl of each), 1 µl, 10x buffer solution , and 5 µl purified water, were mixed, the solutions incubated at 37 °C for 1h, then subjected to agarose gel electrophoresis 90 mA for 40 min, both the digested PCR products and plasmid vector were gel extracted into the same1.5 ml microcentrifuge tube , purified, and vacuum dried for 40 min later re-dissolved in 8 µl purified water, 1 µl ligase, and 1 µl, 10x ligase buffer solution, finally incubated at 16°C overnight for ligation.

## 2.7 Competent cell preparation

Competent cells of *E. coli* strains DH5α and Rosetta were prepared according to the method of Sambrook *et al*[24] A single colony was selected from a fresh LB plate and incubated into 10 ml LB liquid medium, grown overnight at 37°C and sub-cultured (1:100) into a final volume. The sub-culture was incubated at 37°C to an optical density ($O.D.550_{nm}$) of 0.35 was achieved. The cells were chilled on ice for 5 min then centrifuged at 8000 rpm for 5 min at 4°C. The supernatant was removed and the cell pellet was resuspended in 2/5 volumes of competent cell buffer (1): Potassium acetate (30 mM), Rubidium chloride (100 mM), Calcium chloride (10 mM), Manganese chloride (50 mM), and glycerol 15% (v/v). The resuspended cells were chilled on ice for 5 min then centrifuged as before. The supernatant was removed and the cell pellet was resuspended in competent cell buffer (2): MOPS (10 mM), Calcium chloride (75 mM), Rubidium chloride (10 mM), and glycerol 15% (v/v). After a 15 min period on ice the cells were aliquoted (200 µl) into pre-chilled microcentrifuge tubes and stored at -80°C.

## 2.8 Transformation of recombinant plasmid into the *E. coli* DH5α

### 2.8.1 *Recombinant plasmid harbouring nitroreductase gene*

1 µl EcoR1, and 1 µl of 10x buffer solution were added to the ligation mixture (recombinant plasmid) in a suicide cut approach. This eliminates all ligation products that are not recircularised only circularised plasmids will survive in the bacteria. The mixture allowed digesting at 37°C for 1 h, then 200 µl *E coli* DH5α competent cells, an efficient strain of *E. coli* for plasmid continuance pipette into ligation mixture. In order to transform the recombinant plasmid into the competent cells, the mixture was placed on ice for 30 min, to enable the plasmid to stick on the outside of the membrane, then heated to 42°C for exactly 50 sec, to shock the plasmid inside the cell, and then replaced on ice for 2 min. 500 µl of Luria-Bertani broth (LB) was pipetted into the mixture, incubated at 37°C for 45 min, and then plated onto nutrient plates with antibiotic selection overnight at 37°C or until colonies are of the desired size.

### 2.8.2 *Recombinant plasmid harbouring carboxylesterase gene*

200 µl of *E coli* DH5α competent cells was added to the 10 µl ligation mixture, the mixture was then left on ice for 30 min and then heated to 42°C for 50s. The mixture was then returned to ice for 2 min, and subsequently incubated at 37°C for 45 min following 500 µl of LB addition, then plated onto nutrient plates overnight at 37°C.

## 2.9 Screening the colonies

The transformed *E. coli* culture was then spread onto the LB agar (sensitive test agar) supplemented with 50 µg/ml kanamycin in a range of different quantities; 75 µl, 175 µl, 200 µl, and 250 µl. This medium will only allow bacteria carrying the recombinant plasmid to grow, as a kanamycin resistance gene is an integral part of pET 28a(+). Following incubation overnight at 37°C, single colonies where transferred to liquid LB medium (5 ml) containing 50 µg/ml kanamycin, and allowed to incubate overnight at 37°C to further ensure only the resistant cells were grown.

## 2.10 Purification of the high-copy plasmids

The high-copy recombinant plasmids from the 5 ml overnight *E coli* culture in LB medium were isolated after harvesting the cells as a pellet following centrifugation at (13000 rpm) for (1 min) and purified using QIAprep® spin Miniprep kit.[25]

Bacterial cell pellet was re-suspended in 250 µl buffer P1 RNaseA, 250 µl buffer P2 was added and the tubes were mixed thoroughly by inverting 4–6 times, and leaving to stand for 1 min. Then 350 µl buffer N3 was added and the tubes were mixed immediately and thoroughly by inverting 4–6 times, and again leaving stand for 1 min. The high salt concentration of buffer N3 causes denatured proteins, chromosomal DNA, and cellular debris to precipitate, while the smaller plasmid DNA renatures correctly and stays in solution. The resulting solution was then centrifuged for 10 min and a compact white pellet was formed. The supernatant was then decanted to a spin column and centrifuged for 1 min. The flow-through was discarded. The spin column was then washed with 500 µl buffer PB and centrifuged for 1 min. The flow-through was again discarded. Next, the spin column was washed again with 750 µl buffer PE and centrifuged for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min to remove residual wash buffer. The spin column was then placed in a clean 1.5 ml micro-centrifuge tube, and the DNA was eluted by adding 50 µl buffer EB (10 mM Tris·Cl, pH 8.5) to the centre of the spin column, let stand for 1 min and centrifuged for 1 min.

To confirm the success of the ligation, the ligation mixture was digested with the appropriate restriction enzymes (1 µl each) and subjected to agarose gel electrophoresis, allowing the length in base pairs of both the cloned DNA and the original plasmid to be determined.

## 2.11 PCR protocol for the incorporation of the Cys tag sequences

Using purified His-tagged plasmids as the template DNA; the protocols for PCR were repeated using primers (Forward primers) designed to containing six adjacent codons for $Cys_6$ between the $His_6$-tag determined by pET28a (+) and the start codon of the enzymes (NTR and CE).

The same protocols were used for cloning the modified gene, transforming it into *E. coli* DH5α, and for the expression, and purification.

### 2.12 Transformation into the *E. coli Rosetta* strain

2 μl of recombinant plasmids were added into 200 μl competent cells of the *Rosetta* strain of *E. coli* (an efficient bacterium for the expression of heterologous genes), and transformed in the way described previously (section 2.7). The resulting culture was mixed with 500 μl LB medium, incubated at 37°C for 45 min, and then applied to the Petri dishes and allowed to grow at 37°C overnight.

### 2.13 Expression of enzymes

After the overnight incubation the transformed *Rosetta* cells were picked and transferred to a mixture of LB media (5 ml) and 50 μg/ml kanamycin, then grown overnight. The 5 ml of solution was mixed with 500 ml of LB solution and 50 μg/ml kanamycin. The bacteria were grown at 37°C on a rotary shaker, when cultures reached an optical density ($O.D._{600nm}$) of 0.6, expression was induced by the addition of IPTG (2 ml, 0.1 M). Incubation was allowed to continue for an additional 4 hours.

The cells were harvested by centrifuging (8000 rpm for 10 min) and the pellets were re-suspended in 10 ml, 10 mM imidazole solution; consisting of phosphate buffer (PB) (pH 7.4, 6.25 ml, 0.1 M), and imidazole (2M, 0.25 ml), made up to 50 ml with distilled water. The resulting suspensions were then sonicated four times for 30s, and centrifuged (35000 rpm, 5°C, for 45 min). The supernatant was filtered through a 0.4 μm filter to remove any residual particles prior to loading the sample onto the column.

### 2.14 Purification of nitroreductase enzyme

His-tags are often used for affinity purification of His-tagged recombinant proteins expressed in *Escherichia coli*[26] and other prokaryotic expression systems. Nickel-agarose column was used to purify the His-tagged protein, which has affinity toward nickel ions $Ni^{2+}$ that immobilized in a resin, which is packed in a column (HiTrap chelating column). The protein will bind the resin, since it is the only component with a His-tag. The protein was released from the column by eluting it with different concentration of imidazole,[27] (Table 2) which competes with the His-tags for nickel binding.

**Table 2: Preparation of imidazole solutions used to purify NTRs and CEs from the nickel column.**

| Imidazole concentrations | PB 8x stock solution pH 7.4 | Imidazole (2M) | Filtered distilled water |
|---|---|---|---|
| 10 mM | 3.0 ml | 0.12ml | 20.88ml |
| 50 mM | 1.0 ml | 0.20ml | 6.80ml |
| 100 mM | 1.0 ml | 0.40ml | 6.60ml |
| 150 mM | 1.0 ml | 0.60ml | 6.40ml |
| 200 mM | 1.0 ml | 0.80ml | 6.20ml |
| 300 mM | 1.0 ml | 1.20ml | 5.80ml |
| 500 mM | 1.0 ml | 2.00ml | 5.00ml |

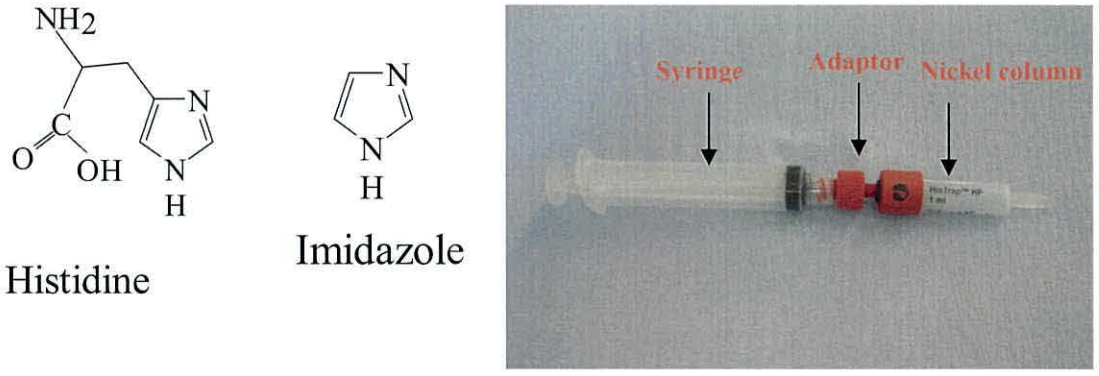


**Figure 2.8: The chemical structure of both Imidazole and histidine (left) and a photograph of nickel column (right)**

The HiTrap chelating nickel column was connected through a Luer adaptor to a syringe used for solutions loading. The ethanol preservative in the pre-packed column was washed out with distilled water (5 ml) at ~ 1 drop/sec. The column was then loaded with nickel salt solution ($NiSO_4$, 0.5 ml, 0.1 M), followed by a further wash with filtered distilled water (5 ml). The column was then equilibrated with binding buffer (10 ml), the samples were then applied, and the flow-through collected to check on gel. The column was then washed again with binding buffer (10 ml) and the flow-through collected. Increasing concentrations of imidazole (50 mM-500 mM) were passed through the column and collected. Finally, the column was washed with the remaining binding buffer (5 ml) and stored below 5 $^{\circ}$C ready for reuse.

The second ml of each elute was subjected to SDS-PAGE to check that the protein was overproduced and that its molecular weight was as expected.

## 2.15 Removal of imidazole from the protein

The purified enzymes loaded into a PD-10 desalting column in order to remove imidazole. PD-10 Desalting Columns contain Sephadex™ G-25 Medium, which allows rapid group separation of high molecular weight substances (protein) from low molecular weight substances (salt). The column top cap removed, the bottom cap cut off and the excess liquid poured off. The column equilibrated with 25 ml, 50 mM, pH7.2phosphate buffer, and the flow-through discarded. 2.5 ml purified enzymes (nitroreductases, and carboxylesterases) loaded into the column, and the flow-through discarded, then the sample eluted with 3.5 ml phosphate buffer.[28]

## 2.16 Preparation for the protein gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most powerful technique used in molecular biology for separating protein mixtures according to their mass: charge ratio. The ionic detergent (SDS) denatures the quaternary, tertiary, and secondary structure of the protein to generate a linear polypeptide chain covered with negatively charged SDS molecules, while mercaptoethanol (β ME) aids the protein denaturation by reducing all disulfide bonds.

To prepare 12% acrylamide, SDS-PAGE the glass plates were cleaned with industrial methylated spirits (IMS) and assembled into gel casting stand. The resolving gel was prepared with doubly de-ionised (DDI) $H_2O$ (3.4 ml), degassed acrylamide (30%, 4.0 ml), Tris-buffer (pH 8.8, 1.5M, 2.5 ml), and SDS (10%, 100 µl), and the stacking gel was prepared in the same way except that the pH of the Tris- buffer was 6.8. When the gel is ready to be polymerized, ammonium persulfate (APS) (10%, 100 µl) and N,N,N',N'-tetramethylethylene diamine (10 µl) were added, initiating gel polymerization. First the resolving gel was poured until it was 1 cm from the top, then water-saturated butanol (500 µl) overlaid the top of the gel to prevent the gel from contact with the air and subsequent crust formation. When the resolving gel was polymerized, the butanol was washed away and the excess distilled water drained with strips of filter paper, the staking gel was poured onto the resolving gel, and a comb was placed into the gel sandwich. When the staking gel was polymerized the comb was removed. The protein samples were mixed

with loading buffer, made up with: electrophoresis buffer (1.0 ml), glycerol (3.0 ml), 0.5% bromophenol blue (0.2 ml), (β ME) and deionised water (5.8 ml), and then heated to 95 °C for 5 min to denature the protein disulfide bond. These samples were then loaded onto the gel well, which was run at 150 V for (1) hour in an electrolyte solution of 1 x SDS made up with (Tris-HCl 3.01 g, glycine 14.41 g, SDS 1 g) made up to 1 L with distilled water. The gel was then stained with Coomassie solution for 30 min made up with Coomassie Blue (1.25 g), methanol (500 ml), and acetic acid (100 ml), made up to 1 L with distilled water. The gel was then de-stained with a mixture of acetic acid (50 ml) and industrial methylated spirits (IMS) (100 ml), made up to 500 ml with distilled water.

## 2.17 Enzyme assays

### 2.17.1 Nitroreductases

Nitroreductase enzymes (NfnB; YdjA; Dde_0086, and Dde_2199) activities were spectrophotometrically determined using a wavelength scan over 250-500 nm, and monitoring the disappearance of NADPH at 340 nm. The reaction mixture contained 500 µl, 0.1 M phosphate buffer pH 7.0, 25 µl, 4 mM NADPH, 10 µl of cell extract in a final volume of 1 ml made up with distilled water and the reaction was initiated by adding 20 µl, 40 mM Dinitroethylbenzene (DNEB) . A baseline run that was absent of enzyme was carried out to test the analysis, and a total of 9 runs performed to characterise the progressive oxidation of the NADH.

### 2.17.2 Carboxylesterases

To demonstrate the effectiveness of the carboxylesterases (*Bacillus subtilis* PnbA, and *Bacillus licheniformis* PnbA) a hydrolysis experiments were carried out using a wavelength scan over 300-550 nm. The reaction mixture contained 0.1 M phosphate buffer pH 8.0, *P*-nitrophenylacetate 20µl, 1mM, and 10µl of cell extract in a final volume of 1 ml. The increase in absorbance around 400 nm as the production of p-nitrophenol was monitored.

## 2.18 Protein concentration

The protein concentration was calculated according to Bovine Serum Albumin (BSA) standard calibration curve. Biuret protein assay reagent was used, and the UV-visible

spectroscopy measurements were taken at 550 nm. Four runs were made for 100 µl of protein.

### 2.19 Specific activity

The specific activity of NTRs was assessed by calculating the rates at different concentrations of analyte in association with the different NTRs concentrations.

### 2.20 Kinetic parameters ($K_m$ and $V_{max}$ values)

In order to calculate the **$K_m$ and $V_{max}$** values, for nitroreductase enzymes activity (µmoles/min/mg) was measured at increasing concentrations of analyte (DNEB for NfnB, and YdjA. 1,2-dinitrobenzene for Dde_0086. 3NBZ for Dde_2199), and the resulting data was analysed by direct linear regression *via* a software package (*Enzpack*).

### 2.21 Stoichiometry

The NADH concentration used in the assay was 200 µM to allow a wider range of concentrations; the reaction has to go through three steps to reach the amine, using one NADH for each step (Fig 2.9) hence allowing the stoichiometry of the reaction to be calculated.

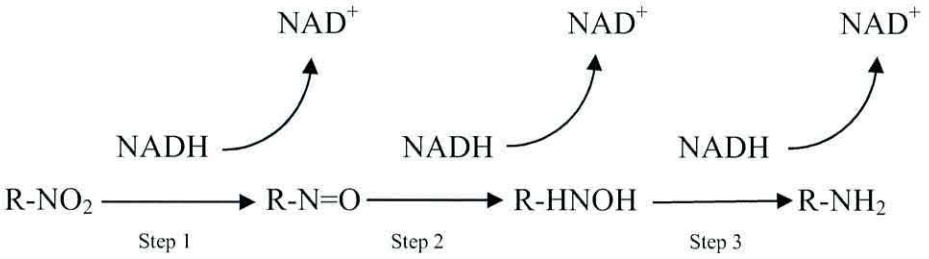


**Figure 2.9: Reduction of nitro-compound to amine through NADH oxidation**

### 2.22 pH and temperature dependence

#### 2.22.1 Nitroreductases

The effect of pH and temperature on the activity of NTR was determined. Assays were carried out spectrophotometrically using a wavelength scan over 250-500 nm for 10 min at a series of different pH (50mM sodium citrate for pH range 5-6, 50 mM phosphate buffer for pH range 7-9, 50 mM CAPS for pH range 10-11) and different temperature values (25°C - 70°C) in 50 mM, pH 7.0 phosphate buffer.

### 2.22.2 Carboxylesterases

The optimum temperature and pH effect on carboxylesterases activity were established at 400 nm for 1 min at a series of different pH (50 mM sodium citrate for pH range 5-6, 50 mM phosphate buffer for pH range 7-9, 50 mM CAPS for pH range 10-11) and different temperature values (25°C –60°C ) in 50 mM , pH 8.0 phosphate buffer.

### 2.23 HPLC condition for quantifying cocaine and benzoylecgonine

Carboxylesterases ability to hydrolyze cocaine was measured by quantifying the amount of benzoylecgonine produced from cocaine by Agilent 1100 series HPLC system. The mobile phase was Isocratic 50% Solvent A: $NaH_2PO_4$ buffer 80mM pH5.5 made up in HPLC grade water, and 50% Solvent B: Acetonitrile. The column used was Phenomenex polar Rp Synergi 4 µm 80 A 250 x 2.0mm, Detector was: UV 320nm, Temperature: 30°C. The flow rate was 0.3ml/min, run time per sample was 15 min, and the injection volume was 5 µl. The standard assay conditions were 100 mM MOPS at pH 7.0, with 2 mM cocaine in a total volume of 1 ml at room temperature. The reaction mixture contained 2 mM cocaine and 20 µl cell extract in 1 ml of 50 mM MOPS buffer, pH 7.0. at room temperature, aliquots were removed at intervals (0 min, 6 hours, 12 hours, and 20 hours). All assay results were compared with those from control assays containing no extract.

### 2.24 Nucleotide sequence accession number

The Genbank accession numbers for the nucleotide sequence of bacterial nitroreductases *nfnb* from *Salmonella typhimurium* $LT_2$; *ydjA* from *Salmonella enterica paratyphi* A (ATCC) 9150, and the complete genome sequences of (*Dde_0086*, and *Dde_2199*) from *Desulfovibrio desulfuricans G20* are AE008722, CP000026, and NC_007519, while the Genbank accession numbers for the nucleotide sequence of bacterial carboxylesterases *pnbA* from *Bacillus subtilis* strain 168, and *pnbA* from *Bacillus licheniformis* ATCC 14580 are NP_391319, and YP_077866.

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# Chapter 3- Bacterial nitroreductases and explosives: Results and discussion

## 3.1 Introduction

Nitroreductases are enzymes that can be characterized by their ability to catalyse the reduction of a wide range of nitro-aromatic compounds. Almost all bacterial nitroreductases occur as a homodimeric flavoprotein that contains one FMN per monomer as a cofactor, and use NAD(P)H as electron donor (Fig. 3.1).

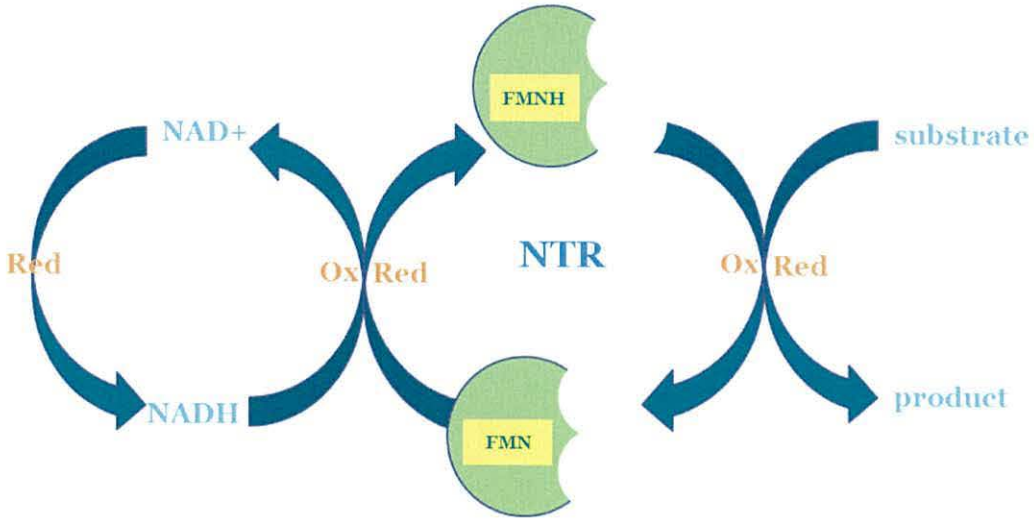


**Figure 3.1: A model of nitroreductase showing the electron transfer steps involved in the reduction of a substrate.**

The ability of NTR to reduce and consequently detect nitro-aromatic compounds, makes them perfect for use in biosensors. The enzyme must first be genetically modified, to be integrated on the surface of the electrode. To enable the enzyme to directly attach to the electrode, cysteine amino acids (cys-tag) are attached to the N-terminus of the enzyme. This allows the enzyme to be in direct contact with the electrode *via* the sulphur molecules on the (cys-tags) bonding to the gold surface; thus forming strong gold thiol bonds, as seen in (Fig. 3.2).

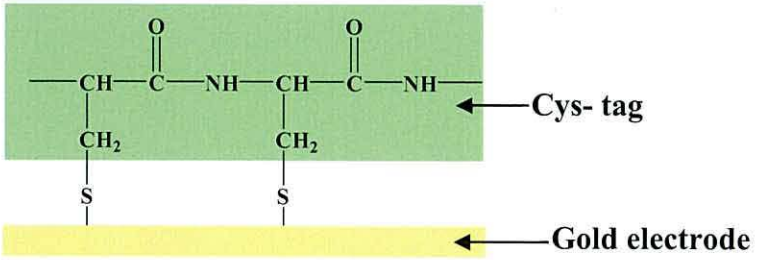


**Figure 3.2: Diagram showing how the cys tags bond to the gold surface of an electrode.**

This chapter describes the isolation of four different bacterial nitroreductases in order to characterize their ability to reduce nitro-compounds, selected for their potential as explosives. These enzymes will subsequently be used as sensor biological recognition elements for explosives detection. PCR was used to isolate the genes of interest, which were then cloned into vector pET 28a(+). After expression using Rosetta *E.coli* cells, the proteins were purified using a high affinity nickel column. The activity of the enzymes was investigated and their activities characterised using a range of substrates containing nitro functional groups. Different bacterial nitroreductases have been chosen (Table 3.1) to increase the possibility of obtaining NTR with a variety of reaction pathways and rates, and hence enable the biosensor to identify and detect nitroaromatics, *via* the simultaneous use of more than one NTR at the sensor surface. A search of microbial genomes yielded many strains which possessed homologues of *nfnB* and a selection was taken due to a range in their similarity.

**Table: 3.1 Amino acid alignment of bacterial nitroreductases**

| Seq | NfnBK12 | NfnBLT2 | YdjA | Dde_0086 | Dde_2199 |
|---|---|---|---|---|---|
| NfnBK12 | **100** | **0.88** | **0.16** | **0.51** | **0.17** |
| NfnBLT2 | | **100** | **0.15** | **0.53** | **0.18** |
| YdjA | | | **100** | **0.14** | **0.13** |
| Dde_0086 | | | | **100** | **0.22** |
| Dde_2199 | | | | | **100** |

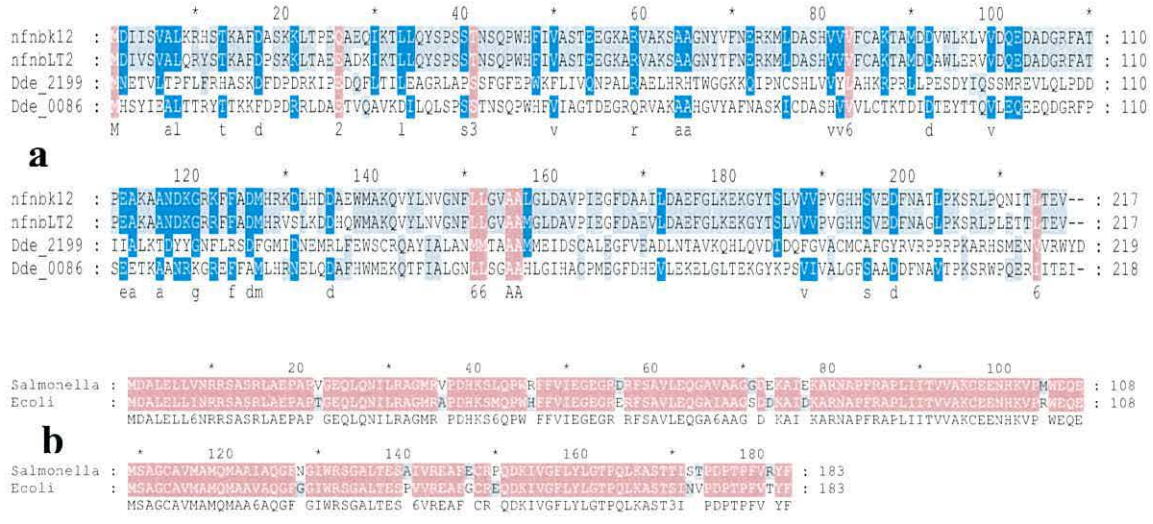


**Figure 3.3: Amino acid alignment of bacterial nitroreductase a: NfnB k12 (line 1) and homologues from *Salmonella typhimurium* $LT_2$ (NfnB), *Desulfovibrio desulfuricans* G20 (Dde_0086 and Dde_2199) b: *Salmonella enteric paratyphi* A (YdjA), and *E coli* (Ec_YdjA), Shading indicates amino acid conservation of 100% (pink), 80% (blue), 60% (grey)**

### 3.2 PCR amplification of bacterial nitroreductases

Genomic DNA was extracted from *Salmonella typhimurium* $LT_2$, *Salmonella enterica paratyphi* A, ATCC 9150, *Desulfovibrio desulfuricans* G20 and PCR amplified using proofstart polymerase (QIGEN) and KOD Hotstart DNA polymerase (Novagen) to isolate four different recombinant and engineered bacterial NTRs (*nfnB* from *Salmonella typhimurium* $LT_2$, *ydjA* from *Salmonella enterica paratyphi* A, ATCC 9150, and *Dde_0086*, and *Dde_2199* from *Desulfovibrio desulfuricans* G20 ), using pre-designed primers (Table 1, Chapter 2).

The primers were designed to anneal to regions at either side of the NTR gene; the primers created restriction sites at either end of the gene to allow its insertion into the plasmid vector.

The first PCR experiment (Fig. 3.4) resulted in the isolation of recombinant NTRs (*nfnB*, *ydjA*, *Dde_0086*, and *Dde_2199*) having a *BamH1* restriction site at the 5` end direction and a *HindIII* restriction site at the 3` end direction.

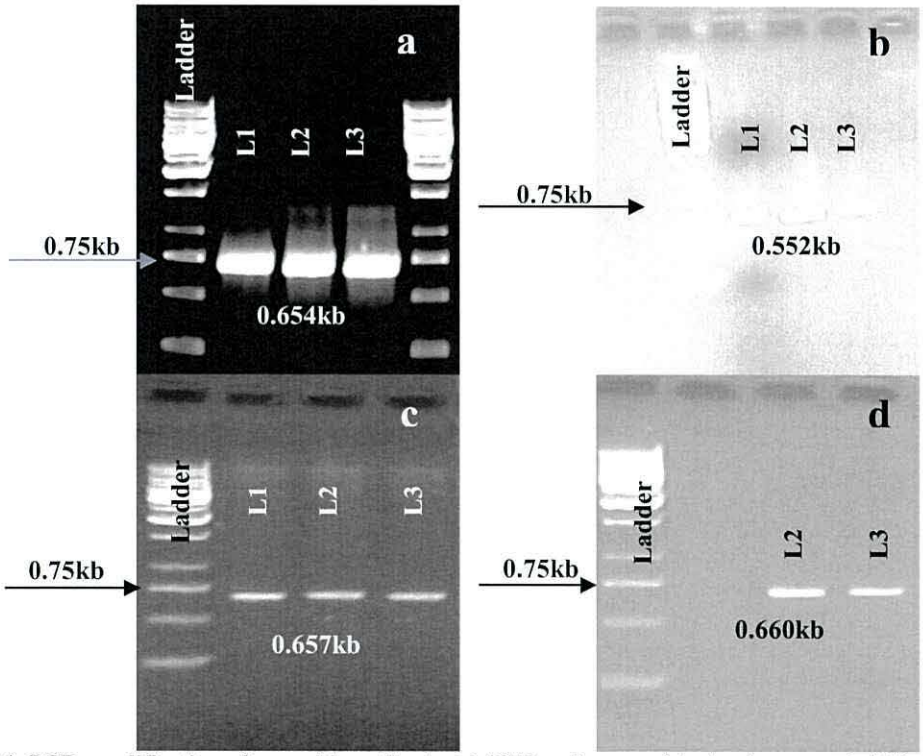


**Figure 3.4: PCR amplification of recombinant bacterial NTRs using proofstart polymerase (*nfnB*) and KOD Hotstart polymerase (*ydjA*, *Dde_0086*, and *Dde_2199*) with pre-designed primers. a: *nfnB* (0.654 kb), b: *ydjA* (0.552kb), c: *Dde_0086* (0.657kb), d: *Dde_2199* (0.660 kb) with forward primer containing BamH1 restriction site and reverse primer containing HindIII restriction site. Ladder: 10 kb. L= lane.**

The second PCR experiment (Fig 3.5) resulted in the isolation of engineered NTRs having six adjacent codons for cysteine between a *BamH1* restriction site and the start codon of the NTR in the 5`end direction and a *HindIII* restriction site in the 3`end direction.

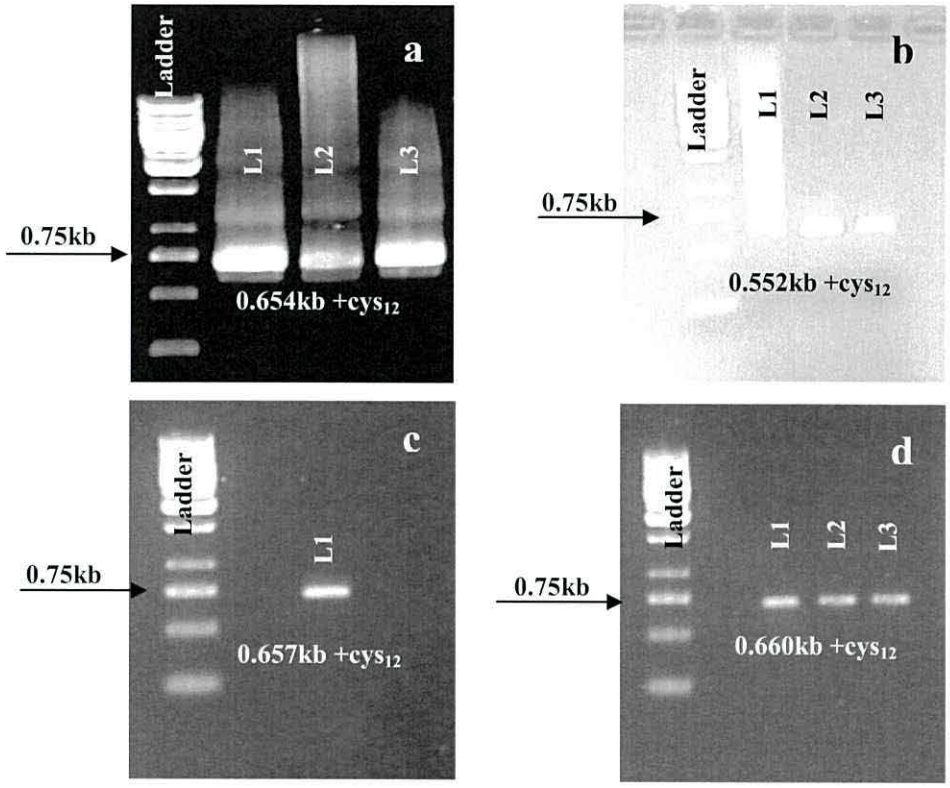


**Figure 3.5: PCR amplification of engineered bacterial NTRs (NTRs-cys$_{12}$) using proofstart polymerase (*nfnB*) and KOD Hotstart polymerase (*ydjA*, *Dde_0086*, and *Dde_21999*) with pre-designed primers. a: *nfnB* (0.654 kb + cys$_{12}$), b: *ydjA* (0.552kb + cys$_{12}$), c: *Dde_0086* (0.657kb + cys$_{12}$), d: *Dde_2199* (0.660 kb + cys$_{12}$) with forward primer containing BamH1 restriction site and reverse primer containing HindIII restriction site. Ladder: 10 kb.**

### 3.3 Ligation of recombinant and engineered bacterial nitroreductases into pET28a(+) vector

Attempts were made to ligate NTRs (*nfnB*, *ydjA*, *Dde_0086*, and *Dde_2199* with *BamH1* and *HindIII*) and engineered NTRs (*nfnB*-cys$_{12}$, *ydjA*-cys$_{12}$, *Dde_0086*-cys$_{12}$, and *Dde_2199*-cys$_{12}$ with *BamH1*and six adjacent codons for cysteine per monomer, and *HindIII*) in a different ligation process into pET28a(+). After an overnight ligation, the plasmid vector was transformed into competent cells of *E. coli* DH5α. A suicide cut with *EcoR1* was used to remove the plasmids that had re-ligated (without insertion). The cells were then grown using kanamycin (50 µg/ml) resistance agar plates (*pET28a(+) carries a kanamycin resistance gene*), thus only cells which have taken up the pET28a(+) vector will

grow successfully. DH5α strains of *E.coli* are more useful for transformation since the number of the transformants obtained is far higher than *E.coli* Rosetta. It has been postulated that an additional layer of the envelope may play an important role comparable to that of the outer membrane.[1] The chemical composition of the peptidoglycan of a highly efficient transformant *E.coli* strain DH5α was analyzed in comparison with a normal and poorly transformant *E.coli* strains revealing a simpler peptidoglycan chemical composition in the DH5α strain. This may be responsible for the simpler architecture of the peptidoglycan which, in turn, may interfere less with the passage of the DNA across the bacterial envelope.[1]

In order to confirm that the cloning vector contained the genes of interest (*nfnB*, *ydjA*, *Dde_0086*, and *Dde_2199*), they were isolated and the genes restricted with *BamH1* and *HindIII*. The resulting fragments were separated using agarose gel electrophoresis. pET28a(+) has *BamH1* and *HindIII* restriction site in the multi-cloning region, therefore a successful transformation when run on a gel would produce two fragments, a 5.3 kb fragment which corresponds to the pET28a(+) and ≈ a (0.552-0.660 kb) fragment which corresponds to NTRs genes. (Fig. 3.6) shows the success of the ligation for recombinant NTRs.

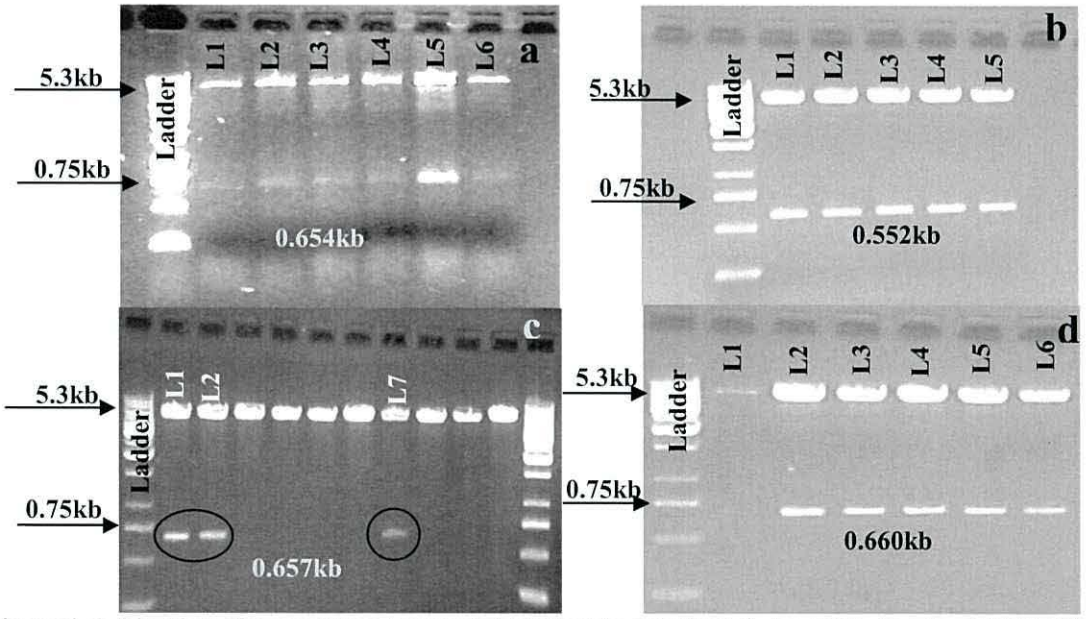


**Figure 3.6: Ligation of recombinant bacterial NTRs into pET28a(+). {a: 5.3kb bands=pET28a(+) vector, 0.654kb bands= *nfnB*. b: 5.3kb bands=pET28a(+) vector, 0.552kb bands= *ydjA*. c: 5.3kb bands=pET28a(+) vector, 0.657kb bands= Dde_0086. d: 5.3kb bands=pET28a(+) vector, 0.660kb bands= Dde_2199} with forward and reverse primers. Ladder =10kb marker.**

The same approach was used to confirm that the plasmid vector pET 28a(+) contained the engineered NTRs (*nfnB*-$cys_{12}$, *ydjA*-$cys_{12}$, *Dde_0086*-$cys_{12}$, and *Dde_2199*-$cys_{12}$). After isolation of plasmids, the plasmid DNA was digested with *BamH1* and *HindIII*. Running the resulting fragments on agarose gel, and estimation of the expected sizes of the fragments (Fig. 3.7) showed the success of the ligation for engineered NTRs.

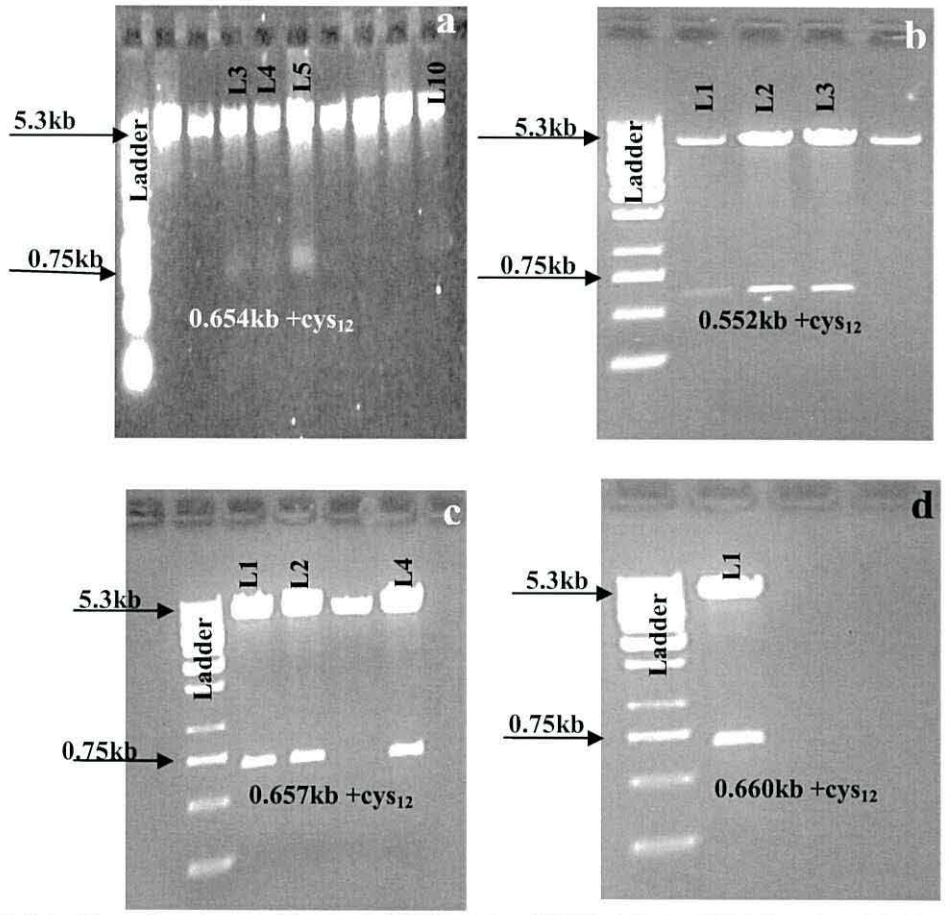


**Figure 3.7: Ligation of engineered bacterial NTRs into pET28a(+). {a: 5.3kb bands=pET28a(+) vector, 0.654kb + $cys_{12}$ bands= *nfnB*-$cys_{12}$. b: 5.3kb bands=pET28a(+) vector, 0.552kb + $cys_{12}$ bands= *ydjA*-$cys_{12}$. c: 5.3kb bands=pET28a(+) vector, 0.657kb + $cys_{12}$ bands= *Dde_0086*-$cys_{12}$. d: 5.3kb bands=pET28a(+) vector, 0.660kb + $cys_{12}$ bands= *Dde_2199*-$cys_{12}$} with forward and reverse primers. Ladder =10kb marker.**

### 3.4 Overproduction of nitroreductase enzymes

In order to overproduce the enzyme, the pET28a(+) vector containing the gene of interest was transformed into Rosetta *E. coli*. This vector contains all of the genetic coding necessary to produce the protein, including a promoter appropriate to the host cell (T7 promoter which is specific to only T7 RNA polymerase, not bacterial RNA polymerase, and also does not occur anywhere in the prokaryotic genome), a *lac* operator which can block transcription, a sequence which codes for ribosome binding, and a sequence which terminates transcription. It is also contains a *lacI* gene (lac repressor protein) . Both the T7 promoter and the *lac* operator are located 5` to the inserted gene, since RNA growth (transcription) is always in the 5`to 3` direction. The T7 RNA polymerase gene was integrated into the *E. coli* host genome, and constructed such that it was under the control of *lac* promoter and operator. The *E. coli* host genome also carries the *lacI* gene (The lac repressor acts both at the lac promoter in the host chromosome to repress transcription of the T7 RNA polymerase gene by the host polymerase and at the T7 lac promoter in the vector to block transcription of the target gene by the T7 RNA polymerase). Once the IPTG (an analogue of lactose) was added to the growing culture, the transcription of T7 RNA polymerase was induced. The T7 RNA polymerase gene on the host cell chromosome has an inducible promoter which is activated by IPTG. This molecule displaces the repressor from the *lac* operator. Since there are *lac* operators on both the gene encoding T7 RNA polymerase, and the target gene, IPTG activates both genes. Therefore, when IPTG is added to the cell, the T7 RNA polymerase is expressed, and quickly begins to transcribe the gene of interest which is then translated as a nitroreductase enzyme. Because T7 is a viral promoter, it transcribes rapidly and profusely for as long as the T7 RNA polymerase is present. The expression of nitroreductase enzyme increased rapidly as the amount of mRNA transcribed from gene of interest increased. Within 3-4 hours the target protein can accumulate to greater than 30-40% of the total cell protein.[2,3,4,5]

The pET-28a(+) carries a ($His_6$) tag at the N-terminal configuration, which is used for purification. The desired gene was inserted into a polylinker site which is downstream to the ($His_6$) tag of the plasmid vector, such that when the gene was expressed, an amino acid sequence of His6 was incorporated into the enzyme. Purification of the desired gene was

achieved using a 1 ml nickel column that retains the enzyme due to its $His_6$ tag. Increasing concentrations of imidazole were passed through the column, and NTR was dissociated from the column allowing the purified protein to be eluted.[6]

It is obvious that the expression of the proteins has been successful (Fig. 3.8 and 3.9), and the proteins have been expressed at the expected molecular weight (25 kDa), increasing quantities of the induced and un-induced fractions demonstrate the successful induction method (Fig. 3.8).

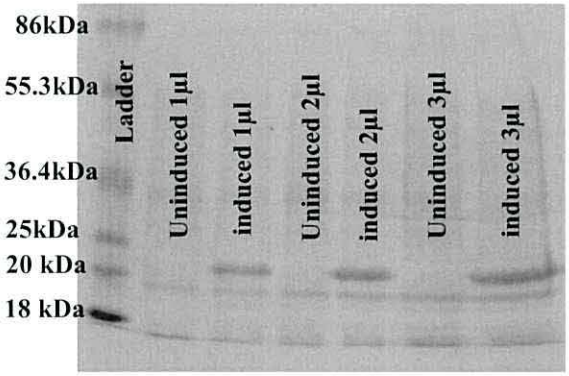


**Figure 3.8: Purification of NfnB. The NfnB gene was over-expressed from pET28a(+), and purified using a nickel column. Proteins were run on SDS-PAGE and compared to molecular markers.**

It is also illustrated that the sample taken at 200 mM imidazole (Fig. 3.9) in the case of NfnB, YdjA; Dde_0086 and Dde_2199 elute the largest quantities of NTRs off the column.

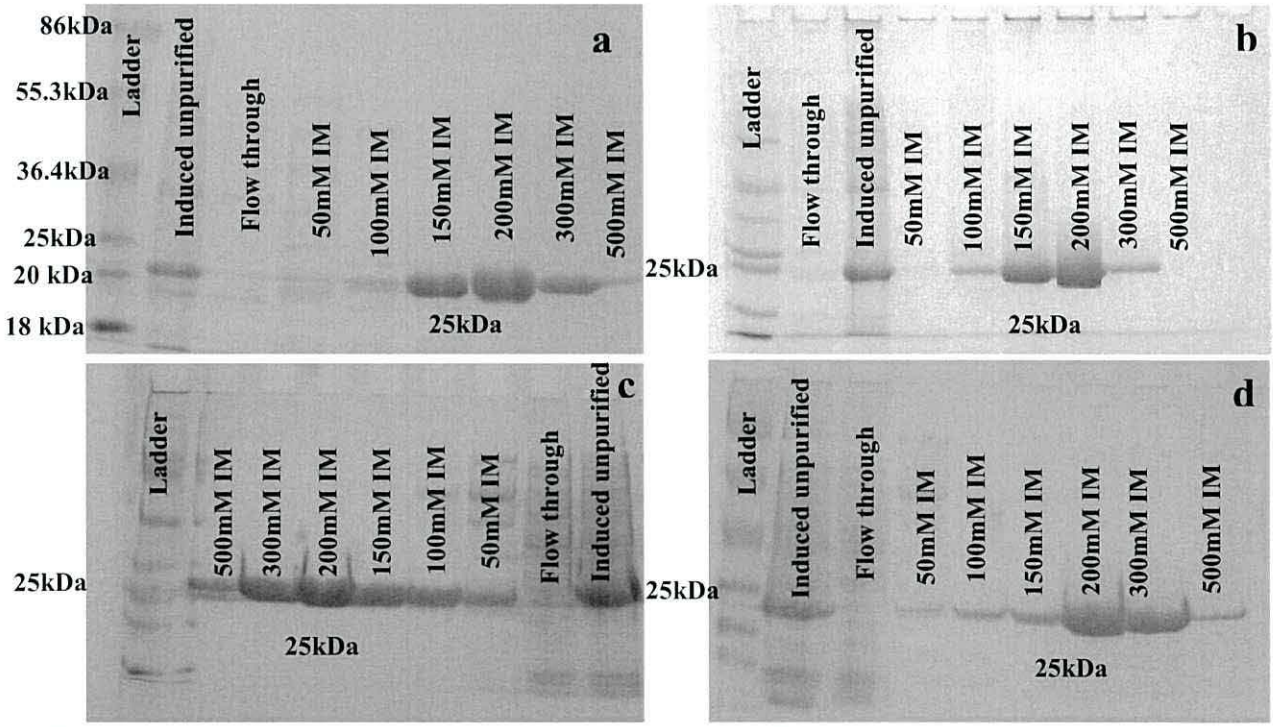


**Figure 3.9: SDS-PAGE of fractions containing the purified bacterial NTRs expressed from a: pET28a(+)-NfnB, b: pET28a(+)-YdjA, c: pET28a(+)-Dde_0086, d: pET28a(+)-Dde_2199.**

The 2nd 1 ml of 200 mM imidazole was found to hold greatest amount of proteins (Fig. 3.10) regarding all recombinant NTRs.

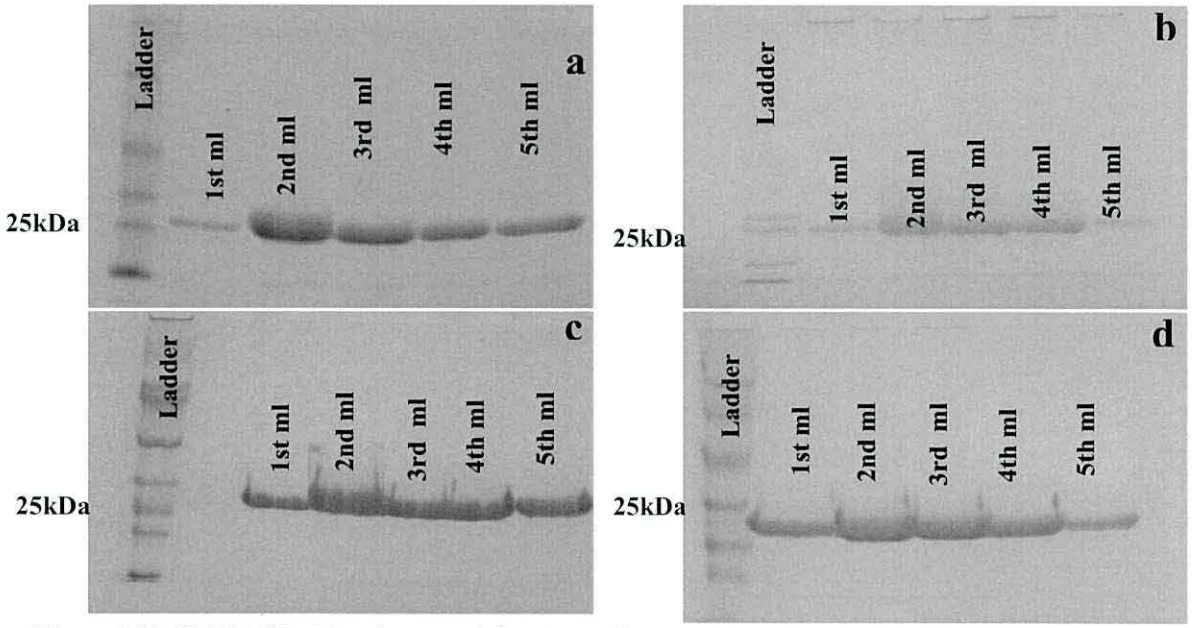


**Figure 3.10: SDS-PAGE of fractions containing the purified bacterial NTRs expressed from a: pET28a(+)-NfnB, b: pET28a(+)-YdjA, pET28a(+)-Dde_0086, d: pET28a(+)-Dde_2199 showing the 200mM imidazole samples.**

The cys-tagged protein (NTRs-cys$_{12}$) required more imidazole concentration to be eluted off the column as the extra binding of the cys-tags, and the high molecular mass bands in the cys-tagged proteins due to the protein dimerisation (Fig. 3.11).

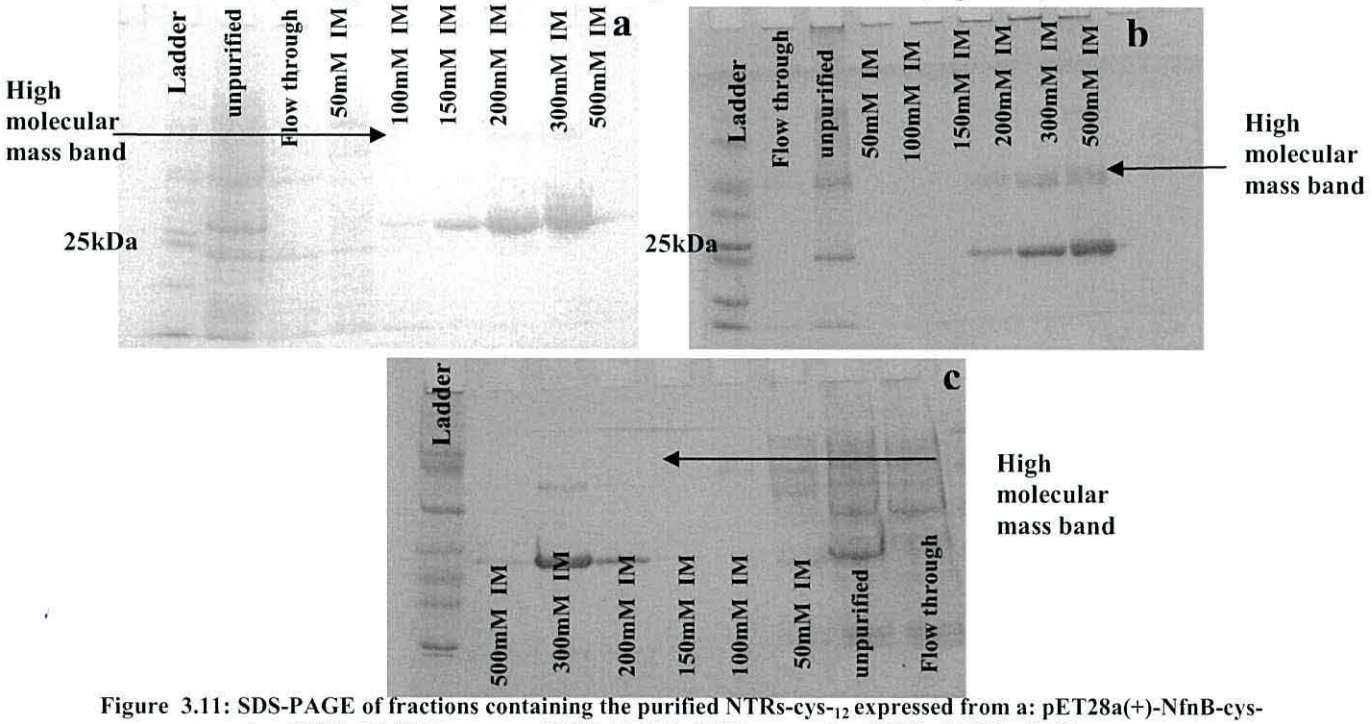


**Figure 3.11: SDS-PAGE of fractions containing the purified NTRs-cys-$_{12}$ expressed from a: pET28a(+)-NfnB-cys-$_{12}$, b: pET28a(+)-YdjA-cys-$_{12}$, pET28a(+)-Dde_0086-cys-$_{12}$, d: pET28a(+)-Dde_2199-cys-$_{12}$.**

Parallel to NTRs the 2nd 1 ml of more concentrated imidazole (300 mM) eluted greatest amount of proteins (Fig. 3.12) in the case of all NTRs- $cys_{12}$.

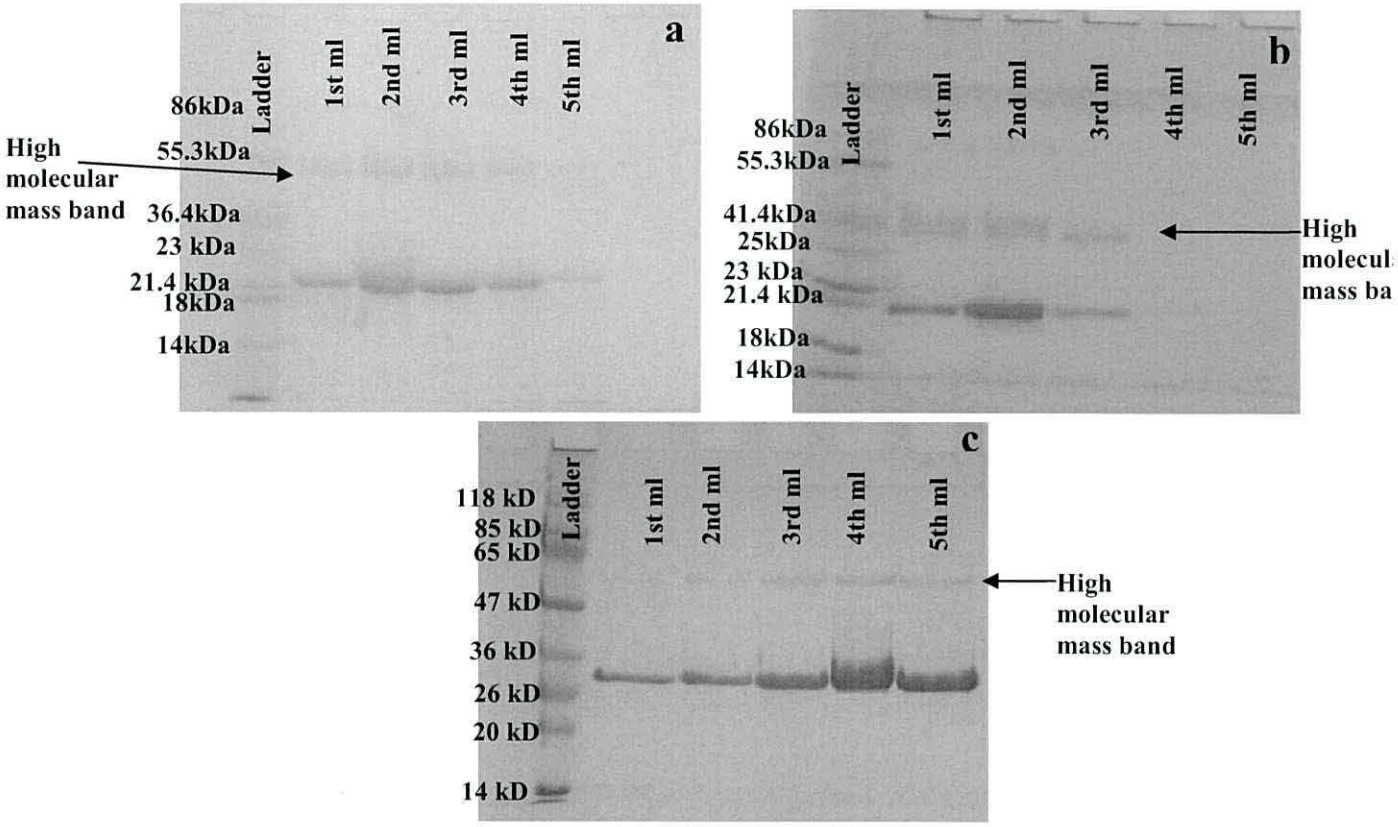


**Figure 3.12: SDS-PAGE of fractions containing the purified engineered NTRs expressed from a: pET28a(+)-NfnB-$cys_{12}$, b: pET28a(+)-YdjA- $cys_{12}$, pET28a(+)-Dde_0086- $cys_{12}$, d: pET28a(+)-Dde_2199 - $cys_{12}$, showing the 300mM imidazole samples.**

All of the enzymes expressed and overproduced successfully except one, this can be seen in the protein gel as a very thin band with the imidazole fractions (Figure 3.13).

Different attempts were made as the unsuccessful overproduction of Dde_2199-cys. Neither incubation of LB mixture ( the transformed E.coli Rosetta cells with Dde_2199-cys- pET28a(+) were picked and transferred into a mixture of 5 ml LB media and 50µg/ml kanamycin, then grown at 37˚C overnight and the 5ml solution was mixed with 500ml LB solution and 50µg/ml kanamycin) at 37 ˚C until the O.D. of 600 nm was 0.6 then inoculated with 2ml, 0.1M IPTG. The incubation was allowed to continue for 4 hours (Fig. 3.13a), nor ligation of Dde_2199 gene (Fig. 3.13b) into the modified pET28a(+) (Where the His tag followed by the cys tag) did not reveal a successful overproduction .

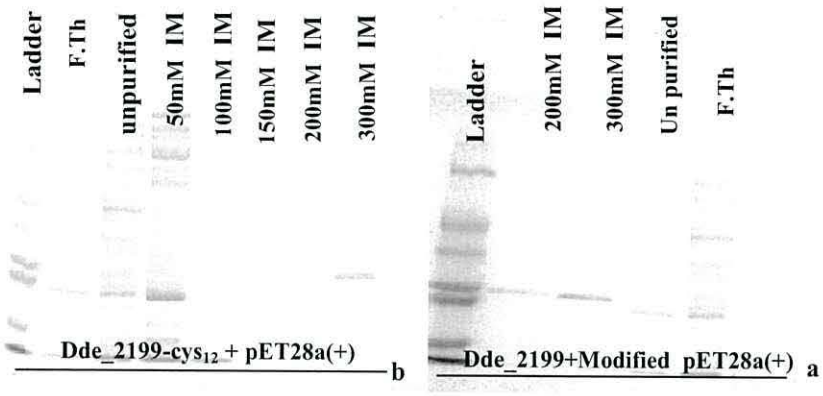


**Figure 3.13: SDS-PAGE of fractions containing a: the purified engineered Dde_2199-cys$_{12}$ expressed from pET28a(+), b: the purified recombinant Dde_2199 expressed from modified pET28a(+) which contains cys-tag, showing the unsuccessful overproduction for both case. F.Th=Flow through.**

The plasmid vector that carries Dde_2199 gene, and the modified vector pET28(+) have been digested with appropriate restriction enzymes (*BamH1* and *HindIII*) and run on agarose gel, the Dde_2199 gene and the modified vector have been gel extracted and purified using QIAquick® PCR purification kit protocol, and religated in order to introduce the Dde_2199 gene into the modified vector (Figs. 3.36 and 3.37) and later over expressed (Fig. 3.14).

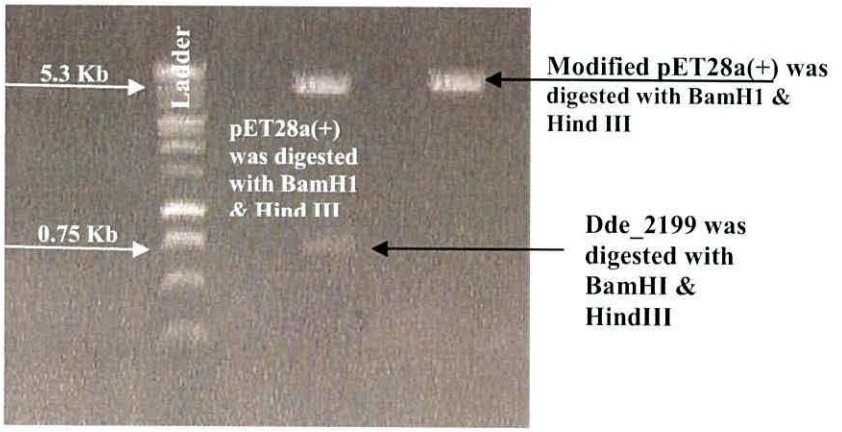


**Figure 3.14: Digestion of (recombinant plasmid pET28a(+) that carries Dde_2199 gene) and (modified pET28a(+)) with BamH1 and HidIII. The bands at 5.3 kb corresponds to pET28a(+), and modified pET28a(+), and the band in 0.75kb corresponds to Dde_2199.**

In order to prove that the cloning vector (modified pET28a(+)) harboured the genes of interest (Dde_2199), they were isolated and the genes restricted with *BamH1* and *HindIII.* The resulting fragments were separated using 0.7% agarose gel electrophoresis. A successful transformation when run on a gel would produce two bands, first a 5.3 kb band

which corresponds to the modified pET28a(+) and ≈ a 0.75kb band which corresponds to Dde_2199. Fig. 3.15 shows the success of the ligation for modified vector and Dde_2199.

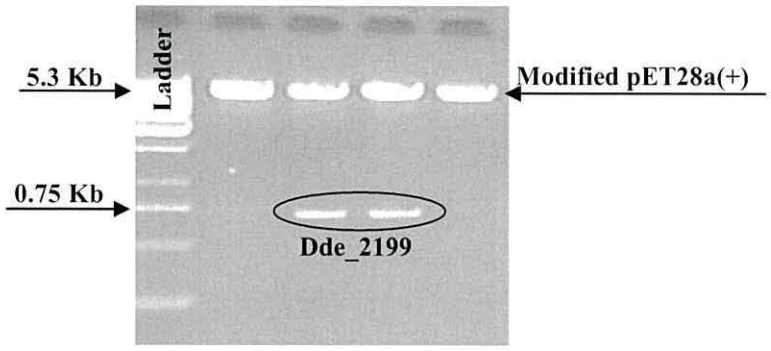


**Figure 3.15: Ligation of Dde_2199 into modified pET28a(+). Lane1, 10kb marker, The band at 5.3 kb corresponds to modified pET28a(+), and the band at the 0.75kb corresponds to Dde_2199.**

The successful over production has been achieved with the incubation of LB mixture (*the transformed E.coli Rosetta cells with Dde_2199-cys- pET28a(+) were picked and transferred into a mixture of 5 ml LB media and 50 μg/ml kanamycin, then grown at 37˚C overnight and the 5ml solution was mixed with 500 ml LB solution and 50 μg/ml kanamycin*) at 37 ˚C until the O.D. of 600 nm was 0.3 and placed at 20˚C until the O.D. of 600 nm was 0.8 then inoculated with IPTG (2 ml, 0.1 M), the incubation was allowed to continue overnight at 20˚C (Fig. 3.16).

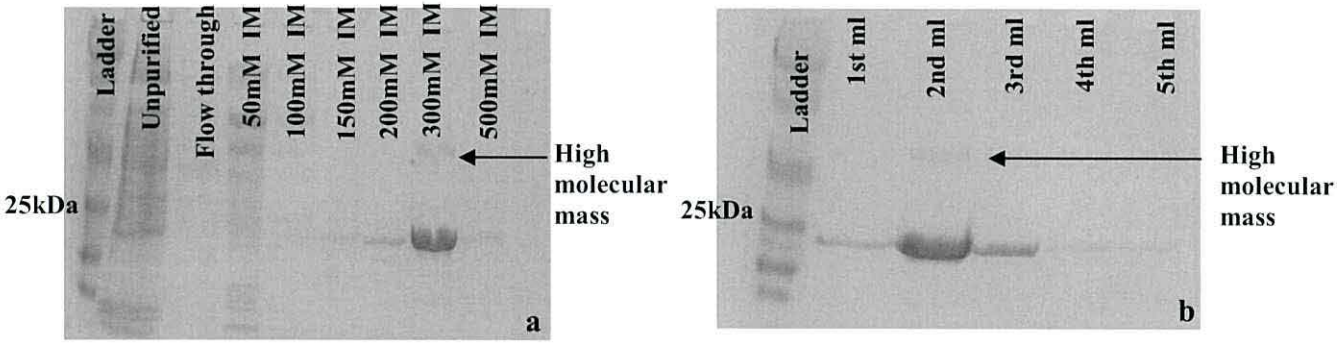


**Figure 3.16: SDS-PAGE of fractions containing the purified Dde_2199 expressed from pET28a(+). a: different imidazole concentrations were used to elute the protein off the column, b: the 300 mM imidazole samples.**

Equivalent to other engineered NTRs, the 300mM imidazole eliminated the greatest quantity of Dde_2199-$cys_{12}$ off the column (Fig. 3.16a), and the 2$^{nd}$ 1 ml of the same concentration contains the greatest amount of protein (Fig. 3.16b).

The SDS-PAGE gels showed that a concentration over 150 mM imidazole is required to remove the protein off the column, and a lower concentrations (50 mM) is required to remove other unwanted fragments. Then 500 mM imidazole is passed through the column to ensure that all of the protein is eluted from the column.

### 3.5 Enzyme assay

To demonstrate the effectiveness of the recombinant NTRs, reduction experiments were carried out using various substrates containing nitro groups; spectra were recorded from 250 nm to 500 nm to monitor the enzymatic activity of the NfnB, and YdjA on 2,4-dinitroethylbenzene (DNEB), Dde_0086 on 1,2-dinitrobenzene (DNB), and Dde_2199 on 3-nitrobenzoate (3-NBZ) for 10 minutes.

A gradual decrease in absorbance with time at 340 nm was found indicating NADH oxidation (Fig. 3.17, 3.18 3.19, and 3.20). A minor durable signal at 410 nm was observed when DNEB (Fig 3.17 and Fig 3.18) and DNB (Fig 3.19) were used as a substrate, which is believed due to the formation of a diazoxy-compound;[7,8] this particular signal was not observed when 3-NBZ was used (Fig 3.20). Regarding poly and di-nitro compounds the reduction of the first nitro group is much faster than others due to the electron deficiency in the aromatic ring, consequently accumulation and condensation of the intermediates; nitroso and hydroxylamino derivatives, hence diazo-compounds formation, while in case of mono-nitrocompounds, nitro group reduction required very low potential so there is a low possibility of diazo-compounds formation.

The continuous, gradual, and regular decrease in absorbance at 340 nm is clear from Fig. 3.17, and the concomitant formation of diazoxy-compounds at 410 nm, when the DNEB reduction is catalysed by the recombinant NfnB-$LT_2$ might indicate good affinity of the enzyme towards the substrate and the enzyme catalytic efficiency.

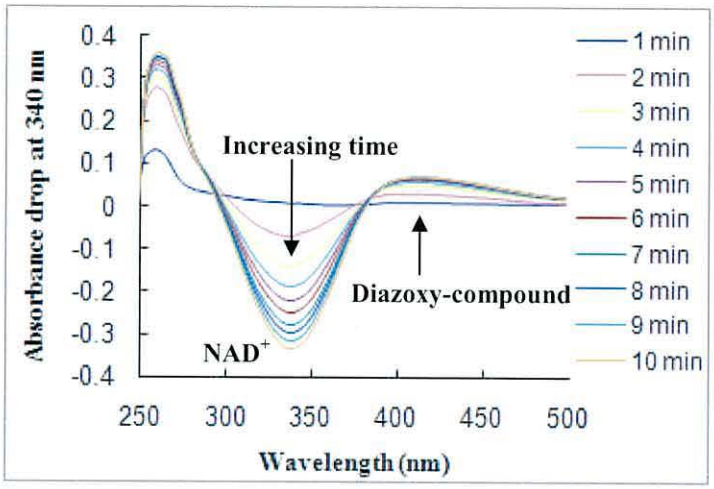


**Figure 3.17: Repetitive scanning of the recombinant NfnB $LT_2$ reduction of DNEB using NADH.**

Once more DNEB reduction by another recombinant NTR (YdjA) was observed (Fig. 3.18). NADH oxidation *via* continuous, gradual and regular decrease in absorbance at 340 nm and diazoxy-compounds production at 410 nm was also observed may also referred to efficiency of the catalytic reaction proceed by the particular enzyme.

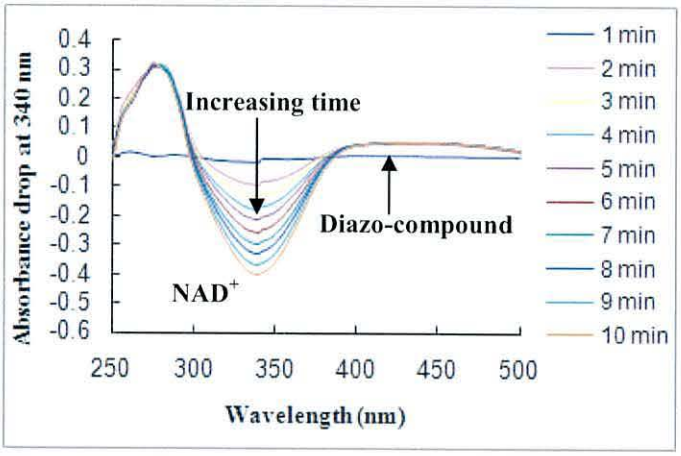


**Figure 3.18: Repetitive scanning of the recombinant YdjA reduction of DNEB using NADH.**

This time irregular gradual decrease in absorbance at 340 nm and slight diazoxy-compounds production at 410 nm were found to be catalyzed by Dde_0086 *versus* DNEB (Fig. 3.19b), this can be explained by the lower affinity and slower reduction rate of the enzyme towards DNEB in comparison with nfnB $LT_2$ (Fig. 3.17) and YdjA (Fig. 3.18). However Dde_0086 showed enhanced response against DNB (Fig. 3.19a) indicating higher reaction rate catalyzed by the enzyme, and this can be confirmed by the activity of Dde_0086 *versus* different nitro-compounds (Fig. 3.22) as Dde_0086 demonstrated higher activity rate with DNB in contrast with other nitro-compounds.

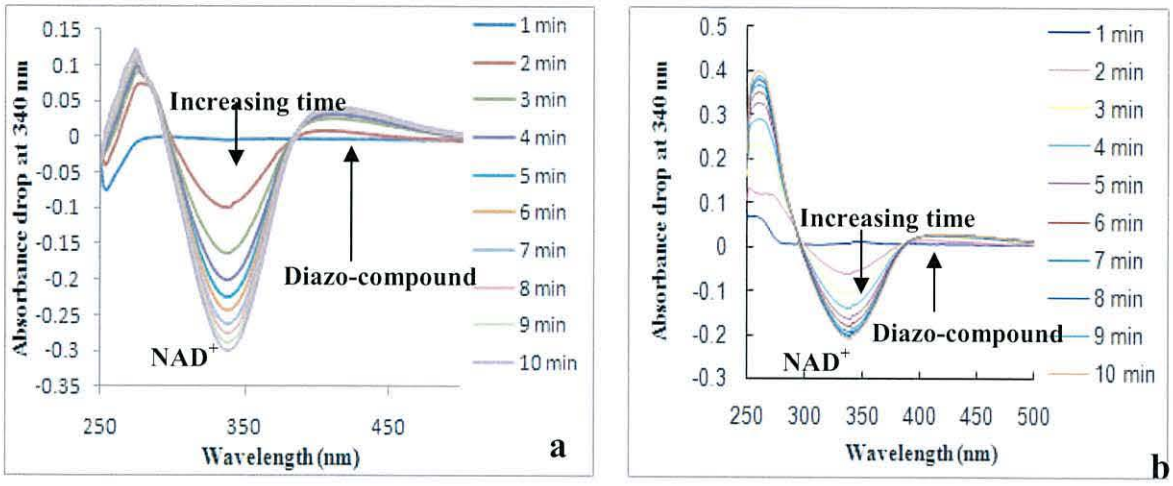


**Figure 3.19: Repetitive scanning of the recombinant Dde_0086 reduction of a: DNB , b: DNEB using NADH.**

Improved activity was recorded for Dde_2199 against 3-NBZ (Fig.3.20) *via* a continuous, gradual and regular decrease in absorbance at 340 nm and diazoxy-compounds production was not observed at 410 nm due to minimal possibility of diazoxy-compounds formation in mononitro-compounds as discussed above.

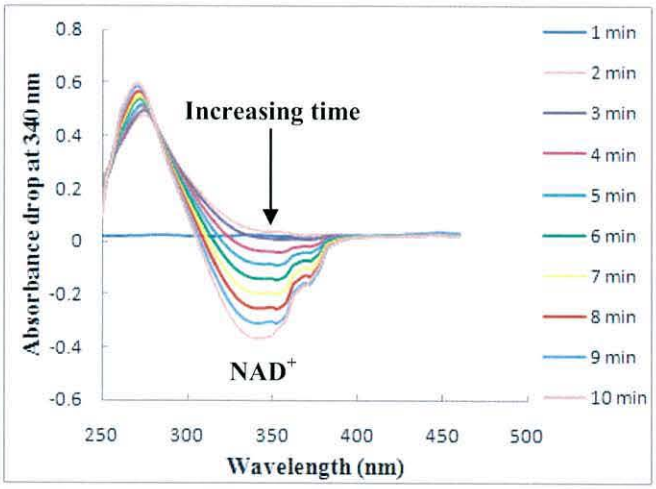


**Figure 3.20: Repetitive scanning of the recombinant Dde_2199 reduction of 3-NBZ using NADH.**

Spectra were also recorded for Nfnb-$cys_{12}$, YdjA-$cys_{12}$, Dde_0086-$cys_{12}$, and Dde_2199 -$cys_{12}$ to evaluate the activity of the engineered NTRs. The oxidation of the NADH was again followed at 340 nm. All the engineered enzymes showed a gradual

decrease with time in the absorbance at 340 nm indicating that they were all successfully expressed in their active conformation.

To confirm that the NTRs are responsible for the NADH oxidation, a series of experiments was performed where the enzyme concentration is increased. If the enzyme is active then an increase in the velocity of the reaction should be seen. Fig 3.21 shows that the oxidation of NADH was indeed due to the NTRs because a straight line relationship between the enzyme concentration and velocity was obtained.

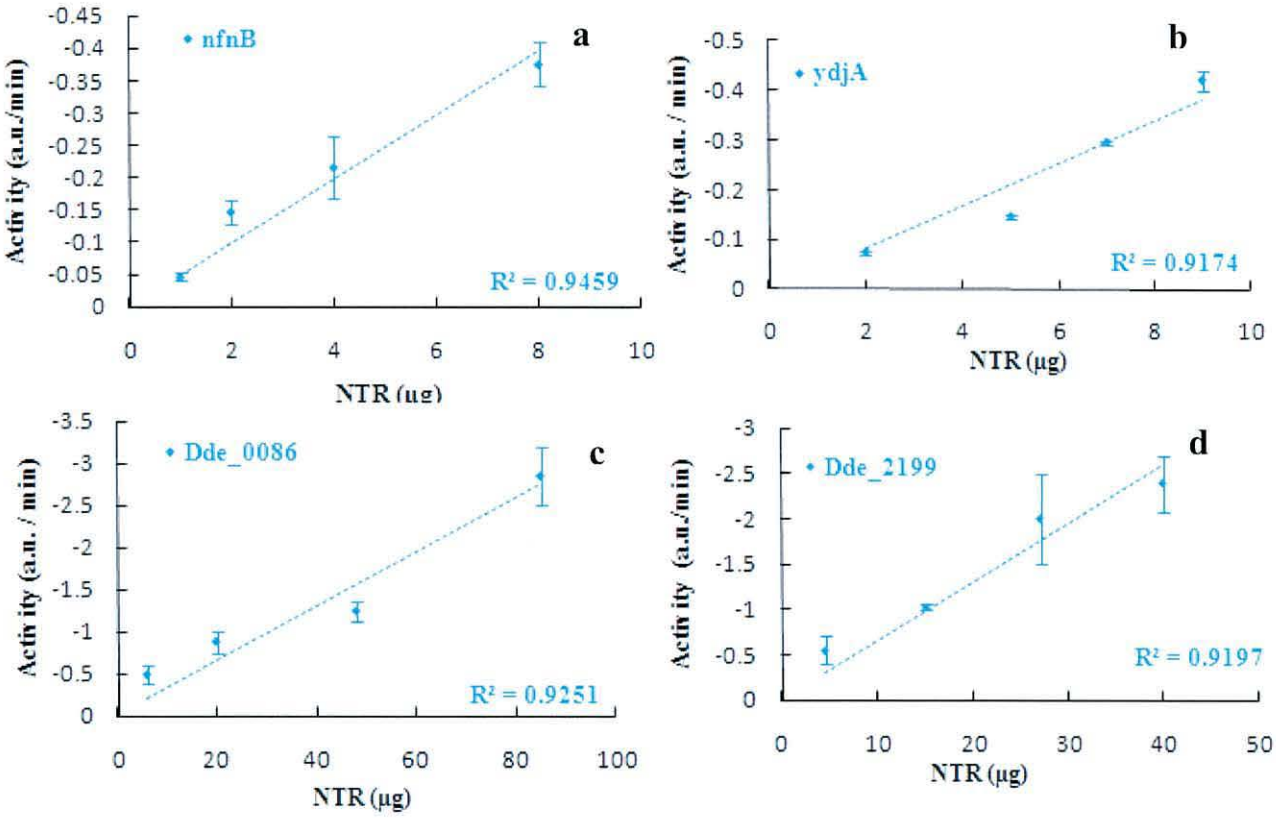


**Figure 3.21: A calibration line of activity / min (340 nm) against µg of recombinant NTRs. a: NfnB, b: YdjA, c: Dde_0086, and d : Dde_2199.**

### 3.6 Enzyme specificity

The specificity of bacterial nitroreductases *versus* substrates containing nitro compounds (explosives) was demonstrated. Unfortunately, the crystal structure of NTRs involved in this study has not been reported yet, to help explain the substrate specificity of the enzymes. Hence the activity of NfnB $k_{12}$ from *E coli* against a broad range of substrates containing nitro compounds were determined, due to homology of the studied NTRs with NfnB from *E. coli* $k_{12}$ which have enormous data published including crystal structure might provide an insight into the substrate specificity of the studied NTRs.

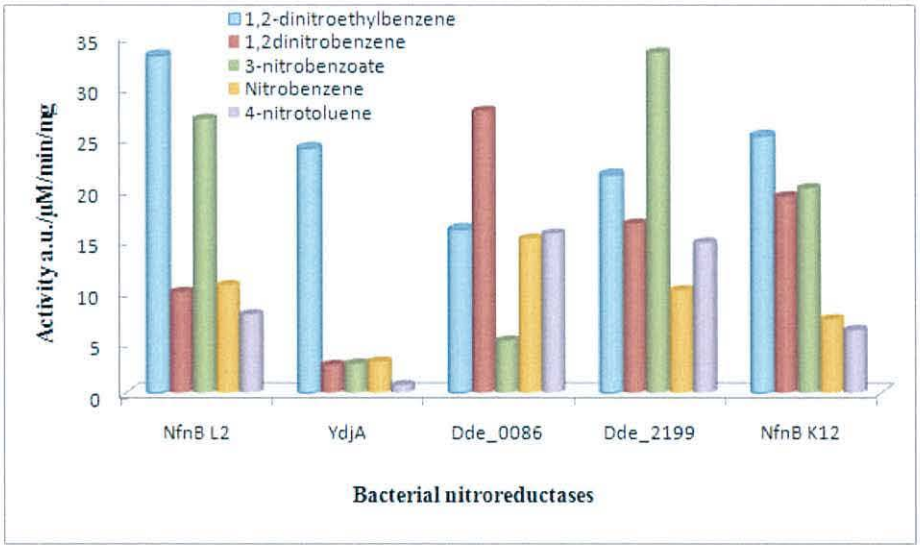


**Figure 3.22: The activity of bacterial nitroreductases against different nitro-compounds.**

It is clear from (Fig 3.22) that bacterial NTRs exhibited broad substrate specificity toward nitro-aromatic substrates, this can be explained by the inherent plasticity of the helix containing the Phe124 residue which shows a pronounced variability in position for accommodating substrates of different sizes.[9,10] The *E. coli* NfnB $k_{12}$ and NfnB $LT_2$ from *Salmonella typhimurium* $LT_2$ approximately exhibited identical pattern of specificity concerned (1,2-DNEB, 3-nitrobenzoate, nitrobenzene, and 4- nitrotoluene), they showed maximum activity with 1,2-DNEB and minimum with 4- nitrotoluene. This might be due to the contribution of identical residues in substrate binding pocket as they share 88% sequence identity.

A different pattern of specificity was observed for YdjA from *Salmonella enterica paratyphi* A and NfnB $k_{12}$ which share 16% sequence identity (Fig 3.22 ), considerable specificity against DNEB was seen, negligible activity was observed concerned other nitro-containing compounds. Conversely, the crystal structure of *E. coli* YdjA (Ec_YdjA) that shares 89% sequence identity with *Salmonella enterica paratyphi* A YdjA revealed that the absence of the two helices in Ec_YdjA which restricts the substrate and cofactor binding in other NTRs, makes a wider and flexible active site.[11] Hence further structural studies with complexes of substrate analogs or inhibitors would be required to obtain a detailed understanding of the substrate-binding mechanism.

The Dde_0086 and Dde_2199 NTRs from *Desulfovibrio desulfuricans* G20 demonstrated diverse model of specificity in contrast with *E. coli* NfnB $k_{12}$ (Fig. 3.22), although the Dde_0086 shares 51% sequence identity with nfnB $k_{12}$ (Table 3.1). This may be due to the participation of different residues in substrate binding cleft. In addition to Phe124, other surrounding aromatic residues may participate in substrate binding, and these residues vary among nitroreductases, suggesting that they may play a role in determining the substrate specificity of the enzymes.[9,10] The substrate is sandwiched between the FMN and Phe124 residue and is held primarily by weak van der Waals and dipole-dipole interactions. Hence, the electron distribution of the FMN may play a major role in substrate orientation, binding affinity and reactivity. In addition, the large change in charge distribution on the flavin on reduction would not only orient the correct substrate but could prevent most substrates from binding to the wrong form of the enzyme as well as helping to eliminate the products of the reaction.[12]

### 3.7 Protein concentrations, specific activities, $K_m$, and $V_{max}$ values

The specific activities of purified, recombinant NTRs and engineered NTRs-$cys_{12}$ against different nitro-aromatic compounds (NACs) were evaluated by estimating the rates of (NACs) reduction with increasing concentrations of substrate (Table 3.2). The resulting data were averaged and the $K_m$ and $V_{max}$ values were calculated from Direct Linear analysis[13] and were obtained with a 68% confidence level (Table 3.2). The Michaelis constant ($K_m$) is essentially how effectively the enzyme binds the substrate, hence affinity,

with a low value indicating a high affinity, and $V_{max}$ is the maximum velocity or rate at which the enzyme catalyzed a reaction.

Since NfnB exhibited an enhanced response towards DNEB (Fig. 3.22), this particular substrate was used to determine the kinetic parameters for NfnB. The increase of the reaction rate catalysed by NfnB and NfnB-$cys_{12}$ by increasing substrate concentrations can be clearly seen (Fig. 3.23). Afterwards any increase in substrate concentration did not affect in the reaction rate due to the saturation of enzyme active site with the substrate roughly (30 µM). The reduction of the reaction rate by cys-tag insertion was also noticed from the same figure. The calculated $K_m$ and $V_{max}$ values regarding NfnB were found to be 18.3 M and 89.12 µmol/min/mg, while NfnB-$cys_{12}$ showed 25.0 µM, and 81.8 µmol/min/mg $K_m$ and $V_{max}$ values respectively indicating that the cys-tag insertion reduced about 33% of enzyme activity as well as decreased the enzyme affinity for the substrate. The $K_m$ value of *Salmonella typhimurium* LT2 NfnB towards DNEB is lower (18.3 µM) in comparison with the $K_m$ value related *E coli* NfnB (27 µM)[14], and YdjA (43.4 µM), hence the high affinity of the enzyme for the substrate in comparison with others.

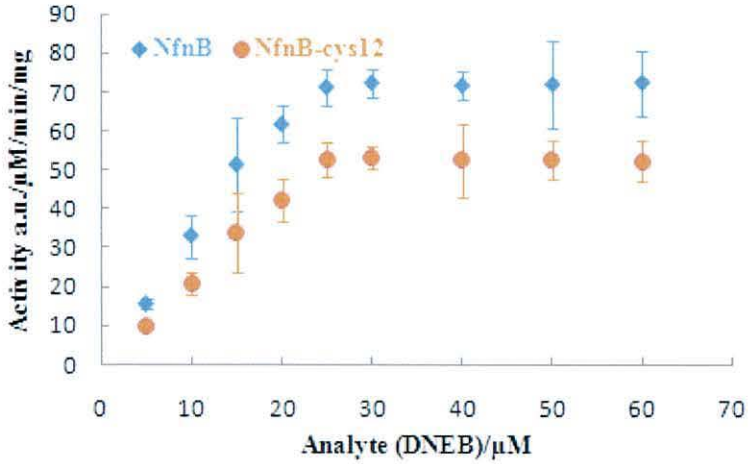


**Figure 3.23 : A chart showing the increasing concentrations of the analyte against activity in µM/min/mg at 340nm, for the purified NfnB and NfnB-$cys_{12}$.**

DNEB was used for estimating YdjA kinetic parameters for to the same reason mentioned above (Fig. 3.22). Similar results were observed concerning recombinant YdjA $K_m$ (43.4 µM) and $V_{max}$ (95.3µmol/min/mg) values and YdjA-$cys_{12}$ ($K_m$ 56.0 µM and $V_{max}$ 92.2µmol/min/mg) as the cys-tag reduced the enzyme activity (26.4%) and the enzyme affinity for the substrate (Fig.3.24).

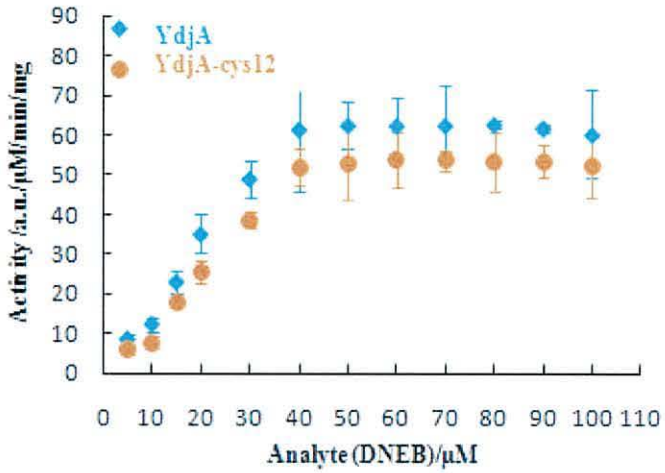


**Figure 3.24 : A chart showing the increasing concentrations of the analyte against activity in µM/min/mg at 340nm, for the purified YdjA and YdjA-cys$_{12}$.**

1,2-DNB was used regarding Dde_0086 as it is showed considerable response with this substrates (Fig. 3.22). It was also found that a low concentration of 1,2-DNB (20 µM) was enough to make the enzyme saturated with the substrate, in addition drove down of reaction rate catalysed by the enzyme considerably *via* cys-tag insertion (Fig. 3.25).

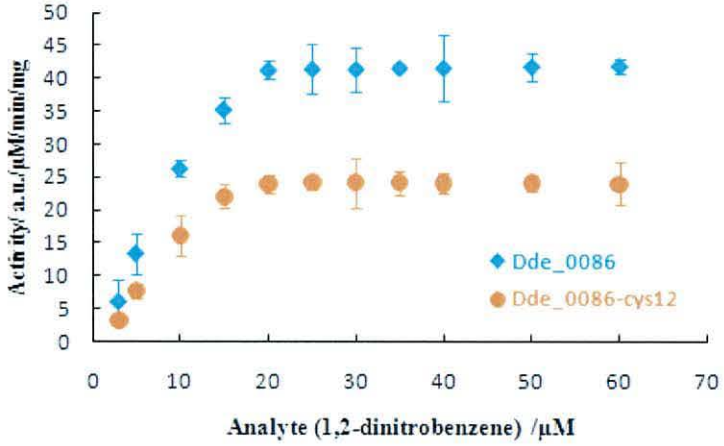


**Figure 3.25 : A chart showing the increasing concentrations of the analyte against activity in µmoles/min/mg at 340nm, for the purified Dde_0086 and Dde_0086-cys$_{12}$.**

Dde_2199 kinetic parameters were estimated with 3-NBZ (Fig. 3.22). The enzyme become saturated with a low substrate concentration approximately 20 µM and no more 3-NBZ was required to increase the reaction rate which is highly reduced by the cys-tag insertion (Fig. 3.26).

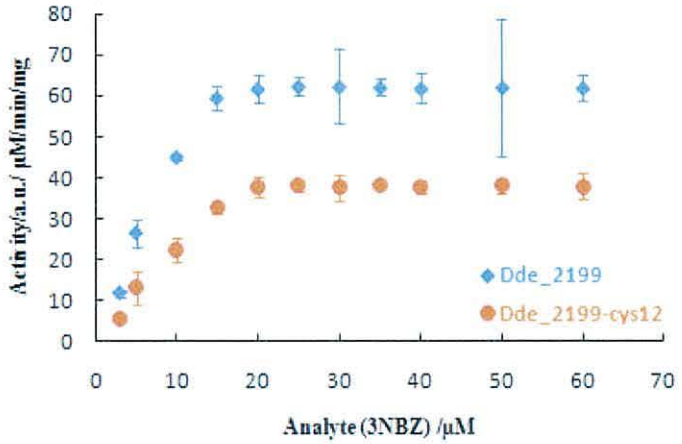


**Figure 3.26 : A chart showing the increasing concentrations of the analyte against activity in µmoles/min/mg at 340nm, for the purified Dde_2199 and Dde_2199-cys$_{12}$.**

The low $K_m$ values found with Dde_0086 (7.7 µM) and Dde_2199 (5.04 µM) toward 1,2-DNB and 3-NBZ respectively suggest that 1,2-DNB and 3-NBZ might be the physiological substrates for these enzymes, although these two enzymes showed enhanced response to wards other NACs (Fig. 3.22). Once more the cys tag insertion affected the reaction rate mediated by these two enzymes and drove down the enzymes activity by (Dde_0086 / 41%) and (Dde_2199/ 49.3%) and reduced the enzymes' affinity for the substrates (Table 3.2).

**Table 3.2: The protein concentrations, Average specific activity, Km, and Vmax values of bacterial nitroreductases.**

| Protein | Average total protein (mg/500ml culture) | Average specific activity (µmol/min/mg) | Km (µM) | Vmax (µmol/min/mg) |
|---|---|---|---|---|
| NfnB | 22.5 | 3.15 | 18.3 (8.19-25.9) | 89.12 (75.3-111) |
| NfnB-cys$_{12}$ | 11.6 | 2.12 | 25.0 (10.1-55.0) | 81.8 (62.7-120) |
| YdjA | 18.3 | 1.29 | 43.4 (16.4-64.0) | 95.3 (76.5-112) |
| YdjA-cys$_{12}$ | 10.4 | 0.95 | 56.0(37.2-108) | 92.2(37.6-132) |
| Dde_0086 | 58.6 | 2.41 | 7.70(2.56-15.3) | 49.5(44.5-56.9) |
| Ded_0086-cys$_{12}$ | 16.8 | 1.43 | 9.71(3.96-16.7) | 28.5(25.3-33.9) |
| Dde_2199 | 28.5 | 4.42 | 5.04(2.65-7.43) | 68.1(65.4-73.8) |
| Dde_2199-cys$_{12}$ | 8.4 | 2.24 | 14.93(5.66-31.2) | 54.0(44.8-67.0) |

### 3.8 Stoichiometry of the enzyme reaction

The reduction of a nitro group to an amine group *via* the nitroso and hydroxylamino-groups is a six electron reduction. Thus each reduction step must use one pair of electrons indicating that one NADH molecule is required for each step. [15,16]

To confirm the stoichiometry of the reaction and the identity of the products, the stoichiometry of the reaction between NADH and nitroaromatic substrates was calculated from the total drop in absorption available when using 200 μM of NADH; this drop is equivalent to -1.2 a.u. Hence -1.2 corresponds to -0.006 a.u., the absorbance drop for 1μM of NADH.

The recorded absorbance drop for 20 μM of DNEB equalled –0.123419 a.u. (Fig. 3.27) consequently when this drop is divided by -0.006 a.u. this yields the concentration of NADH (20.6 μM reacting for every 20 μM of analyte present; hence one μM of NADH is oxidized for every one μM of analyte present.

The resulting data achieved for NfnB (Fig. 3. 27) shows a correlation of 0.97 with a slope of 1.02 therefore, the ratio of analyte (DNEB) to NADH is 1:1. This stoichiometry was also confirmed with the NfnB-cys$_{12}$ (correlation of 0.99 and slope 0.99). Hence, the NfnB must reduce the nitro group to nitroso.

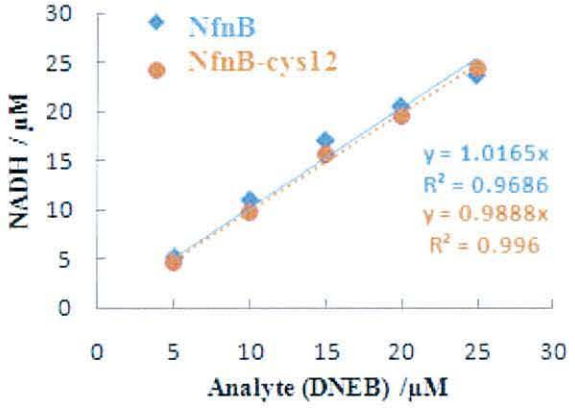


**Figure 3.27 : A chart showing the calculated concentrations of the 2,4-dinitroethylbenzene/ NfnB against the concentration of NADH used during the reaction at 340 nm**

The stoichiometry of the YdjA enzymatic reaction against DNEB was found to be 1:1 μmol of substrate/ μM of NADH (correlation of 0.99 with a slope of 0.94) and the result confirmed with the YdjA-cys$_{12}$ (correlation of 0.99 with a slope of 0.9). Therefore, the YdjA must reduce the nitro group to nitroso (Fig. 3.28).

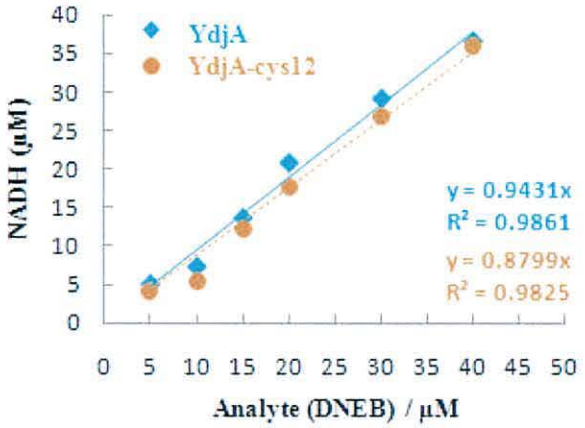


**Figure 3.28 : A chart showing the calculated concentrations of the 2,4-dinitroethylbenzene /YdjA against the concentration of NADH used during the reaction at 340 nm**

The molar ratio of NADH oxidized to DNB reduced (µM NADH/ µM DNB) was found to be 2.7 µM (correlation of 0.98) hence Dde_0086 must reduce the nitro group to amino-group. A similar result was obtained regarding Dde_0086-$cys_{12}$ (correlation of 0.99 with a slope of 2.5) (Fig. 3. 29).

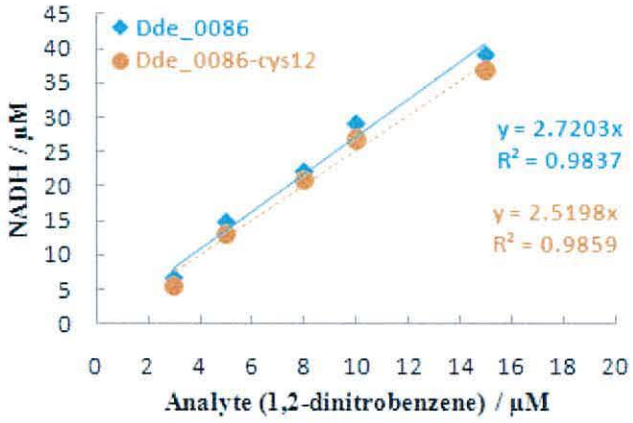


**Figure 3.29 : A chart showing the calculated concentrations of the DNB/Dde_0086 against the concentration of NADH used during the reaction at 340 nm**

The rate of oxidation of NADH was 2 times that of the reduction of the 3-NBZ with a correlation of 0.96 and a slope of 2.3. This stoichiometry was supported *via* the result of Dde_2199-$cys_{12}$ (correlation of 0.99 with a slope of 2.0), hence Dde_2199 must reduce the nitro group to a hydroxylamino-group (Fig. 3. 30).

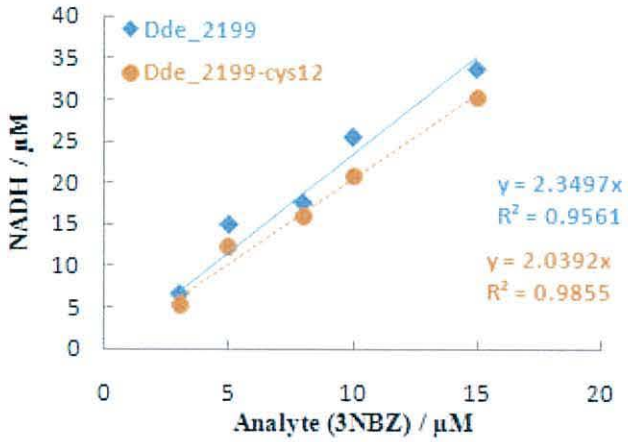


**Figure 3.30 : A chart showing the calculated concentrations of the 3-NBZ/ Dde_2199 against the concentration of NADH used during the reaction at 340 nm**

### 3.9 pH and temperature dependence

The effects of pH and temperature on the activity of NTRs were examined. Assays were carried out spectrophotometrically using a wavelength scan over 250-500 nm for 10 min at a series of different pH (50mM sodium citrate for pH range 5-6, 50mM phosphate buffer for pH range 7-9, 50mM CAPS for pH range 10-11) and different temperature values (25°C -70°C) in 50 mM phosphate buffer. The measured activity was estimated from the absorbance drop at 340 nm. The optimum activity of purified NfnB was found to be at pH 7.0 which decreases as the pH varies in both directions (Fig. 3.31).

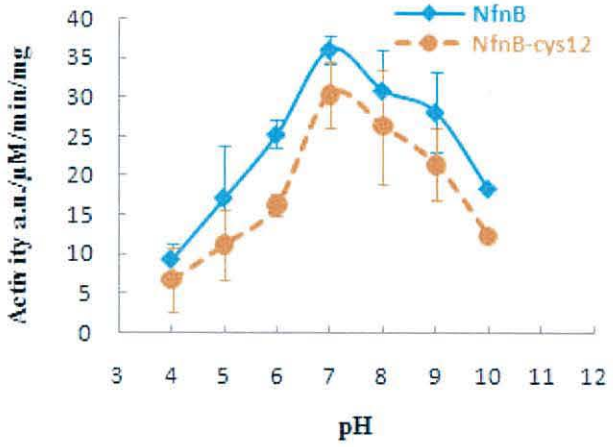


**Figure 3.31: The effect of pH on the activity of NfnB and NfnB-cys$_{12}$.**

YdjA showed a considerable reductase activity over a wide pH range (7-9) with maximal catalytic activity at pH 7.0 (Fig. 3.32), and the enzyme sharply reduced its activity at low acidic pH (3.0) and high basic pH (10.0).

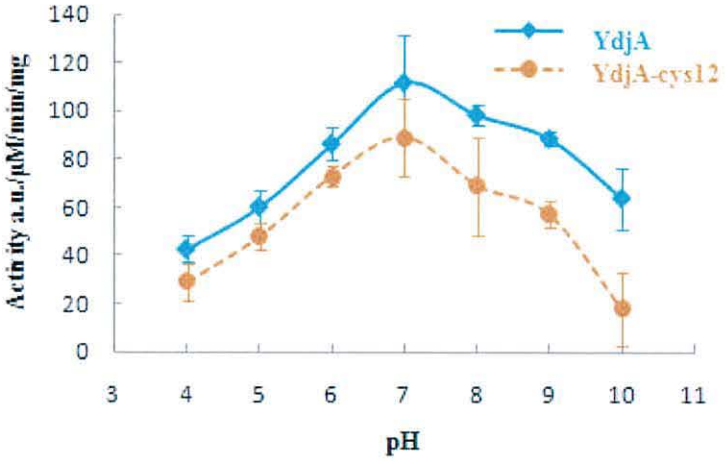


**Figure 3.32: The effect of pH on the activity of YdjA and YdjA-cys$_{12}$.**

The Dde_0086 exhibited high reductase activity under acidic and alkaline conditions and retained high level of its activity even after incubation at pHs of 3–11for 10 min (Fig. 3.33). This is in agreement with the published value[17] of *Bacillus subtilis* WU-S2B that showed high stability under acidic and alkaline conditions and retained 90% of its activity even after incubation at a wide pH range (4-12) for 24h. The high level of packing of the two subunits could be related with the intrinsic stability of the enzyme, and may be an advantage for a potential use in clinical or biotechnological applications. However, further studies and structural analysis will be required to understand the biological function of this enzyme and its potential applications.[18]

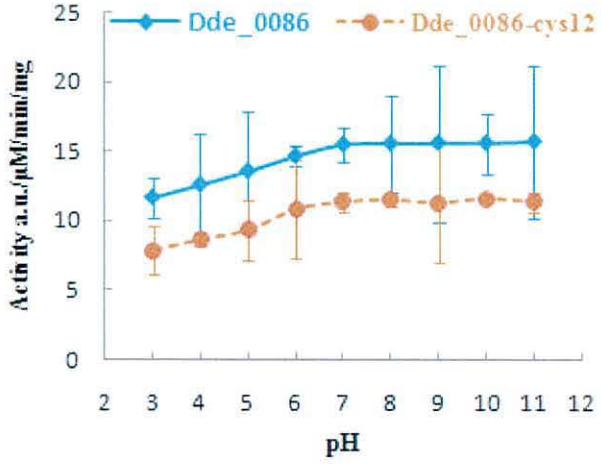


**Figure 3.33: The effect of pH on the activity of Dde_0086 and Dde_0086-cys$_{12}$.**

Parallel to NfnB and YdjA, Dde-2199 demonstrated a substantial catalytic efficiency within a broad pH range (7-9) with a maximal activity at pH (7.0). The enzyme reductase rate reduced as the pH varying in both directions, additionally the enzyme activity extremely reduced at pH 3.0 and 11.0 (Fig 3.34). The optimum activity of the NTRs is at pH.7.0 which is in agreement with the published value.[19]

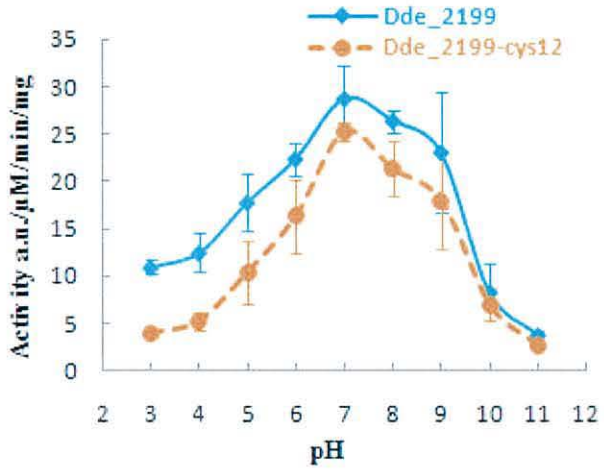


**Figure 3.34: The effect of pH on the activity of Dde_2199 and Dde_2199-cys$_{12}$.**

Similar results (pH dependence) were observed for NTRs-cys (NfnB, Fig. 3.31, YdjA, Fig. 3.32, Dde_0086, Fig. 3.33, and Dde_2199, Fig. 3.34) albeit with decreased levels of activity indicating that cys-tag insertion had no effect on pH /activity profile.

The effect of temperature on NTRs stability was determined. Purified NTRs were incubated in a heating blocks over a temperature range from 25 to 70°C. After 5 min incubation, the protein was added to an assay mixture, and the enzyme reaction was carried out spectrophotometrically using a wavelength scan over 250-500 nm for 10 min. The effects of temperature on the activity of NTRs were studied at pH 7.0.

NfnB in this study was relatively stable at temperatures 50°C and below. It retained 74% of its activity at 55°C, and totally lost its activity when incubated at 65°C for 5 min. (Fig. 3.35). This is in agreement with the nitroreductase activity of white rot fungus *Phanerochaete chrysosporium*.[20]

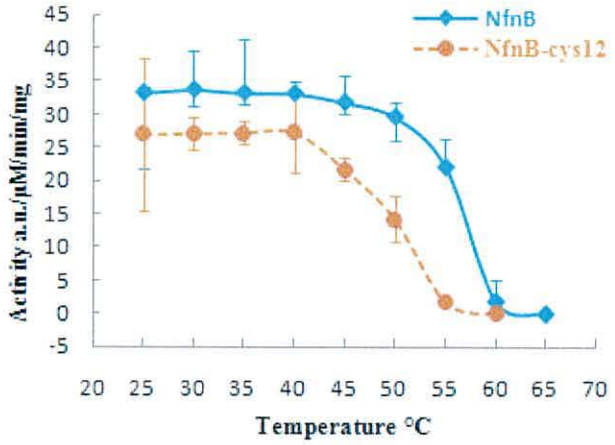


**Figure 3.35: The effect of temperature on the stability of NfnB and NfnB-cys$_{12}$.**

The activity of YdjA was not affected by incubation in the temperature range (25°C-40°C), and the activity gradually decreased as the temperature increased above 40°C. Moreover, ydjA preserves 55% of its original activity at 55°C, the optimal temperatures of several bacterial nitroreductases were shown to be 40°C or less.[16,21,22]

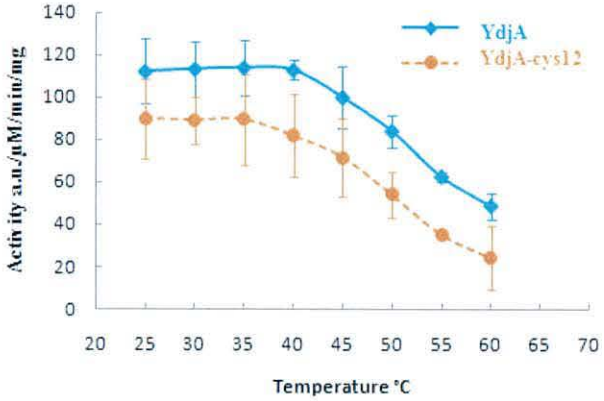


**Figure 3.36: The effect of temperature on the stability of YdjA and YdjA-cys$_{12}$.**

Dde_0086 and Dde_2199 (Fig. 3.37) exhibited high stability over wide temperature ranges (25–55) °C and they retained 52% and 83.5% respectively of their activity even after incubation at 60 °C; a complete loss of activity was noticed for both enzymes at 70°C (Fig. 323), these are in agreement with the published value that showed the maximum activity at temperatures > 50°C.[17, 23] The high reductase activity for Dde_0086 and Dde_2199 over a wide temperature range (25–55 °C) indicates that these enzymes may be crucial in the desulfurising ability of *Desufovibrio desulfuricans* G20.

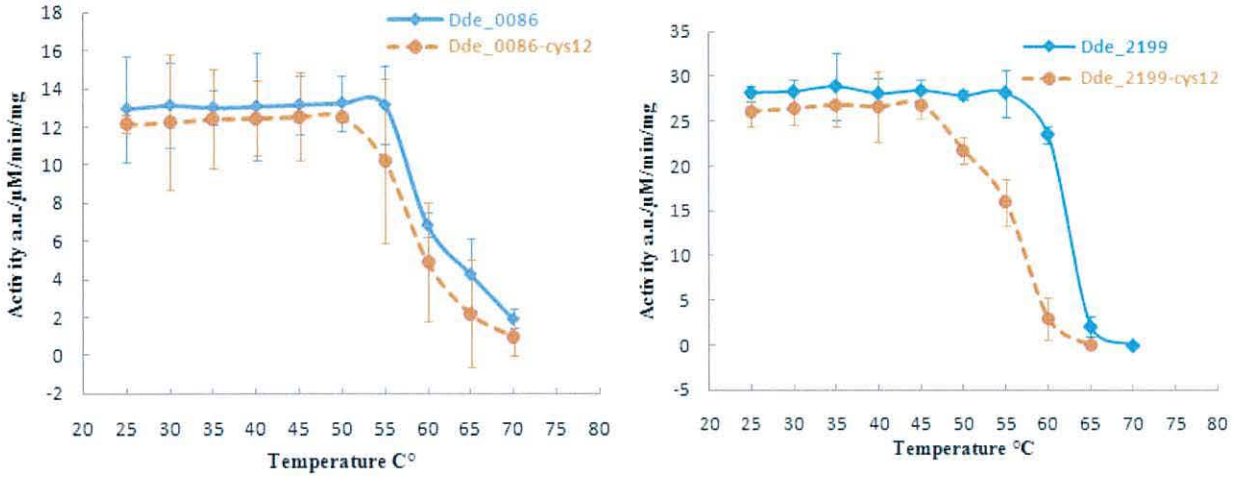


**Figure 3.37: The effect of temperature on the stability of Dde_0086, Dde_0086 –$cys_{12}$, Dde_2199 and Dde 2199 –$cys_{12}$.**

Unexpectedly the optimal temperatures for stability of the engineered NTRs (NTRs-cys) were lower than the corresponding values described for recombinant NTRs (Fig 3.23). (NTRs-cys) showed a different temperature-activity profile, the maximum temperature for stability being shifted from (50 to 40°C), (40 to 35°C), (55 to 50°C), (55 to 45°C), for NfnB, YdjA, Dde_0086, and Dde_2199 respectively indicating that the presence of a cys-tag affect on the temperature stability of the engineered bacterial enzyme. It is possible that the engineered enzyme does not fold into an optimal conformation when expressed in *E. coli*. This may also have an effect on the temperature/activity profile of the native nitroreductase.

### 3.10 Conclusion

The results revealed that NTRs genes (*nfnB*, *ydjA*, *Dde_0086*, and *Dde_2199*) which were obtained from *Salmonella enterica serovar typhimurium* strain LT2, *Salmonella enteric* subsp. *Enterica serover paratyphi* A strain ATCC 9150, and *Desulfovibrio desulfuricans* strain G20 respectively were successfully isolated using PCR amplification. The primers were designed to have restriction sites that allowed the efficient incorporation of the genes into the plasmid vector, pET-28a(+). The plasmid vector was transformed into Rosetta, a strain of *E.coli*, because it is an efficient bacterium for the expression of the enzyme. The protein was isolated using a nickel column because the engineered protein contains a positively charged six-residue histidine tag. Purification was achieved by competitive removal from the nickel column using imidazole. The presence of the NTRs and their activities were confirmed by exposing the various substrates containing nitro groups (2,4-DNEB, 1,2- DNB, 3-NBZ, NB, 4-NT) to increasing concentrations of the enzymes. In the second set of experiments, the protocol for PCR was repeated using primers designed to contain six adjacent codons for cysteine to enable the enzyme to adhere to the electrode surface. The same protocols were used for cloning the modified NTR gene, transforming it into *E. coli* Rosetta strain, and confirming the enzyme activity.

The optimum pH (7.0) and optimum temperature range for enzyme stability (25-55C°) for the NTRs were demonstrated along with $K_m$ and $V_{max}$ values. The stoichiometry was also assessed revealing that the bacterial NTRs react through different reaction pathways, NfnB and YdjA oxidizing one μM of NADH to every μM of DNEB; Dde_0086 required three μM of NADH to reduce one μM of DNB, hence reducing the nitro group to amino group, while Dde_2199 oxidized two μM of NADH to reduce one μM of 3-NBZ.

Although the cys-tag insertion did not block the enzyme activity, and did not restrict substrate entrance into the active site, it was shown to have an effect on enzyme activity, protein concentration, and the optimum temperature range for enzyme stability.

It is clear from (Fig 3.38) that cys-tag insertion had an effect on the protein concentrations regarding all engineered NTRs. It reduced expression about 48.4%, 43.2%, 71.3%, and 70% in the case of NfnB, YdjA, Dde_0086, and Dde_2199 respectively.

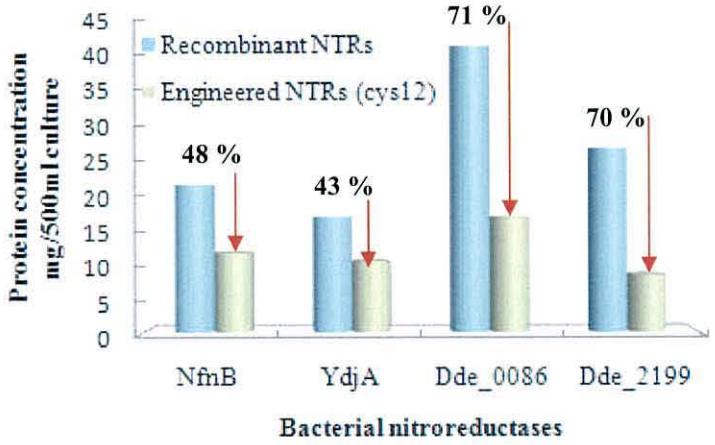


**Figure 3.38: The effect of cys-tag insertion on the protein concentration of bacterial NTRs.**

Once again the cys-tag insertion revealed its effect on enzyme properties. It is reduced the enzymes specific activities nearly by (27-50%), in the case of NfnB it reduced about 33% and 27%, 41%, 50% in the case of YdjA, Dde_0086, and Dde_2199 respectively (Fig 3.39).

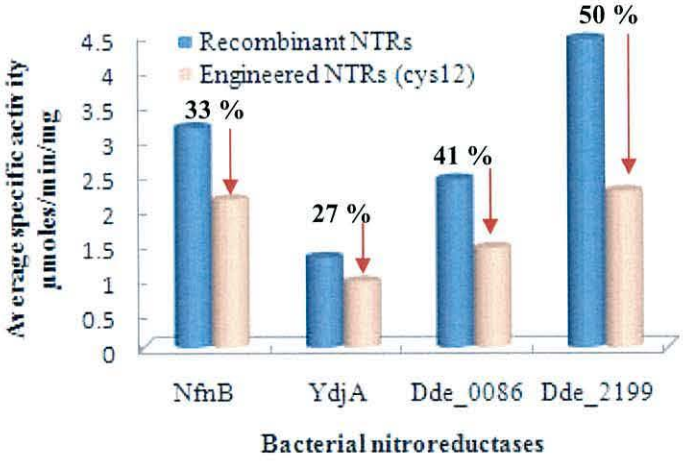


**Figure 3.39: The effect of cys-tag insertion on the activity of bacterial NTRs.**

The optimum temperature range for enzyme stability was also affected by cys-tag insertion; it is obvious from Fig 3.40 how cys-tag reduced the optimum temperature range.

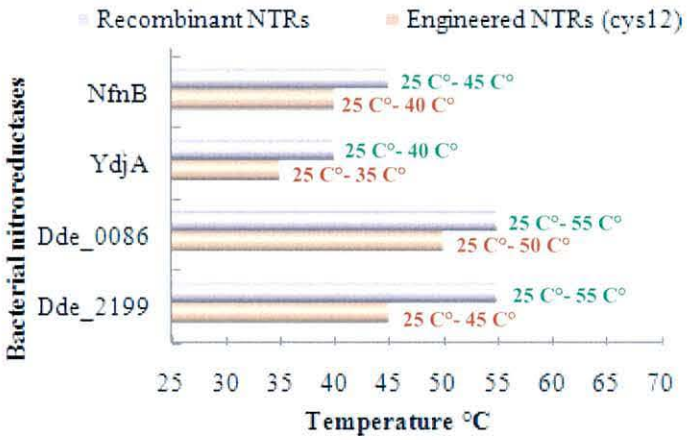


**Figure 3.40: The effect of cys-tag insertion on the optimum temperature range for enzyme stability of bacterial NTRs.**

Bacterial NTRs showed different activity profiles against various nitro-aromatic compounds, different reaction pathways and stoichiometry ratio. These will allow the biosensor to detect as well as identify different nitro- aromatic explosives, through immobilization more than one NTRs at the sensor surface which is the main purpose of this study.

### 3.11 References


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# Chapter 4- Bacterial nitroreductases and TNT

## 4.1 Introduction

2,4,6-Trinitrotoluene (TNT) is one of the most stable and recalcitrant of high explosives and does not occur naturally in the environment. It's comparatively simple and economical to produce, its stability, and its ability to be melted and cast as well as press-moulded into shells have supported its widespread use in weapons.[1] TNT has accumulated in the environment due to its recalcitrance which is generally attributed to the symmetric localization of the nitro groups of the aromatic ring, which restricts attack by dioxygenase enzymes implicated in the microbial metabolism of aromatic compounds. Contamination by this nitroaromatic compound is a typical environmental problem at many sites of earlier explosive production, handling, or storage.[2] TNT shows a high inherent toxic potential as reported by different studies on mammals including humans, aquatic (algae, fish, bacteria) and terrestrial organisms (plants, soil invertebrates).[3,4] Furthermore, evidence of a mutagenic and carcinogenic potential is reported.[5,6] clearly illustrating that explosive compounds should be removed from contaminated sites. TNT biodegradation has been considerably studied in various microorganisms.[7,8] Although the degree of TNT transformation and the main products depend on the type of microorganism and the environmental conditions, initial reactions of TNT degradation are reductive rather than oxidative owing to nitro groups, which reduce the electron density of the aromatic ring, and inhibit oxidative attack by electrophilic oxygenases.[9] TNT`s initial reduction metabolism is likely to go on through either one-electron or two-electron mechanisms. The nitro group reduction via a one-electron mechanism, catalyzed by oxygen-sensitive (type II) nitroreductases generates a nitro anion radical, which reacts with oxygen to form superoxide and regenerates the parent nitro substituted compound. Oxygen-insensitive (type I) nitroreductases catalyze a two-electron reduction of nitro group substituted compounds, generating the corresponding nitroso, hydroxylamine, and amino derivatives.[10,11] Due to the great needs in public security and environmental protection there has been a great driving force in the development of sensors for the detection of TNT by various approaches,[12,13,14] of which electrochemical sensors are mainly suited for TNT detection since the electron-deficient nitro compounds are easily electrochemically reduced. Additionally electrochemical sensors meet the requirements of a high-speed and

on the-spot detection due to the inexpensive and portable devices which can be made from these sensors.

### 4.2 Chemistry of TNT

The three nitro-groups with a nucleophilic aromatic ring structure make TNT susceptible to reductive attack but resistant to oxygenase attack from aerobic organisms. In most current reports the reductive mechanism predominates in TNT degradation. The $\pi$ electrons from the TNT aromatic ring are removed by the electronegative nitro groups, which makes the nucleus electrophilic. The nitro group exists as a resonance hybrid. Because the oxygen atoms are more electronegative than the nitrogen atom, the polarization of the nitrogen-oxygen bond causes the nitrogen atom to carry a partial positive charge and to serve as an electrophile. Therefore, the most common reaction of the nitro group in biological systems is reduction, which can occur either by one-electron or two-electron mechanisms.[15]

TNT reduction can be achieved by two pathways. The first one includes a reduction of one or two nitro groups to a hydroxylamino group followed by formation of different amino-substituted metabolites such as amino-dinitrotoluenes and diamino-nitrotoluenes and tetranitroazoxytoluenes (AZTs) *via* the hydroxylamino-dinitrotoluenes (HADNTs) intermediate, although complete reduction of TNT to triaminotoluene (TAT) appears to require strict anaerobic conditions.[16,17] The second metabolic pathway involves removal of TNT nitro groups as nitrite ions, which is initiated by hydride attack on the aromatic ring. Vorbeck *et al.* first reported that a *Mycobacterium* sp. strain HL reduced TNT to a hydride-Meisenheimer complex. [18]

Nitro group and the amino group are comparatively stable. The reduction of the nitro group to the amine includes a sequence of reactions produces highly reactive intermediates. Conversely, the nitroso and hydroxylamino groups are electrophiles, which can interact with biomolecules to cause toxic, carcinogenic, and mutagenic effects.[19] Complete reduction of the nitro group to an amino group appears to decrease the mutagenic effect of the compound.[20]

### 4.3 Biodegradation of TNT

Poly-nitro nitroaromatic compounds (TNT) are difficult to degrade. When microbes are able to deal with these chemicals they degrade them at a very slow rate. The slow rate of degradation can result from the compounds` intrinsic toxicity, as well as from their limited solubility.

#### 4.3.1 Anaerobic degradation of TNT

The reactions of TNT degradation in anaerobic systems almost completely involve the reduction of nitro groups to aromatic amines through a six-electron transfer mechanism.[21] The reduction of nitro-groups to nitroso derivatives, hydroxylamines, or amines is catalysed by NTRs through the consecutive addition of electron pairs donated by co-substrates. Anaerobic degradation has the potential advantages of rapid reduction at low redox potential that minimize substrate oxidative polymerization due to the absence of oxygen. Therefore TNT anaerobic degradation systems have received enormous attention to get more efficient rates of removal, since azoxynitrotoluene products are not formed.[22,23] Anaerobes rarely draw out complete conversion of TNT into $CO_2$ or methane,[21] hence participation of a consortium of bacteria is required to achieve complete TNT degradation[24] The earliest proof of the anaerobic metabolism of TNT was reported by McCormick et al.[30] who illustrated that crude extracts of *Veillonella alkalescens* could reduce TNT to triaminotoluene (TAT). Additionally anaerobic cultures of *Desulfovibrio* and *Clostridium* have been reported to degrade TNT through triaminotoluene intermediate,[25] and a variety of other bacteria have been shown to reduce TNT under anaerobic conditions; *Escherichia coli* (TNT Reduction to TAT),[26] *Lactobacillus sp.* (TNT Reduction to TAT),[26] *Methanococcus sp. strain B (TNT Reduction to DANT),*[27] *Pseudomonas sp. strain JLR11* (TNT as nitrogen source; TNT as final electron acceptor),[28,29] *Veillonella alkalescens* (reduction of TNT to TAT),[30] *Sphingomonas capsulate* (Amine metabolites),[31] *Klebsiella sp.*C1 (reduced to hydroxylamino dinitrotoluenes, aminodinitrotoluenes and to nitrite *via* denitration),[32] *Methylobacterium sp.* BJ001(reduced to amino-dinitrotoluenes with no significant release of $CO_2$).[33]

### 4.3.2 Aerobic degradation of TNT

Oxygenase reactions are unidentified for TNT, due to the electrophilic character of the nitro groups. Therefore, even in the presence of oxygen, this explosive must be transformed by reductive metabolism. Under aerobic condition bacteria try to transform the TNT via reducing one or more nitro groups on the aromatic ring to hydroxylamino or amino groups. The TNT partially reduced forms (hydroxylamino- and nitrosodinitrotoluenes) are condensed in the presence of oxygen to produce recalcitrant tetranitroazoxytoluenes (e.g. 2,2'-azoxy, 4,4'- azoxy, 2,4'-azoxy) which resist further microbial metabolic degradation and cause a higher rate of mutation, toxicity, and genotoxicity than TNT.[34,35] These degradation reactions remove TNT but produce extremely recalcitrant products that are not metabolised by most of the microbes that produce them.[36]

The nucleophilic attack by a hydride ion to form a hydride-Meisenheimer complex, followed by the release of nitrite, is another reductive degradation mechanism mediated by aerobic bacteria. Where oxygenolytic attack under aerobic conditions is difficult due to their electron deficient nature, the electrophilic reaction is easy. The complex re-aromatizes after the release of nitrite anion.[37] Several bacteria show their ability to degrade TNT aerobically; *Pseudomonas* (TNT elimination via hydride-Meisenheimer complex formation and transform to dinitrotoluene, nitrotoluene and toluene),[38] *Rhodococcus erythropolis* (catalyze ring hydrogenation, hydride and dihydride TNT-Meisenheimer complexes formation),[39] *Pseudomonas fluorescens* (reduction by addition of hydride to form dihydride Meisenheimer complex to catalyze reduction of nitro group).[40]

### 4.3.3 Degradation of TNT by fungi

It is well known that fungi are also able to degrade TNT; many fungi were reported to partially degrade TNT *via* reducing it to amino aromatics.[41] The wood white rot fungus *Phanerochaete chrysosporium*, white rot fungi, and litter decay fungi were investigated for their capability to transform TNT. The importance in white rot fungi arises from their ability to degrade a diverse group of environmentally persistent and toxic chemicals. They produce a complex system of extracellular peroxidases, small organic molecules, and hydrogen peroxide for the degradation of lignin. The ligninolytic system is non-specific

and can biodegrade a wide range of synthetic chemicals including nitroaromatic compounds.[25]

As in other organisms, fungal degradation of TNT starts with a reduction producing aminodinitrotoluenes (ADNT), which is catalysed by a plasma-membrane reduction system and independent of ligninolytic system expression. Further degradation is achieved via the production of lignin peroxidase and Mn-dependent peroxidase.[42,43] These fungi are able to mineralize TNT, they use extracellular enzymes, and ligninolytic system to mineralize TNT. They need reduced nitroaromatics to start the metabolic pathway, and therefore TNT has to be reduced to ADNT before mineralization can progress.[44] Different fungi were found to degrade TNT; *Phanerochaete chrysosporium* (hydroxylamino dinitrotoluene formation),[45] *Litter decaying fungus* (concentration decreased to 95% in 300 days with no reduced intermediate formation),[46] *Nematoloma forwardii* (complete mineralization through conversion of 2- amino-4,6 dinitrotoluene by manganese peroxidase),[47] *Phlebia radiata* (reduce and mineralize TNT by manganese dependent peroxidase),[48] *Phanerochaete chrysosporium* (mineralized under lignolytic condition with formation of 9 different acetylated products).[49,50]

### 4.3.4 Degradation of TNT by plants

Plants appear to deal with TNT via the chemical transformation of TNT by reductive and oxidative processes, TNT metabolites are subsequently conjugated with plant-derived glucose, malonate or glutathione, and sequestered within plant tissues or polymers rather than being mineralized to carbon dioxide and nitrogen.[51,52] TNT prefers reductive transformation reactions in plants due to the electron-withdrawing properties of the nitro groups. Both the 2- and the 4- isomers of HADNT and ADNT intermediates have been detected in plants.[53,54]

Phytoremediation of TNT, is often a slow and incomplete process, potentially causing accumulation of toxic metabolites, therefore plants have been genetically modified to overcome the inherent limitations of plant detoxification capabilities. Bacterial genes encoding enzymes (nitroreductase) involved in the breakdown of TNT have been introduced in higher plants, resulting in significant enhancement of plant tolerance, uptake, and detoxification performances.[55,56]

### 4.4 Bacterial oxygen-insensitive nitroreductases involved in TNT degradation

The nitroreductases are a group of enzymes that catalyze the reduction of nitro groups on aromatic compounds, including TNT. Certain bacteria are known to hold oxygen-insensitive nitroreductase, which transfers two electrons of NAD(P)H to reduce TNT. *E coli.* nitroreductases NfsA and NfsB have been reported for their ability to reduce TNT.[57]

***Pseudomonas sp.***

In 2001, a TNT-degrading nitroreductase with a monomeric molecular mass of 27 kDa was obtained from *Pseudomonas aeruginosa* strain isolated from TNT contaminated soil. The enzyme rapidly degraded TNT to 4-hydroxylamino-2,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene and required NADH as a co-substrate.[58]

In *Pseudomonas putida* JLR11 (TNT-degrading strain) two different nitroreductases PnrA and PnrB have been identified. According to sequence analysis studies PnrA was suggested to be close to NfsA subgroup A nitroreductases, whereas PnrB is close to subgroup B nitroreductases and they are expressed constitutively. PnrA catalyzes the reduction of different compounds including 2,4,6-trinitrotoluene, 2, 4,-dinitrotoluene, 3-nitrotoluene 3-and 4-nitrobenzoate, 3,5-dinitrobenzamide and 3,5-dinitroaniline, and the $V_{max}/K_m$ parameter (6400 μmol/min/mg/5 mM) revealed that TNT is the most efficient substrate for this enzyme. Recently it has been reported that *Pseudomonas putida* JLR11 carries out multiple enzymatic attacks on TNT releasing ammonium.[59]

More recently a TNT nitroreductase PnrB from *Pseudomonas* sp. HK-6 which is able to use TNT as a sole nitrogen source has been cloned and expressed in *Escherichia coli*. The enzyme showed an apparent $V_{max}$ and $K_m$ for TNT (12.6 μmol/min/mg and 2.9 mM), complete TNT degradation was achieved by wild-type strain, while a PnrB mutant degraded only 10% of TNT . This result showed that PnrB might play the major role in the TNT degradation pathway.[60]

***Clostridium acetobutylicum***

Two different oxygen insensitive nitroreductases NitA (31 kDa) and NitB (23kDa) have been characterized in *Clostridium acetobutylicum*. They share 46% similarity in amino acids sequences and their biochemical characteristics are highly similar to NfsA the major nitroreductase in *Escherichia coli*. Both enzymes revealed a broad substrate specificity and showed no flavin reductase activity. NitA uses NADH as electron donor, while NitB can utilize both with preference for NADH. NitA had a specific activity for TNT reducing over eight fold more effectively than NitB. The nitroreductases showed higher relative expression on induction with TNT compared to the uninduced control.[61]

***Klebsiella sp.***

*Klebsiella sp.* Strain C1 NAD(P)H nitroreductases have the ability to metabolize TNT by two reduction pathways (denitration and nitro group reduction) and the Nitroreductase I, with the highest catalytic activity of TNT has been purified and characterized. The N-terminal amino acid sequence of this enzyme does not show high similarity with nitroreductases from other enteric bacteria.[62,63] Unlike other nitroreductases that preferentially reduce TNT nitro groups in the *para* position, Nitroreductase I has the ability to reduce *ortho* isomers (2-hydroxylaminodinitrotoluene and 2-aminodinitrotoluene) more easily than *para* isomers (4-hydroxylaminodinitrotoluene and 4-aminodinitrotoluene). The enzyme does not reduce the *para* position nitro group while, the *ortho* position nitro group of 2,4-dinitrotoluene is reduced to generate 2-hydroxylamino-4-nitrotoluene. More recently Nitroreductase II has been shown to have a higher substrate affinity and specific activity for TNT reduction than other nitroreductases.[64]

The main objective of this chapter is to the activity and specificity of bacterial NTRs *versus* TNT, as well as the stoichiometry of the enzymatic reaction to confirm the reductive pathways followed by each enzyme.

## 4.5 Enzyme assay

To calculate the enzymatic activity of bacterial NTRs (NfnB $LT_2$, YdjA, Dde_0086, Dde_2199, and NfnB $K_{12}$) to TNT, the oxidation rates of NADH were measured in accordance with changes in absorbance at 340 nm.

NfnB $K_{12}$ (Fig. 4.1) demonstrated high catalytic efficiency towards TNT, the continuous and gradual decrease in absorbance at 340 nm, and the associated formation of azoxy-dimer compounds at 410 nm, which might be referred to the good affinity of the enzyme towards the substrate and the enzyme catalytic efficiency.

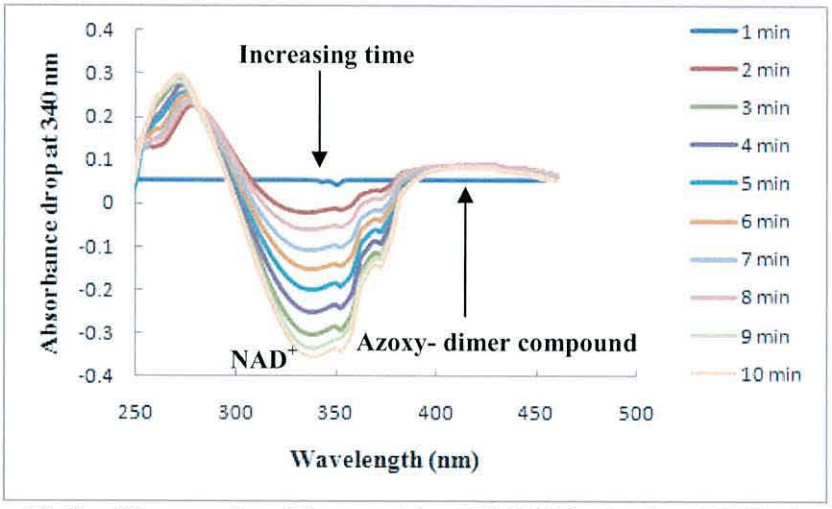


**Figure 4.1: Repetitive scanning of the recombinant NfnB K12 reduction of TNT using NADH.**

Acceptable TNT reduction by another recombinant NTR, NfnB$LT_2$ was observed (Fig. 4.2). NADH oxidation was seen *via* gradual and regular decrease in absorbance at 340 nm and a significant production of azoxy-dimer compounds at 410 nm was also observed, which can be explained by effective catalytic reaction proceeding by the particular enzyme, and leading to the accumulation of intermediates, and hence azoxy-dimer production.

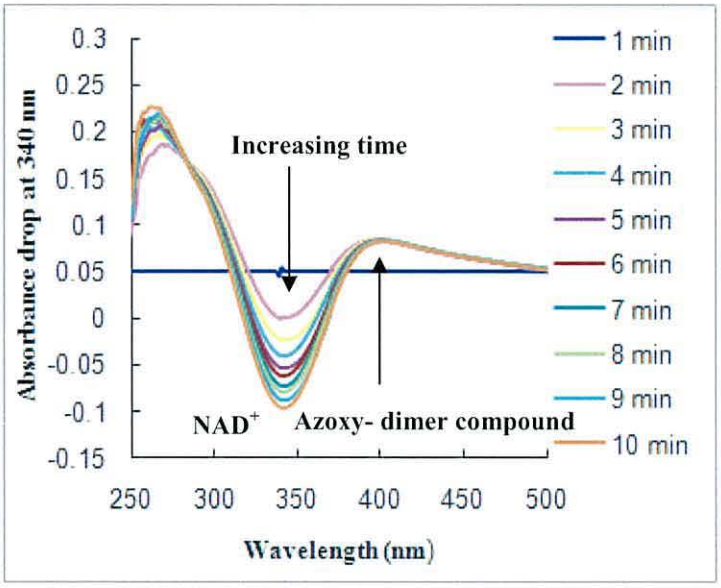


**Figure 4.2: Repetitive scanning of the recombinant NfnB LT2 reduction of TNT using NADH.**

TNT degradation at very low rate was catalyzed by YdjA and very slight production of azoxy-dimer compounds was noticed at 410 nm because of the minimal reduction rate carry on by the enzyme. Therefore there is a low possibly of intermediate accumulation and condensation (Fig. 4.3).

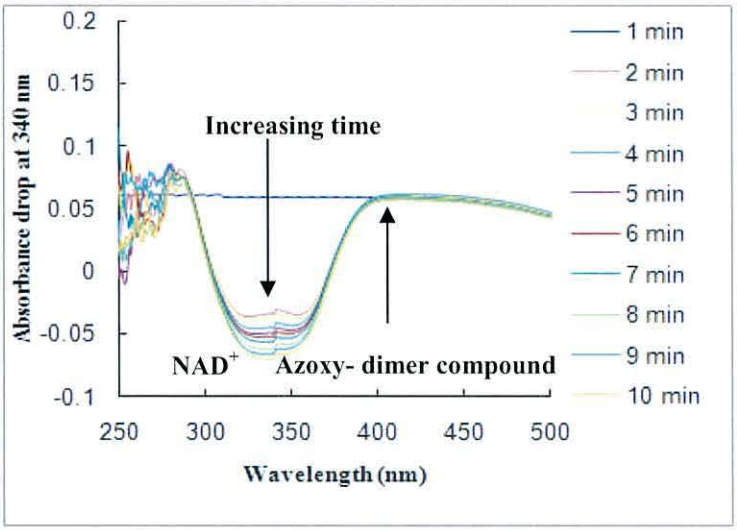


**Figure 4.3: Repetitive scanning of the recombinant YdjA reduction of TNT using NADH.**

Although Dde_0086 catalysed TNT reduction effectively (Fig. 4.4), a minor signal at 410 nm corresponding to azoxy-dimer formation was made. This can be explained by the slow reaction rate catalyzed by the enzyme, and therefore minimal intermediate accumulation; although there might be an affinity of the enzyme to the TNT.

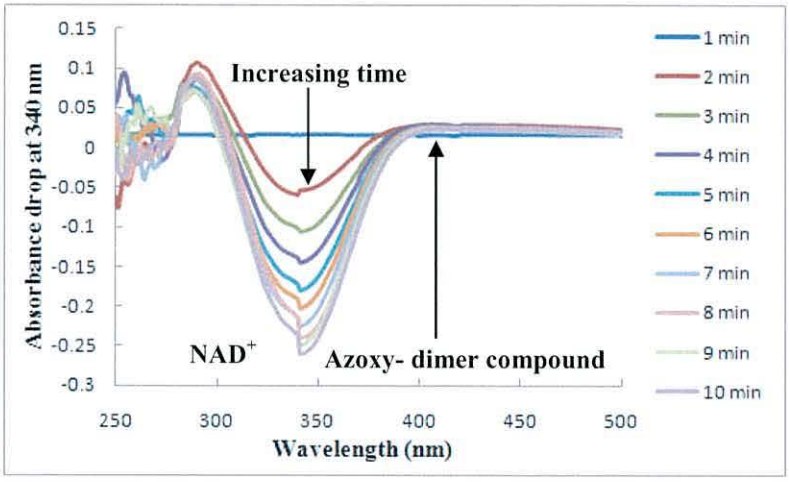


**Figure 4.4: Repetitive scanning of the recombinant Dde_0086 reduction of TNT using NADH.**

High non-regular TNT reduction was found to be caused by Dde_2199, and a low azoxy-dimer formation at 410 nm due to the same reason mentioned above.

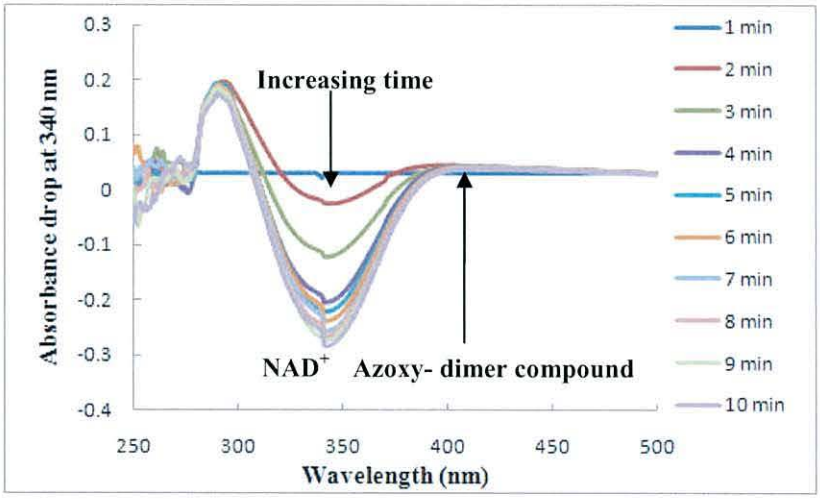


**Figure 4.5: Repetitive scanning of the recombinant Dde_2199 reduction of TNT using NADH.**

All bacterial NTRs showed considerable activity towards TNT with different reaction rates, while YdjA exhibited a negligible response compared to other NTRs. A gradual decrease with time in absorbance at 340 nm was noticed indicating NADH oxidation. A durable signal at 410 nm was observed regarding all NTRs reduction of TNT, which is believed due to the formation of azoxy-dimer compounds owing to the condensation of partially reduced intermediates in the presence of oxygen.[8,57]

Spectra were also recorded for Nfnb-$cys_{12}$, YdjA-$cys_{12}$, Dde_0086-$cys_{12}$, and Dde_2199 -$cys_{12}$ to demonstrate the activity of the engineered NTRs. The oxidation of the NADH was again monitored at 340 nm. All the engineered enzymes showed a gradual decrease with time in the absorbance at 340 nm, and the formation of an azoxy-dimer compound at 410 nm was also noticed.

### 4.6 Enzyme specificity

Bacterial NTRs specificity against TNT was demonstrated. Unfortunately, as mentioned before in chapter 3 due to the rare or non-existent experimental evidence concerning all bacterial NTRs involved in this study, therefore the activity of NfnB $k_{12}$ from *E coli* against TNT was determined to facilitate explain the substrate specificity of the enzymes, due to homology of the studied NTRs with *E. coli* $k_{12}$ for which a great deal of data has been published including NTRs enzymes involved in the reduction of TNT as well as reductive transformation pathways of TNT.

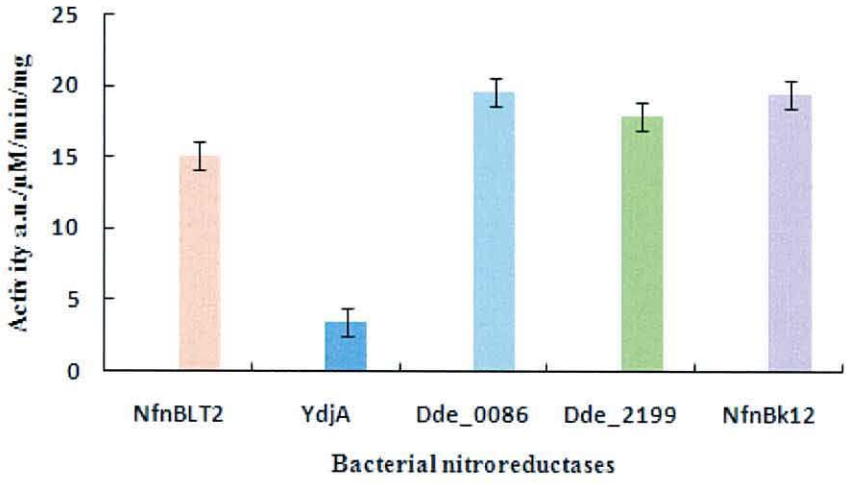


**Figure 4.6: The activity of bacterial nitroreductases against TNT.**

It has been shown that *E. coli* has multiple enzymes that attack TNT, and site directed mutagenesis study revealed that a single mutation, NfsA mutation, NfsB mutation, or NEM mutation (responsible for the release of nitrite from the nitroaromatic ring) provided a growth yield and TNT transformation capacity similar to that of the wild-type strain. The limited TNT consumption when NEM was combined with either NfsA or NfsB indicates that NEM reductase could also play a role in TNT metabolism in *E. coli* and the triple mutant strain in which the three genes had been inactivated, the inactivated was not able to metabolize TNT and did not grow. This result confirms that NfsA, NfsB and NEM reductase all play a role in TNT metabolism in *E. coli*.[65]

It is obvious from Fig 4.6 that bacterial NTRs except YdjA exhibited considerable activity against TNT and in a pattern identical to that of NfnB $K_{12,}$ although they share various similarity rates (chapter 3, Table 3.1), this might be due to the participation of similar or identical residues in active site cleft, which accommodate the TNT. Further studies regarding crystal structure of bacterial NTRs in a complex with the TNT might establish the direct link between the NTRs and TNT reduction or to identify other responsible enzymes.

Negligible activity was observed for YdjA *versus* TNT (Fig. 4.6), although studies reported that the lack of two helices around the active site, which restrict substrate binding, make the enzyme able to accommodate a wide range of substrates, hence additional studies regarding YdjA-TNT structure might provide further insight into YdjA substrate specificity.

The ability of *Desulfovibrio* sp. to use TNT as a nitrogen source for growth and electron acceptor have been extensively studied.[66,67,68,69] These studies revealed the possibility of using this isolate to decontaminate the sites contaminated with different nitro-compounds under anaerobic conditions, making this organism of great interest as bioremediation and biological recognition elements for TNT biosensor. Up to now there is no experimental evidence regarding the role of enzymes responsible for TNT degradation in *Desulfovibrio* sp., and these are the first results about NTRs degrading TNT *Desulfovibrio desulfuricans G20.*

## 4.7 Stoichiometry of the enzyme reaction

As described in chapter 3 the reduction of a nitro group to an amine group *via* the nitroso and hydroxylamino-groups is a six electron reduction. Thus each reduction step must use one pair of electrons indicating that one NADH molecule is required for each step.

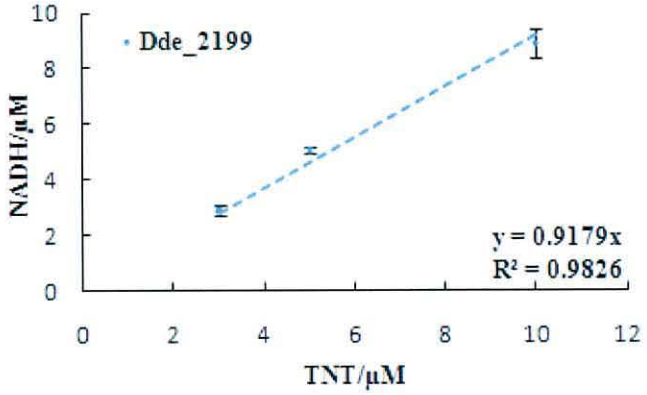


**Figure 4.7 : A chart showing the calculated concentrations of TNT/ Dde_2199 against the concentration of NADH used during the reaction at 340 nm.**

The recorded absorbance drop for 5 μM of TNT equalled –0.03039 a.u. (Fig. 4.7) consequently when this drop is divided by -0.006 a.u. this yields the concentration of NADH (5.1 μM) reacting for every 5 μM of TNT present; hence one μM of NADH is oxidized for every one μM of TNT present.

The resulting data achieved for NfnB demonstrated a correlation of 0.98 with a slope of 1.96 therefore. The ratio of TNT to NADH is 1:2 hence, and the NfnB must reduce the nitro group to hydroxylamino. The stoichiometry of the YdjA enzymatic reaction against TNT could not be estimated due to the negligible response of this enzyme towards TNT. (Fig. 4.7)

The molar ratio of NADH oxidized to TNT reduced (μM NADH/ μM TNT) was found to be 0.90 (correlation of 0.98), and 0.92 (correlation of 0.98) concerning Dde_0086 and Dde_2199 respectively, hence Dde_0086 and Dde_2199 must reduce the nitro group to the nitroso intermediate.

The different reductive pathways followed by bacterial NTRs in TNT degradation pave the way to develop a biosensor for TNT and other nitro-compounds detection and identification, which is the main objective of this study.

## 4.8 Conclusions

All bacterial NTRs except YdjA exhibited significant activity towards TNT with different reaction rates catalysed by the different enzymes. They showed the same pattern of specificity towards TNT in a model identical to that of NfnB $K_{12,}$ except YdjA.

NfnB $LT_2$ and *Desulfovibrio desulfuricans G20* NTRs (Dde_0086 and Dde_2199) followed different reductive transformation pathways concerning TNT degradation.

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# Chapter 5- Cocaine and Bacterial carboxylesterases Results and Discussion

## 5.1 Introduction

The key purpose of this chapter was to develop biological recognition elements for a sensor for cocaine detection by way of isolation two different bacterial carboxylesterases (CEs) PnbA from *Bacillus subtilis* subsp. subtilis strain 168 designated in this study as PnbA1, and PnbA from *Bacillus licheniformis* ATCC 14580 designated as PnbA2, based on the role of human liver carboxylesterases hCE to hydrolyze cocaine into its main metabolites; ecgonine methyl ester and benzoic acid, or benzoylecgonine and methanol. Then confirm their activity *via* the catalysis of cocaine into its main metabolites, followed by engineering of the enzymes through integration of the cys-tag (6 cysteine amino acids) at the enzyme N-terminus to allow the enzyme to be immobilised on the biosensor electrode surface and subsequently study the effect of cys-tag insertion on hydrolysis reaction pathways achieved by the enzyme.

Amino acids alignments of hCE-1, hCE-2, PnbA1, and PnbA2 (Fig. 5.1) have shown that PnbA1, and PnbA2 were revealed to have 58% sequence identity. PnbA1 shares 33% sequence identity with hCE-1, and 34% with hCE-2, while PnbA2 exhibits 30% sequence identity with hCE1-1 and hCE-2.

CEs proliferate in higher eukaryotes, but appear infrequently in bacteria and lower eukaryotes.[1] To date there is no experimental evidence concerning cocaine metabolism by bacterial carboxylesterases, however the capability of hCE-1[2] and hCE-2[3] to hydrolyze cocaine, and the crystal structures of hCE1 in complex with the cocaine analogue homatropine have been determined.**Error! Bookmark not defined.**[4]

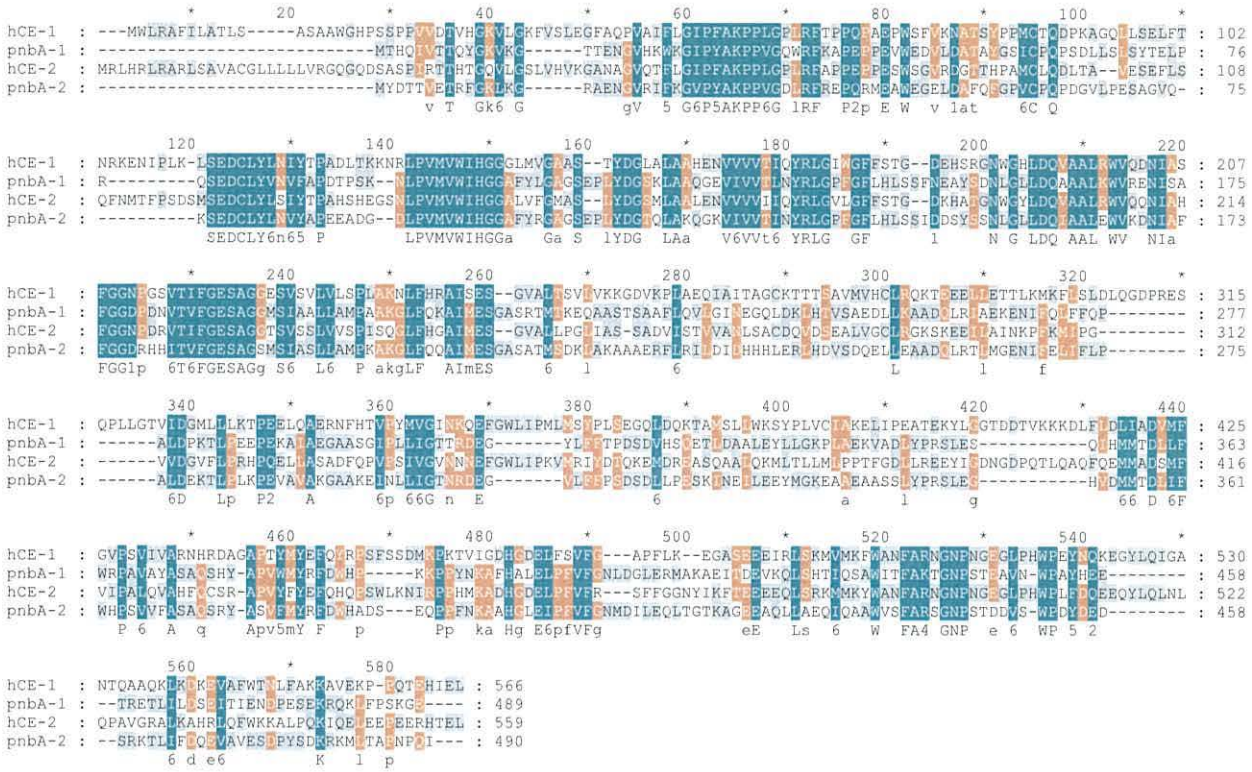

**Figure 5.1: Amino acid alignment of hCE-1, hCE-2, PnbA1, and PnbA2 using clustalX2. Shading indicates amino acid conservation of 100% (green), 80% (orange), and 60% (grey).**

## 5.2 PCR amplification of bacterial carboxylesterases

Genomic DNA from *Bacillus subtilis* subsp. subtilis strain 168, and *Bacillus licheniformis* ATCC 14580 were extracted and PCR amplification using Phusion™ High-Fidelity DNA Polymerase with predesigned primers (Chapter 2 Table 1) was used to isolate two different bacterial carboxylesterase enzymes, *pnbA1* from *Bacillus subtilis* subsp. subtilis strain 168 and *pnbA2* from *Bacillus licheniformis* ATCC 14580.

The first PCR experiment (Fig. 5.2) resulted in the isolation of CEs genes(*PnbA1* and *PnbA2*) having a *BamH1* restriction site in the 5` end regarding both enzymes, and a *HindIII* restriction site and *SacI* in the 3` end regarding *pnbA1* and *pnbA2* respectively.

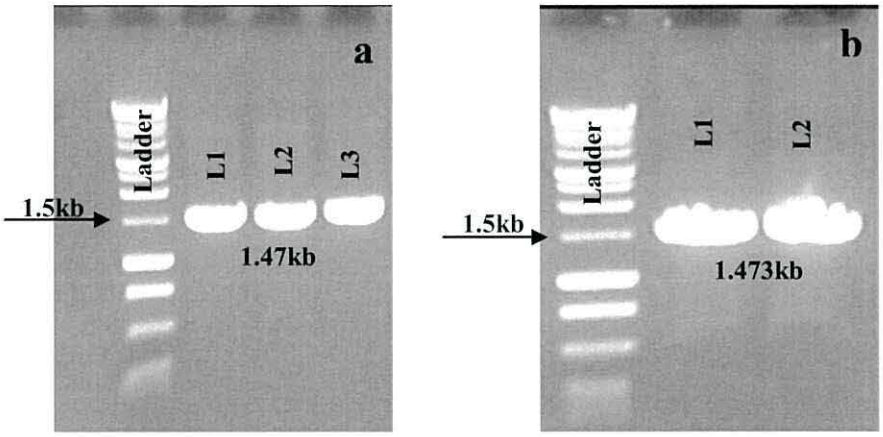


**Figure 5.2: PCR amplification of recombinant bacterial carboxylesterases using Phusion™ High-Fidelity DNA polymerase with pre-designed primers. a: *pnbA1* (1.47 Kb), b: *pnbA2*. (1.473 Kb) with forward primer containing BamH1 restriction site and reverse primer containing HindIII restriction site regarding *pnbA1*, and forward primer containing BamH1 restriction site and reverse primer containing *SacI* restriction sit regarding *pnbA2*. Ladder: 10 kb. L= lane.**

The primers were designed in a manner to match two regions at both side of the CEs gene, and helped in the isolation of the region containing the gene of interest; the primers generated restriction sites at either end of the gene to allow its successful insertion into the plasmid vector. The *pnbA2* reverse primer comprised *SacI* restriction site instead of *HindIII* due to the involvement of *HindIII* restriction site sequences within *pnbA2* gene sequence, which would digest the gene of interest, hence disrupt gene cloning.

The second PCR experiment resulted in the isolation of modified CEs genes having six adjacent codons for cysteine (cys-tag) (Figure 5.3). The success of the experiments was confirmed *via* 0.7% agarose gel electrophoresis, and a molecular weight marker (10 kb) was analysed by electrophoresis with PCR products to estimate the sizes of unknown fragments.

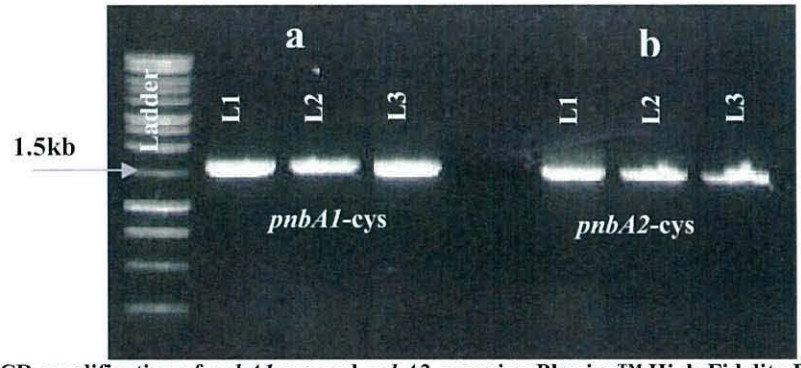


**Figure 5.3: PCR amplification of *pnbA1*-cys and *pnbA2*-cys using Phusion™ High-Fidelity DNA polymerase with pre-designed primers. a: *PnbA1*-cys (1.47 kb) with forward primer containing cys-tag, and BamH1 restriction site and reverse primer containing HindIII restriction site. b: *pnbA2*-cys (1.473kb) with forward primer containing cys-tag, and BamH1 restriction site and reverse primer containing *SacI* restriction sit.**

### 5.3 Ligation of recombinant and engineered bacterial carboxylesterases into pET28a(+)

A gel extraction kit was used to ligate the CEs genes into the plasmid vector due to failure of different attempts that made for ligation (as described in Chapter 3 for NTR ligation).

The plasmid vector pET28a(+) and purified PCR products (*pnbA1*, *pnbA2*,) were run in a agarose gel then gel extracted, purified, vacuum dried, and resuspended in PCR water and digested with *BamH1* and *HindIII* for *pnbA1*, while *BamH1* and *SacI* were used for *pnbA2*, and ligated in different ligation reaction. After an overnight ligation, the plasmid vector was transformed into competent cells of *E. coli* DH5α , a suicide cut with *EcoR1* was used to remove the plasmids that had re-ligated (without insertion). To prove that the cloning vector contains the insert, the resulting vectors were digested with appropriate enzymes and the resulting fragments were run in 0.7% agarose gel electrophoresis. Figure 5.4 illustrates the success of the ligation process for recombinant CEs; hence a successful ligation when run on a gel would produce two fragments one corresponds to the vectors and the second corresponds to the insert.

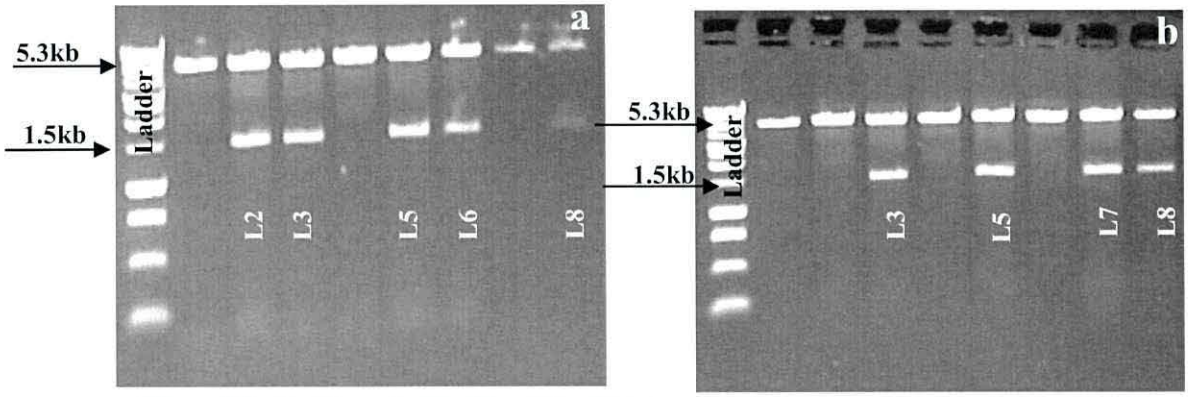


**Figure 5.4: Ligation of recombinant bacterial carboxylesterases into pET28a(+). {a: 5.3kb bands=pET28a(+) vector, 1.47kb bands= *pnbA1*. b: 5.3kb bands=pET28a(+) vector, 1.473kb bands= *pnbA2*} with forward and reverse primers. Ladder =10kb marker.**

Once again a gel extraction kit was used to ligate the engineered CEs into the plasmid vector due to the same reason described above, and in the same way confirmed that the plasmid vector pET 28a(+) contained the engineered CEs (*pnbA1*-cys, and *pnbA2*-cys), after isolation of plasmids, restriction of the genes with *BamH1, HindIII*, and *SacI* running the resulting fragments on agarose gel, and estimation the expected sizes of the fragments. Figure 5.5 shows the success of the ligation for engineered CEs.

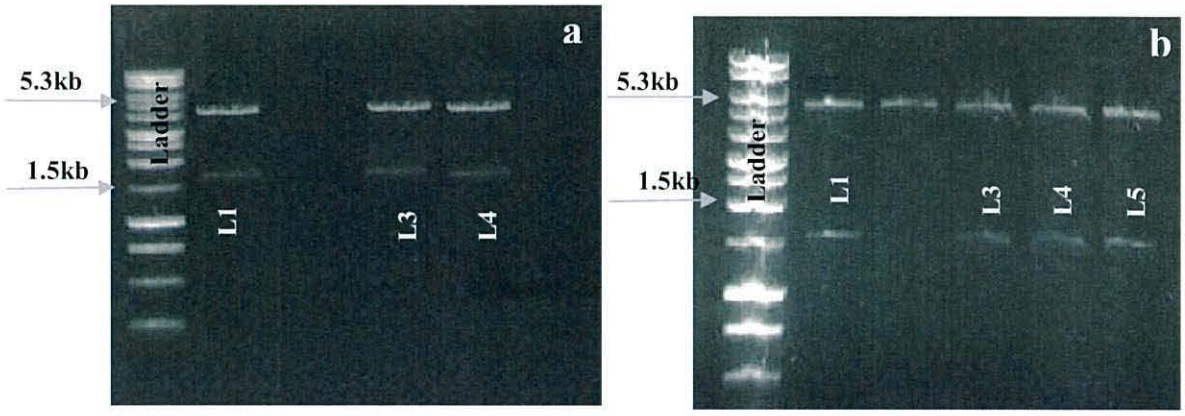


**Figure 5.5: Ligation of engineered bacterial carboxylesterases into pET28a(+). {a: 5.3kb bands=pET28a(+) vector, 1.47kb bands= *pnbA1*-cys. b: 5.3kb bands=pET28a(+) vector, 1.473kb bands= *pnbA2*-cys} with forward and reverse primers. Ladder =10kb marker.**

## 5.4 Overproduction of carboxylesterase enzyme

In order to overproduce the enzyme, the pET28a(+) vectors containing recombinant carboxylesterases *pnbA1*, and *pnbA2* genes were transformed into Rosetta *E.coli* in different transformation processes and let to overproduce. It is clear from Figure 5.6 that the induction of the proteins has been successful and the proteins have been expressed at the expected molecular weight. It is also illustrated that the samples taken at 100 mM imidazole elutes the largest quantities of proteins off the column.

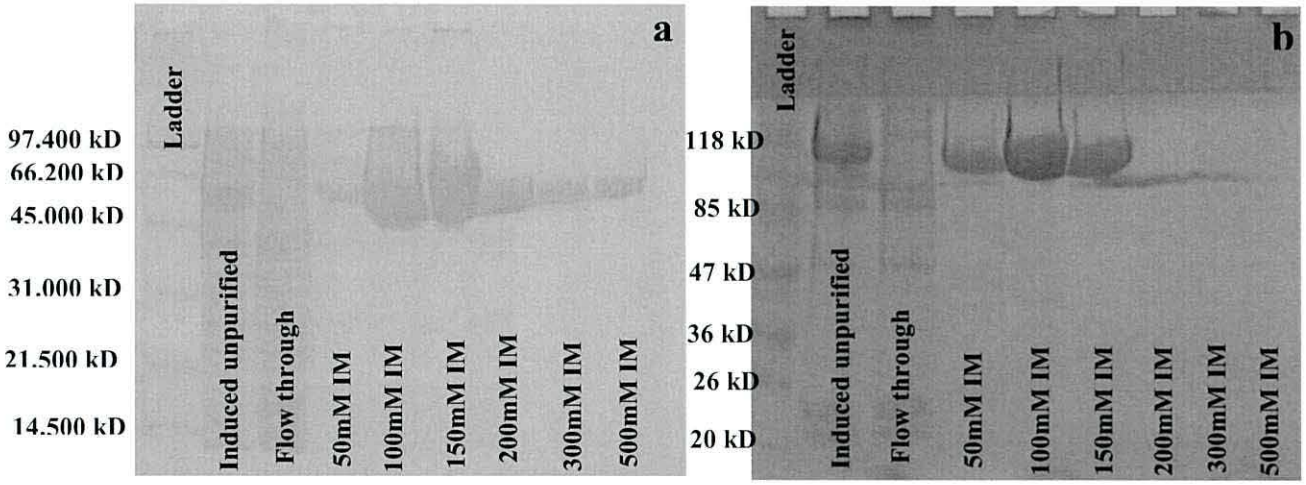


**Figure 5.6: SDS-PAGE of fractions containing the purified bacterial CEs expressed from a: pET28a(+)-PnbA1, b: pET28a(+)-PnbA2.**

Within all imidazole concentrations, the 2nd 1ml of 100mM imidazole was found to contain the greatest amount of proteins (Fig. 5.7) regarding both recombinant CEs.

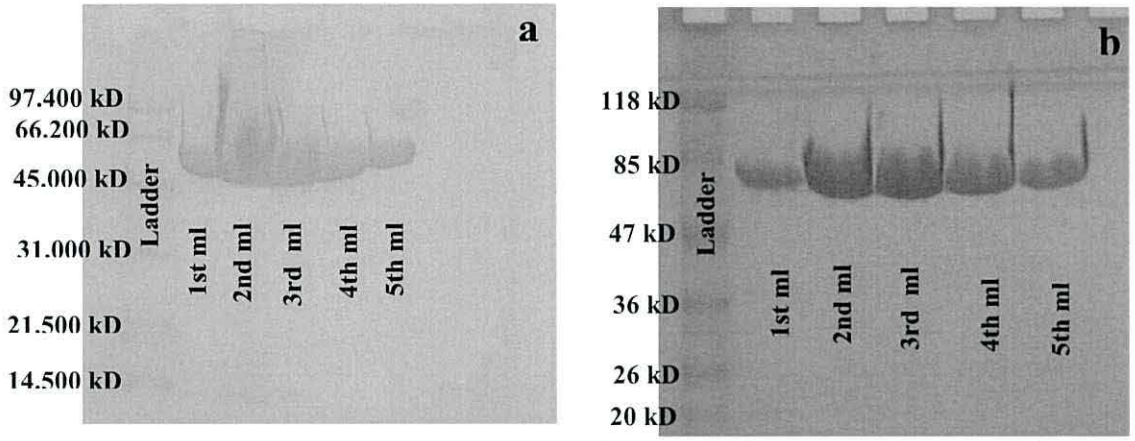


**Figure 5.7: SDS-PAGE of fractions containing the purified bacterial CEs expressed from a: pET28a(+) PnbA1,b: pET28a(+)-PnbA2 showing the 100 mM imidazole samples.**

The cys-tagged protein required a higher imidazole concentration to be eluted due to the extra binding of the cys-tags, and the high molecular mass bands in the cys-tagged proteins attributed to the protein dimerisation (Fig.5.8).

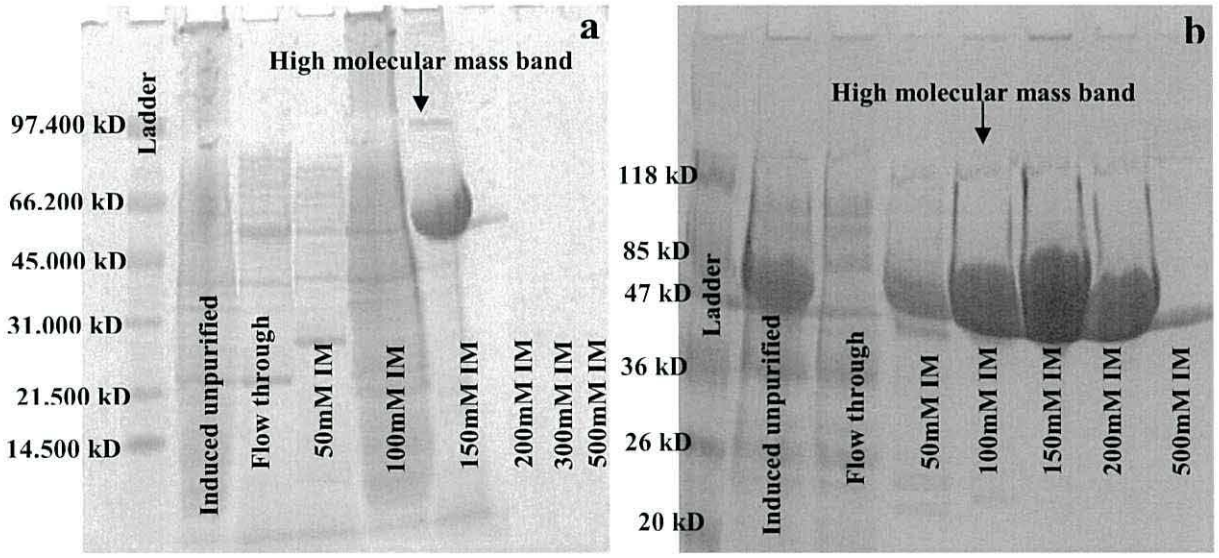


**Figure 5.8: SDS-PAGE of fractions containing the purified CEs-cys expressed from a: pET28a(+)-PnbA1-cys, b: pET28a(+)-PnbA2-cys.**

Analogous to recombinant CEs, the 2nd 1 ml of more concentrated (150 mM) imidazole eluted greatest amount of proteins (Fig. 5.9) concerned both engineered CEs.

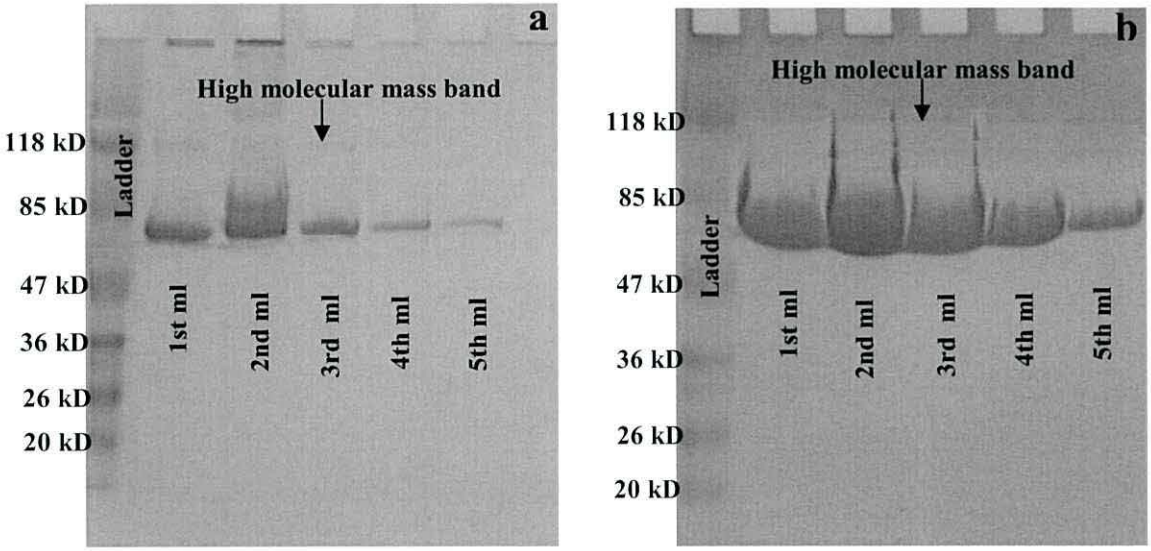


**Figure 5.9: SDS-PAGE of fractions containing the purified engineered CEs expressed from a: pET28a(+)-PnbA1-cys, b: pET28a(+)-PnbA2-cys, showing the 150mM imidazole samples.**

The SDS-PAGE gels showed that a concentration of over 150 mM imidazole is required to remove the protein off the column, and a lower concentration (50 mM) is required to remove other unwanted fragments. Then 500 mM imidazole is passed through the column to ensure that all of the protein is eluted from the column.

### 5.5 Enzyme assay.

To demonstrate the effectiveness of the carboxylesterases hydrolysis experiments were carried out using a wavelength scan over 300-500 nm. The reaction mixture contained 1 mM phosphate buffer pH 8.0, *para*-nitrophenylacetate (20 µl, 1 mM), 10 µl of cell extract in a final volume of 1ml. At time 0 (blue line) the reaction cuvette and the reference cuvette were equivalent. The addition of purified carboxylesterases (PnbA1, PnbA2) enzymes to the reaction cuvette in a different hydrolysis reactions initiated the reaction, and the appearance (positive absorbance) of the *para*-nitrophenol at 400 nm was monitored appropriately. The experiment was determined after 9 scans, and the sequential increase in absorbance at approximately 400 nm was due to the liberation of *para*-nitrophenol from para-nitrophenylacetate as *para*-nitrophenol has a strong absorbance at 400 nm (Fig. 5.10).

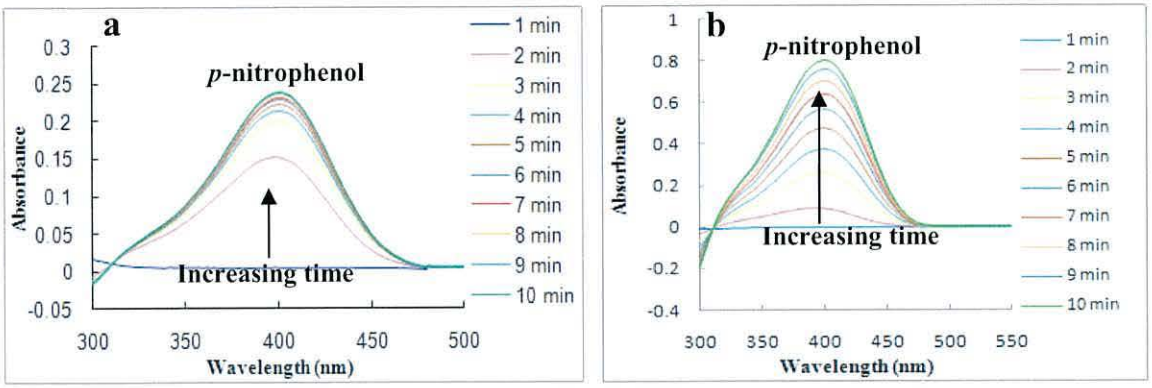


**Figure 5.10 : Repetitive scanning of the recombinant carboxylesterases hydrolyzing *para*-nitrophenylacetate (*p*-NPA). a: PnbA1, b: PnbA2. The largest increase in absorbance can be seen at 400nm, the optimum absorbance of *p*-nitrophenol. The contents of the sample and reference cuvette were the same except that the reference cuvette did not contain any enzyme.**

It is obvious from Figure 5.10a that there is a considerable increase in absorbance at 400 nm related to 2$^{nd}$ (pink line) and 3$^{rd}$ (yellow line), while a slight absorbance increase was observed related to other lines with increasing time. These might be explained by the low affinity of the substrates towards the enzyme, or even a minimal reaction rate catalyzed by the enzyme. Whereas the increase in absorbance at 400 nm considering PnbA2 (Fig. 5.10b) with increased time increased gradually and approximately at the same rate and these might be due to the high affinity of the substrate and a great reaction rate proceeded by the enzyme. These results related to both enzymes (PnbA1 and PnbA2) have been confirmed by the hydrolysis reaction catalysed by the engineered CEs (PnbA1-cys and PnbA2-cys). Once again PnbA1-cys (Fig. 5.11a) exhibited unequal hydrolysis reaction rates, great absorbance increase at 400 nm for the first two lines (pink and yellow) and a faint increase for the other lines with increasing time, a regular reaction rate demonstrated by PnbA2-cys (Fig. 5.11b) .

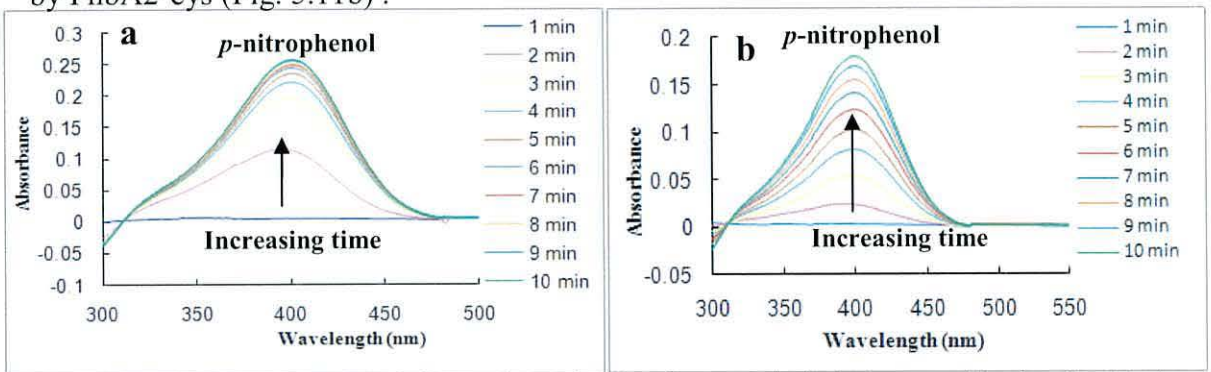


**Figure 5.11 : Repetitive scanning of the engineered carboxylesterases hydrolyzing *para*-nitrophenylacetate (*p*-NPA). a: PnbA1-cys, b: PnbA2-cys. The largest increase in absorbance can be seen at 400nm, the optimum absorbance of *p*-nitrophenol. The contents of the sample and reference cuvette were the same except that the reference cuvette did not contain any enzyme.**

## 5.6 Protein concentration

The protein concentration was calculated according to a Bovine Serum Albumin (BSA standard) calibration curve, and the Biuret Protein Assay was used to determine the protein concentration based on the colour change of the copper in the Biuret reagent in the presence of peptide bonds. (Table 1)

**Table 1: The protein concentrations of purified recombinant and engineered carboxylesterases.**

| Protein | Average total protein mg/ml | Average total protein mg/500ml culture |
|---|---|---|
| PnbA1 | 5.9 | 20.65 |
| PnbA1-cys | 5.4 | 15.12 |
| PnbA2 | 6.2 | 24.8 |
| PnbA2-cys | 5.9 | 17.7 |

For an unknown reason Table 1 and Figure 12 show that cys-tag insertion decreased protein expression about 26.8% for PnbA1, and 28.6 regards PnbA2.

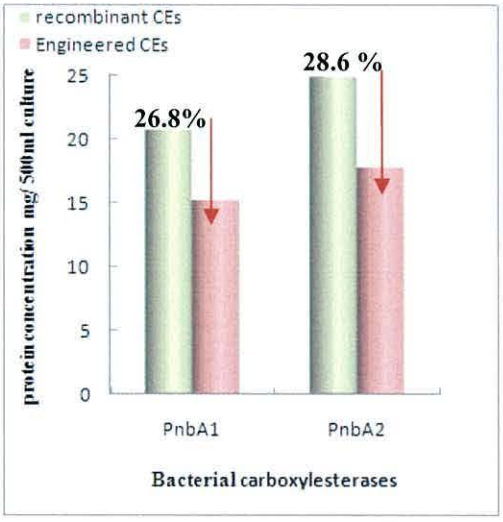


**Figure 5.12: Effect of cys-tag insertion on protein expression.**

The activity of the purified carboxylesterases against increasing enzyme concentrations has been shown in Figures 5.13a and 5.13b, the use of increasing amount of carboxylesterases resulted in a corresponding linear increase in the rate of *p*-nitrophenol production, indicating that the CEs is responsible for the *p*-nitrophenol production.

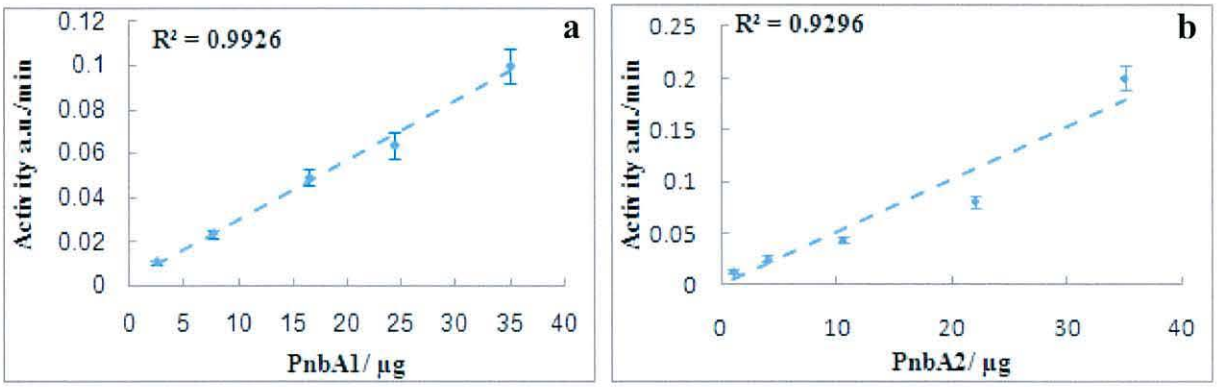


**Figure 5.13: A calibration line of activity / min (400 nm) against µg of a: PnbA1, b: PnbA2.**

## 5.7 pH and temperature dependence

The optimum pH and temperature dependence were estimated for the carboxylesterase activity (PnbA1 and PnbA2) at a series of different pH and temperature values. The enzymatic assays were conducted using different buffers for various pH ranges: 50mM sodium citrate (pH range 5 – 6), 50 mM phosphate buffer (pH range 7 – 9), and 50 mM CAPS (pH range 10 – 11), and the measured activity was represented as µM/min/mg.

Both enzymes were active at a broad range of pH from 6.0 to 11.0 (Figures 5.14, and 5.15), but more favourable in a basic pH, and a broad maximal activity for both enzymes between pH 8.0 and 9.0 was found. These results are in agreement with published values [5] for carboxylesterases from *Geobacillus stearothermophilus*. However, both enzymes were completely inactivated at acidic pH( 5.0) and these results are in agreement with published values[6] for other carboxylesterases with complete inactivation at acidic pH<5.0.

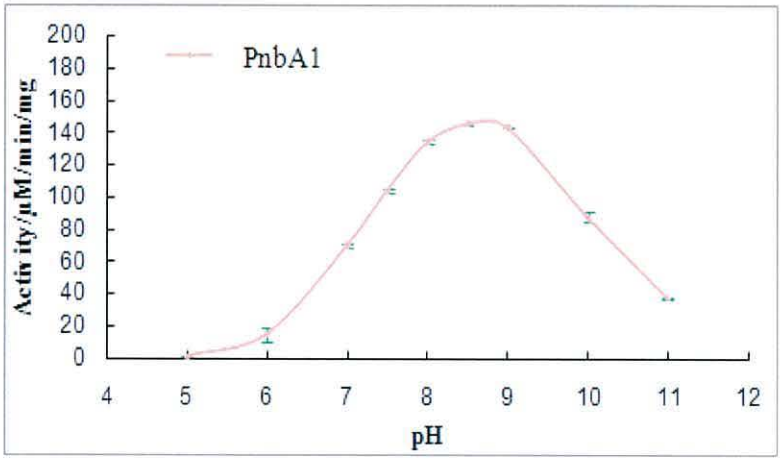


**Figure 5.14: The effect of pH on the activity of PnbA1 from rates of absorption at 400 nm.**

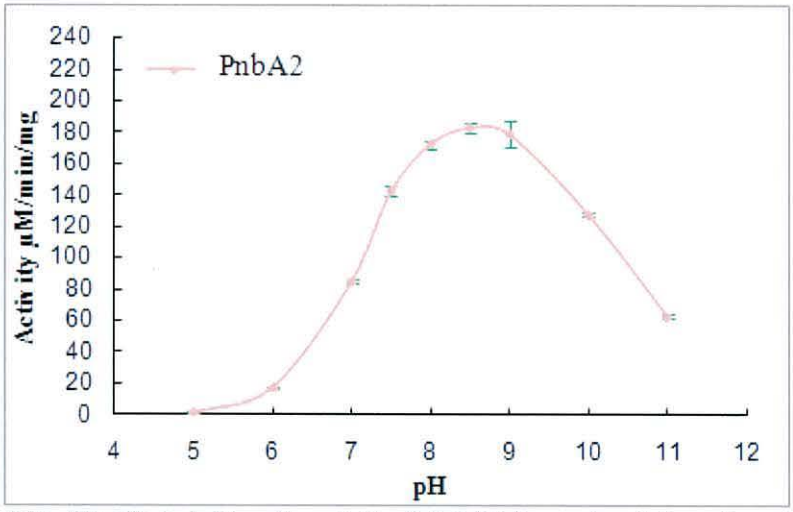


**Figure 5.15 : The effect of pH on the activity of PnbA2 from rates of absorption at 400 nm.**

The thermostability of PnbA1 and PnbA2 was investigated over a temperature range of 25–60°C in 50 mM phosphate buffer pH 8.0 (Figures 5.16 and 5.17). The derived results indicate that PnbA1 remained stable at temperatures ranging from 25°C to 45°C when incubated for 10 min at pH 8.0. However, stability of the enzyme decreased sharply above 45°C, this result is in agreement with published value[7] for *Bacillus* sp. BP-7 type B carboxylesterase, while PnbA2 remained stable at temperatures ranging from 25 to 40 °C when incubated at pH 8.0 for 10 min and stability of the enzyme decreased gradually above 40 °C, this result is in agreement with the published value.[6]

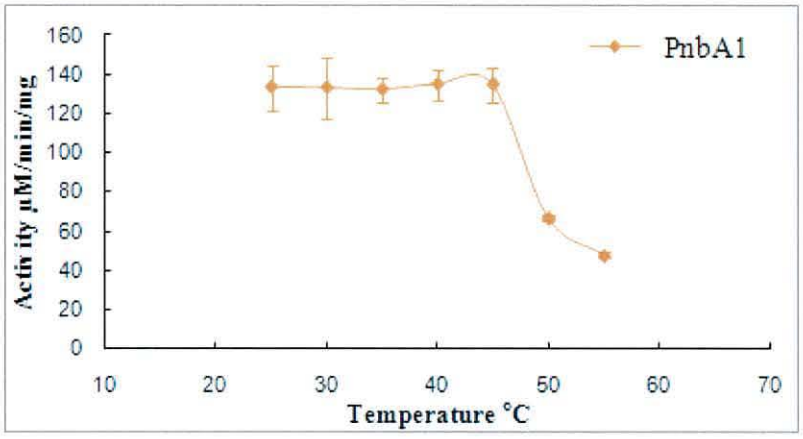


**Figure 5.16 : The effect of temperature on the activity of PnbA1 from rates of absorption at 400 nm.**

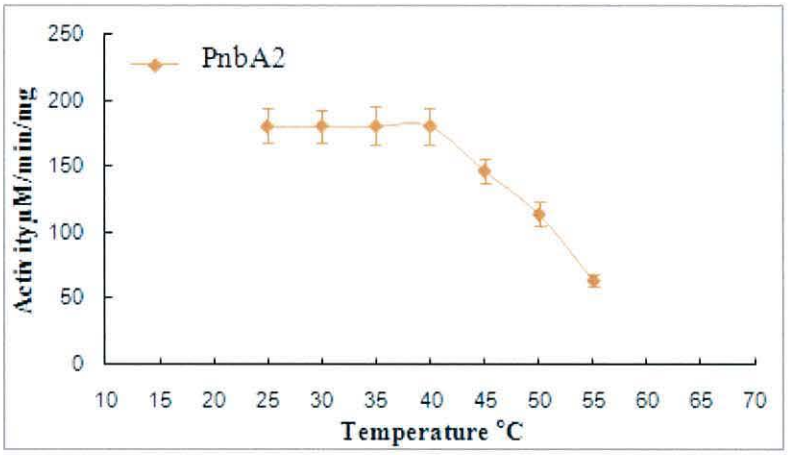


**Figure 5.17 : The effect of temperature on the activity of PnbA2 from rates of absorption at 400 nm.**

As mentioned above the activity of PnbA1 sharply decreased when the temperature move up 40°C (Fig. 5.16), while PnbA2 activity gradually decreased with temperature increase, and the enzyme retained a considerable part of its activity even at a temperature of 55°C (Fig 5.17), indicating that PnbA1 has a lower denaturing temperature than that of PnbA2. This property (thermostability) of the enzyme can be exploited for different biotechnological purposes specifically gene therapy due to the role of the enzyme in cancer therapy, which requires active enzyme at 37°C and some enzymes once isolated cannot remain active at the particular temperature.

### 5.8 Cocaine breakdown by bacterial carboxylesterases

Carboxylesterase activity regarding cocaine was measured by quantifying the amount of benzoylecgonine produced from cocaine using HPLC. The reaction mixtures were 100 mM MOPS at pH 7.0, with 2 mM cocaine, and 20µl cell extract in a total volume of 1 ml incubated at room temperature. Aliquots were removed at intervals (0 min, 6 hours, 12 hours, and 20 hours). All assay results were compared with those from control assays containing no extract.

The two compounds were detected by UV absorbance at wavelengths of 230 nm due to maximum absorption of both compounds at this wavelength, and the peak positions for cocaine and benzoylecgonine were confirmed by using the pure compounds. The identity of each compound was established by comparing the retention times and UV spectra obtained from samples with those obtained from standards. Cocaine, and benzoylecgonine were well resolved and the retention times were approximately 4.4 min, and 2.0 min respectively.

Figure 5.18 shows the typical elution profile of a control assay (2 mM cocaine in 100mM MOPS pH 7.0) containing no enzyme. Cocaine was distinctly separated with retention time of 4.2 min, and remained stable during 20 hours at room temperature when incubated within 100 mM MOPS pH7.0.

It is noticeable (Fig.5.18) the stability of cocaine during 20 hours at room temperature and100mM MOPS buffer pH 7.0 and it is remains stable for 24 hours at the same condition after that spontaneous degradation of cocaine will start even in a buffer with pH 7.0.

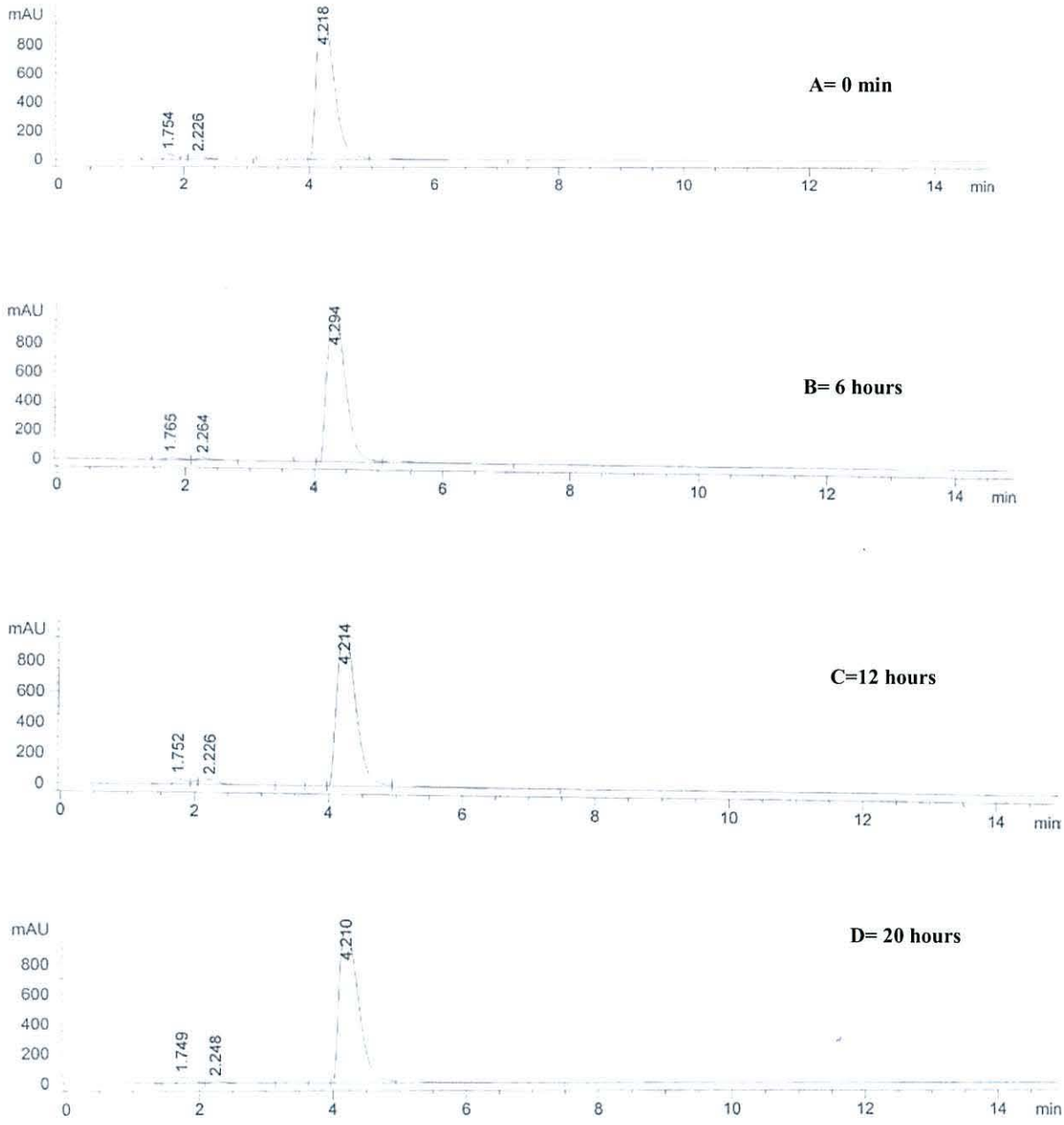


**Figure 5.18 : Control assay of 2mM cocaine in 100mM MOPS buffer pH 7.0, aliquots were taken at intervals (A=0 min, B=6 hours, C=12 hours, and D=20 hours).**

The efficiency of PnbA1 to hydrolyse cocaine was established using the same reaction conditions (100 mM MOPS at pH 7.0, with 2 mM cocaine, and 20µl cell extract in a total volume of 1 ml) and Figure 5.19 shows the typical profile elution of cocaine hydrolysis and the concomitant formation of benzoylecgonine by bacterial carboxylesterases PnbA1 during 20 hours, with approximate retention times of 4.6 min and 2.0 min.

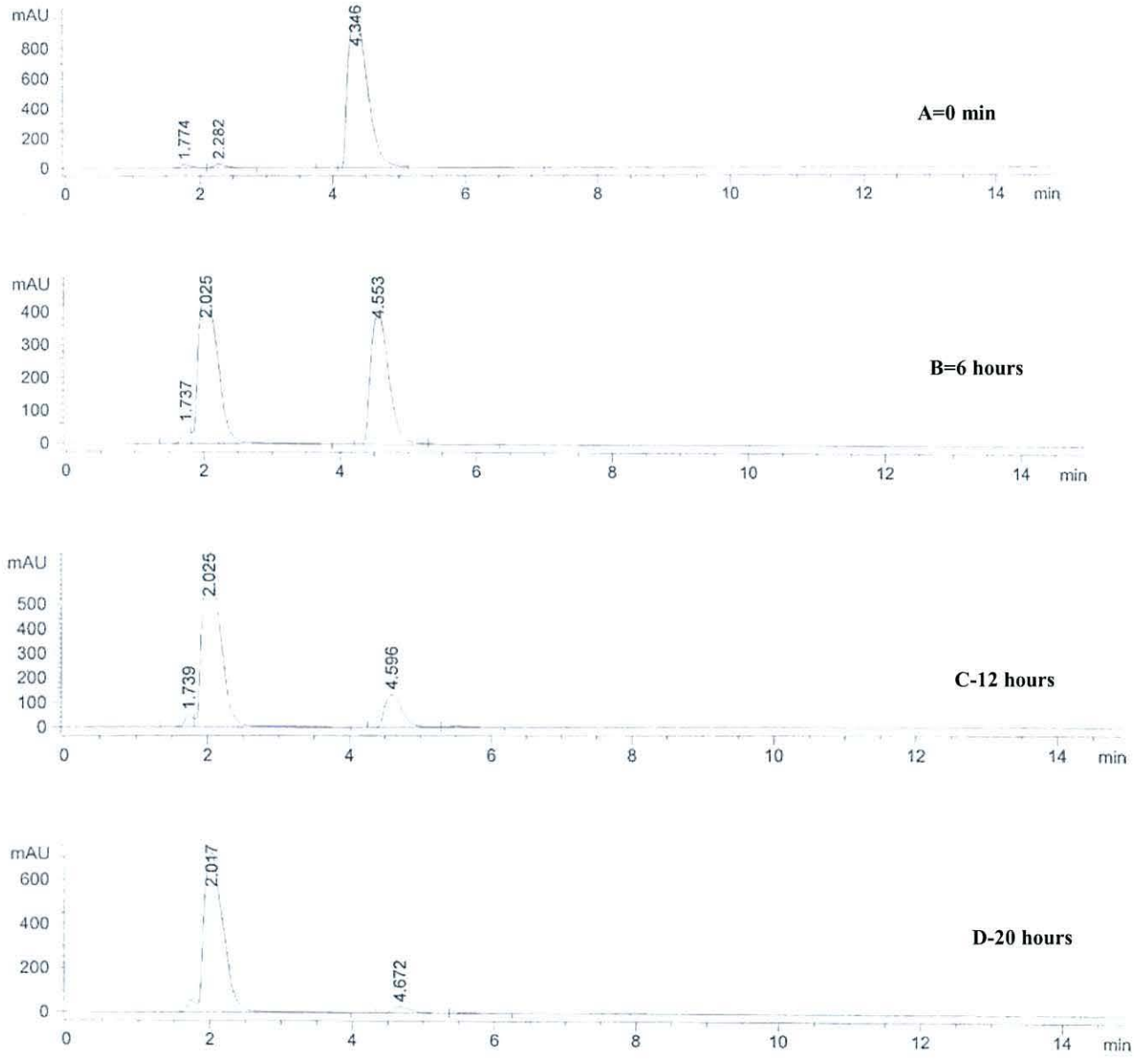


**Figure 5.19 : Breakdown of cocaine with the concomitant production of benzoylecgonine by PnbA1. Samples removed at 0 min, 6, 12, and 20 hours from an incubation of 2 mM cocaine with cell extract.**

PnbA2 was able to break down 2 mM cocaine within 20 hours at room temperature, Figure 5.20 shows the typical profile elution of cocaine hydrolysis and the concomitant formation of benzoylecgonine.

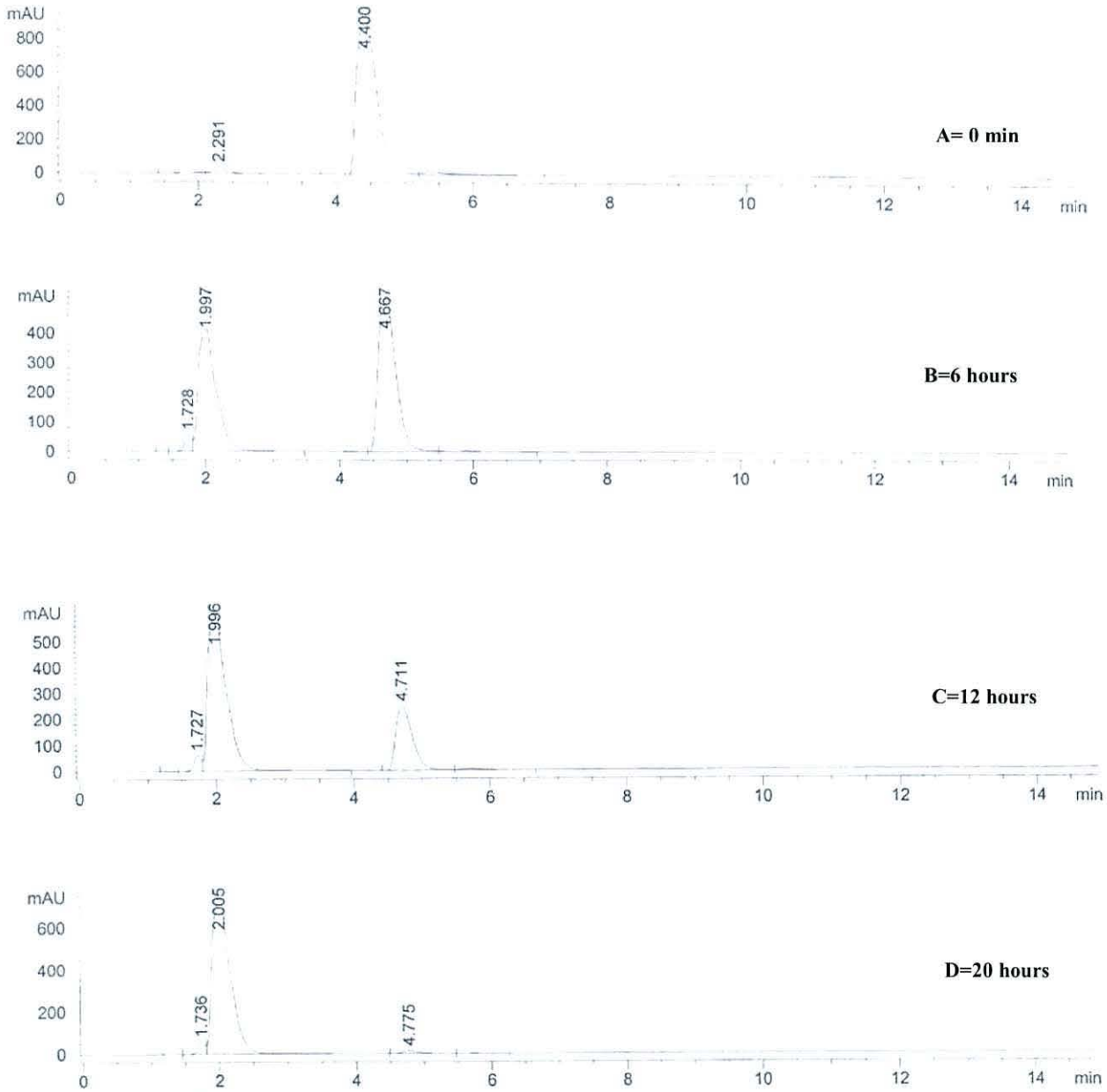


**Figure 5.20 : Breakdown of cocaine with the concomitant production of benzoylecgonine by PnbA2. Samples removed at 0 min, 6, 12, and 20 hours from an incubation of 2 mM cocaine with cell extract.**

Using the same procedure the capability of engineered CEs (PnbA1-cys and PnbA2-cys) to hydrolyze cocaine was established.

Different reaction conditions have been used to enhance the catalytic efficiency of the enzymatic reaction, and all the assays for recombinant and engineered CEs were done at room temperature and 30°C with and without shaking. Identical results were obtained related to shaking and standing (without shaking) reaction condition, while 30°C was found to speed up the reaction progress.

Figures 5.19 and 5.20 clarified that **a**: at 0= time there is only one peak with retention time roughly 4.4 min. This peak represents cocaine as demonstrated by the control sample. At 0 = min the reaction mixture contains 100 mM MOPS pH 7.0, with 2 mM cocaine, the reaction initiated by addition of fresh purified CEs enzyme, **b**: later after 6 hours it is clear from the same figure that the cocaine peak at retention time 4.6 was driven down due to the cocaine break down, and the appearance of a new peak with retention time 2.0 min representing the production of benzoylecgonine, the main cocaine metabolite, as confirmed by the control sample. Hence the ability of the enzyme to break down cocaine and formation of its main metabolites, c: after 12 hours a great drop in the cocaine peak and noticeable increase in the benzoylecgonine peak were found indicating the continuous enzymatic reaction, d: next after 20 hours there was no cocaine peak and a greater peak (in comparison with the peak after 12 hours) with retention time 2.0, min signifying break down of all the cocaine into benzoylecgonine, hence the continuous catalytic efficiency of the enzyme.

Carboxylesterases are serine esterase involved in both drug metabolism and activation. The substrate specificity of CEs is significantly different. Some of them namely human CE-1(hCE-1) essentially hydrolyzes a substrate with a small alcohol group and large acyl group, but sometimes act on structurally distinct compounds with either a large or small alcohol group due to its wide active pocket. In contrast, other CEs specifically hCE-2 identifies a substrate with a large alcohol group and small acyl group due to the occurrence of conformational interference in the active pocket. The different catalytic efficiencies of hCE-1 and hCE-2 are associated with the relative size of the substrate substituents *versus* that of the enzyme active sites.

hCE-1 and hCE-2 catalyse the hydrolysis of cocaine; cocaine has two ester groups which can be hydrolyzed (Figure 5.21). The hCE-1 enzyme hydrolyzes the methyl ester of cocaine. This suggests that the hCE-1 acyl sites recognizes large multi-ring substituents, and its alcohol site accommodates smaller methyl groups (Figure 5.21.b). In contrast, hCE-2 prefers the benzoyl ester of cocaine. This suggests that the hCE-2 acyl site recognizes the relatively smaller benzoyl groups and its alcohol site recognizes the bulky multi-ring groups (Figure 5.21.a), and the substrate specificity of cocaine esterase (CocE) resembles that of hCE-2.

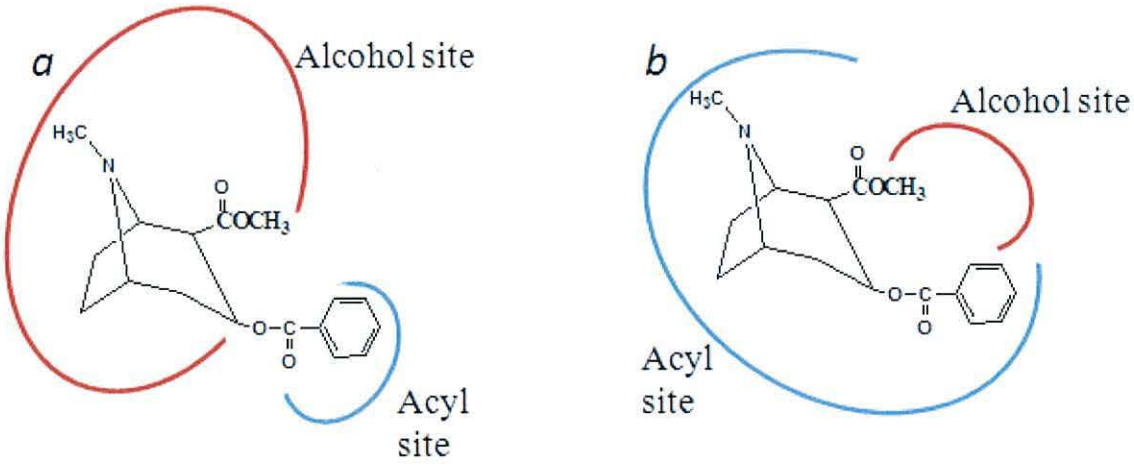


**Figure 5.21: The chemical structure of cocaine as recognized by different carboxylesterases. *a*, CocE and human carboxylesterase hCE-2 specifically cleave the benzoyl group from cocaine by forming an acyl-enzyme intermediate with the benzoyl group and releasing the ecgonine methyl ester as the alcohol group. *b*, Human carboxylesterase hCE-1 specifically cleaves methanol from cocaine by forming an acyl-enzyme intermediate with the benzoylecgonine group and releasing the methanol as the alcohol group.[8]**

These results revealed that bacterial carboxylesterases hydrolyzed cocaine into benzoylecgonine rather than ecgonine methyl ester which results as a consequence of hydrolysis methyl ester of cocaine and this is explained that PnbA-1, and PnbA-2 resemble hCE-1 in cocaine metabolism. These results pave the way to use bacterial carboxylesterases as a model of hCEs, since proteins from bacterial origin are relatively easy to overexpress, purify, and engineer.

## 5.9 Conclusions

The results demonstrate the successful isolation, cloning, expression, and purification of recombinant (*pnbA1*, and *pnbA2*), and engineered (*pnbA1*-cys, and *pnbA2*-cys) bacterial carboxylesterases. Enzyme activity was confirmed spectrophotometrically *via* hydrolysis of *p*-nitrophenylacetate into *p*-nitrophenol and acetic acid. Enzyme capability to hydrolysis cocaine into benzolyecgonine and methanol was quantified using HPLC. Both enzymes showed broad maximal activity between pH (8.0, 8.5, and 9.0), PnbA1 temperature stability ranging between (25-45°C), however PnbA2 stability range was (25-40°C). Insertion of cys-tag at the N-terminal of the enzyme did not limit entrance to the active site which is located at the base of a cavity with dimensions 20 Å by 13 Å by 18 Å, and did not prevent substrate hydrolysis, although it reduced protein concentrations.

Cys-tag insertion reduced the protein concentration around 27-29% roughly concerning PnbA1 and PnbA2 respectively.

Bacterial carboxylesterases pnbA1 and pnbA2 mimic hCE1 and not hCE2 in its reaction pathways hydrolyzing cocaine into benzoylecgonine and methanol rather than ecgonine methyl ester and benzoic acid.

These results are the first experimental evidence confirming the capability of bacterial carboxylesterase to hydrolyse cocaine into its main metabolites, therefore opening up the possibility to use these enzymes in numerous biotechnological applications in addition to a cocaine biosensor.

## 5.10 References


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[2] Redinbo MR, Bencharit S, Potter PM (2003) human carboxylesterase 1: from drug metabolism to drug discovery. *Biochem. Socie.Transa.* **31**:620.

[3] Pindel EV, Kedishvili NY, Abraham TL, Brzezinski MR, Zhang J, Dean RA, Bosron WF (1997) Purification and Cloning of a Broad Substrate Specificity Human Liver Carboxylesterase That Catalyzes the Hydrolysis of Cocaine and Heroin. *J. BIOL. CHEM.* **272**: 14769.

[4] Imai T (2006) Human carboxylesterase isozymes: catalytic properties and rational drug design. *Drug metabol. Pharmaco.* **21**: 173.

[5]Ewis HE, Abdelal AT, Lu C-D (2004) Molecular cloning and characterization of two thermostable carboxyl esterases from Geobacillus stearothermophilus. *Gene.* **329**: 187.

[6] Maqbool Q-U-A, Johri S, Rasool S, Riyaz-ul-Hassan S, Verma V, Nargotra A, Koul S, Qazi GN (2006) Molecular cloning of carboxylesterase gene and biochemical characterization of encoded protein from *Bacillus subtilis* (RRL BB1). *J.Biotechnol* **125**:1-10.

[7] Prim N, Javier Pasto FI , Diaz P (2001) Cloning and Characterization of a Bacterial Cell-Bound Type B Carboxylesterase from *Bacillus* sp. BP-7. *Cur.Microbiol.* **42**:237.

[8] William FB, Thomas DH (2002) Lessons from a bacterial cocaine esterase. *Natu. Struc.Bio.* **9**: 4.

## Chapter 6 - Overall conclusions and future works

In this study, biosensor recognition elements *via* isolation and manipulation four different bacterial nitroreductases genes (nfnB, ydjA, Dde_0086, and Dde_2199) that were obtained from *Salmonella enterica serovar typhimurium* strain LT2, *Salmonella paratyphi A*, and *Desulfovibrio desulfuricans G20* respectively have been developed for the detection of explosives. The NTRs genes were isolated using PCR amplification, and the primers were designed to have restriction sites that allowed the efficient incorporation of the gene into the plasmid vector, pET-28a(+). The plasmid vector was transformed into *Rosetta*, a strain of *E.coli*, because it is an efficient bacterium for the expression of the enzyme. The proteins were isolated using a nickel column because the engineered protein contains a positively charged six-residue histidine tag. Purification was achieved by competitive removal from the nickel column using imidazole. The presence of the NTRs and their activity were confirmed by exposing various nitro compounds (2,4-dinitroethylbenzene, 1,2-dinitrobenzene, nitrobenzene, 3-nirtro-benzoate, 4-nitrotoluene, and 2,4,6-trinitrotoluene) to increasing concentrations of the enzyme. In the second experiment the protocol for PCR was repeated using primers designed to containing six adjacent codons for Cysteine at the 3` end of the NTRs to enable the enzyme to adhere to the electrode surface (gold) by formation of strong thiol bonds, the same protocols were used for cloning the modified NTRs genes, transforming it into *E. coli Rosetta* strain, and confirming the enzyme activity. The enzymes showed maximum activity at pH 7.0 and preferred temperature range (25°C - 55°C).

In the second part of this study two different bacterial Carboxylesterases (pnbA1 and pnbA2) attained from *Bacillus subtilis* and *Bacillus licheniformis* have been developed as biological recognition elements for cocaine biosensors. They were isolated, modified, and characterized. Both enzymes show their ability to hydrolyse cocaine into benzoylecgonine and methanol rather than ecgonine methyl ester and benzoic acid; they mimic hCE-1 in their reaction pathways to hydrolyse cocaine. Both enzymes remain active at a broad pH range (6.0 -11.0) however, more favourable in a basic pH, and a broad maximal activity between pH (8.0-9.0) have been found. pnbA1 remained stable at wide temperatures range (25°C to 45°C) while, pnbA2 remained

stable at temperatures ranging from (25 to 40). The wide pH and temperature range that the enzyme showed for their activity pave the way to use these enzymes in numerous biotechnological applications beside cocaine biosensors as certain of them required particular pH and temperature.

Manipulation of NTRs and CEs enzymes to develop sensor biological recognition elements *via* cys-tag integration at the enzymes N-terminus to make them able to stay at the electrode surface, did not significantly reduce the enzymes activities, and did not restrict substrate entrance into the active site and product release from there, which is the main problem in developing enzymes for biosensor purpose, although the particular tag reduced the enzyme specific activity, protein concentration, optimum temperature range for enzyme activity, and did not affect the optimum pH for enzyme activity.

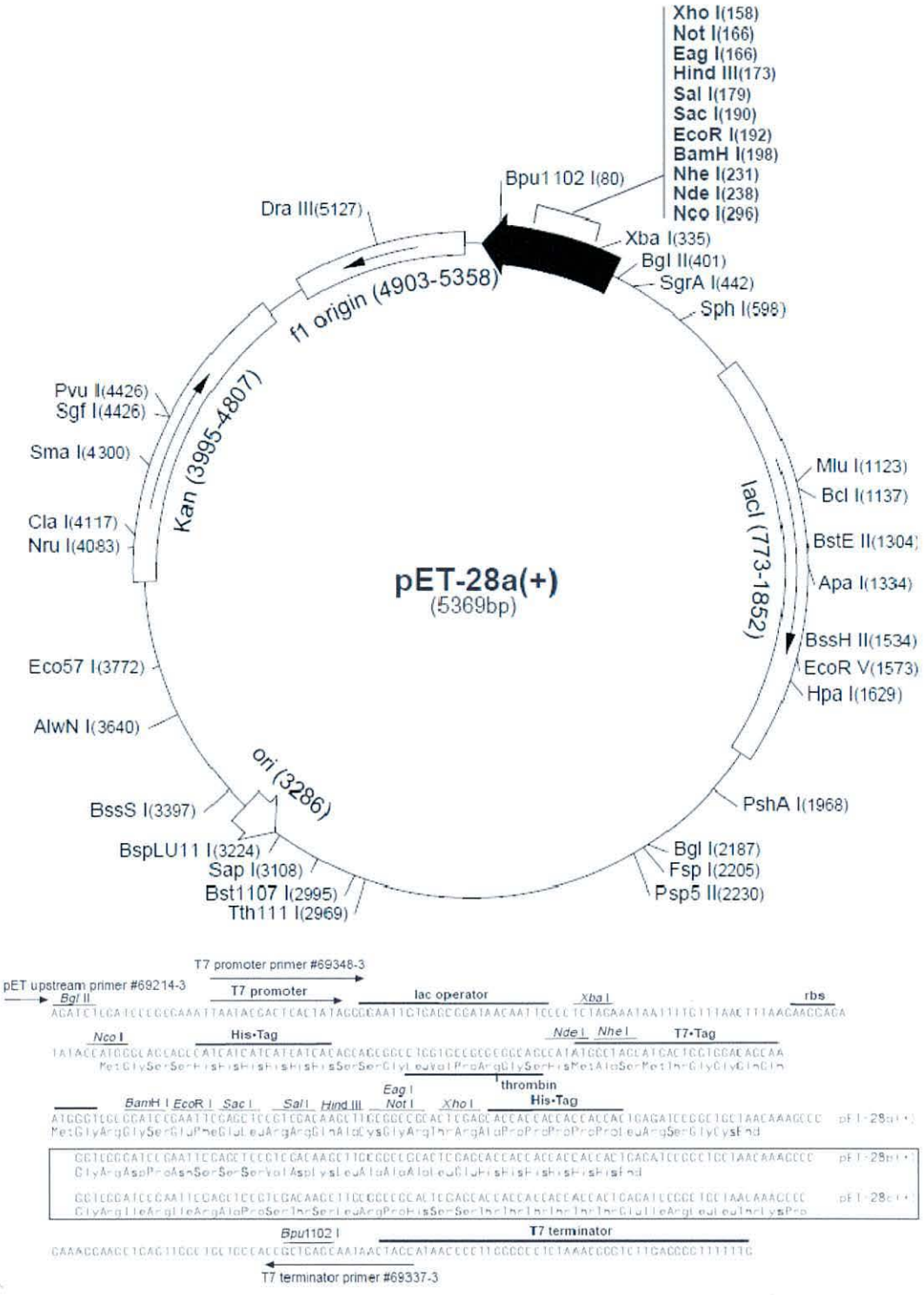
In order to evaluate the electrochemical activities of NTRs and CEs, immobilisation of these enzymes onto electrode surface is necessary. The four different bacterial NTRs which have been successfully isolated, purified, and characterised can be immobilised on the same electrode to develop a biosensor with a various level of detection of many different nitro-aromatic compounds.

To investigate the effect of the cyc-tag and the His-tag on the enzyme activity, the position of cys-tag can be changed from the N-terminus into the C-terminus either by integrating the cyc-tag into the pET28a(+) or at the enzyme C-terminus as well as remove the His-tag following purification.

Resolving the crystal structure of the studied enzymes will verify the effect of cys-tag on the three dimensional conformation of the enzymes. The crystal structure of the NTRs with various nitro-compounds involved in this study will give an insight into understanding the different responses exhibited by the different NTRs versus different substrates.

Isolation of other CEs enzymes due to their role in cancer therapy, detoxification of chemical weapons (Sarin, Tabun and Soman), and numerous organophosphate and carbamates used as insecticides and drugs, would be advantageous towards the development of a biosensor for the potential to sense more than one analyte.

## Appendix 1: pET-28a(+) plasmid map




*pET-28a(+) plasmid map, with the sequence of cloning T7 region shown in detail*[1]

### Reference

[1] Novagen technical literature *pET-28a-c(+)* vector TB074 12/98 (2003)

## Appendix 2: Publication proof sequencing

The sequence for *nfnB* from Wild type strain along with sequencing results for *nfnB*-pET28a(+).

*nfnb1* = *nfnb*- pET28a(+)
*nfnb2* = *nfnb* from Wild type strain

```
                 10         20         30         40         50         60         70
nfnb1    CAGGAAATTT CTGAGCCGTC TCTCTCCCTT AATTGGCGAA TTCCTTGCAC TTAGGAGGCA GCCTCAGTAG
nfnb2    ---------- ---------- ---------- ---------- ---------- ---------- ----------

                 80         90        100        110        120        130        140
nfnb1    TTAGTTCGAG GGATCGTTGA GCACCCGCCC GCCGCAAGAA TTGTGCATTG CAAGAAGATT GACGCCCCAA
nfnb2    ---------- ---------- ---------- ---------- ---------- ---------- ----------

                150        160        170        180        190        200        210
nfnb1    CAGTTCCCCC  CGGCCACCGG GGCCTTGCCA CCCATACCCA CGCCGAAAAC AAGCGCTTCAT GAGCCCGAA
nfnb2    ----------  ---------- ---------- ---------- ---------- ----------  ---------

                220        230        240        250        260        270        280
nfnb1    GTGGCGAGGC CCGATCTTCC CCATCGGTGA TGTCGGCCGA TATAGGCGCC AGCAACCGCA CCTGTGCCGC
nfnb2    ---------- ---------- ---------- ---------- ---------  ---------- ----------

                290        300        310        320        330        340        350
 nfnb1   CGGTGATGCC GGCCACGATG CGTCCGGCGT AGAGGATCGA GATCTCGATC CCGCGAAATT AATACGACTC
 nfnb2   ---------- ---------- ---------- ---------- ---------- ---------- ----------

                360        370        380        390        400        410        420
nfnb1    ACTATAGGGG AATTGTGAGC GGATAACAAT TCCCCTCTAG AAATAATTTT  GTTTAACTTT AAGAAGGAGA
nfnb2    ---------- ---------- ---------- ---------- ----------  ---------- ----------

                430        440        450        460        470        480        490
nfnb1    TATACCATGG GCAGCAGCCA TCATCATCAT CATCACAGCA GCGGCCTGGT GCCGCGCGGC AGCCATATGG
nfnb2    ---------- ---------- ---------- ---------- ---------- ---------- ----------

                500        510        520        530        540        550        560
nfnb1    CTAGCATGAC TGGTGGACAG CAAATGGGTC GCGGATCCTT TATGGATATC GTTTCTGTCG CCTTACAGCG
nfnb2    ---------- ---------- ---------- ---------- -ATGGATATC GTTTCTGTCG CCTTACAGCG

                570        580        590        600        610        620        630
nfnb1    CTACTCCACT AAGGCGTTCG ATCCCAGCAA AAAACTGACC GCCGAAGAAG CGGATAAAAT AAAAACACTA
nfnb2    CTACTCCACT AAGGCGTTCG ATCCCAGCAA AAAACTGACC GCCGAAGAAG CGGATAAAAT AAAAACACTA

                640        650        660        670        680        690        700
   nfnb1 CTACAGTACA GCCCCTCCAG CACCAATTCC CAGCCGTGGC ACTTTATTGT CGCCAGTACG GAAGAAGGCA
   nfnb2 CTACAGTACA GCCCCTCCAG CACCAATTCC CAGCCGTGGC ACTTTATTGT CGCCAGTACG GAAGAAGGCA
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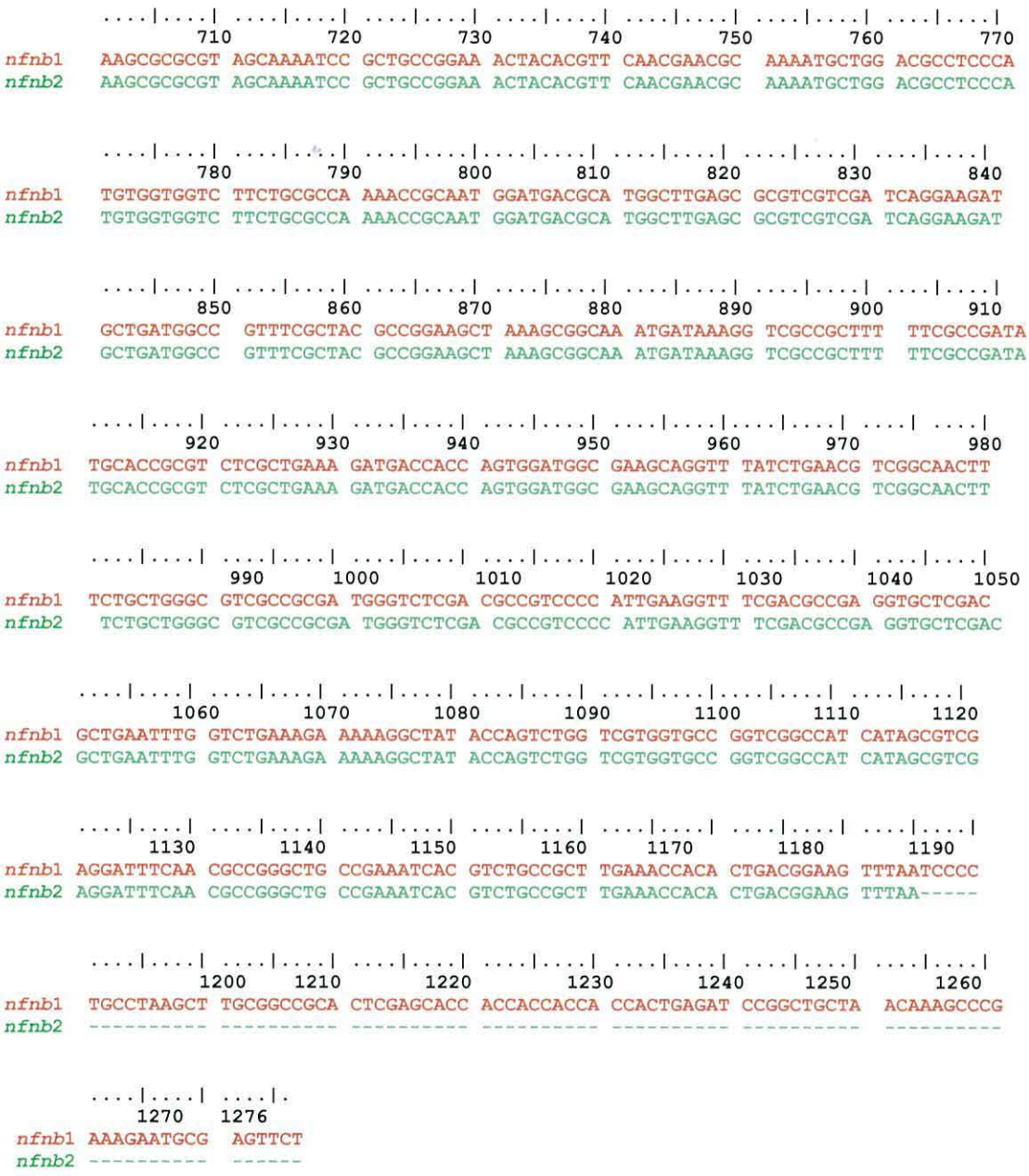
710 720 730 740 750 760 770
nfnb1 AAGCGCGCGT AGCAAAATCC GCTGCCGGAA ACTACACGTT CAACGAACGC AAAATGCTGG ACGCCTCCCA
nfnb2 AAGCGCGCGT AGCAAAATCC GCTGCCGGAA ACTACACGTT CAACGAACGC AAAATGCTGG ACGCCTCCCA
780 790 800 810 820 830 840
nfnb1 TGTGGTGGTC TTCTGCGCCA AAACCGCAAT GGATGACGCA TGGCTTGAGC GCGTCGTCGA TCAGGAAGAT
nfnb2 TGTGGTGGTC TTCTGCGCCA AAACCGCAAT GGATGACGCA TGGCTTGAGC GCGTCGTCGA TCAGGAAGAT
850 860 870 880 890 900 910
nfnb1 GCTGATGGCC GTTTCGCTAC GCCGGAAGCT AAAGCGGCAA ATGATAAAGG TCGCCGCTTT TTCGCCGATA
nfnb2 GCTGATGGCC GTTTCGCTAC GCCGGAAGCT AAAGCGGCAA ATGATAAAGG TCGCCGCTTT TTCGCCGATA
920 930 940 950 960 970 980
nfnb1 TGCACCGCGT CTCGCTGAAA GATGACCACC AGTGGATGGC GAAGCAGGTT TATCTGAACG TCGGCAACTT
nfnb2 TGCACCGCGT CTCGCTGAAA GATGACCACC AGTGGATGGC GAAGCAGGTT TATCTGAACG TCGGCAACTT
990 1000 1010 1020 1030 1040 1050
nfnb1 TCTGCTGGGC GTCGCCGCGA TGGGTCTCGA CGCCGTCCCC ATTGAAGGTT TCGACGCCGA GGTGCTCGAC
nfnb2 TCTGCTGGGC GTCGCCGCGA TGGGTCTCGA CGCCGTCCCC ATTGAAGGTT TCGACGCCGA GGTGCTCGAC
1060 1070 1080 1090 1100 1110 1120
nfnb1 GCTGAATTTG GTCTGAAAGA AAAAGGCTAT ACCAGTCTGG TCGTGGTGCC GGTCGGCCAT CATAGCGTCG
nfnb2 GCTGAATTTG GTCTGAAAGA AAAAGGCTAT ACCAGTCTGG TCGTGGTGCC GGTCGGCCAT CATAGCGTCG
1130 1140 1150 1160 1170 1180 1190
nfnb1 AGGATTTCAA CGCCGGGCTG CCGAAATCAC GTCTGCCGCT TGAAACCACA CTGACGGAAG TTTAATCCCC
nfnb2 AGGATTTCAA CGCCGGGCTG CCGAAATCAC GTCTGCCGCT TGAAACCACA CTGACGGAAG TTTAA-----
1200 1210 1220 1230 1240 1250 1260
nfnb1 TGCCTAAGCT TGCGGCCGCA CTCGAGCACC ACCACCACCA CCACTGAGAT CCGGCTGCTA ACAAAGCCCG
nfnb2 ---------- ---------- ---------- ---------- ---------- ---------- ----------
1270 1276
nfnb1 AAAGAATGCG AGTTCT
nfnb2 ---------- ------

The sequence for *nfnB* from Wild type strain along with sequencing results for *nfnB*-cys- pET28a(+).

*nfnb1* = *nfnb*-cys- pET28a(+)
*nfnb2* = *nfnb* from Wild type strain

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50         60         70
nfnb1       GGGATCGGAC GTCATTCCCC TCTAGAATAA TTTTGTTTAA CTTTAAGAAG  GAGATATACC ATGGGCAGCA
nfnb2       ---------- ---------- ---------- ---------- ----------  ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      80         90        100        110        120        130        140
nfnb1       GCCATCATCA TCATCATCAC AGCAGCGGCC  TGGTGCCGCG CGGCAGCCAT ATGGCTAGCA TGACTGGTGG
nfnb2       ---------- ---------- ----------  ---------- ---------- ---------- ----------

                ....|....|  ....|....| ....|....| ....|....| ....|....| ....|....|....|....|
                     150        160        170        180        190        200        210
nfnb1       ACAGCAAATG GGTCGCGGAT CCTGTTGCTG TTGCTGTTGC TTTATGGATA TCGTTTCTGT CGCCTTACAG
nfnb2       ---------- ---------- ---------- ---------- ---ATGGATA TCGTTTCTGT CGCCTTACAG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     220        230        240        250        260        270        280
nfnb1       CGCTACTCCA CTAAGGCGTT CGATCCCAGC AAAAAACTGA  CCGCCGAAGA AGCGGATAAA ATAAAAACAC
nfnb2       CGCTACTCCA CTAAGGCGTT CGATCCCAGC AAAAAACTGA  CCGCCGAAGA AGCGGATAAA ATAAAAACAC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     290        300        310        320        330        340        350
nfnb1       TACTACAGTA CAGCCCCTCC AGCACCAATT CCCAGCCGTG GCACTTTATT GTCGCCAGTA CGGAAGAAGG
nfnb2       TACTACAGTA CAGCCCCTCC AGCACCAATT CCCAGCCGTG GCACTTTATT GTCGCCAGTA CGGAAGAAGG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     360        370        380        390        400        410        420
nfnb1       CAAAGCGCGC GTAGCAAAAT CCGCTGCCGG AAACTACACG TTCAACGAAC GCAAAATGCT GGACGCCTCC
nfnb2       CAAAGCGCGC GTAGCAAAAT CCGCTGCCGG AAACTACACG TTCAACGAAC GCAAAATGCT GGACGCCTCC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     430        440        450        460        470        480        490
nfnb1       CATGTGGTGG TCTTCTGCGC CAAAACCGCA ATGGATGACG CATGGCTTGA GCGCGTCGTC GATCAGGAAG
nfnb2       CATGTGGTGG TCTTCTGCGC CAAAACCGCA ATGGATGACG CATGGCTTGA GCGCGTCGTC GATCAGGAAG

                ....|....|....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     500        510        520        530        540        550        560
nfnb1       ATGCTGATGG CCGTTTCGCT ACGCCGGAAG CTAAAGCGGC AAATGATAAA GGTCGCCGCT TTTTCGCCGA
nfnb2       ATGCTGATGG CCGTTTCGCT ACGCCGGAAG CTAAAGCGGC AAATGATAAA GGTCGCCGCT TTTTCGCCGA

                ....|....| ....|....| ....|....| ....|....|....|....| ....|....| ....| ....|
                     570        580        590        600        610        620        630
nfnb1       TATGCACCGC GTCTCGCTGA AAGATGACCA CCAGTGGATG GCGAAGCAGG TTTATCTGAA CGTCGGCAAC
nfnb2       TATGCACCGC GTCTCGCTGA AAGATGACCA CCAGTGGATG GCGAAGCAGG TTTATCTGAA CGTCGGCAAC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     640        650        660        670        680        690        700
nfnb1       TTTCTGCTGG GCGTCGCCGC GATGGGTCTC GACGCCGTCC CCATTGAAGG TTTCGACGCC GAGGTGCTCG
nfnb2       TTTCTGCTGG GCGTCGCCGC GATGGGTCTC GACGCCGTCC CCATTGAAGG TTTCGACGCC GAGGTGCTCG
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          ....|....| ....|....| ....|....| ....|....| ....|....|....|....| ....| ....|
                   710        720        730        740        750        760        770
nfnb1      ACGCTGAATT TGGTCTGAAA GAAAAAGGCT ATACCAGTCT GGTCGTGGTG CCGGTCGGCC ATCATAGCGT
nfnb2      ACGCTGAATT TGGTCTGAAA GAAAAAGGCT ATACCAGTCT GGTCGTGGTG CCGGTCGGCC ATCATAGCGT

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    780        790        800        810        820        830        840
nfnb1      CGAGGATTTC AACGCCGGGC TGCCGAAATC ACGTCTGCCG CTTGAAACCA CACTGACGGA AGTTTAATCC
nfnb2      CGAGGATTTC AACGCCGGGC TGCCGAAATC ACGTCTGCCG CTTGAAACCA CACTGACGGA AGTTTAA---

         ....|....|  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                850        860        870        880        890        900        910
nfnb1    CCTGCCTAAG  CTTGCGGGCC GCACTCGAGC ACCACCACCA CCACCACTGA GATCCGGCTG CTAACAAAGC
nfnb2    ----------  ---------- ---------- ---------- ---------- ---------- ----------

         ....|....| ....|....| ....|....| ....|....|....|....| ....|....| ....|....|
                920        930        940        950        960        970        980
nfnb1    CCGAAAGGAA GCTGAGTTGG CTGCTGCCAC CGCTGAGCAA TAACTAGCAT AACCCCTTGG GGCCTCTAAA
nfnb2    ---------- ---------- ---------- ---------- ---------- ---------- ----------

         ....|....| ....|....|....|....| ....|....| ....|....| ....|....| ....|....|
                990       1000     1010       1020       1030       1040       1050
nfnb1    CGGGTCTGAG GGGTTTTTGC TGAAAGGAGA CTATATCCGG ATGGCGATGG GACGCGCCCT  GTAGCGCGCA
nfnb2    ---------- ---------- ---------- ---------- ---------- ----------  ----------

        ....|....| ....|....| ....|....| ....|....| ....|....|  ....|....| ....|....|
              1060       1070       1080       1090       1100      1110       1120
nfnb1     TAGCGCGTCG ATGTGTTGTT ACGCGCAGCG TGACGCTACA CTGCAGCGCT AGCGTCGGCT TCATCGATTC
nfnb2     ---------- ---------- ---------- ---------- ---------- ---------- ----------

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....| ....|
                1130       1140       1150       1160       1170       1180       1190
nfnb1     TAGTTCGTTC TCGCCACGGT  TCGCCGCATT  CGTCAGCCTA TCGGGGACTC CGTAAGGGTT CCGAAATTAA
nfnb2     ---------- ----------  ----------  ---------- ---------- ---------- ----------

          ....|.
            1196
nfnb1     GGGGTC
nfnb2     ------
```

The sequence for *ydjA* from Wild type strain along with sequencing results for *ydjA* - pET28a(+).

*ydjA1* = *ydjA*- pET28a(+)
*ydjA2* = *ydjA* from Wild type strain

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|....|....| ....|....|
                       10         20         30         40         50         60         70
ydjA1            CATAGGACCT GCCTGATACA CCCGTTCGTA GGGCCCTTCG TTTTGCAGAA CGAACGTTCG AAGTCAATAC
ydjA2            ---------- ---------- ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       80         90        100        110        120        130        140
ydjA1            AGAACATTCT GCGGTCGGGA TGCGTGTTCG GATCATAAGT CTTTACAGCG TGGCGTTTTT TGTGATGGAC
ydjA2            ---------- ---------- ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      150        160        170        180        190        200        210
ydjA1            GAGAGGGAGC GCGATCGCTC AGCGTCGTGC TGATCGAGGC GCTGTCGCTG CCGTACGCCG ATGAGAAAGC
ydjA2            ---------- ---------- ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....|....|....| ....|....| ....|....|
                      220        230        240        250        260        270        280
ydjA1            GATCGAAAAA GCGCGTAAGT CGCCGTTTCG CGCGCCGCTG ATCATTACCG TGTGCGAAAT GCGAAGAAAA
ydjA2            ---------- ---------- ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      290        300        310        320        330        340        350
ydjA1            CCATAAGGTT CCCGTGTGGG AACAGGAGAT GTCTGCCGGA TGCGCGGTGA TGGCGATGCA AATGGCGGCG
ydjA2            ---------- ---------- ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      360        370        380        390        400        410        420
ydjA1            ATTGCGCAAG GGTTTAATGG TATCTGGCGT AGCGGCGCAT TAACGGAGAG CGCTATCGTG CGGGAAGCCT
ydjA2            ---------- ---------- ---------- ---------- ---------- ---------- ----------

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      430        440        450        460        470        480        490
ydjA1            TTGAGTGCCG CCCGCAGGAT AAAATTGTTG GTTTTCTCTA TCTCGGCACG CCGCAGCTCA AAGCGTCAAC
ydjA2            ---------- ---------- ---------- ---------- ---------- ---------- ----------

                 ....|....|....|....| ....|....| ....|....| ....|....| ....|....|  ....|....|
                      500        510        520        530        540        550        560
ydjA1            GACGATCAGT ACCCCCGACC CCACGCCGTT TGTGCGTTAT TTCTGACGCT GAGGGATAAA  ACTGTCAAGC
ydjA2            ---------- ---------- ---------- ---------- ---------- ----------  ----------

                 ....|....| ....|....| ....|....| ....|....|....|....| ....|....| ....|....|
                      570        580        590        600        610        620        630
ydjA1            TGCTGGATCC AATCAAATGG ATGCACTAGA ACTGCTGGTC AACCGTCGTA GCGCCTCTCG TCTCGCAGAA
ydjA2            ---------- ------atgg atgcactaga actgctggtc aaccgtcgta gcgcctctcg tctcgcagaa

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      640        650        660        670        680        690        700
ydjA1            CCGGCGCCTG TCGGAGAGCA ATTACAGAAC ATTCTGCGGG CGGGAATGCG TGTTCCCGAT CATAAATCTC
ydjA2            ccggcgcctg tcggagagca attacagaac attctgcggg cgggaatgcg tgttcccgat cataaatctc
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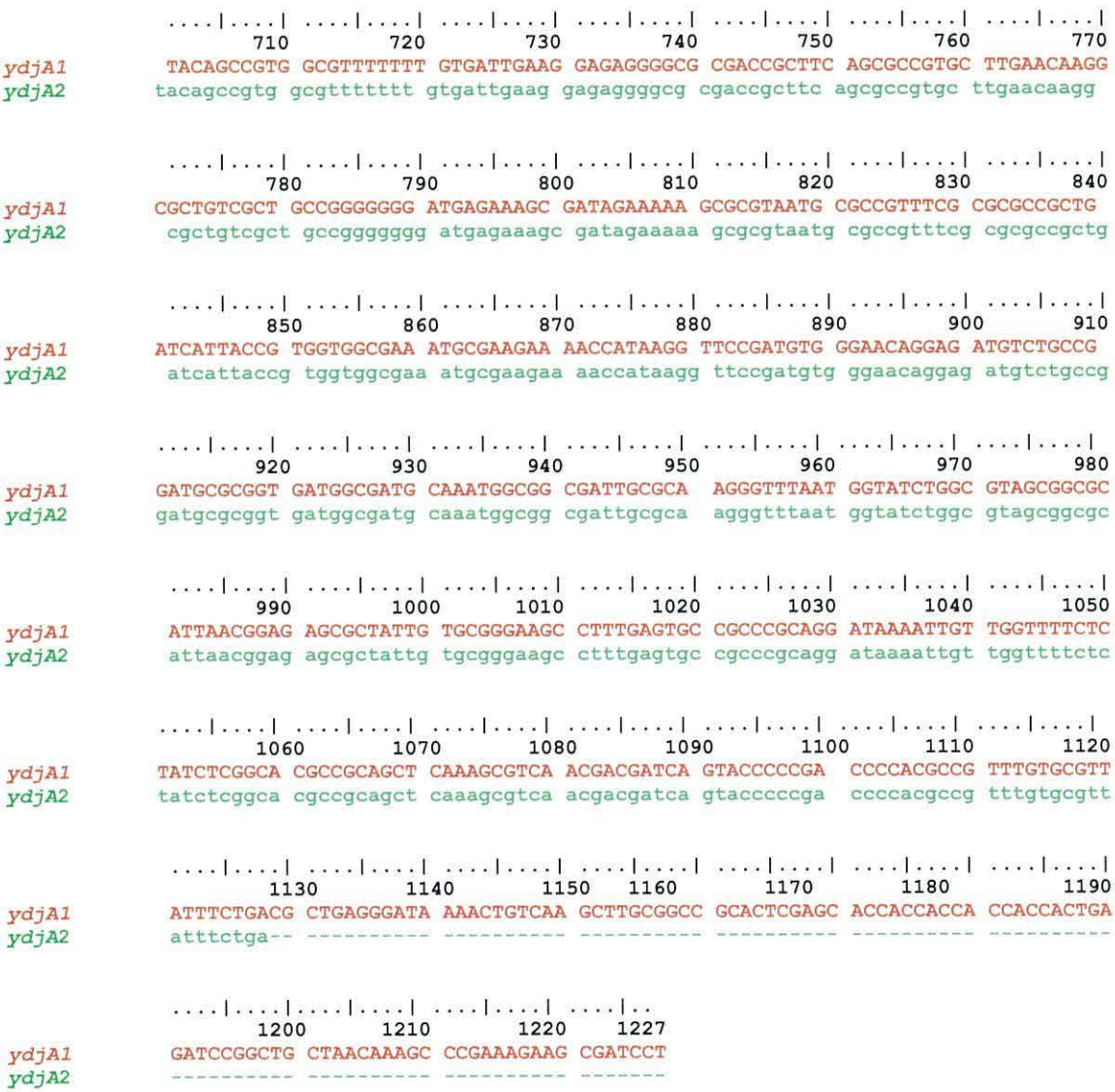
710 720 730 740 750 760 770
ydjA1 TACAGCCGTG GCGTTTTTTT GTGATTGAAG GAGAGGGGCG CGACCGCTTC AGCGCCGTGC TTGAACAAGG
ydjA2 tacagccgtg gcgtttttt gtgattgaag gagaggggcg cgaccgcttc agcgccgtgc ttgaacaagg
780 790 800 810 820 830 840
ydjA1 CGCTGTCGCT GCCGGGGGGG ATGAGAAAGC GATAGAAAAA GCGCGTAATG CGCCGTTTCG CGCGCCGCTG
ydjA2 cgctgtcgct gccgggggggg atgagaaagc gatagaaaaa gcgcgtaatg cgccgtttcg cgcgccgctg
850 860 870 880 890 900 910
ydjA1 ATCATTACCG TGGTGGCGAA ATGCGAAGAA AACCATAAGG TTCCGATGTG GGAACAGGAG ATGTCTGCCG
ydjA2 atcattaccg tggtggcgaa atgcgaagaa aaccataagg ttccgatgtg ggaacaggag atgtctgccg
920 930 940 950 960 970 980
ydjA1 GATGCGCGGT GATGGCGATG CAAATGGCGG CGATTGCGCA AGGGTTTAAT GGTATCTGGC GTAGCGGCGC
ydjA2 gatgcgcggt gatggcgatg caaatggcgg cgattgcgca agggtttaat ggtatctggc gtagcggcgc
990 1000 1010 1020 1030 1040 1050
ydjA1 ATTAACGGAG AGCGCTATTG TGCGGGAAGC CTTTGAGTGC CGCCCGCAGG ATAAAATTGT TGGTTTTCTC
ydjA2 attaacggag agcgctattg tgcgggaagc ctttgagtgc cgcccgcagg ataaaattgt tggttttctc
1060 1070 1080 1090 1100 1110 1120
ydjA1 TATCTCGGCA CGCCGCAGCT CAAAGCGTCA ACGACGATCA GTACCCCCGA CCCCACGCCG TTTGTGCGTT
ydjA2 tatctcggca cgccgcagct caaagcgtca acgacgatca gtacccccga ccccacgccg tttgtgcgtt
1130 1140 1150 1160 1170 1180 1190
ydjA1 ATTTCTGACG CTGAGGGATA AAACTGTCAA GCTTGCGGCC GCACTCGAGC ACCACCACCA CCACCACTGA
ydjA2 atttctga-- ---------- ---------- ---------- ---------- ---------- ----------
1200 1210 1220 1227
ydjA1 GATCCGGCTG CTAACAAAGC CCGAAAGAAG CGATCCT
ydjA2 ---------- ---------- ---------- -------

The sequence for YdjA from Wild type strain along with sequencing results for YdjA-cys-pET28a(+).

ydjA1 = ydjA-cys-pET28a(+)
ydjA2 = ydjA from Wild type strain

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       10         20         30         40         50         60         70
ydjA1      GGTCGGCGAA CATTCCCGTC TAGAAATAAT TTTGTTTAAC TTTAAGAAGG  AGATATACCA TGGGCAGCAG
ydjA2      ---------- ---------- ---------- ---------- ----------  ---------- ----------

                ....|....| ....|....| ....|....|....|....| ....|....| ....|....| ....|....|
                       80         90        100        110        120        130        140
ydjA1      CCATCATCAT CATCATCACA GCAGCGGCCT GGTGCCGCGC GGCAGCCATA TGGCTAGCAT GACTGGTGGA
ydjA2      ---------- ---------- ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      150        160        170        180        190        200        210
ydjA1      CAGCAAATGG GTCGCGGATC CTGTTGCTGT TGCTGTTGCA ATCAAATGGA TGCACTAGAA CTGCTGGTCA
ydjA2      ---------- ---------- ---------- ---------- -----atgga tgcactagaa ctgctggtca

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      220        230        240        250        260        270        280
ydjA1      ACCGTCGTAG CGCCTCTCGT CTCGCAGAAC CGGCGCCTGT CGGAGAGCAA TTACAGAACA TTCTGCGGGC
ydjA2      accgtcgtag cgcctctcgt ctcgcagaac cggcgcctgt cggagagcaa ttacagaaca ttctgcgggc

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      290        300        310        320        330        340        350
ydjA1      GGAATGCGT GTTCCCGATC ATAAATCTCT ACAGCCGTGG CGTTTTTTTG TGATTGAAGG AGAGGGGCGC
ydjA2      ggaatgcgt gttcccgatc ataaatctct acagccgtgg cgttttttg tgattgaagg agaggggcgc

                ....|....| ....|....| ....|....| ....|....| ....|....|  ....|....| ....|....|
                      360        370        380        390        400        410        420
ydjA1      GACCGCTTCA GCGCCGTGCT TGAACAAGGC GCTGTCGCTG CCGGGGGGGA TGAGAAAGCG ATAGAAAAAG
ydjA2      gaccgcttca gcgccgtgct tgaacaaggc gctgtcgctg ccgggggga tgagaaagcg atagaaaaag

                ....|....| ....|....| ....|....|....|....| ....|....| ....|....|   ....|....|
                      430        440        450        460        470        480        490
ydjA1      CGCGTAATGC GCCGTTTCGC GCGCCGCTGA  TCATTACCGT GGTGGCGAAA TGCGAAGAAA ACCATAAGGT
ydjA2      cgcgtaatgc gccgtttcgc gcgccgctga  tcattaccgt ggtggcgaaa tgcgaagaaa accataaggt

                ....|....|....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     500        510        520        530        540        550        560
ydjA1      TCCGATGTGG GAACAGGAGA TGTCTGCCGG ATGCGCGGTG ATGGCGATGC AAATGGCGGC GATTGCGCAA
ydjA2      tccgatgtgg gaacaggaga tgtctgccgg atgcgcggtg atggcgatgc aaatggcggc gattgcgcaa

                ....|....| ....|....| ....|....| ....|....|....|....| ....|....| ....|....|
                      570        580        590        600        610        620        630
ydjA1      GGGTTTAATG GTATCTGGCG TAGCGGCGCA TTAACGGAGA GCGCTATTGT GCGGGAAGCC TTTGAGTGCC
ydjA2      gggtttaatg gtatctggcg tagcggcgca ttaacggaga gcgctattgt gcgggaagcc tttgagtgcc

                ....|....| ....|....|....|....| ....|....|....| ....| ....|....| ....|....|
                      640        650       660         670        680        690        700
ydjA1      GCCCGCAGGA TAAAATTGTT GGTTTTCTCT ATCTCGGCAC GCCGCAGCTC AAAGCGTCAA CGACGATCAG
ydjA2      gcccgcagga taaaattgtt ggttttctct atctcggcac gccgcagctc aaagcgtcaa cgacgatcag
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      710        720        730        740        750        760        770
ydjA1           TACCCCCGAC CCCACGCCGT TTGTGCGTTA TTTCTGACGC TGAGGGATAA AACTGTCAAG CTTGCGGCCG
ydjA2           taccccgac cccacgccgt ttgtgcgtta tttctga--- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      780        790        800        810        820        830        840
ydjA1           CACTCGAGCA CCACCACCAC CACCACTGAG ATCCGGCTGC TAACAAAGCC CGAAAGGAAG CTGAGTTGGC
ydjA2           ---------- ---------- ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      850        860        870        880        890        900        910
ydjA1           TGCTGCCACC GCTGAGCAAT AACTAGCATA ACCCCTTGGG GCCTCTAAAC GGGTCTTGAG GGGTTTTTTG
ydjA2           ---------- ---------- ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      920        930        940        950        960        970        980
ydjA1           CTGAAAGGAG GAACTATATC TGGATTGGCG AATGGGACGC CGCTCTGTAG CGGCGCATTA AGCGCGGCGG
ydjA2           ---------- ---------- ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      990       1000       1010       1020       1030       1040       1050
ydjA1           GTGTGGGTGG TTACGCGCAG CGTGACCGCT ACACTTGCCA GCGCCCTAGC GCCCGCCTCC TTTCGCTTTC
ydjA2           ---------- ---------- ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     1060       1070       1080       1090       1100       1110       1120
ydjA1           TTCCCTTCCT TTCTCGCACG ATCGCGCTTC CCGTCAGCTC TAATCGGGGC  TCCTTAGGGT TCGATTAGTG
ydjA2           ---------- ---------- ---------- ---------- ----------  ---------- ----------

                ....|....| ....|....| ....|....|....|....| ....|....| ....|....| ....|....|
                     1130       1140       1150       1160       1170       1180       1190
ydjA1           CTTACGCACC TCGACCCAAA AACTGATAGG GTGATGTTCA CGTACTGGCA TCCCCTGATG ACGGTTTCGC
ydjA2           ---------- ---------- ---------- ---------- ---------- ---------- ----------

                  ....|....| ....|....| ....|....| ....|....| ....|....
                       1200       1210       1220       1230       1239
ydjA1           CGTTGGACGG  TGGGAGTCCA CGTCTTAATA GGGGAACCTC TTGGTTTCA
ydjA2           ----------  ---------- ---------- ---------- ---------
```

**The sequence for Dde_0086 from Wild type strain along with sequencing results for Dde_0086- pET28a(+).**

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Dde_0086-1 =  Dde_0086- pET28a(+)
Dde_0086-2 =  Dde_0086 from Wild type strain

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50         60         70
Dde_0086-1    GGGTCGACGG TACATTCCCC TCTAGAATAA TTTTGTTTAA CTTTAAGAAG GAGATATACC ATGGGCAGCA
Dde_0086-2    ---------- ---------- ---------- ---------- ---------- ---------- ----------

                 ....| ....| ....|....|....|....|....|....| ....|....| ....|....| ....|....|
                         80         90        100        110        120        130        140
Dde_0086-1 GCCATCATCA TCATCATCAC AGCAGCGGCC TGGTGCCGCG CGGCAGCCAT ATGGCTAGCA TGACTGGTGG
Dde_0086-2 ---------- ---------- ---------- ---------- ---------- ---------- ----------

                ....|....|....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        150        160        170        180        190        200        210
Dde_0086-1 ACAGCAAATG GGTCGCGGAT CCGTCATGCA TAGCTATATT GAAGCTCTTA CCACCAGATA CACCACAAAA
Dde_0086-2 ---------- ---------- -----atgca tagctatatt gaagctctta ccaccagata caccacaaaa

                ....|....| ....|....| ....|....| ....|....|....|....| ....|....| ....|....|
                       220        230        240        250        260        270        280

Dde_0086-1 AAGTTTGATC CGGACCGCAG GCTGGACGCC GAAACCGTGC AGGCGGTGAA GGATATTCTG CAACTCAGTC
Dde_0086-2 aagtttgatc cggaccgcag gctggacgcc gaaaccgtgc aggcggtgaa ggatattctg caactcagtc

                 ....|....| ....|....|....|....| ....|....| ....|....| ....|....| ....|....|
                        290        300        310        320        330        340        350
Dde_0086-1 CTTCTTCGAC CAACTCTCAG CCGTGGCATT TTGTCATTGC CGGTACGGAT GAAGGCAGGC AGCGCGTGGC
Dde_0086-2 cttcttcgac caactctcag ccgtggcatt ttgtcattgc cggtacggat gaaggcaggc agcgcgtggc

                ....|....| ....|....| ....|....| ....|....| ....|....|....|....| ....|....|
                        360        370        380        390        400        410        420
Dde_0086-1 AAAGGCGGCA CACGGGGTTT ACGCATTTAA CGCATCAAAA ATCTGCGATG CTTCTCATGT TGTGGTGCTG
Dde_0086-2 aaaggcggca cacggggttt acgcatttaa cgcatcaaaa atctgcgatg cttctcatgt tgtggtgctg

                 ....|....| ....|....| ....|....|....|....| ....|....| ....|....| ....|....|
                        430        440        450        460        470        480        490
Dde_0086-1 TGCACAAAAA CGGATATCGA CACGGAATAC ACCACACAGG TGCTTGAGCA GGAAGAGCAG GACGGTCGTT
Dde_0086-2 tgcacaaaaa cggatatcga cacggaatac accacacagg tgcttgagca ggaagagcag gacggtcgtt

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|....|....|
                        500        510        520        530        540        550        560
Dde_0086-1 TTCCTTCTGA AGAAACGAAG GCCGCCAACA GAAAAGGCCG TGAGTTTTTT GCCATGCTGC ACCGTAACGA
Dde_0086-2 ttccttctga agaaacgaag gccgccaaca gaaaaggccg tgagtttttt gccatgctgc accgtaacga

                ....|....| ....|....| ....|....| ....|....|....|....| ....|....| ....|....|
                        570        580        590        600        610        620        630
Dde_0086-1 GTTGCAGGAC GCTTTTCACT GGATGGAGAA GCAGACGTTT ATCGCCCTTG GTAATCTGTT GTCCGGTGCG
Dde_0086-2 gttgcaggac gcttttcact ggatggagaa gcagacgttt atcgcccttg gtaatctgtt gtccggtgcg

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        640        650        660        670        680        690        700
Dde_0086-1 GCACATCTGG GCATACACGC ATGCCCCATG GAAGGCTTTG ATCACGAAGT GCTGGAAAAA GAGCTGGGAT
Dde_0086-2 gcacatctgg gcatacacgc atgccccatg gaaggctttg atcacgaagt gctggaaaaa gagctgggat
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          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....| ....|
                  710        720        730        740        750        760        770
Dde_0086-1 TGACGGAGAA AGGGTACAAG CCCTCGGTCA TTGTGGCTCT GGGGTTTAGT GCCGCTGATG ATTTCAATGC
Dde_0086-2 tgacggagaa agggtacaag ccctcggtca ttgtggctct ggggtttagt gccgctgatg atttcaatgc

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  780        790        800        810        820        830        840
Dde_0086-1 GGTGACACCC AAATCCCGCT GGCCTCAGGA ACGGATAATA ACCGAAATAT GACGTGCGGC AGCAATGAAG
Dde_0086-2 ggtgacaccc aaatcccgct ggcctcagga acggataata accgaaatat ga-------- ----------

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|....|....|
                  850        860        870        880        890        900        910
Dde_0086-1 CTTGCGGCCG CACTCGAGCA CCACCACCAC CACCACTGAG ATCCGGCTGC TAACAAAGCC  CGAAAGGAAG
Dde_0086-2 ---------- ---------- ---------- ---------- ---------- ----------  ----------

          ....|....| ....|....| ....|....| ....|....|....|....| ....|....| ....|....|
                  920        930        940        950        960        970        980
Dde_0086-1 CTGAGTTGGC TGCTGCCACC GCTGAGCAAT AACTAGCATA ACCCCTTGGG GCCTCTAAAC  GGGTCTTGAG
Dde_0086-2 ---------- ---------- ---------- ---------- ---------- ---------- ----------

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  990        1000       1010       1020       1030       1040       1050
Dde_0086-1 GGGTTTTTTG CTGAAAGGAG  GACTATATCC AGGATTGGCG ATGGGACGCG CCCTGTAGCG CGCATTAAGC
Dde_0086-2 ---------- ---------- ---------- ---------- ---------- ---------- ----------

          ....|....| ....|....| ....|....| ....|....| ....|....|  ....|....| ....|....|
                  1060       1070       1080       1090       1100      1110       1120
Dde_0086-1 GCGCAATGTG TGTTACGCGC AGCGTGACCG CTACACTTGC AGCGCCCTAG CGCCCGCTCC TTTCGCTTTC
Dde_0086-2 ---------- ---------- ---------- ---------- ---------- ---------- ----------

          ....|....| ....|....| ....|....|  ....|....| ....|....| ....|....| ....|....|
                  1130       1140       1150      1160       1170       1180       1190
Dde_0086-1 TTCCCATCTT TCTCGCCACG ATCGCAGGCT  TCCGTCAGCT CTAATCAGGG GACTCCGTTT AAGTCGAATT
Dde_0086-2 ---------- ---------- ----------  ---------- ---------- ---------- ----------

            ....|....| ....|....| ....| ....| ....|....| ....|....| ....|.
                    1200       1210       1220       1230       1240   1246
Dde_0086-1     TAAGGCTTTA CGGCACCTCG AACCCCAAAA AACCTTGAAT ATAAAAGGGG TGAATG
Dde_0086-2     ---------- ---------- ---------- ---------- ---------- ------
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The sequence for Dde_0086 from Wild type strain along with sequencing results for Dde_0086-cys- pET28a(+).

```
Dde_0086-1 =  Dde_0086-cys- pET28a(+)
Dde_0086-2 =  Dde_0086 from Wild type strain

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      10         20         30         40         50         60         70
Dde_0086-1 GTCGAACGCT ACATTCCCCT CTAGAATAAT TTTGTTTAAC TTTAAGAAGG  AGATATACCA TGGGCAGCAG
Dde_0086-2 ---------- ---------- ---------- ---------- ----------  ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     80         90        100        110        120        130        140
Dde_0086-1 CCATCATCAT CATCATCACA GCAGCGGCCT  GGTGCCGCGC GGCAGCCATA TGGCTAGCAT GACTGGTGGA
Dde_0086-2 ---------- ---------- ---------- ---------- ---------- ---------- ----------

                ....|....|....|....| ....|....| ....|....| ....|....| ....| ....| ....| ....|
                    150        160        170        180        190        200        210
Dde_0086-1 CAGCAAATGG GTCGCGGATC CTGTTGCTGT TGCTGTTGCG TCATGCATAG CTATATTGAA GCTCTTACCA
Dde_0086-2 ---------- ---------- ---------- ---------- --atgcatag ctatattgaa gctcttacca

                ....|....| ....|....| ....|....| ....|....|....|....| ....|....| ....|....|
                    220        230        240        250        260        270        280
Dde_0086-1 CCAGATACAC CACAAAAAAG TTTGATCCGG ACCGCAGGCT GGACGCCGAA ACCGTGCAGG CGGTGAAGGA
Dde_0086-2 ccagatacac cacaaaaaag tttgatccgg accgcaggct ggacgccgaa accgtgcagg cggtgaagga

                ....|....| ....|....|....|....| ....|....| ....|....| ....|....| ....|....|
                    290        300        310        320        330        340        350
Dde_0086-1 TATTCTGCAA CTCAGTCCTT CTTCGACCAA CTCTCAGCCG TGGCATTTTG TCATTGCCGG TACGGATGAA
Dde_0086-2 tattctgcaa ctcagtcctt cttcgaccaa ctctcagccg tggcattttg tcattgccgg tacggatgaa

                ....|....| ....|....| ....|....| ....|....| ....|....| ....| ....| ....|....|
                    360        370        380        390        400        410        420
Dde_0086-1 GGCAGGCAGC GCGTGGCAAA GGCGGCACAC GGGGTTTACG CATTTAACGC  ATCAAAAATC TGCGATGCTT
Dde_0086-2 ggcaggcagc gcgtggcaaa ggcggcacac ggggtttacg catttaacgc  atcaaaaatc tgcgatgctt

                ....|....| ....|....| ....|....|....|....| ....|....| ....|....| ....|....|
                    430        440        450        460        470        480        490
Dde_0086-1 CTCATGTTGT GGTGCTGTGC ACAAAAACGG ATATCGACAC GGAATACACC ACACAGGTGC TTGAGCAGGA
Dde_0086-2 ctcatgttgt ggtgctgtgc acaaaaacgg atatcgacac ggaatacacc acacaggtgc ttgagcagga

                 .... |.... | .... |.... | .... |.... | ....  |....| ....|....| ....| ....|
                    500        510           520           530        540        550
Dde_0086-1 AGAGCAGGAC  GGTCGTTTTC     CTTCTGAAGA   AACGAAGGCC  GCCAACAGAA  AAGGCCGTGA
Dde_0086-2 agagcaggac  ggtcgttttc     cttctgaaga   aacgaaggcc  gccaacagaa  aaggccgtga

                  ....|....| ....|....| ....|....| ....|....| ....|....|....|....| ....|....|
                    560        570        580        590        600        610        620
Dde_0086-1 GTTTTTTGCC ATGCTGCACC GTAACGAGTT GCAGGACGCT TTTCACTGGA TGGAGAAGCA  GACGTTTATC
Dde_0086-2 gttttttgcc atgctgcacc gtaacgagtt gcaggacgct tttcactgga tggagaagca  gacgtttatc

                  ....|....| ....|....| ....|....|....|....| ....|....| ....|....| ....|....|
                    630        640        650        660        670        680        690
Dde_0086-1 GCCCTTGGTA ATCTGTTGTC CGGTGCGGCA CATCTGGGCA TACACGCATG CCCCATGGAA GGCTTTGATC
Dde_0086-2 gcccttggta atctgttgtc cggtgcggca catctgggca tacacgcatg ccccatggaa ggctttgatc
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             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    700        710        720        730        740        750        760
Dde_0086-1 ACGAAGTGCT GGAAAAAGAG CTGGGATTGA CGGAGAAAGG GTACAAGCCC TCGGTCATTG TGGCTCTGGG
Dde_0086-2 acgaagtgct ggaaaaagag ctgggattga cggagaaagg gtacaagccc tcggtcattg tggctctggg

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|....|....|
                    770        780        790        800        810        820       830
Dde_0086-1 GTTTAGTGCC GCTGATGATT TCAATGCGGT GACACCCAAA  TCCCGCTGGC CTCAGGAACG GATAATAACC
Dde_0086-2 gtttagtgcc gctgatgatt tcaatgcggt gacacccaaa  tcccgctggc ctcaggaacg gataataacc

              ....|....| ....|....|....|....| ....|....| ....|....| ....|....| ....|....|
                    840        850       860        870        880        890        900
Dde_0086-1 GAAATATGAC GTGCGGCAGC AATGAAGCTT GCGGCCGCAC TCGAGCACCA CCACCACCAC CACTGAGATC
Dde_0086-2 gaaatatga- ---------- ---------- ---------- ---------- ---------- ----------

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    910        920        930        940        950        960        970
Dde_0086-1 CGGCTGCTAA CAAAGCCCGA AAGGAAGCTG AGTTGGCTGC TGCCACCGCT  GAGCAATAAC TAGCATAACC
Dde_0086-2 ---------- ---------- ---------- ---------- ----------  ---------- ----------

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   980        990       1000       1010       1020       1030       1040
Dde_0086-1 CCTTGGGGCC TCTAAACGGG TCTTGAGGGG  TTTTTTGCTG AAAGGAGGAA CTATATCCGG ATTGGCGAAT
Dde_0086-2 ---------- ---------- ----------  ---------- ---------- ---------- ----------

              ....|....|....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   1050      1060       1070       1080       1090       1100      1110
Dde_0086-1 GGGACGCGCT CTGTAGCGGC GCATAAGCGC GCAGATGTGT GATTACGCGC AGCGTGACGC TACACTTGCA
Dde_0086-2 ---------- ---------- ---------- ---------- ---------- ---------- ----------

            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   1120       1130      1140       1150    1160       1170          1180
Dde_0086-1 GCGCCCTAGC  GCCCGCTCTT TCGCTTTCTT CCGTCATTTC  TCGCCACGTA CGACAGCCTT CCCGTCAGCT
Dde_0086-2 ----------  ---------- ---------- ----------  ---------- ---------- ----------

            ....|....| ....|....|....|....| ....|....| ....|....| ....|....| ....|....|
                   1190      1200       1210       1220       1230       1240       1250
Dde_0086-1 CTAATTCGGG ACTCCTTTAG TACGATTAGG CCTTAACGGT AACTCTCGGA ACTCCAAAAA AACTGTGATA
Dde_0086-2 ---------- ---------- ---------- ---------- ---------- ---------- ----------


Dde_0086-1 G
Dde_0086-2 -
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The sequence for *Dde_2199* from Wild type strain along with sequencing results for *Dde_2199*-pET28a(+).

*Dde_2199-1* = *Dde_2199*-pET28a(+)
*Dde_2199-2* = *Dde_2199* from Wild type strain

```
                ....|....| ....|....| ....|....| ....|....| ....|....|  ....|....| ....|....|
                       10         20         30         40         50         60         70
Dde_2199-1      CTAGAATAAT TTTGTTTAAC TTTAAGAAGG AGATATACCA TGGGCAGCAG  CCATCATCAT CATCATCACA
Dde_2199-2      ---------- ---------- ---------- ---------- ----------  ---------- ----------

                ....|....| ....|....| ....|....|  ....|....| ....|....| ....|....| ....|....|
                       80         90        100        110        120        130        140
Dde_2199-1      GCAGCGGCCT GGTGCCGCGC GGCAGCCATA  TGGCTAGCAT GACTGGTGGA CAGCAAATGG GTCGCGGATC
Dde_2199-2      ---------- ---------- ----------  ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|  ....|....|
                      150        160        170        180        190        200        210
Dde_2199-1      CGCCATGAAT GAGACAGTCC TTACCCCTTT CCTGTTCCGG CATGCCAGCA AGGATTTCGA TCCCGACAGA
Dde_2199-2      ----atgaat gagacagtcc ttacccctttt cctgttccgg catgccagca aggatttcga tcccgacaga

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      220        230        240        250        260        270        280
Dde_2199-1      AAAATTCCCG AAGACCAGTT TCTGACCATT CTGGAAGCAG GCAGACTCGC GCCCAGTTCC TTCGGCTTTG
Dde_2199-2      aaaattcccg aagaccagtt tctgaccatt ctggaagcag gcagactcgc gcccagttcc ttcggctttg

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      290        300        310        320        330        340        350
Dde_2199-1      AACCGTGGAA GTTTCTGATA GTGCAGAACC CCGCCCTGCG CGCCGAGCTG CACCGGCACA CATGGGGCGG
Dde_2199-2      aaccgtggaa gtttctgata gtgcagaacc ccgccctgcg cgccgagctg caccggcaca catggggcgg

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      360        370        380        390        400        410        420
Dde_2199-1      CAAAAAGCAG ATTCCCAACT GCAGCCATCT GGTTGTCTAT CTGGCCCACA  AACGCCCGCG TCTGCTGCCG
Dde_2199-2      caaaaagcag attcccaact gcagccatct ggttgtctat ctggcccaca  aacgcccgcg tctgctgccg

                ....|....| ....|....| ....|....|....|....| ....|....| ....|....| ....|....|
                      430        440        450        460        470        480        490
Dde_2199-1      GAAAGCGATT ATATACAGTC TTCCATGCGT GAGGTGCTGC AACTGCCCGA CGACATCATA GCCCTGAAAA
Dde_2199-2      gaaagcgatt atatacagtc ttccatgcgt gaggtgctgc aactgcccga cgacatcata gccctgaaaa

                ....|....|....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      500        510        520        530        540        550        560
Dde_2199-1      CGGACTATTA  CGGCAACTTC CTGCGCAGCG ATTTCGGCAT GATAGATAAC GAGATGCGGC TTTTTGAATG
Dde_2199-2      cggactatta  cggcaacttc ctgcgcagcg atttcggcat gatagataac gagatgcggc tttttgaatg

                ....|....| ....|....| ....|....| ....|....|  ....|....| ....|....| ....|....|
                      570        580        590        600        610        620        630
Dde_2199-1      GTCGTGCAGG CAGGCGTACA TAGCGCTGGC CAACATGATG ACAGCGGCAG CCATGATGGA AATCGACAGC
Dde_2199-2      gtcgtgcagg caggcgtaca tagcgctggc caacatgatg acagcggcag ccatgatgga aatcgacagc

               ....|....| ....|....|  ....|....| ....|....| ....|....| ....|....| ....|....|
                      640        650        660        670        680        690        700
Dde_2199-1 TGCGCACTGG AAGGCTTTGT TGAAGCGGAC CTGAACACCG CCGTGAAGCA GCACCTTCAG GTGGATACGG
Dde_2199-2 tgaagcggac ctgaacaccg ccgtgaagca gcaccttcag gtggatacgg tgcgcactgg aaggctttgt
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          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  710        720        730        740        750        760        770
Dde_2199-1 ATCAGTTCGG TGTTGCCTGC ATGTGTGCCT TTGGCTACAG AGTGCGCCCC CCGCGGCCCA AAGCCCGCCA
Dde_2199-2 atcagttcgg tgttgcctgc atgtgtgcct ttggctacag agtgcgcccc ccgcggccca aagcccgcca

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  780        790        800        810        820        830        840
Dde_2199-1  CAGCATGGAA AACGTGGTAC GCTGGTACGA TTAATCTGCC GCAAAGACAA GCTTGCGGCC GCACTCGAGC
Dde_2199-2  cagcatggaa aacgtggtac gctggtacga ttaa------ ---------- ---------- ----------

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  850        860        870        880        890        900        910
Dde_2199-1  ACCACCACCA CCACCACTGA GATCCGGCTG CTAACAAAGC CCGAAAGGAA GCTGAGTTGG CTGCTGCCAC
Dde_2199-2  ---------- ---------- ---------- ---------- ---------- ---------- ----------

          ....|....| ....|....| ....|....| ....|....|
                  920        930        940        950
Dde_2199-1  CGCTGAGCAA TAACTAGCAT AACCCCTTGG GGCTCTAAAC
Dde_2199-2  ---------- ---------- ---------- ----------
```

The sequence for *Dde_2199* from Wild type strain along with sequencing results for *Dde_2199*-cys-pET28a(+).

```
Dde_2199-1   =  Dde_2199-cys-pET28a(+)
Dde_2199-2   =  Dde_2199 from Wild type strain


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50         60         70
Dde_2199-1   TAGAATAATT TTGTTTAACT TTAAGAAGGA GATATACCAT GGGCAGCAGC  CATCATCATC ATCATCACAG
Dde_2199-2   ---------- ---------- ---------- ---------- ----------  ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       80         90        100        110        120        130        140
Dde_2199-1   CAGCGGCCTG GTGCCGCGCG GCAGCCATAT  GGCTAGCATG ACTGGTGGAC AGCAAATGGG TCGCGGATCC
Dde_2199-2   ---------- ---------- ----------  ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       150        160        170        180        190        200        210
Dde_2199-1   TGTTGCTGTT  GCTGTTGCGC CATGAATGAG ACAGTCCTTA CCCCTTTCCT GTTCCGGCAT GCCAGCAAGG
Dde_2199-2   ----------  ---------- -atgaatgag acagtcctta ccccttcct gttccggcat gccagcaagg

                ....|....| ....|....| ....|....| ....|....|....|....| ....|....| ....| ....|
                       220        230        240        250        260        270        280
Dde_2199-1   ATTTCGATCC CGACAGAAAA ATTCCCGAAG ACCAGTTTCT GACCATTCTG GAAGCAGGCA GACTCGCGCC
Dde_2199-2   atttcgatcc cgacagaaaa attcccgaag accagtttct gaccattctg gaagcaggca gactcgcgcc

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       290        300        310        320        330        340        350
Dde_2199-1   CAGTTCCTTC GGCTTTGAAC CGTGGAAGTT TCTGATAGTG CAGAACCCCG CCCTGCGCGC CGAGCTGCAC
Dde_2199-2   cagttccttc ggctttgaac cgtggaagtt tctgatagtg cagaaccccg ccctgcgcgc cgagctgcac

                ....|....| ....|....| ....|....| ....|....| ....|....|....|....| ....|....|
                       360        370        380        390        400       410        420
Dde_2199-1 CGGCACACAT GGGGCGGCAA AAAGCAGATT CCCAACTGCA GCCATCTGGT  TGTCTATCTG GCCCACAAAC
Dde_2199-2 cggcacacat ggggcggcaa aaagcagatt cccaactgca gccatctggt  tgtctatctg gcccacaaac

                 ....|....| ....|....| ....|....|....|....| ....|....| ....|....| ....|....|
                       430        440        450        460        470        480        490
Dde_2199-1   GCCCGCGTCT GCTGCCGGAA AGCGATTATA TACAGTCTTC CATGCGTGAG GTGCTGCAAC TGCCCGACGA
Dde_2199-2   gcccgcgtct gctgccggaa agcgattata tacagtcttc catgcgtgag gtgctgcaac tgcccgacga

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       500        510        520        530        540        550        560
Dde_2199-1   CATCATAGCC CTGAAAACGG ACTATTACGG CAACTTCCTG CGCAGCGATT TCGGCATGAT  AGATAACGAG
Dde_2199-2   catcatagcc ctgaaaacgg actattacgg caacttcctg cgcagcgatt tcggcatgat  agataacgag

                 ....|....| ....|....| ....|....| ....|....|....|....| ....|....| ....|....|
                       570        580        590        600        610        620        630
Dde_2199-1    ATGCGGCTTT TTGAATGGTC GTGCAGGCAG GCGTACATAG CGCTGGCCAA CATGATGACA GCGGCAGCCA
Dde_2199-2    atgcggcttt ttgaatggtc gtgcaggcag gcgtacatag cgctggccaa catgatgaca gcggcagcca

                ....|....| ....|....|....|....| ....|....| ....|....| ....|....| ....|....|
                       640        650        660        670        680        690        700
Dde_2199-1   TGATGGAAAT CGACAGCTGC GCACTGGAAG GCTTTGTTGA AGCGGACCTG AACACCGCCG TGAAGCAGCA
Dde_2199-2   tgatggaaat cgacagctgc gcactggaag gctttgttga agcggacctg aacaccgccg tgaagcagca
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                ....|....| ....|....| ....|....| ....|....| ....|....|....|....| ....|....|
                      710        720        730        740        750        760        770
Dde_2199-1      CCTTCAGGTG GATACGGATC AGTTCGGTGT TGCCTGCATG TGTGCCTTTG GCTACAGAGT GCGCCCCCCG
Dde_2199-2      ccttcaggtg gatacggatc agttcggtgt tgcctgcatg tgtgcctttg gctacagagt gcgcccccg


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      780        790        800        810        820        830        840
Dde_2199-1      CGGCCCAAAG CCCGCCACAG CATGGAAAAC GTGGTACGCT GGTACGATTA ATCTGCCGCA AAGACAAGCT
Dde_2199-2      cggcccaaag cccgccacag catggaaaac gtggtacgct ggtacgatta a--------- ----------


                ....|....|....|....| ....|....| ....|....| ....|....| ....|....| ....| ....|
                      850        860        870        880        890        900        910
Dde_2199-1      TGCGGCCGCA  CTCGAGCACC ACCACCACCA CCACTGAGAT CCGGCTGCTA ACAAAGCCCG AAAGGAAGCT
Dde_2199-2      ----------  ---------- ---------- ---------- ---------- ---------- ----------


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      920        930        940        950        960        970        980
Dde_2199-1      GAGTTGGCTG CTGCCACCGC TGAGCAATAA CTAGCATAAC CCCTTGGGGC CTCTAAACGG GTCTTGAGGG
Dde_2199-2      ---------- ---------- ---------- ----------  ---------- ---------- ---------


                ....|....| ....|
                      990   995
Dde_2199-1      TTTTTGCTGA AAGAG
Dde_2199-2      ---------- -----
```