Chapter I:

Introduction and Review of Literature

1.1. Basic Introduction

Hydrocarbons have played very important role in the development of modern human society. This can easily be understood by looking at its very prevalent and direct use as fossil fuel or the use of hydrocarbon derivatives in numerous products like toothpaste, medicines, cosmetics, plastics, pesticides, fertilizers, preservatives, artificial flavoring agents etc [1-5]. The vast availability of crude oil reserve and their ever expanding roles in our day to day life have also brought ecological problems to the fragile biosphere on this earth by way of polluting the native environment. The oil spill, caused by the discharge of liquid petroleum hydrocarbon into the environment due to the natural or anthropogenic reasons is adding severity to such environment harming every year.

The estimated crude oil spillage considering only the transportation during the last 45 years is found to be around 20 million tons. Further, the oil spilt due to accidents is also massive, estimated to be approximately 142 million tons [6]. Hundreds of chemicals of different properties and toxicities are present in crude oil and these chemicals if mixed with soil can bring about soil physicochemical properties to affect adversely its fertility status. Crude oil constituents even may penetrate down the subsurface to cause ground water pollution.

Hydrocarbon pollution is one of the significant problems the world is facing today. Aliphatic as well as polycyclic aromatic hydrocarbons (PAHs) are present everywhere in the environment which needs timely

appraisal and treatment to mitigate their harmful effects on life and biodiversity [7,8]. There are several remediation techniques involving approaches like chemical, mechanical or biological. Being cost effective and environment-friendly, bioremediation is considered best method for remediation of hydrocarbons. Bioremediation involves engagement of biocatalysts in the process of removal of these pollutants. A biocatalyst may be a microorganism or an enzyme which facilitate conversion of pollutants to simpler forms ultimately their mineralisation. One of the key enzymes involved in CH activation is Cytochrome P450 monooxygenases (CYP450), which initiates transformation of both aliphatic as well as polycyclic aromatic hydrocarbons. This property of CYP450 has been well exploited for the development of novel strategies and technologies in environmental bioremediation [9-12]. The same property can also be used in detection and quantification of hydrocarbon in a given sample of interest. Presently, procedure for doing such a job is cost intensive and time consuming involving series of experiments and instruments such as GC-MS, HPLC. There are also sensors developed for this purpose which uses principles of high-cost electromagnetic radiation or temperature dependent gas.

Cytochrome P450 monooxygenases can hydroxylate hydrocarbons. The property of hydroxylation of hydrocarbon by CYP450 can be used as a tool for developing new biosensors for hydrocarbon detection. This includes

working in room temperature, providing a cost-efficient, easy to analysis, tool for monitoring of crude oil contamination.

1.2. Introduction to Cytochrome P450

Cytochrome P450 (CYP450) monooxygenases are heme-containing enzymes that form a superfamily responsible for catalysis of varieties of lipophilic substrates to hydrophilic metabolites via oxidation [13]. They can activate drugs, carcinogens, pesticides, xenobiotics and hydrocarbons [14] via hydroxylation, epoxidation, oxidation of heteroatoms, and dealkylation. [15]. The history of Cytochrome P450 dates back to the late 1940s. The evidence of the enzyme was seen during the investigation of a few unusual reactions involving molecular oxygen [16]. Osamu Hayaishi reported an enzyme which could catalyse catechol to muconic acid by oxidative cleavage and termed the enzyme as "pyrocatechase" [17]. Further experimental evidences of their occurenece were provided in 1955 by Axelrod [18] and Brodie *et al.* [19] in liver endoplasmic reticulum. The reported enzyme was claimed to perform oxidation of xenobiotics. Garfinkel and Klingenberg in 1958, reported about a carbon monoxide (CO) binding pigment in liver microsome, having an absorption peak at 450nm [20,21]. This pigment was further classified as a hemoprotein of b-type class by Omura and Sato in 1964 [22], and eventually named as Cytochrome P450 due to its characteristic absorption peak at 450 nm. Since then there has been growing interest for the enzyme due to its diversity

and versatility of actions making it one of the most prominent topics in the field of biochemical research.

The enzyme CYP450 is found in almost every organism of the world. There are more than 20000 isozymes of the enzyme, identified in the genomes of various organisms [23]. Most of the prokaryotic CYP450s are simple and usually soluble, whereas eukaryotic counterparts are mostly membrane-bound [24,25]. The membrane-bound CYP450s are embedded in the lipid bilayer via an N-terminal trans-membrane helix with an estimated depth of 1-2 nm [26].

1.2.1. Microbial Cytochrome P450

Most of the microbial CYP450s are soluble and are without the N-terminal membrane anchor [24]. Almost all the microbial CYP450s need an additional Class I redox partner system (soluble Ferredoxin and ferredoxin reductase), whereas the fewer number of CYP450s are also driven by class II and class III redox partners (Flavin mononucleotide (FMN) containing flavodoxin) [27,28]. Although for the majority of the CYP450s the Class I redox partner is essential for the monooxygenases activity, exceptions have also been reported showing various outliers. The BM3 (BM3, CYP102A1) of *Bacillus megaterium* fuses with a soluble fatty acid hydroxylase to form a soluble self-sufficient Cytochrome P450 reductase [29]. In the early 2000s another self-sufficient prokaryotic CYP450 had been reported. In this case, the CYP450 was found to be fused with an NAD(P)H-dependent flavin-containing and 2Fe-2S cluster-containing

reductase. This group of fused CYP450s were found to be catalysing thiocarbamate herbicides [30]. The discovery of these self-sufficient CYP450s have provided a significant push for microbial CYP450s for use in various applications such as production and catalysis of complex molecules and biosensors for detection.

Microbial CYP450 enzymes are found to be more stable and highly active than the mammalian counterparts [29]. Due to its more soluble nature, the bulk production and isolation of the microbial CYP450s are more convenient for both laboratories and industries [31-33]. These recombinant and reconstituted self-sufficient CYP450s have also been reported with more functionalities and better activities [34] against various substrates such as drugs [34], hydrocarbons [35], explosives [36] and sterols [37].

1.2.2. Structure and mechanism of reactions of CYP450

Although there have been thousands of reported CYP450s and number of CYP450 genes, the overall topology and fold are common for all [38]. The core of the enzyme is formed of three parallel helices named D, L, I and an antiparallel helix E respectively [39,40]. The prosthetic heme group lies in between the distal I helix and proximal L helix. The prosthetic group is bound to the neighbouring Cysheme-ligand loop. This loop has a conserved amino acid sequence of FxxGx(H/R)xCxG for all the CYP450s. The cysteine residue proximal or "fifth" ligand to the heme iron is conserved. The sulfur ligand is thiolate^{52} and responsible for the characteristic soret peak at 450 nm for Fe-CO complex. The I helix contains

a signature amino acid sequence (A/G) - Gx(E/D)T at the middle of the helix and forms a wall of the heme pocket. In the active site there lies a highly conserved threonine residue followed by an acidic group, which is believed to be involved in catalysis [40-44].

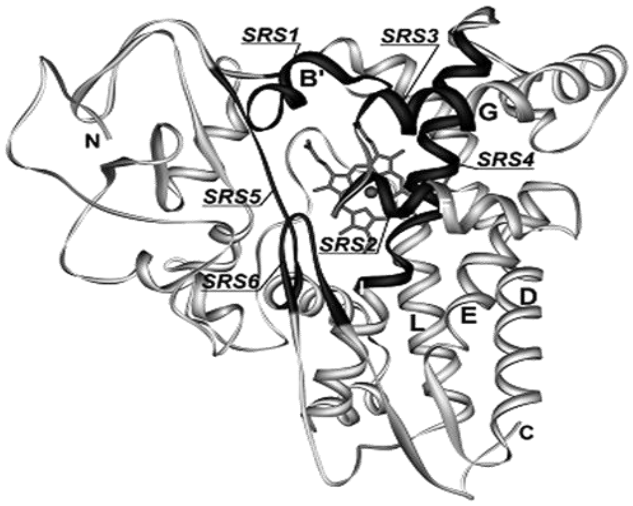

Fig.1.1: The ribbon structure of Cytochrome P450 along with the SRS regions and α helices (in capital letters) (Source: Denisov *et.al.*)

Further, Gotoh reported that there are mainly six structural recognition/binding site for substrate recognition, termed "Substrate recognition sites" (SRS) [45]. Which are B′ helix region (SRS1), parts of the F and G helices (SRS2 and SRS3), a part of I helix (SRS4), the K helix â2 connecting region (SRS6), and the â4 hairpin (SRS5) site (figure1) [40,45]. The SRS predetermines the substrate specificity, and mutation in the SRS region affects the substrate specificity. Though it seems that the catalytic core of the enzyme is highly conserved, there is plenty of structural diversity to allow the huge number of substrates it acts upon with variable specificity.

The CYP450s are monooxygenases but are involved in a large number of different stereo and regio-specific reactions [46]. They are mostly involved in mixed function oxidation-reduction reactions. During the monooxygenation reactions, one oxygen atom combines with the substrate and the other oxygen atom combines with two hydrogen atoms donated

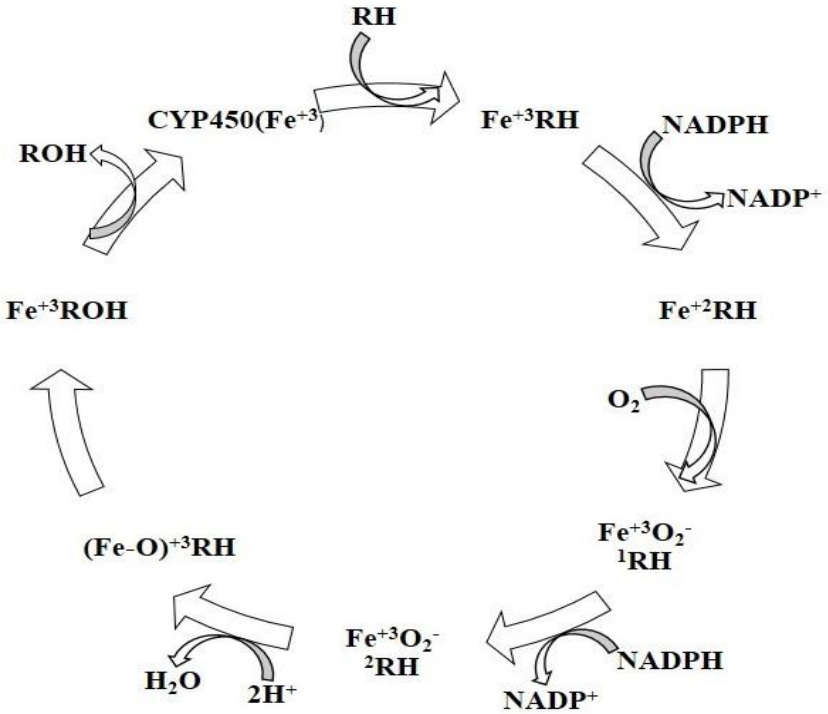

Fig.1.2: (RH) binds to CYP450 near the distal region of heme moiety (Fe in Fe3+ state). Electrons flow from NADPH via NADPH-P450 reductase (FAD/FMN reductase) and reduces the Fe^{+3} to Fe^{+2}. $Fe^{+2}RH$ rapidly attacks an Oxygen (O_2) molecule to form $Fe^{+2}O_2^{-}RH$. $Fe^{+2}O_2^{-}RH$ being not very stable gradually changes to $Fe^{+3}O_2^{-}RH$. Which follows a second reduction of the compound to form $Fe^{+3}O_2^{-2}RH$. The O_2^{-2} from the compound binds with two protons from the reaction mixture and gets cleaved to form H_2O and $(Fe^{+3}\text{-}O)^{+3}RH$. Eventually the iron bound oxygen is transferred to the substrate RH to form ROH. Summarising the entire reaction the hydrocarbon substrate (RH) is being hydroxylated to ROH via the monooxygenase activity of the Cytochrome P450.

by two NAD(P)H to form water [16]. The catalytic reaction cycle occurring during the process is summarised in figure 1.2.

1.2.3. *Hydrocarbon hydroxylation by Cytochrome P450*

The chemical transformation of the region and stereoselective hydroxylation of hydrocarbon C-H bond is complicated. Whereas, in biological systems, this reaction can efficiently be catalysed by some metalloenzymes without any external energy. Cytochrome P450 monooxygenase being one of the most prominent metalloenzymes is also involved in hydroxylation of a considerable number of different hydrocarbons. These diverse group of enzyme can hydroxylate both simple aliphatic to complex Polyaromatic hydrocarbons (PAH).

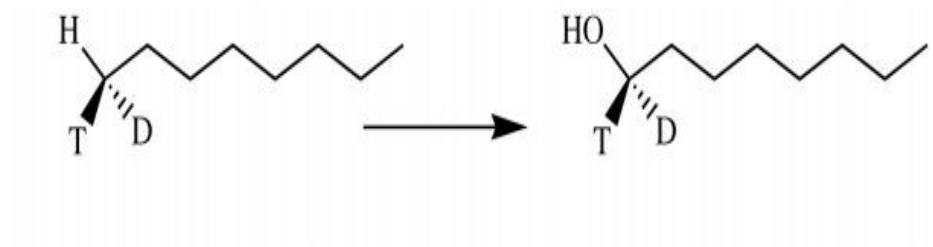

Fig.1.3: the retention of the stereochemistry during hydroxylation of octane to 1-octanol. (Source: Shapiro *et.al.*)

In most of the cases, the mechanism of hydrocarbon hydroxylation of C-H bonds typically proceeds with the retention of stereochemistry at the reacting carbon. In 1958, investigators reported the retention of configuration at the reacting carbon for hydroxylation of cholesterol and pregnane-3, 20-dione [47,48]**.** Further, the similar mechanism of retention of configuration has also been reported for acyclic compounds. The

conversion of octane to 1-octanol by rat liver microsome CYP450s maintains the same stereochemistry during the process of hydroxylation (figure 1) [49]. Similarly, for the conversion of geraniol by *Catharanthus roseus* CYP450s [50] the stereochemistry is maintained. Even though these studies try to explain the general mechanism of action as the retention of stereochemistry, there are reports of other mechanisms as well. Groves *et al.* gave the radical rebound mechanism to explain the hydroxylation of norbornene by liver microsome with loss of the stereochemistry [51].

1.3. Enzyme immobilisation

Enzyme immobilisation is the process by which enzymes are confined to or localised in a defined region of space with the retention of their enzymatic activity. Enzyme immobilisation can be carried out using reversible interactions with the support such as physical adsorption, ionic linkages, and affinity binding or by the formation of stable covalent bonds [52]. The essential components of an enzyme immobilisation system includes the enzyme, the matrix and the mode of attachment [53]. Based on this, enzyme immobilisation techniques can be classified into three different types: by attachment to carriers, by crosslinking and by encapsulation or inclusion formation [54]. Amongst them, encapsulation is one of the best methods for the immobilisation of the enzymes as it can prevent the effects of negative influences such as the aggression and denaturation of the enzymes by external agents such as proteases. It can

also provide the natural cellular environment for the enzyme, making it more stable and active [55].

1.3.1. Immobilisation of CYP450s

It has been mentioned that CYP450 have a huge potential owing to its versatility of substrates. However, the practical use of the enzyme is limited due to its low stability outside its native cellular environment [56]. Enzyme immobilisation have been used to increase the stability of an enzyme as well as for better product separation [54,57]. The first attempt to immobilise the enzyme cytochrome P450 was made in 1978 by Burner and Losgen, in BrCN Sepharose 4B. However, the resulting hydroxylation activity was very low. Azari and Wiseman in 1980 reported the immobilisation studies for *S. cerevisiae* CYP450s [58]. King *et al.* in 1988 immobilised the CYP450 enzymes from *S. cerevisiae* via entrapment in calcium alginate with retention of 84% activity for up to two weeks [59]. Reports regarding the use of mammalian CYP450s are more as compared to microbial CYP450s [60,61]. In the following table 1, the different reports regarding the use of microbial CYP450s have been listed along with the different immobilisation matrices.

Table 1.1: Immobilisation of Microbial CYP450s

Year	CYP450 used	Immobilisation matrix and technique used	Remarks
1980[58]	S. cerevisiae CYP450s		
1988[59]	S. cerevisiae CYP450s	calcium alginate, entrapment	Retention of 84% activity up to two weeks.
2000[62]	Streptomyces griseus	ionic exchange matrix DE52	85% retention. No significant loss of

	(CYP105D1)		activity has been observed during 1 day of reaction at 25°C with co-factor recycling, reduced turnover.
2003[63]	P450 BM-3 from B. megaterium	anion exchangers such as DEAE and SuperQ sol-gel matrix derived from tetraethoxyorthosilicate (TEOS)[64]	Products of the reaction also adsorbed to these matrices. long-term activity (half-life of 30 days at 25°C),
2006[65]	Bacterial P450 BM-3	Immobilised on glassy carbon electrodes modified with PDDA, Adsorption	Six-fold increase in product.
2009[66]	Bacterial P450 BM-3 mutant	Polypyrrole, entrapment	Polypyrrole acted as electron mediators.
2010[67]	Bacterial P450 BM-3 mutant	mesoporous molecular sieves.	Higher activity than the soluble form.
2011[68]	Bacterial BM3 M7 mutant	Immobilised on DEAE-650S, further entrapped with k-carrageenan together with zinc dust.	Retention of 76% activity for 10 reaction cycles. When used in bioreactor worked for five days.
2014[69]	Bacterial CYP450 BM3	Entrapment in poly(3-hydroxybutyrate) granules	Higher stability and catalytic activity as compared to free P450 against changes of pH, temperature, and concentrations of urea and ions.
2015[61]	Bacterial CYP450	Immobilisation by crosslinking by employing the self-assembling nature of two proteins.	Formation of a simple water-insoluble gel after the crosslinking.
2016[70]	Bacterial CYP102A1	Immobilisation on metal surfaces by using selectively binding peptides (indium tin oxide or gold binding peptides)	Higher activity than the soluble form.
2017[71]	Bacterial P450 BM-3 mutant	Ni2+-functionalized magnetic nanoparticles, adsorption and cross-linking-adsorption	88% retention of activity after five days. Easy separation.

1.4. Introduction to Biosensors

A biosensor can be a device, module or a system with the purpose of detection or quantification involving a biological component such as protein, enzyme, or an antibody. Along with the biological component it also comprises a non-biological component for transferring the biological or chemical signal to a detectable output. The International Union of Pure and Applied Chemistry (IUPAC) in IUPAC gold book has defined biosensor as "A device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals"[72].

The history of the first biosensor can be dated back to the late 50s to early 1960s. Professor Leland C Clark Jr., from the University of Alabama, USA is known as the father of the concept of "Biosensor". In 1956 he first invented oxygen electrode, followed by an amperometric enzyme electrode for glucose (1962). Here, he entrapped glucose oxidase (GOX) on Clark oxygen electrode using a dialysis membrane. He also correlated the relation between the oxygen consumption and glucose concentration making it the first reported biosensor [73]. Updike and Hicks described an enzyme electrode based biosensor with GOX deposited in an oxygen electrode in 1967 [74]. It is to be noted that this was the first report of a functional biosensor which paved the way for other researchers to get

interested in enzyme-based biosensors. Further, in 1969, the first potentiometric biosensor was reported for urea using urease [75].

Since the first report of the glucose sensor by Clark *et al.* in 1962, there has been a gradual increase in number of research regarding biosensors. Different approaches have been taken by different researchers for the development of biosensors. Thermal enzyme probes, which relies on thermal transducers were first introduced in 1974 [76,77]. This was followed by the introduction of biosensor where optical fibres were used as the transducing element [78]. Divies first gave the concept of "microbial electrodes" using microbial cell in 1975 for alcohol measurement [79]. In the following years the concept of biosensors diversified extensively incorporating various tools and techniques. This involved a wide variety of strategies with different biological entities such as enzymes, nucleic acids, antibodies, cells in various combination of alternative transducing elements such as piezoelectric, ISFTEs , magnetic beads, optical, thermometric etc. [80-83]. These many combinations of biological elements along with transducing elements have generated a wide variety of biosensors having numerous applications in the field of healthcare, biomonitoring of the environment, food and beverages processing, defence and security [82,84-87].

1.4.1. Basic components of a Biosensor

A biosensor must have two basic component, one is the biological receptor, and the other one is the transducer. Along with these two

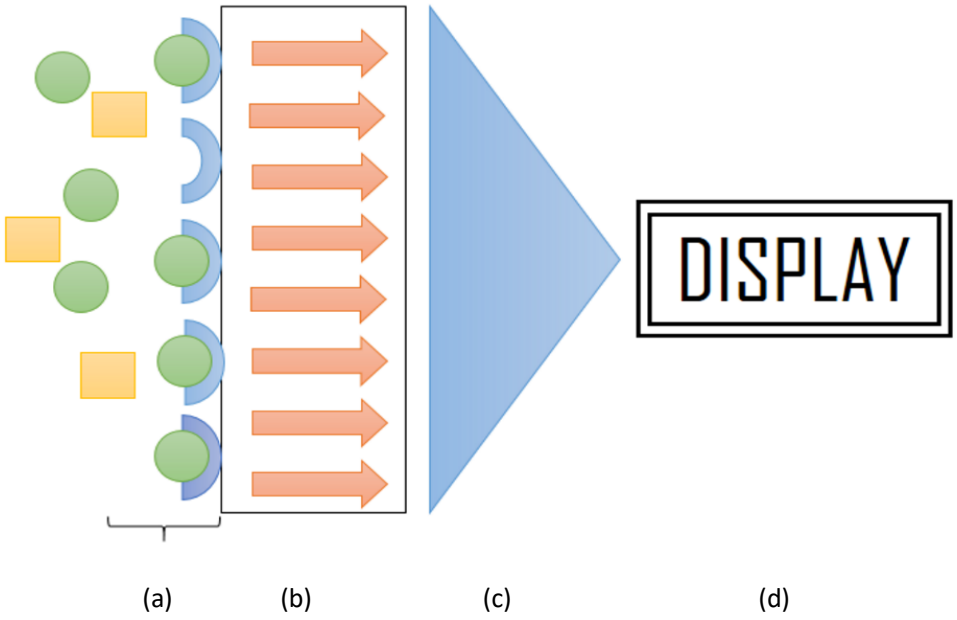

Fig.1.4: A schematic diagram of a biosensor, where (a) is the biological receptor along with two types of analyte molecules (green and orange), (b) is transducer, (c) represents amplifier/processor and (d) is the Display.

components, a biosensor may also have an amplifier-processor and a display (Figure 1). Biological receptors or in short bioreceptor is the sensing element of a biosensor. They usually interact with the analyte in some specific manner, generating some form of signal, henceforth they are the element responsible for the overall specificity of the device. In figure 1(a) two types of analytes can be seen green and orange but the bioreceptors are only specific towards the green analytes, and hence the biosensor becomes specific. Bioreceptors can be of different types, such as enzyme, multi-enzyme system, antibody, deoxyribonucleic acid (DNA), bio-membrane component, organelle, bacterial or another cell, or whole slices of mammalian or plant tissues etc. [79,83,88-90].

The transducer is the next major component in a biosensor, responsible for the conversion of the biological interaction into some detectable signal such as current, voltage etc. It can be of various types, such as electrochemical, optical, calorimetric and piezoelectric [90]. The choice and method of transduction depend on the physicochemical interaction between the analyte and the bioreceptor. Though the methods of transduction can vary, it can be divided into two groups; chemical transduction (amperometry, potentiometry, conductimetry and optical detection) and physical transduction (gravimetric detection and colourimetry) [91]. Based on different types of transducers there can also be different types of biosensors, viz. potentiometric, amperometric, conductimetric, optical, colourimetric, mechanical and piezo-electric biosensors [92]. The biosensors with potentiometric, amperometric and conductimetric transducers are collectively termed as electrochemical biosensor [93]. As this study focuses mainly on an Ion Sensitive Field Effect Transistor (ISFET) based potentiometric biosensor with Cytochrome P450 (CYP450), this review of literature will also focus mainly on different aspects of potentiometric biosensor relating ISFET and sensors associated with CYP450.

The signal generated from the transducer is usually low and noisy. Hence it requires an amplifier for signal amplification. The signal generated may require additional processing, for example, conversion of parameters

(current to concentrations) where the processor comes in to affect and finally output is presented in the display.

1.4.2. Electrochemical biosensor

An electrochemical biosensor is a sensor which acts by electrochemical oxidation and reduction reaction of the analyte with the bioreceptor. These chemical reactions generate or consume ions or electrons. This can be correlated to amount concentration, or analyte level in the sample [93,94]. In an electrochemical biosensor, a biochemical layer is coated to an electronic-ionic conducting or semiconducting material, and hence they are also termed as a chemically modified electrode (CME) [95,96]. Depending upon the measuring electrical parameters electrochemical biosensor are separated as potentiometric, amperometric and conductimetric biosensor [93]. A brief comparative table (table 1.2) has been provided discussing the above mentioned three sensors followed by a detailed study regarding potentiometric electrochemical biosensor with respect to ISFET.

Table 1.2: Types of electrochemical biosensors based on different transducers along with characteristics:

Characteristics	Potentiometric	Amperometric	Conductimetric
Transducing elements	Ion sensitive electrode (ISE) [97], Ion Sensitive Filed Effect Transistor (ISFET) [98], Enzyme Field Effect transistor	Metal electrodes [99], carbon electrodes, chemically modified electrodes.	Interdigitated electrode, Metal electrode.

	(ENFET) [81], Metal electrode [99].		
Measured parameters	Potential/Voltage	Current	Conductance/Resistance
Analyte for detection	Ions such as H^+, K^+, Ca^{+2}, Na^+,CO_2, NH_3, OH^- [93,99,100]	O_2, Sugar, Alcohol.	Urea, Charged Species, Oligonucleotides.
Bioreceptor	Biological Ionphores [101], Enzyme [81], antibodies [88], whole cell [102], membrane receptors[103], lipid bilayer, hydrophobic membrane, Small protein molecules, and peptides, aptamer [104].	Enzyme, antibodies, whole cell [102], membrane receptors, lipid bilayer, hydrophobic membrane, Small protein molecules, and peptides, aptamer, Oligonucleotide duplex.	Enzyme, antibodies, whole cell [102], membrane receptors, lipid bilayer, hydrophobic membrane, Small protein molecules, and peptides, aptamer.
Sensitivity	High	High	Low
Equation	Nerst Equation	Cottrell Equation	Incremental resistance

1.4.3. Potentiometric biosensors and Ion Sensitive Field Effect Transistor (ISFET)

As the name suggests, the potentiometric biosensors are associated with potentiometric transducers, where at the surface of the transducing element an electric potential is formed due to the change in distributions of either ions or electrons. In a potentiometric biosensor, the primary signal generated is due to the ions accumulated/depleted at the ion-sensitive surface. Depending upon the need and variability a

potentiometric biosensor can have variable transducing elements, such as Ion-Sensitive electrode (ISE) [97], Ion Sensitive Field Effect Transistor (ISFET) [98], Enzyme Field Effect transistor (ENFET) [81], and Metal electrodes [99].

The idea of Ion Sensitive Field Effect Transistors (ISFET) was introduced by Bergveld in 1970 [105] for the measurement of ion activities around a nerve. It was followed by the report of Matsuo and Wise on a similar device with Silicon Nitride sub-gate layer [106] for bio-potential recording. ISFET is a derivative of Metal Oxide Semiconductor Field Effect Transistor (MOSFET). Ion Sensitive Field Effect Transistors are semiconductor amplifier devices with functional similarities to junction transistors but with a higher input resistance [113]. ISFETs operate in such a similar way

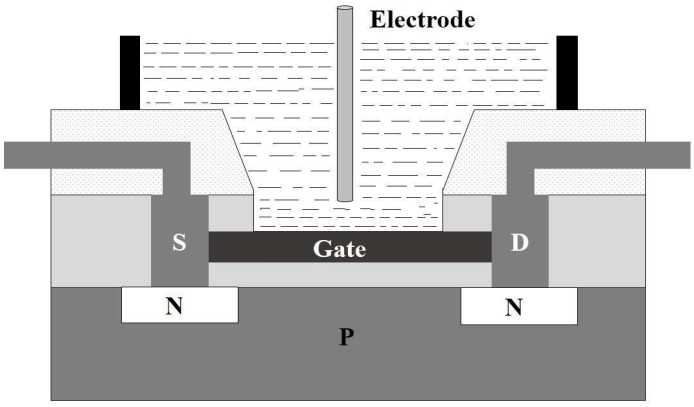

Fig.1.5: Schematics of an ISFET device, there are mainly three components in an ISFET, the source (S), the drain (D) and a gate (G), along with those three there is a reference electrode connected via the electrolyte. Here, N and P denotes the P type and N type semiconductor.

to a (MOSFET) that ISFETs can also be called Gate modified MOSFET [114]. The only difference is that in ISFET, the metal gate is substituted by an ion-sensitive surface, a reference electrode and an electrolyte (Figure 2). The working principle is also similar with a MOSFET, to be described in chapter 4. In last 48 years, this idea of ISFET has grown massively with numerous applications in the field of DNA sequencing [107,108] and various sensors such as ChemFET [109], Enzyme FET [81], DNAFET [110], ImmunoFET [111,112] etc

1.5. Cytochrome P450 in biosensors

The catalytic activities of the enzyme Cytochrome P450 with various applications have attracted a large number of researchers since the late seventies of the last century [115-117]. In the early era, for the enzyme cytochrome P450, intact hepatocytes or microsomes were used as the biological transducing element. In the year 1978, Renneberg *et al.* presented the first report of liver microsome based enzyme electrode involving Cytochrome P450 [116]. This followed a vast number of biosensors involving Cytochrome P450 based on variation in CYP species, technique, substrate and Co-substrate, immobilisation techniques, electrodes and electronic component [88,118-120].

It has been already mentioned that CYP450 can act on a wide range of substrates [13,121,122]. Hence biosensor associated with CYP450 can also have a verity of substrates ranging from pesticides to medicines. The different biosensors associated with CYP450 or its derivative have been

listed in table 1.3 in chronological order, focusing only on the electrochemical sensors.

Table 1.3: Different biosensors involving cytochrome P450 and its derivatives:

Year	Bioreceptor: Cytochrome P450 type	Transducer, type of biosensor	Substrate used and remarks
1978 [116,123]	Microsomes from rat liver	Paraffined spectral carbon, Amperometric, Electrochemical sensor.	Aniline, used for the comparison of the reaction catalysed by CYP450 and Horse redox peroxidases (HRP).
1996 [119]	Recombinant cytochrome P450 101	Edge plane pyrolytic graphite, Electrochemical	Camphor, Binding is seen, but complete catalysis is not described.
1997 [124]	Cytochrome P450cam CYP101	Antimony doped Tin Oxide.	A bioreactor for Camphor, not working without the mediator Pdx.
1998 [125]	Cytochrome P450cam CYP101	Gold electrode, Multilayers with PEI, PDDA, PSS—Au	Styrene, proteins were mono-layered one over another, post the 2nd layer there wasn't much electrochemistry.
1999 [126]	Surface cysteine-free mutant CYP101	Gold electrode, Cyclic voltammetry, electrochemical.	No substrate but limited O2 reduction.
1999 [127]	Cytochrome P450cam CYP101	Au-MPS—PDDA, DDAB, multilayers carbon cloth	cis-β-methylstyrene, measurement in solution.
2000 [128]	Cytochrome P450 2B4 and 1A2	rhodium–graphite electrodes, electrochemical	Aminopyrine, aniline, 7-ethoxyresorufin, and 7-pentoxyresorufin, riboflavin is required for catalysis.
2002 [129]	rat CYP1A1 P450 monooxygenase	ISFET, Voltage output, electrochemical	Dichlorophenols, a fusion protein of rat CYP1A1 and yeast NADPH-cytochrome P450 oxidoreductase used as the bioreceptor.

2002 [130]	Mutated bacterial Bacterial cytochrome $P450_{cam}$	Nano-crystalline Sb-doped tin oxide Electrode, Spectroelectrochemistry	Camphor and styrene Phenosafranine used as Mediator
2002 [131]	Human/bacterial CYP450 CYP102, CYP2E1	Glassy carbon (GC) disc, CV, Electrochemical	Pesticides, Fusion protein have been used.
2003 [132]	bacterial cytochrome P450cin (CYP176A)	DDAB-EPG electrode, CV, potentiometry	pH dependent: 6 < pH < 10
2003 [133]	CYP1A2	Multilayers with PSS - Carbon cloth, CV	Styrene, temperature dependent.
2004 [134]	Human Cytochrome CYP2E	Glassy carbon (GC) and the gold electrode, Chronoamperometry.	p- nitrophenol
2004 [135]	Microbial Cytochrome P450 CYP119	Didodecyldimethylammonium bromide (DDAB) film on a plastic formed carbon (PFC), Electrochemical.	No catalysis, electrochemistry observed up to 80◦C.
2004 [136]	CYP101	4,4′-dithiodipyridin modified gold electrode, Spectroelectrochemistry	No catalysis, enzymes retain its native state during electrolysis.
2005 [137]	CYP1A2 and CYP3A4 microsomes	Pyrolytic graphite, electrochemical	Styrene,
2005 [138]	human P450s 2C9, 2C18 and 2C19	DDAB modified Pyrolytic graphite, Electrochemical	Torsemide, warfarin, and tolbutamide showed low signals even in anaerobic CV. Similar work has been reported by other researchers as well [139,140].
2006 [141]	Bacterial Cytochrome P450 BM3	In sodium dodecyl sulfate (SDS) surfactant films on the surface of basal-plane graphite (BPG) electrodes, Electrochemical	Styrene
2008 [142]	CYP3A4 fusion protein	Glassy carbon modified with Poly-diallyldi-methylammonium	Erythromycin

		chloride(PDDA) electrode, Electrochemical	
2008 [143]	CYP2B4	Graphite electrode with modification by gold nanoparticles and DDAB, Electrochemical.	Benzphetamine, Similar work has been reported by Shumyantseva *et al.* [145].
2008 [144]	Cytochrome P450scc (CYP11A1)	Rhodium-graphite electrode modified with Multi-walled carbon nanotubes (MWCNT), Electrochemical.	Cholesterol, the detection linearity is enhanced with MWCNTs. Carrara *el al.* also reported similar work with graphite or gold electrode for substrates Benzphetamine, naproxen and cyclophosphamide [146].
2009 [66]	Microbial CYP102 (P450BM-3) Mutant	Platinum and glassy carbon modified with polypyrrole	No catalysis, Compared chemical vs. electrochemical polymerization of polypyrrole for effect on enzyme catalysis. Alonso-Lomillo also reported a polypyrrole modified gold electrode for phenobarbital [148].
2009 [147]		Gold electrode modified with Amine coupling via EDC/NHS to a mixed SAM of OT and MUA	Warfarin, further gold electrodes with similar modification for different substrates have been reported by others [149,150].
2010 [151]	CYP2E1 Single-Cysteine Mutants	Gold electrode modified with DTME SAM	p-nitro-phenol
2012[152]	CYP1A2, CYP2B6, CYP3A4	Multi-walled carbon nanotubes (MWCNT) fixed carbon paste screen printed graphite electrode.	Anticancer drugs cyclophosphamide, ifosfamide, ftorafur and etoposide. sensitivity values in the range of 8–925 nA/μM
2013[153]	Human CYP450 2C9	polyacrylamide (PAM) hydrogel films cast on GC electrodes (GCE)	Bis-phenol A, Sensitivity of 18.21 μA/mM

2013 [154]	cytochrome P450 2B4	The enzyme was screen-printed onto the surface of SPCEs	Cocaine, reproducibility of 5.91%, repeatability 10.45% and detection limit 0.2 mM
2014[155]	cytochrome P4501A2	Drop casting of MWCNTs and CYP P4501A2 on a graphite screen-printed electrode (SPE)., CV	Naproxen an anti-inflammatory compound. The sensor is capable of precisely monitoring the real-time delivery of NAP for 16 h.
2014[156]	cytochrome P4502D6	screen-printed Ag/AgCl electrode, Electrochemical	Codeine, detection limit of detection of 4.9 μM
2016[157]	Cytochrome P450 BM3 expressed in E. coli cells	The ag-agcl reference electrode, CV	Prepared for inhibition studies of imidazole, metyrapone and 1-aminobenzotriazole (ABT). Imidazole was found to be the most potent inhibitor; further the sensor does not need enzyme purification.

1.6. Present scenario of Hydrocarbon detection

Hydrocarbon detection has always been an essential subject for research. Various methods have been developed each with its advantages and disadvantages. The gravimetric methods are easier to use but time-consuming and involve sample loss due to heat or infrared. Further, these techniques also require a large amount of sample with the involvement of expansive instruments such as a spectrophotometer, centrifuge etc. [158]. Gas chromatography (GC) alone and GC along with mass spectroscopy (MS) have been successfully utilised for identification and quantification of hydrocarbon samples. However, they suffer from disadvantages of expenditure. The machines also require expertise to operate making them very specific.

The sensor technologies developed for hydrocarbon sensing are mostly gas sensors. As the name suggests they can sense gas and hence they involves high energy consuming processes for conversion of samples to gaseous form. In table 1.4 the few important methods of hydrocarbon sensing has been tabulated along with its advantages and disadvantages.

Table 1.4: Methods of hydrocarbon sensing.

Methods	Advantages	Disadvantages
Gravimetric methods: Environmental Protection Agency(EPA) method 418.1	Works in room temperature, the sensitivity of 1 mg/l	Involves toxic chemical Freon 113, spectrophotometer, time-consuming
EPA Method 1664 [158]	No- toxic chemicals, with a sensitivity of 5-1000 mg/l	Time-consuming, Works only on those materials which can be extracted by n-hexane, sample loss due to high temperature, involves centrifuge, vacuum pump, ice bath.
EPA method 9070		
Methods involving GS/MS	Accuracy with a huge number of compounds.	High cost and technical knowledge is required.
Various sensors Gas sensors		Most of them need heat for sample processing, non-functional in room temperature.
Infra-red sensors	Limited number of substrates	Expensive, requires complicated machineries

1.7. Aims and objective of the study

Based on the above observation it can be summarised that there is a high demand for development of easy monitoring of hydrocarbons in room temperature without much consumption of time and energy and also in a cost-effective way. Keeping that in mind, this study attempts to develop

an ISFET based biosensor involving Cytochrome P450 for in situ monitoring of remedial measures of hydrocarbon pollution. Further, the developed biosensor may also be helpful for sensing petroleum reserves. To facilitate the above aim, the following four objectives were taken for the study.

1. Screening and identification of CYP450 producing bacteria from oil contaminated soil samples of Assam.
2. Partial purification and characterisation of Cytochrome P450.
3. Fabrication and characterisation of the Cytochrome P450 ENFET.
4. Improvement of biosensor stability and study of biosensor output with respect to pH, temperature, and co-factor.

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