Chapter - I

Introduction
1.1 General impact.

From primeval days both human and animal were and still exclusively depend on nature. The continuous changes in pre and post biotic ages, ecosystem followed system of order and disorder. The imbalance of a nature due to modern civilization and progress of human being through more complicated activities seems to be a great challenge. Extreme industrialization, modern scientific development, modern cultivation of farms has led the more guanine problem of concern. The environmental pollution due to this involves direct or indirect threat to every living organism. During the ages of development the living organisms have acquired the capacity to adapt themselves to the changes in the ecosystem. The adaptation process has helped the living organism to acquire the capacity to modulate their biological phenomenon and biological system so as to make their survival continue.

The revolution of industries was in a period from 18th to 19th century, makes major turning point in human history almost every nooks and corner of human being and marked turning point in earth’s ecology and human relationship with their environment. The transition of human society from being a hunter-gatherer to one of agriculture jump-started the ability of humans to use the resources of the earth more sufficiently. Industrialization resulted in water, air and soil pollution, thus so harmful waste increased astronomically high. As industrial revolution dramatically changed each and every aspect of human life and lifestyles impacted on natural resources, public health begin to register world psyche until 1960’s after nearly 150 years from its beginning.

The production of fuels such as coal and crude oil including petroleum when used into new steam engines found very effective to high speed and capacity. Steam power which in the front of technology found to be the biggest advancements revolutionized many industries including manufacturing and textiles. Petroleum-derived contaminants were most prevalent sources of environmental deterioration among some in the industrialized world. Hydrocarbon molecule, which makes crude oil and petroleum products are highly toxic to many organisms, including humans [1]. Production of chemicals in large scale is important during the industrial development. Hence, pollution is the persistent problem, which pollutes the water, air and soil.
Chapter I

Introduction

The introduction of polluted contaminant into the environment due to anthropogenic activities resulted to natural disasters. Pollution has a detrimental effect on ecosystem, making every living thing virtually impossible to sustain life. Pollution causes instability, disorder, harm or discomfort to the ecosystem i.e. physical systems or living organisms. Pollution can take the form of xenobiotic, pollutants the elements of pollution, can be foreign substances; when naturally occurring and they are considered contaminants when they exceed natural levels [2]. All are major users of energy that produce large amounts of waste products and pollution. Other industries have less potential impact but are still considered highly problematic when they come to pollution. These industries include the textile, leather tanning, paint, plastics, pharmaceutical, paper and pulp-industries.

One of among basic needs which is most and fashionable nowadays is clothing, having source from textile industry. The World Bank estimates that 17 to 20 percent of industrial water pollution comes from textile dyeing and treatment. Water pollution is a prominent environmental problem witnessed across the globe. India being a developing country, since its independence has maintained a democratic tradition and country with quite developed environmental policy and need for environmental protection is written in the Indian constitution. After globalization rapid economic growth achieved by some of the developing countries, adversely affected the quality of the environment. A growing environmental concern took the form of waste reduction, prudent use of resources and the development of environmentally safe products [3].

The defense against these adverse changes in the ecosystem and environment, the living system has adapted the biological mechanism, the defense mechanism against environmental pollution, medication and other foreign compounds take place mainly in the liver of the animals. The mixed function oxidase system is major enzymatic defense mechanism, well established in animals. The enzyme system involved in the biotransformation of xenobiotics is mainly confined to endoplasmic reticulum. Endoplasmic reticulum with rough and smooth type is isolated as microsomal, are sealed vesicles of ruptured endoplasmic reticulum during homogenization.

Rough endoplasmic reticulum is the site for enzyme synthesis, which is more active on smooth endoplasmic reticulum, formed by loss of ribosome from rough types. Endoplasmic reticulum consists of an intracellular lipid membrane which can channel and compartmentalize
materials inside the cell. The structural and the lipoproteinous nature of endoplasmic reticulum seem to be best suited for the enzymes to reside on it and to provide a structural framework for enzyme reactions. The whole structure seems to have evolved at least partly because of the requirement of organisms to detoxified foreign molecules, especially lipophilic to which they are naturally and continually exposed. But the basic physiological role seems to be the metabolism of endogenous substrates. These enzyme complexes metabolize or oxidize a variety of drug and pollutants; such as polycyclic aromatic hydrocarbon (PAH), pesticides and steroids [4, 5].

The microsomal monooxygenase system metabolizes most foreign lipophilic compounds, which enter the body to more polar and readily extractable products. This enzymes system embedded in endoplasmic reticulum consists of flavoprotein, NADPH cytochrome P-450 reductase and group of hemoproteins. This system also requires molecular oxygen for its activity [6, 7, and 8] and NADPH as an electron donor. This system catalyses the incorporation of an oxygen atom into lipophilic substrate molecules and reduction of other oxygen atom to water [1], resulting into an increase hydrophilicity of the molecules. The stiochiometry of the reaction can be described as follows; where SH is the substrate and EH is electron donor.

\[
SH + EH + H^+ + O_2 \rightleftharpoons SOH + E + H_2O
\]

Apart from main site of liver, the presence of this system is also reported in various tissues like kidney, intestine, adrenal, spleen, testis, ovary, placenta, brain, skin etc [1-9]. The presence of cytochrome P-450 monooxygenase system is not limited in wide species of animals fish [10] and birds [11] but also present in yeast and bacteria [12], fungi and plants [13].

Bacteria have unique feature in rapidly adapting to limited nutrient supplies and occupying hostile environment. The metabolic diversity and plasticity of bacteria in the face of environmental insults and limitations provide an immense reservoir of exploitable regulatory devices and biochemical activities [14, 15]. Among these abilities bacteria have a potential to biodegrade and hence to remove a wide variety of man-made aromatic compounds discharged through urban and industrial activities [16, 17]. The enzymes system employed by the microorganisms for the degradation of xenobiotics is microbial biotransformation enzymes. This involves mixed function oxidase system i.e. cytochrome P-450 containing monooxygenase and dioxygenases, other enzymes like azoreductases, laccases, hydrolases, etc.
1.2 Cytochrome P-450

The term cytochrome P-450 is used to refer to a group of proteins that apparently requires a sulphur atom ligand to the iron and forms carbon monoxide complex in reduced state. Grafinkel [18] and Klingenberg [19] first described the pigment from liver microsomal fraction giving a characteristic carbon monoxide absorption difference spectrum with the peak at 450 nm. Ryan and Engel [20] further provided the evidence for its possible relation with enzymatic hydroxylation. Omura and Sato 1964 [21] later named the substance cytochrome P-450 from the position of absorption of CO-complex, it was first shown to be a hemoproteins, it has its prosthetic group a noncovalently bound iron protoporphyrin IX, a form of heme found in b-type cytochromes, like cytochrome b, hemoglobin and myoglobin. The ability of those hemoproteins with an available 6th coordination position to bind small molecules such as cyanide, carbon monoxide, ethyl isocyanide etc, has provided means for their analysis and quantification. In the case of cytochrome P450, the binding of carbon monoxide permitted demonstration of its involvement in the monooxygenase reaction [22].

Involvement of cytochrome P-450 in the reaction of mixed function oxidase system and activation of oxygen was established by Estabrook et al [23]. Solubilisation of the hemoprotein resulted into the loss of the typical absorption of CO-complex at 450 nm (figure 1). With concomitant shift to 420 nm, this was referred as catalytically inactive P-450 or cytochrome P-420. Presence of cytochrome P-450 is not confined to mammalian species. It is also found in birds [23, 24], fishes [25], plants and bacteria [26].

This was demonstrated to be a hemoproteins of the b-type class in 1964 [21, 22] which was named cytochrome P-450 after the strong feature in its absorption spectrum.
Cytochrome P-450 monooxygenase system is highly nonspecific. Though the reason for this is unknown, the enzymes in the system with endogenous substrate and important functions in the synthesis of steroids or hormones are more specific. Well-defined products are produced for the functioning of the organisms. However, in the biotransformation of the xenobiotics, introduction of the oxygen atom at any position in the substrate molecule is usually sufficient to increase the hydrophilic properties of these compounds. This reaction facilitates its excretion from the body.

Several investigators have attempted to find out the possible explanation for the substrate non-specificity of this enzyme. It was found that several forms of cytochrome P-450 (isozymes), with slightly different but overlapping substrate specificities exist [28-29]. The relative distribution of different forms of cytochrome P-450 is attributed to genetic and environmental influence [30]. Sex, species, age, strains, nutritional status and exposure of organism to various chemicals explained the multiplicity of the cytochrome P-450 [26, 31]. It is well known that various cytochrome P-450 so far characterized are different from each other by molecular weight, amino acids composition and terminal amino acids [26-33]. Even antibodies prepared against electrophoretically homogenous form of particular cytochrome P-450 do not cross react with other forms of cytochrome P-450. However, the spectral properties are almost identical due

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**Fig. 1:** Absorption spectrum of cytochrome P-450 CO complex showing the characteristic Soret peak at approximately 450 nm for P-450 [27].
to small but significant differences between the various forms [14-13]. The heme is bound to
cytochrome P-450 to form a single polypeptide chain with molecular weight varying from 40
kDa to 60 kDa. The similarity in the optical spectra could be explained on the basis of essentially
identical coordination sphere of heme [34].

1.3 Cytochrome P-450 monooxygenases

Although initially the microsomal drug and xenobiotic-metabolizing enzymes were
referred as mixed function oxidases; recent years the term monooxygenases became the more
recognized one. Cytochromes P-450 got name from their character as hemoproteins with unusual
spectral properties displaying typical absorption maxima of the reduced CO bound complex at
450 nm. Cytochrome stands for a hemoproteins, P for pigment and 450 reflects the absorption
peak of the CO complex at 450 nm. The ability of reducing P-450 to produce an absorption peak
at 450 nm upon CO binding is still used for the estimation of the P450 content (Omura and Sato,
1964) [22].

Cytochrome P450 monooxygenases are versatile biocatalysts which, that introduces
oxygen into a vast range of molecules, catalyze diverse reactions in a regio and stereo selective
manner. These properties have been used for drug development, bioremediation, the synthesis of
fine chemicals and other useful compounds. P-450 monooxygenases have pivotal roles in
primary, secondary metabolic pathways and drug degradation. Moreover, oxygenases may be
enantio, chemo or regio selective and thus can produce optically pure compounds in reactions
where chemical catalysts fail; oxygenases are being exploited in various industrial processes [35-
37]. Cytochromes P-450s are ubiquitously distributed enzymes, which were discovered about 50
years ago and which possess high complexity and display a broad field of activity. They are
hemoproteins encoded by a super family of genes converting a broad variety of substrates and
catalyzing a variety of interesting chemical reactions. This enzyme family is involved in the
biotransformation of drugs, the bioconversion of xenobiotics, the metabolism of chemical
carcinogens and the biosynthesis of physiologically important compounds such as steroids, fatty
acids, eicosanoids, fat-soluble vitamins, bile acids, the conversion of alkanes, terpenes and
aromatic compounds as well as the degradation of herbicides and insecticides. There is also a
broad versatility of reactions catalysed by Cytochromes P450 such as carbon hydroxylation,
heteroatom oxygenation, dealkylation, epoxidation, aromatic hydroxylation, reduction and dehalogenation [38-42].

Monooxygenases belong to a family of enzymes that introduce one atom of dioxygen into a wide range of substrates. For degradation of the aromatic compounds fission of the ring mediated by dioxygenases is necessary. Cytochromes P-450 constitutes a super family of heme monooxygenases with more than 8000 isozymes identified in organisms representing all biological kingdoms [43]. All Cytochromes P-450 share a common fold, have a molecular weight of 45–60 k Da and contain a single b-type hem (iron protoporphyrin IX); prosthetic group is deeply buried inside the protein globule [44-45]. Even though the sequence conservation among P-450 proteins of different families may be less than 20%, their general topography and structural fold are highly conserved and point to a common mechanism of oxygen activation. In contrast to that, the most variable regions are represented by the flexible substrate recognition regions (SRS) [46], which enable P-450s to be the most versatile biological catalyst known to a variety of substrates.

**Fig. 2:** Assignment of Cytochromes P-450 to enzyme groups. P-450 is associated with the pink colored subdivisions [47].

Environmental chemical’s metabolism is highly dependent on particular xenobiotic metabolizing enzymes (XME) (Fig. 2), their isoforms and their polymorphic variants. In phase I a polar functional group is introduced into the molecule, rendering suitable substrate for phase II metabolism. Phase II metabolism consists of conjugation of phase I metabolites or xenobiotics
that already possesses a suitable functional group with water-soluble endogenous metabolites such as sugars, amino acids, sulfate or glutathione. The isoforms of cytochrome P-450 are the most important of the phase I enzymes in the metabolism of xenobiotics and in the introduction of functional groups for phase II metabolism.

Almost all P-450s are end terminal monooxygenases that utilize electrons derived from cofactors NADH or NAD (P) H. For catalytic activity P-450s must be associated with redox partner proteins that transfer electrons from NAD(P)H to the P-450 heme center via a flavin adenine dinucleotide FAD or flavin mononucleotide (FMN) containing cytochrome P-450 reductase (CPR) (Fig. 3, general steps in P-450) [50].

1.4 Cytochrome P-450 dependent monooxygenase

Generally the steps involved in the hydroxylation reactions are:
1. Binding of substrate to the oxidized cytochrome P-450.
2. Reduction of NAD(P)H or NADH cytochrome P-450 ferredoxin reductase by NAD(P)H or NADH.
3. Reduction of cytochrome P-450 substrate complex by ferredoxin.
4. Addition of oxygen molecule to reduced cytochrome P-450 substrate complex.
5. Reduction of oxygenated reduced cytochrome P-450 substrate complex by another electron, probably from NAD (P)H or NADH cytochrome P-450 reductase.
6. Decomposition of oxygenated reduced cytochrome P-450 substrate complex to hydroxylated substrate oxidized cytochrome P-450 and water.
1.5 Constituent of monooxygenase system

Monooxygenases belong to a family of enzymes that introduce one atom of dioxygen into a wide range of substrates. For degradation of the aromatic compounds fission of the ring mediated by dioxygenases is necessary.

![Diagram of monooxygenase system](image)

**Figure 3:** General steps in CYP-450 [48]

This family of enzymes also inactivates and causes the development of tolerance to many therapeutic agents and drugs [49-50]. In some fungi and bacteria, P-450 enzymes are used to initiate the oxidation of hydrocarbons or other recalcitrant compounds for use as carbon sources for growth [51].

These P-450s dependent monooxygenases systems broadly classed into three types based on protein components; Figure 4-A illustrates three-protein components occur in mammalian cells of mitochondria (involved in steroid metabolism), bacteria having P-450s dependent monooxygenases systems. The mitochondrial P-450s are intrinsic membrane proteins associated electron-transfer components are isolated in soluble form. The bacterial proteins, including the P-450s components, are generally soluble fraction of cytoplasm [52-53].

The steroid hydroxylase P-450s isolated from adrenal cortex mitochondria require the both a reductase, which contains FAD and interacts directly with NADPH, and a small ferredoxin like protein (adrenodoxin), which shuttles electrons between reductase and the P-450
cytochrome [54]. Figure 4-B depicts the two-protein P-450s dependent monooxygenase system found in endoplasmic reticulum of higher animal cells and also in other eukaryotic cells [47] as compare number of distinct species, this is the largest class of P-450s in the mammals [42, 55-56].

Figure 4: Comparisons of Three protein system mitochondrial, two protein system microsomal, and one protein system of bacterial P-450 monooxygenase [57].

So-called “microsomal” P-450 systems have only one electron-transfer protein interacts with NADPH and transfers electrons directly to cytochrome P-450 species. Protein, NADPH-P-450 oxidoreductases, contains both FAD and FMN (but no iron sulfur center) and is an integral membrane protein exists in only major form in contrast to the multitude of distinct P-450 species. Figure 2C, illustrates the known example to date of a one-protein P-450-dependent monooxygenase system, which isolated from Bacillus megaterium [58]. This single soluble polypeptide contains FAD, FMN and P-450 heme moiety (but no Fe-S center) initially reduction is by NADPH and afterwards all electron transfers.

A remarkable feature of the P-450s is the manipulation of the same basic structure and chemistry to achieve an enormous range of functions in organisms as diverse as bacteria and man. Electron spin resonance spectroscopy suggested that P-450 is a low spin ferric hemoproteins [59] with a thiol residue as an axial haem ligand [60-62]. This is lead to explain the unusual sort peak position and its perturbation upon the binding of substrates and other chemicals in terms of charge transfer modulated by the Fe-S bond [63-65]. Raman spectroscopy
provided confirmation of the presence of a Fe-S bond and identified this as a covalently bonded cysteine residue [66]. In 1985, a full structure of P-450cam (CYP101), a bacterial P-450 from *Pseudomonas putida*, was obtained (figure-5) [67-68]. Subsequently, crystal structures have been obtained for the P-450<sub>terp</sub> [69] and P-450<sub>BM3</sub> enzymes [70] as well as for P-450<sub>cam</sub> in complex with substrate [71], carbon monoxide [72], inhibitors [73-74] and substrate analogues [75-77].

![Cytochrome P-450 heme containing protein structure Schlichting I](image)

**Figure 5:** Cytochrome P-450 heme containing protein structure Schlichting I [78].

1.6 Gene organization and evolutionary aspect

P-450 superfamily genes are subdivided and classified as recommendations of a nomenclature committee [79], on the basis of phylogenetic criteria, gene organization and amino-acid identity. The root symbol CYP is followed by a number for families and a number of genes; e.g. CYP4U2. There are also designations for clades of CYP families (clans, which can be defined as group of genes that clearly diverged from a single common ancestor; clans are named from the lowest family number in the clade) [81] and for specific alleles of a gene (in humans) [81-82].

The diversity of the cytochrome P-450 superfamily arose by an extensive process of gene duplication and by probable, but less well documented cases of gene amplifications, conversions, genome duplications, gene loss and lateral transfers. “Fossils” evidence of these processes can be found by careful sequence aliments as well as from the presence of many P-450 gene clusters in most organisms. These gene clusters can contain up to 15 P-450 genes, orientation and sequence similarly of which sometimes allows a reconstruction leading formation of the cluster.
The origin of P-450 superfamily lies in prokaryotes before the advent of eukaryotes and before the accumulation of molecular oxygen in the atmosphere. *Escherichia coli* no P-450 gene, *Mycobacterium tuberculosis* has 20 [83], baker’s yeast has 3 P-450 genes [84]. Only P-450 gene family, CYP51 is conserved across phyla, plants to fungi and animals.

1.7 Classification of cytochrome P-450

Cytochrome P-450 enzymes are classified according to their protein sequence similarity [85] and divided into gene families and subfamilies. 265 families have now been identified, of which 18 families and 43 subfamilies are from mammals [86]. Cytochrome P-450 enzymes can be divided into four classes on the basis of the redox partner Fig. 5. Class I P-450s use a flavoprotein which contains FAD and iron-sulfur protein as electron transporters and this class is found in some bacteria and most mammalian mitochondrial P-450s. Class II P-450, need a flavoprotein which contains FAD and FMN as electron transporters and this class is found in microsomal. Class III P-450s can gain electrons from the substrate without interacting with redox partners (self-contained system) and can be found in bacteria, mammals and plants [87, 88]. Class IV P-450 has recently been identified. In these, the substrate has a flavoprotein reductase domain, an iron-sulfur cluster and a cytochrome P-450 enzyme [89].

![Figure 6: From the homepage of S.K. Chapman (June 2005) [90]](image)

In the last decade, CYP450 studies have been expanded rapidly and hence it was essential to classify the CYP450s to families and subfamilies members of the CYP4A family are responsible for the metabolism of fatty acids to ω and ω-1 hydroxylated forms, which is a
preferable site for all CYP4 family [91] and are involved in drug biotransformation like CYP2D6, 2C19 and 3A4 [92]. CYP4 family is divided into subfamilies which of three subfamilies (CYP4A, CYP4B and CYP4F) are identified in mammalian species. Furthermore, two uncharacterized new members of the CYP4 family have been identified in this laboratory and named CYP4X1 and CYP4Z1.

1.8 CATALITIC CYCLE OF CYTOCHROME P-450s:

![Proposed catalytic cycle of Cytochrome P-450 enzymes monooxygenases (Denisov et al., 2005) [93-94].](image)

Cytochrome P-450s are usually monooxygenase, catalyzed the insertion of one of the atoms of molecular oxygen into a substrate, the second atom of oxygen being reduced to water.
Chapter I

Introduction

The most frequently catalyzed reaction is hydroxylation (O - insertion) using the very reactive and electrophilic iron-oxo intermediate, these steps can be summarized as follow:

**Step 1:** Also called resting state, this step is the faster, the substrate binds to the active site of the enzyme to Fe\(^{III}\) group. Fig.7.1, which is on the opposite of peptide chain; binding of the substrate induces a change in the conformation of the active site and displacing a H\(_2\)O molecule, [95] changing the state of the heme iron from low-spin to high-spin gives rise to a change in the spectral properties of the enzyme, with an increase in absorbance at approximate 390nm and a decrease at ~ 420nm, which is referred to as "type-I" (see inset graph in figure) [96]. Some substrates cause an opposite change in spectral properties, which is a reverse type-I, Inhibitors and certain substrates that bind directly to the heme iron give rise to type-II difference spectrum, with a maximum at ~ 430nm and a minimum at ~ 390nm (see inset graph in figure). This complex remains stable, allowing the degree of binding to be determined from absorbance measurements [97].

**Step 2:** Which is independent of whether substrate is bound. The electrons come from NADPH via the accessory flavoprotein NADPH P-450 reductase, with the flow going from the reductase prosthetic group FAD to FMN to the P-450 [98-101], (Fig. 7.2). There appear to be few exceptions to NADPH P-450 reductase as the electron donor. Unnatural donors such as ferredoxins and flavodoxins will work in artificial systems but only relatively slowly [102-103].

**Step 3:** Ferrous P-450 binds O\(_2\). This complex is unstable and can generate ferric Auto-oxidation (O\(_2^{-}\)) [104-107]. This inference has come from some work with bacterial P-450s [108-109] (Fig. 7.3). If carbon monoxide (CO) binds to reduce P-450, the catalytic cycle is interrupted as shown in figure 5. This reaction yields the classic CO difference spectrum with a maximum at 450 nm.

**Step 4:** A second electron enters in the system, this may come from NADPH P-450 reductase or, in some cases, also call oxo-P-450 complex from cytochrome b\(_5\) (CYTb\(_5\)) [110-111] (Fig. 7.4). (CYTb\(_5\) is rather passive in supplying the first electron, probably because the first reduction is thermodynamically and more difficult than the second and in rare cases can lead to auto-oxidation shunt (step 2) [16,115-117]).
Step 5: A proton is added Fe$^{II}$-O-OHR. The peroxo group formed in step 4 is rapidly protonated twice by local transfer from surrounding amino-acid side chains (Fig. 7.5).

Step 6: the O-O bond is cleaved, generating H$_2$O (with the addition of a H$_2^+$ proton) and an entity shown as FeO$^{3+}$. This high-valent complex can be written as Fe$^V$=O. With reference to the precedent of peroxidases shunt to step 2, at the most, this step is stable and it has been extensively characterized [115]. The most general view is that, the iron is Fe4 and the porphyrin ring is one-electron deficient (Fe$^{4+}$ O$_2$-porph$^+$) (Fig. 7.6). The FeO$^{3+}$ complex can be used to rationalize most of the P-450 reactions.

Step 7: It is called reactive intermediate. In this step the electron-deficient complex either abstracts a 2 hydrogen atom or a 2 electron from the substrate and form hydrogen peroxide to (Step 2) else forms a sigma complex with the substrate Fe$^{III^+}$=O$_2$H$_2$R. (Fig. 7.7).

Step 8: There with complex collapse of the intermediate or intermediate pair in generates the product (Fig. 7.8). (In the case of a hydrogen abstraction mechanism, this step is referred to as the “oxygen rebound”) and the product dissociates from the enzyme.

However, the point should be made as, this is may a complex version and that the system is dynamic and the steps do not necessarily proceed in a linear order around the cycle.

1.9 Classification of electron transport systems for Cytochromes P-450s

After the discovery of cytochromes P-450, two main classes described on the basis of the redox partners involved. The adrenal mitochondrial P-450 systems get electrons from NADPH via adrenodoxin reductase adrenodoxin [116] and the liver microsomal P-450s receive electrons from NADPH via FAD or FMN-containing P-450 reductase [117,118]. The camphor hydroxylase [119,120], the first bacterial P-450 system discovered was found that system is analogously organized to the mitochondria and electrons are transferred from NADH via a FAD-containing reductase (putidaredoxin reductase) and an iron–sulfur protein of the [2Fe–2S] type (putidaredoxin) to CYP101.
• **Class I**

Bacterial cytochrome P-450 and mitochondrial P-450 systems from eukaryotes are of Class I, P450 type systems. Even though phylogenetically not elated [121-122], both groups composition of three separate proteins in common: a FAD-containing reductase, transfers reduction equivalents from NADH or NADPH to the ferredoxin, which in turn reduces the cytochrome P-450 itself. In bacteria, all three proteins are soluble, Figure 6-A in eukaryotes, only the ferredoxin is a soluble protein of the mitochondrial matrix, whereas the reductase and cytochrome P-450 are membrane associated and membrane-bound to the inner mitochondrial membrane, respectively Figure 8-B [123-124]. The bacterial cytochromes P-450 are involved in the catabolism of compounds used as carbon source [125,137-139], the metabolism of xenobiotics [126], fatty acids [127] and the production of biologically active secondary metabolites such as antibiotics or antifungal [128-129,136-139].

The electrons necessary for the P-450 catalyzed reactions, which are usually provided by NADH and transferred via a NADH-dependent FAD-containing reductase and a ferredoxin of the [2Fe–2S] type [139,143] In *Bacillus subtilis*, a [4Fe–4S] cluster ferredoxin was suggested as a potential redox partner of CYP107H (P450BioI) [130]. The prototype of bacterial P-450 system is the P450cam system from *Pseudomonas putida*, cytochrome P-450 (CYP101) catalyzes the step 5-exo hydroxylation in the breakdown of D-camphor, enabling the bacterium to grow and use D-camphor as only carbon source [131-132]. The flavoprotein of the system putidaredoxin reductase is a FAD containing, strictly NADH-dependent ferredoxin reductase lacking the NADP-binding sequence lacking the NADP-binding sequence [68, 133] **Error! Bookmark not defined.**
Table 1: Classification of Cytochrome P-450 depending on the topology of the protein components involved in the electron transfer to the P-450 enzyme [49]

<table>
<thead>
<tr>
<th>Class</th>
<th>Source</th>
<th>Electron transport chain</th>
<th>Localization/remarks</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Bacterial</td>
<td>NAD(P)H • [FdR] • [Fdx] • [P-450]</td>
<td>Cytosolic, soluble</td>
<td>[134-136]</td>
</tr>
<tr>
<td>II</td>
<td>Bacterial</td>
<td>NADH • [CPR] • [P-450]</td>
<td>Cytosolic, soluble; <em>Streptomyces carbeophilus</em></td>
<td>[138]</td>
</tr>
<tr>
<td></td>
<td>Microsomal A</td>
<td>NADPH • [CPR] • [P-450]</td>
<td>Membrane anchored, endoplasmic reticulum</td>
<td>[139]</td>
</tr>
<tr>
<td>III</td>
<td>Bacterial</td>
<td>NAD(P)H • [FdR] • [Fdx] • [P450]</td>
<td>Cytosolic, soluble, <em>Citrobacter braakii</em></td>
<td>[140-141]</td>
</tr>
<tr>
<td>IV</td>
<td>Bacterial</td>
<td>Pyruvate, CoA • [OFO] • [Fdx] • [P450]</td>
<td>Cytosolic, soluble, <em>Sulfolobus kadoi</em></td>
<td>[142-143]</td>
</tr>
<tr>
<td>V</td>
<td>Bacterial</td>
<td>NADH • [FdR] • [Fdx–P-450]</td>
<td>Cytosolic, soluble, <em>Methylococcus capsulatus</em></td>
<td>[144]</td>
</tr>
<tr>
<td>VI</td>
<td>Bacterial</td>
<td>NAD(P)H • [FdR] • [Fdx–P-450]</td>
<td>Cytosolic, soluble, <em>Rhodococcus rhodochrous strain 11Y</em></td>
<td>[145-146]</td>
</tr>
<tr>
<td>VII</td>
<td>Bacterial</td>
<td>NADH • [PFOR–P-450]</td>
<td>Cytosolic, soluble, <em>Rhodococcus sp strain NCIMB9784</em> <em>Burkholderia sp.</em> <em>Ralstoni ameliilidurans</em></td>
<td>[91]</td>
</tr>
<tr>
<td>VIII</td>
<td>Bacteria, fungi</td>
<td>NADPH • [CPR–P-450]</td>
<td>Fungi Cytosolic, soluble, <em>Bacillus megaterium</em>, <em>Fusarium oxysporum</em></td>
<td>[91,147]</td>
</tr>
<tr>
<td>IX</td>
<td>Only NADH dependent, fungi</td>
<td>NADH • [P-450]</td>
<td>Cytosolic, soluble, <em>Fusarium oxysporum</em></td>
<td>[148]</td>
</tr>
<tr>
<td>X</td>
<td>Independent in plants/mammals</td>
<td>[P-450]</td>
<td>Membrane bound, endoplasmic reticulum</td>
<td>[149-150]</td>
</tr>
</tbody>
</table>

Fdx (iron–sulfur-cluster); FdR, Ferredoxin reductase (FAD); CPR, cytochrome P-450 reductase (FAD, FMN); Fldx, Flavodoxin (FMN); OFOR, 2-oxo acid: ferredoxin oxidoreductases (thiamin pyrophosphate, [4Fe–4S] cluster); PFOR, phthatate family oxygenase reductase (FMN, [2Fe–2S] cluster).

Fdx\(^a\) containing iron–sulfur-cluster of [2Fe–2S], [3Fe–4S], [4Fe–4S], [3Fe–4S]/[4Fe–4S] type.
Schematic organization of different cytochrome P-450 systems (Fig. 8A to 8K) [47]

8A- Class I, bacterial system;

8B- class I, mitochondrial system;

8C- Class II microsomal system

8D- Class III, bacterial system; e.g., P-450cin

8E- Class IV, bacterial thermophilic system,

8F-Class V bacterial [Fdx]-[P-450] fusion system
Chapter I

Introduction

8G- Class VI, bacterial [Fldx]–[P-450] fusion

8H- Class VII, bacterial [PFOR]-[P-450] fusion

8I- Class VIII bacterial [CPR] [P-450] fusion system, 8J- Class IX soluble eukaryotic P450nor

8 K- Independent eukaryotic systems, P450TxA.
• **Class II**

Class II, Cytochromes P-450 commonest in eukaryotes, performs extremely diverse catalytic reactions, Figure 7-C in mammals and responsible for the oxidative metabolism of both endogenous and exogenous compounds.

• **Class III**

A novel class of P-450 systems reported in 2002 [141], which is strongly evocative of the classical bacterial system and also significant differences. Three components P-450 systems characteristic of most of the bacterial and mitochondrial systems: Electrons are transferred from the primary electron donor i.e. NAD(P)H via NAD(P)H-dependent FAD-containing ferredoxin reductase and redox protein to the cytochrome P-450. But unlike in the class II electron transfer protein CPR. In this novel system, the two redox centers FAD and FMN belong to separate proteins, Figure 8-D [141-142].

In Bacillus subtilis, there is another cytochrome P-450 designated as CYP107H (P-450BioI) [130,152]. Nevertheless, it cannot be ruled out that CYP107H belongs to the bacterial class I cytochrome P-450s as there are also experimental data indicating that the only B. subtilis ferredoxin (Fer) is able to relay reduction equivalents to CYP107H [133].

• **Class IV**

The soluble CYP119 (EC 1.14.14.) has been identified in the extreme acidothermophilic archaeon *Sulfolobus solfataricus* [165]. Firstly discovered and intensively characterized thermophilic cytochrome P-450 [153]. It does not obtain its reducing equivalents from an NAD(P)H-dependent flavoproteins [154]. The reconstituted catalytic systems (Figure 8-E) have been tested with lauric acid. Since until now the physiological substrate for CYP119 is not known [154].

• **Class V**

Novel class of P-450 systems consist two separate protein components unknown putative NAD(P)H-dependent reductase and a cytochrome P-450-ferredoxin-fusion protein. Figure 6-F, which is in contrast to the classical three-components class I system. Only known example of this novel class is the sterol 14α-demethylase CYP51 (MCCYP51FX, EC 1.14.13.70) from *Methylococcus capsulatus* which is the only cytochrome P-450 existent in *M. capsulatus* show a unique primary structural organization [145]. In this enzyme, a P-450 heme-monooxygenase
domain is fused at the C-terminus to a [3Fe–4S] type ferredoxin domain via an alanine-rich linker region, which is thought to act as a flexible hinge interacts between the two domains.

- **Class VI**

The class VI cytochrome P-450 system is comprises subunits of a putative NAD(P)H-dependent flavoprotein reductase and a flavodoxin-P-450-fusionprotein, which standing in between the P-450BM3 and P-450cin systems. These principally use the same redox centers FAD, FMN and heme although differ in characteristics, also the number of separate proteins comprising the system. The first example of the novel class VI P450s is the cytochromeP-450-like gene from *Rhodococcus rhodochrous* strain 11Y (designated as xplA), in which the cytochrome P-450 is fused to a flavodoxin domain at its N terminus. The product of the constitutively expressed gene was shown to be responsible for the degradation of the widely used military explosive (RDX) Figure 8-G [155-156].

- **Class VII**

Bacterial fusion system of class VII constitutes totally novel class of P-450 systems. Structural organization is a quite unique kind, a cytochrome P-450 is C-terminally fused to a reductase domain, usually not associated with P-450 systems a phthalate dioxygenase reductase domain Figure 8-H. The class VII cytochrome P-450 firstly reported is cytochrome CYP116B2 (P450RhF) from *Rhodococcus* sp strain NCIMB 9784 [157], whose cytochrome P450 domain with high homology (55%), [158] to class I CYP116.

- **Class VIII**

Class VIII contains P-450 proteins which are fused to their eukaryotic like diflavin reductase partner cytochrome P-450 reductase (CPR) in a single polypeptide chain (Figure 8-I) and therefore are catalytically self-sufficient as monooxygenases. In diverse prokaryotes and lower eukaryotes, Cytochromes P-450 of this type has been discovered [127]. Soil bacterium *Bacillus megaterium* is the most intensively studied member of this class, which is hydroxylase type the cytosolic fatty acid hydroxylase flavocytochrome CYP102A1 (P450BM3, EC 1.14.14.1). P-450BM3 has been even so used a model of the mammalian hepatic P-450 enzymes due to its structure, which operate a similar electron transfer system using separate, membrane-embedded, P-450 and reductase enzymes [127]. Miura and Fulco [159] were first isolated the P-450BM3 and was the first P-450 discovered as a fusion (N-terminal) to its NADPH-cytochrome P-450 reductase (EC 1.6.2.4), which has been proved in subsequent investigations [91-93]. CYP102A1
is a fully soluble single polypeptide [93-94] with a molecular weight of 119 kDa [91]. It is composed of a heme-containing P-450 oxygenase domain, connected to a diflavin reductase domain containing cofactors FAD, FMN [91] and related to mammalian cytochrome P-450 reductase by sequence, structure, and function [92].

- **Class IX**

  Using NADH as electron donor, P-450_{nor} catalyzes independent of other electron transfer proteins (Figure 8-J). Class IX introduces up till now only the nitric oxide reductase (EC 1.7.99.7) as a special case of a cytochrome P-450. Among heme-thiolate proteins the imperfect filamentous fungus *Fusarium oxysporum* was identified as a P-450 with particular features as nitric oxide reductase (CYP55, P-450_{nor}). This enzyme localized in mitochondrial and cytosolic fractions of the cells represents the first and so far only soluble eukaryotic P-450 protein [95-96].

- **Class X**

  Monooxygenases when functions as P-450s require consecutive delivery of two electrons via different types of redox proteins, some P-450s catalyze substrate conversion using an independent intramolecular transfer system (Fig. 7-K). This class X of P-450 systems spans the independent cytochromes P-450 allene oxide synthase (EC 4.2.1.92), fatty acid hydroperoxide lyase (4.2.1.92), divinyl ether synthase (4.2.1.92), prostacyclin synthase (EC 5.3.99.4), and thromboxane synthase (EC 5.3.99.5).

  During the past years the diverseness of electron transport chains in this concerning family of proteins has been identified

1.10  **Bacterial Cytochrome P-450s**

Bacterial cytochromes P-450s are often soluble enzymes and are involved in critical metabolic processes. Three examples that have contributed significantly to structural and mechanistic studies are listed here, but many different families exist. Cytochrome P-450_{cam} (CYP101) originally from *Pseudomonas putida* has been used as a model for many cytochromes P-450 and was the first cytochrome P-450, three-dimensional protein structure solved by X-ray crystallography. This enzyme is part of a camphor-hydroxylating catalytic cycle consisting of two electron transfer steps from putidaredoxin, a 2Fe-2S luster-containing protein cofactor. In bacteria, cytochromes P-450 vary qualitatively and quantitatively from one species to another.
and even in a given species under different growth conditions, as illustrated for cytochrome P-450 by Guengerich et al. [160].

Cytochrome P-450 eryF (CYP107A1), originally from the actinomycete bacterium *Saccharopolyspora erythraea* [161], is responsible for the biosynthesis of the antibiotic erythromycin by C6-hydroxylation of the macrolide 6-deoxyerythronolide B. Cytochrome P-450 BM3 (CYP102A1) from the soil bacterium *Bacillus megaterium* catalyzes the NADPH-dependent hydroxylation of several long-chain fatty acids at the ω–1 through ω–3 positions. Unlike almost every other known CYP (except CYP505A1, cytochrome P-450 foxy), it constitutes a natural fusion protein between the CYP domain and an electron donating cofactor. Thus, BM3 is potentially very useful in biotechnological applications [162-163].

Cytochrome P-450 119 (CYP119), isolated from the thermophilic archa *Sulfolobus acidocaldarius* [164], has been used in a variety of mechanistic studies [165]. Because thermophilic enzymes evolved to function at high temperatures, they tend to function more slowly at room temperature (if at all) and are therefore excellent mechanistic models.

Many microorganisms have expressed their genes to synthesize different enzymatic proteins for the degradation and detoxifying of toxic compounds using their own defense mechanisms by hydroxide or oxidize them, like (Cytochrome a, b, bs, C, P-450) in this presence of various cytochrome scientists have reported Cytochrome as has located in soluble fraction of the bacteria, like CYP-450 by R. G. Bartsch, 1968 [166], Kelly et al. 2003 [167], and Sawada 2004 [168]. Electron transport chain of *Streptomyces carbophilus* [169], *citrobacter braakii* [170], *Sulfolobusto kadaii* [171], *Methyllococcus capsulatus* [172], *Rhodococcus rhodocrous* [173], *bacillus megaterium* [174] and *Fusarium oxysporum* [175], have been also reported from neutrophilic bacteria.

Alkaliphiles are the extremophiles organisms that thrives in an extreme environment have adapted their physiological character towards alkaline pH and maintain internal pH by pumping hydrogen ions in and out at the exact rate. Davidson et al had attemptt to purify two soluble cytochromes from the alkalophile *Bacillus firmus* [179]. Lewis, R.J 1980: has mentioned that Alkaliphiles have much higher cytochrome contents than conventional bacteria and than their own non-alkalophilic mutant derivatives [177]. Davidson et al had attemptt to purify two soluble cytochromes from the alkalophile *Bacillus firmus* [178]. Yumoto et al 1991 and his grope have purified and characterized two membrane-bound c-type cytochromes from a facultative
alkalophilic *Bacillus* [179], the comparative studies of 10 facultative alkaliphilic bacteria, were studied by Isao Yumoto et al. 1997, and two obligate alkaliphiles having relatively high total cytochrome contents of type-b, type-c and a minimum from type-a [180].

Cytochrome P-450 enzymes can be expressed as recombinant proteins in many different systems including mammalian cell culture, yeast, baculovirus, *Escherichia coli* and *Salmonella typhimurium*. Bacterial expression systems seem to be a good quality alternative which provides high yield expression of protein with least procedural cost. Barnes et al, in 1991, first time obtained high level P450 expression in E. coli via the modification of the N-terminal c DNA sequence encoding N-terminus and an increase of AT-enriched codons usage of *Haemophilus influenza* Rd KW20 [181]. The complete P450 catalytic cycle in bacterial system was obtained by co expression of CYP and its redox partner, NADPH-cytochrome P-450 reductase [182]. Most bacterial systems for P-450 expression use vectors (like pCW) containing promoters derived from the inducible lacoperon (such as tac and trc) where isopropyl-β-D-thiogalactoside is used for the induction of protein expression [183]. Furthermore, other conditions can be varied with the aim of enhancing the expression of P450s [184]. Genetic promiscuity, rapid growth rate and ability to use wide range of chemicals as a source of carbon and energy permits them to degrade environmentally hazardous chemicals.

### 1.11 The role of cytochrome b₅

Cytochrome *b₅* is involved in many mammalian P4-50 reactions, particularly in steroid metabolism. It may donate the second electron to the oxyferrous P-450 during catalysis, but is known to act as a structural effectors of P-450 function as evidenced by the ability of apo (heme-free) *b₅* to stimulate activity of cytochrome P-450 3A4 [185]. Cytochrome *b₅* has allosteric roles but redox functions may also be operative and the precise role is probably dependent on the P-450 system involved. Binding of *b₅* frequently perturbs spin state equilibrium in favor of the high spin form.

Until recently, the interaction between cytochrome *b₅* and bacterial P-450s (P-450BM3 also binds *b₅*) was considered to reflect merely an evolutionary link between redox partner in prokaryotic and eukaryotic P-450s, since *b₅* was thought to be exclusively a eukaryotic redox protein. However, some recent studies indicate that bacteria may also encode cytochromes *b₅*,
indeed the first three-dimensional structure of a bacterial cytochrome \( b_3 \) has been reported [186]. Ferric cytochrome \( b_5 \) forms a complex with ferric cytochrome P-450 in the reconstituted system, causing a configurational change in the cytochrome P-450 indicative of low to high spin transition [187]. The magnitude of the spectral alteration varied for different forms of cytochrome P-450 [188]. The interaction between these two hemoproteins has been studied by a number of investigators. Substrates markedly increase the affinity between cytochrome P-450 and cytochrome \( b_5 \) and vice versa [188]. Substrate is also reported to increase the affinity of cytochrome P-450 for NADPH-cytochrome P-450 reductase [189].

### 1.12 Localization of CYP-450

CYP-450s in prokaryotes are soluble proteins. Class I P-450s require both an FAD- containing NAD(P)H-reductase and an iron-sulfur redoxin as electron donors. Prokaryotes classes require only an FAD/FMN-containing NADPH-P-450 reductase, which is fused to the P-450 protein. P-450s often confer on prokaryotes the ability to catabolize compounds used as carbon source or to detoxify xenobiotics. Other functions described for prokaryotic P-450 include fatty acid metabolism and biosynthesis of antibiotics.

Class I and class II P-450s from all organisms participate in the detoxification or sometimes the activation of xenobiotics. They have been shown to contribute carcinogenesis and are essential determinants of drug and pesticide metabolism, tolerance, selectivity and compatibility [24-28]. P-450s that actively metabolize xenobiotics often have their expression induced by exogenous chemicals [25, 28-30]. P-450 from class III is self-sufficient and does not require molecular oxygen or an external electron source.
Figure 9 - Secondary and tertiary structures of P-450 proteins [190]. (a) Topology diagram showing the secondary structure and arrangement of the secondary structural elements of a typical P-450 protein (CYP102) [191]. Blue boxes, α helices; groups of cream arrows outlined with dotted lines, β sheets; lines, coils and loops. The sizes of the elements are not in proportion to their length in the primary sequence. (b) A ribbon representation of the distal face of the folded CYP2C5 protein showing its putative association with the ER membrane (purple) [192].

There are usually around four β sheets and 13 α helices defining one domain that is predominantly β sheets and one that is predominantly α helices. The first domain is often associated with substrate recognition and the access channel, the second with the catalytic center. Adapted from [192] (b) A ribbon representation of the distal face of the folded CYP2C5 protein showing its putative association with the ER membrane (purple) 'Error! Bookmark not defined.' Helices and sheets are labeled as in (a). Heme is in orange, the substrate in yellow. The α domain is on top left, the β domain more closely associated with the membrane at bottom right. Epitopes not accessible for antibody binding when the protein is associated with the ER are shown in red (numbers give their position in the primary sequence). The transmembrane amino-terminal segment, removed for crystallization, and an additional 11 residues that are disordered in the crystal structure, are not shown. The 1st helix above the heme, close to the substrate-binding site.

The heme-binding loop is visible behind the heme protoporphyrin. The conserved Gln-X-X-Arg structure in the K helix is also at the back and so is not readily visible. The proximal (back) face of the protein is involved in redox partner recognition and electron transfer to the active site; protons flow into the active site from the distal face (front). The substrate access
channel is usually assumed to be located in close contact of the membrane between the F-G loop, the A helix, β strands 1-1 and 1-2. More pictures showing other aspects of the structure, including reductase and substrate-binding, can be viewed at [193, 194]. Another picture (a model) of membrane-bound P-450 including the transmembrane domain can be seen at [195-196].

1.13 Function of cytochrome P-450

Cytochrome P-450 plays an important role in the metabolism of endogenous chemicals and the detoxification of natural toxins, drugs and xenobiotics. The main function of CYP450 is the monooxygenase activity whereby it introduces one atom of oxygen into a substrate but there are others functions depending on the substrate such as (1) N-hydroxylation, (2) N-oxidation, (3) sulfoxidation, (4) oxidative de-aminations, (5) N-dealkylation (6) S-dealkylations, (7) O-dealkylations, (8) epoxidation, (9) peroxidation, (10) desulfuration, (11) dehalogenation (12) aliphatic and aromatic hydroxylation [197]. CYP-450 enzymes catalyze many steps of sterol biosynthesis, metabolism of fatty acids, sterols, and the vast majority of drugs and xenobiotics. Furthermore, they are responsible for the metabolism of xenobiotics to metabolites responsible for toxic or carcinogenic effects [198].

The typical function of cytochrome P-450 is the mono-oxygenation of various substrates by introducing oxygen into a wide variety of substrates and structural classes of compounds (Porter and Coon 1991) [199]. Although some parts of this mechanism are poorly understood, it is still accepted by most workers to date. The cytochrome P-450 system is responsible for biotransformation of drugs, environmental pollutants, pesticides and industrial chemicals [200], Fig. 8 which illustrates the functional roles of cytochrome P-450s in most living cell system.
The vast number of catalytic reactions is due to the diversity and overlapping specificities of CYP-450 enzymes. For example, CYP1, CYP2 and CYP3 are responsible for metabolism of exogenous compounds, whereas other families such as CYP4 are responsible mainly for endogenous metabolism with some exogenous substrates such as phthalate ester. CYP-450s from 13 mammalian gene families are involved in the metabolism of endogenous substrates that are physiologically important in the biosynthesis of thromboxanes (CYP5), prostacyclins (CYP8A), steroid hormones (CYP11, CYP17, CYP19, CYP21), bile acids (CYP7A, CYP8B, CYP27A), calcitriol (CYP27A and CYP27B) [202-204].

Fig 10. The functional roles of CYP-450s. The Figure from Simpson (Simpson 1997)
1.14 General cytochrome P-450s reactions:

Cytochrome P-450 enzymes catalyze thousands of different reactions. Although microsomal monooxygenase reactions are basically similar in the role played by molecular oxygen and in the supply of electrons are therefore classified on the basis of the overall chemical reaction catalyzed. Bacterial cytochrome P-450 generally catalyzes the oxidation, reduction, hydrolysis and conjugation reactions with various xenobiotic substrates such as dyes and drugs.

1. Aromatic Hydroxylation

Highly reactive intermediates of aromatic hydroxylations, such as arene oxides, can also be produced:

\[
\begin{align*}
&\text{Cl} \\
\text{Cl} &\text{OH}
\end{align*}
\]

2. Aliphatic hydroxylation

Simple aliphatic molecules such as n-butane, n-pentane, and n-hexane, as well as alicylic compounds such as cyclohexane are known to be oxidized to alcohols;

\[
\begin{align*}
\text{R-CH}_3 &\rightarrow \text{R-CH}_2\text{OH}
\end{align*}
\]

3. Dealkylation: O-, N- and S-Dealkylation

Probably the best known example of O-dealkylation is the demethylation of p-nitroanisole, N-dealkylation reaction in metabolism of drugs. S-dealkylation is believed to occur with a number of thioethers;

\[
\begin{align*}
\text{R-NH-CH}_3 &\rightarrow \text{(R-NH-CH}_2\text{O) \rightarrow R-NH}_2 + \text{HCHO} \\
\text{R-OCH}_3 &\rightarrow \text{(R-O-CH}_2\text{OH) \rightarrow R-OH + HCHO}
\end{align*}
\]
4. **N-Oxidation**

N-oxidation can occur in a number of ways, including hydroxylamine formation;

\[
(CH_3)_3N \rightarrow (CH_3)_3N-OH^+ \rightarrow (CH_3)_3NO + H^+ 
\]

5. **Oxidative deamination**

Reaction indicates that, it is probably not an attack on the nitrogen but rather on the adjacent carbon atom, giving rise amine, which eliminates ammonia, producing a ketone;

\[
R_2CH-NH_2 \rightarrow R_2C-(OH)NH_2 \rightarrow R_2C=O + NH_3 
\]

6. **S-Oxidation**

Thioethers oxidized by monooxygenases to sulfoxides and further oxidized to sulfones;

\[
R-S-R' \rightarrow (R-SOH-R') + R-SO-R' + H^+ 
\]

7. **Epoxidation**

Epoxidation is an extremely important microsomal reaction because not only stable and persistent. Epoxides are formed but highly reactive areneoxides produced;

![Naphthalene Epoxide](image)

8. **Desulfuration**

The phosphorothionates \([(R_{10})_2P(S)OR_2]\), owe their insecticidal activity and their mammalian toxicity to an oxidative reaction, the P=S group is converted to P=O;

\[
(OC_2H_5)_2P-O-\text{NO}_2 \rightarrow (OC_2H_5)_2P-O-\text{NO}_2 + [S] 
\]
Chapter I

Introduction

Reductive type of metabolism

A number of functional groups, such as nitro, diazo, carbonyl, disulfide sulfoxide, alkene, pentavalent arsenic are susceptible to reduction.

1. Azo reduction

Requirements for azo reduction are similar to those for nitro reduction, namely anaerobic conditions and NADPH;

\[
R_N N_R \rightarrow R_NH_2 + R_NH \]

2. Nitro reduction

Convincing evidence has been presented that this reaction sequence is catalyzed by nitroreductase:

\[
\text{Nitroreductase} \quad \text{NADH} \rightarrow \text{NAD}^+ \\
\text{2-nitrophenol} \rightarrow \text{2-aminophenol}
\]

3. Aldehyde and Ketone reduction

In addition to the reduction of aldehyde and ketone through the reverse reaction, a family of aldehyde reductases also reduces these compounds:

\[
\text{Cl-CHO} \rightarrow \text{Cl-CH}_2\text{OH}
\]

Among CYPs there are isomerases, reductases, and dehydrases and in addition to oxidation, they contribute to various other reactions listed in Table-1 (Guengerich [205], Danielson [206], and Bernhardt [207]). The reactions mainly lead to the conversion of harmful xenobiotics less toxic by increasing their solubility, which is the first step in preparation of excretion (Denisov et al 2005) [208]. However, exceptions can be found among the listed reactions.
1.15 Modulation of mixed function oxidase system

Miller et al. [209] and Brown et al. [210] reported the inductive effects of foreign compounds on liver microsomal enzymes and were further examined in more detail by Remmer et al. [211]. Then many other investigators researched phenomenon thoroughly [212-214] and found its result with increased biosynthesis of microsomal xenobiotic metabolizing enzymes, which was termed as induction. Cytochrome P-450 consists of several forms, which possesses different specificities for a given substrate [215-216]. The different forms of cytochrome P-450 are induced by different inducers [217-218]. The alteration of any specific forms of cytochrome P-450 may thus be responsible for the metabolism of certain substrate. The mechanism underlying the inductive effect of different xenobiotics may vary from each other and this forms the basis of classification of enzyme inducers. The differences in the type of inducers are with respect to their carbon monoxide different spectrum, ethyl isocyanide differences spectrum, substrate specificity and genetic control of cytochrome P-450 induction.

Phenobarbital, a broad spectrum inducer, causes increase in electron transport components like cytochrome P-450, NADH cytochrome-c-reductase, increase in cytochrome P450 substrate complex, its rate of reduction and other enzymes involved in drug metabolism [219-220]. Polycyclic hydrocarbons, such as 3-methyl cholanthrene and a narrow spectrum inducer, induce the formation of another variety of cytochrome P-450 called cytochrome P-448. It has different affinities for various substrates than the usual form D.D.T., polychlorinated biphenyls, halogenated hydrocarbons and organophosphates are potent stimulators of microsomal enzymes [221-222]. Various environmental factors alter the activities of the cytochrome P-450 [223]. Some substances inhibit the metabolism of the chemical by combining reversibly with cytochrome P-450 [224]. Some inhibitors, such as piperonyl butoxide are converted to metabolites that apparently have a higher affinity for the cytochrome P-450 than does the parent compound [225]. Some chemicals cause the destruction of cytochrome P-450 e.g. carbon tetrachloride causes the destruction of cytochrome P-450 [226], presumably by causing the peroxidation of microsomal lipids. Some salts, like nickel chloride and cobalt chloride inhibit ALA-synthetase [227-229], which are the rates of limiting enzyme in the heme biosynthesis.
They induce the heme oxygenase, which is the heme-degrading enzyme. As a result, the rate of enzymatic degradation of cytochrome P-450 is decreased [230]. The allylic compounds, such as ally isopropyl acetamide, cause the destruction of cytochrome P-450 by an unknown mechanism [231].

1.16 Mechanisms of induction of cytochromes P-450

Most cytochrome P-450 systems are inducible and many excellent reviews have covered and elaborated this subject profoundly [232-234]. Many other eukaryotic and prokaryotic P-450 systems are induced by essentially unknown mechanisms [235-236]. A less graceful but more universally applicable classification scheme involves categorizing induction by the chemical (or biochemical) nature of the inducer (i.e. polycyclic aromatics, phenobarbital, ethanol, PCN-glucocorticoids etc.) a system used to advantage by Okey et, al., in his recent review emphasizing mammalian liver P-450s [237]. This method is too specific and found to be useful in categorizing bacterial systems.

Three types of induction and classification scheme have been developed on that basis among P-450 producing bacteria, which include (a) substrate induction, (b) nonsubstrate induction by exogenous or endogenous substances and (c) induction by environmental factors. The substrate induction includes true substrates and substrate analogs, i.e. substances that not metabolized by the enzyme. Analogs mimic a substrate by interacting with the enzyme at the substrate-binding site and like a true substrate, cause induction presumably by the same mechanism. In bacteria, these substrate-type inducers are usually exogenous (i.e. "Xenobiotic") but may be endogenous and, by definition, do not include oxygen. The second category involves induction mediated by a substance (again, excluding oxygen) that is neither a substrate nor a substrate analog and may be a xenobiotic or a cell product. The final category includes factors such as temperature, pressure, light, oxygen tension, pH and the depletion of carbon sources leading to a release of catabolite repression.
1.17  **Inducible cytochrome P-450 system involved in oxidation.**

Several bacterial systems which are specific for initiating the oxidation of various hydrocarbons and in each case they are substrate induced due to xenobiotics, which include P-450\textsubscript{oct} from a species of *Corynebacterium*, now classified as *Rhodococcus rhodochrous* ATTC19067 [54, 237-238]. P-450\textsubscript{oct} was obtained as a particulate preparation by ammonium sulfate fractionation of a cell-free extract from *R. rhodochrous*. In combination with NADH and a soluble fraction containing a flavoprotein, it catalyzed the conversion of n-octane to l-octanol [238]. P-450\textsubscript{oct} as measured by its reduced CO difference spectrum, was induced about eightfold when grown in a medium containing octane as a sole carbon source [239]. The P-450\textsubscript{oct} system merits further investigation, especially since the crude system appears to resemble the microsomal P-450 monooxygenases more than the soluble, three-protein component typical of many bacteria. Cytochrome P-450\textsubscript{non} from *A. calcoaceticus*, which hydroxylates and is induced by alkanes from C\textsubscript{6} to C\textsubscript{16}, is more characteristic of most bacterial or mitochondrial P-450s. Catalytic activity requires three proteins including the P-450 component, which was soluble in the presence of Triton X-100 but denatured when the detergent was removed [239], an iron-sulfur protein (a ferredoxin) and an NADH-specific ferredoxin reductase [240].

As with many bacterial systems that oxidize hydrocarbons, the enzymes for the p-cymene pathway are apparently encoded on transmissible plasmids [241]. P-450\textsubscript{cyc} was isolated as a NADPH-dependent cyclohexane hydroxylases activity from the specie of *Xanthobacter* that had been grown on cyclohexane as a sole carbon source; only alicyclic hydrocarbons closely related to cyclohexane supported growth [242]. Sariaslani [54] reported a personal communication from one of the discoverers that the P-450\textsubscript{cyc} system contains three proteins to convert cyclohexane to cyclohexanol.

Another generally recognized difference between prokaryotic and eukaryotic P-450s are pertaining to function; most known bacterial P-450-dependent systems initiate the oxidation of recalcitrant carbon compounds so that the hosts can utilize them as sole carbon sources for growth. Some lower eukaryotes certain yeasts [243], also employ P450-dependent systems in
this way but, among most fungi as well as in higher eukaryotes, cytochrome P-450s are involved in specific pathways of sterol or other lipid synthesis or, as in the mammalian liver microsomal systems, in detoxification reactions. This apparent general difference in function is partly artifactual, however, since many of the bacterial systems were discovered by enrichment techniques that depending on the ability of an organism to use a component of the growth medium as a carbon source [54] \{Error! Bookmark not defined.\}. Finally, bacterial P-450s may be sorted and categorized based on the type of induction. Using the three broad categories of induction set forth in this review, most bacteria as well as the preponderance of eukaryotic P-450s can apparently be classified as substrate inducible [233]. Nevertheless, the enormous differences in the levels of organization between eukaryotic and prokaryotic cells e.g., the extremely complex receptor-mediated system that regulates the substrate-induction of microsomal P-450s involved in the oxygenation of aromatic hydrocarbons [234-244] cannot exist in prokaryotic cells that lack endoplasmic reticulum and a nucleus; substrate induction of P-450s in bacteria must be a fundamentally different process.

1.18 Mechanism of camphor hydroxylation by electron transport system CYP-450\textsubscript{cam}

Electron flow in cytochrome P-450\textsubscript{cam} system occurs as follows: NADH reduces FAD component of NADH putidaredoxin reductase to FADH\textsubscript{2}, which reduces the Fe cluster of putidaredoxin, which shuttles electrons from NADH putidaredoxin reductase to the P-450 component. The electron transfer proteins are all soluble and do not appear to be membrane associated. According to the Protein Data Bank [245], monooxygenase has sequence similarity, or family, is known as FAD binding protein. The monooxygenase protein is composed of approximately 398 amino acid residues, splitting into 4 identical chains, these chains consist of both α-helix, 12 helices characterized by 123 residues and β sheet, 18 pleated sheets characterized between 94-96 residues [246].
Fig. 11. Mechanism of camphor hydroxylation by the P450\textsubscript{cam} monooxygenase system incorporating the enzymatic NADH-regeneration system.

1.19 Alkaliphilic bacteria:

Alkaliphiles have been thriving everywhere on our planet, only a few microbiologists have shown interest in this microbial domain. These organisms are grown optimally under alkaline conditions, typically exhibiting one or more growth optima within the pH range 8–11 and which typically grows slowly or not below pH 7 (cf. ACIDOPHILE which grown at high acidic pH). Alkaliphiles include a range of bacteria e.g. certain Bacillus sp. (including B.alcalophilus, B.firmus and B.pasteurii), Ectothiorhodospira abdelmalekii, Exiguobacterium aurantiacum, species of Natronobacterium, Natronococcus, Thermomicrobium roseum, certain fungi and the organisms occur e.g. in natural alkaline lakes and in water made alkaline by the effluents from certain industrial processes such as rayon manufacture [247].

Fig. 12. pH range 1–12 of bacterial growth.
Chapter I

Natural alkaline environments are characterized by high concentrations of free or complexed \( \text{Na}_2\text{CO}_3 \) and usually by high concentrations of \( \text{NaCl} \). A number of alkaliphiles have an obligate requirement for \( \text{Na}^+ \) an ion important e.g. in SYMPORT processes; in at least some *flagellated* alkaliphiles, flagellar rotation is driven by SODIUM MOTIVE FORCE. However, in some species capable of growth at neutral pH, \( \text{Na}^+ \) is required only under non-alkaline conditions. Fig (13) shows the cell wall of alkaliphilic bacteria [248].

![Figure 13](image)

**Fig. 13.** The of obligate alkalophilic bacteria respiration requirement for \( \text{Na}^+ \) and \( \text{H}^+ \) ion.

The first alkaliphiles bacteria from the alkaline crater lake of Lonar in Maharashtra state, India reported was *Burkholderia cepacia* MCMB-821 which was isolated from the sediment soil and grown at pH 9 in the presence of 2% salt and lactose as the electron donor in 2006 [249]. Alkaliphilic bacteria need extra energy in order to maintain the intracellular pH at near neutral values [250]. Those bacteria have not been fully studied yet, but in view of their extreme environment, they are expected to possess an even more active respiratory machine than neutrophilic bacteria. The later representatives of Alkaliphilic, aerobic, chemo-litho-autotrophic sulfur bacteria have recently been discovered in Lonar lakes of Buldana district, Maharashtra state, India, that have cytochrome P-450_{cam} content up to 2 n mol/mg of in 24 h under aerobic conditions [251]. Others strain are found to be obligate alkaliphiles, these microorganisms utilize a limited number of reduced sulfur compounds as electron donors while growing optimally at pH 10.0 to 12.0, but cannot grow at neutral pH (6.7- 7.4).
Microorganisms that occupy extreme pH environments have resulted in the definition of an unusual group termed alkaliphiles. Alkaliphiles grow optimally at pH 9.0 and above, with extreme alkaliphiles growing optimally above pH 10.0. Obligate alkaliphiles are incapable of growing at near neutral pH values. Facultative alkaliphiles exhibit significant growth at near neutral pH but also exhibit deficits in growth yield or growth rates relative to neutrophilic bacteria at same pH [252-254]. Therefore, the term “alkaliphile” is used for microorganisms that grow optimally or very well at pH 9.0 or above.

1.19.1 Distribution and types of Alkaliphiles

Alkaliphiles are mainly of two physiological groups of microorganisms i.e. alkaliphiles and haloalkaliphiles. Alkaliphiles require an alkaline pH of 9.0 or more for their growth and have an optimal growth pH of around 10, whereas haloalkaliphiles require both an alkaline pH 9.0 and high salinity [up to 33% (wt/vol) NaCl] [255]. Alkaliphiles classified into two main groups, alkaliphiles (also called alkalophiles) and alkalitolerants. The term alkaliphiles (alkali from Arabic; soda ash and phile; loving) is generally restricted to those microorganisms that actually require alkaline media for growth. Bacteria are categorized as alkaliphilic when optimal growth takes place at pH 9.0 or greater. Alkaliphiles can be further categorized as obligate, if pH 8.5 to 9.0 is absolutely required for growth, or as facultative, if growth can occur near neutral pH [256]. Alkaliphilic bacteria are of heterogeneous group, from eubacteria, such as Bacillus sp. to archaeabacteria, and Natronobacterium sp. Most of the alkaliphilic organisms are aerobic or facultatively anaerobic, few are strictly anaerobic & among aerobic organisms only a 1-10% occurrence of isolating alkaliphiles.

1.19.2 Alkaliphilic bacteria cytochrome

Adaptation mechanisms of microorganisms to alkaline environment have been studied over the last three decades in many laboratories all over the world. In spite of considerable contribution of scientists to the study of alkalophile, the energy coupling principles are not yet clear in a variety of the alkalophilic bacterial representatives [257]. One of the common features
Chapter I  

Introduction

of alkalitolerants and alkalophilic bacteria is a multiple magnification of cytochrome content in membranes as compared with neutrophilic bacteria. Bacillus firmus RAB [258], a c-type cytochrome, numerous cytochrome b species, and a terminal oxidase of the cytochrome aa3 type have been detected. Although one of the minor b-type cytochromes might be a cytochrome o [259]. Obligately alkalophilic bacteria, which grow optimally at pH values of 10 or higher, are confronted by a special set of bioenergetic problems and demand with respect to pH homeostasis and ATP synthesis [259-260].

Fig. 14. Full view of Lonar lake boldana distic, M.S, India

1.19.3 Alkaline Crater Lake of Lonar:

Alkaline Lonar Lake in India is one of the unique ecosystem and wonder on the earth being formed by meteorite impact on basaltic rock. It is situated in village Lonar in the Buldana district of Maharashtra state, India (Latitute 19° 58”, Longitude 76° 36”) in formerly volcanic Deccan-trap geological region (Fig. 14). It is an almost circular depression with its longest and shortest diameters being 1875m, respectively with a raised rim of about 30 m and depth of 135m [261]. Based on geological studies, it is postulated that the lake was originated as a meteorite impact crater about 50 to 60 thousand years ago.

Lonar Lake generated due to the meteor impact on basaltic rock and this is unique on the earth. The water is alkaline with pH of 11-11.5; this high alkalinity is due to the high concentration of sodium carbonate [262] and has been exploited for manufacture of soda since fairly ancient days. Review of literature reveals that the alkalinity of the lake water in terms of carbonate was 7.52% while salinity was 30.87% in 1910-1960 [263]. The salinity of lake is now
lowered down to 7.9% (Malu et al. 2000) [264], the literature survey indicates that most of the analyses of lake have been limnological rather than microbiological. Single report on bioremediated of phenol by alkaliphilic bacteria isolated from Lonar lake sediment [265]. Primary accounts of bacterial diversity of Lonar lake ecosystem which include some of the biochemical identification of some isolate have been recorded by [266].

1.20 Aims of the thesis

Our laboratory has Purified one novel Alkaliphilic and two obligate Alkaliphiles bacterial cytochrome P-450\textsubscript{cam}, known as CYP-450\textsubscript{cam} or CYP-450\textsubscript{alks}. However, nothing is known of the properties and functionality of this CYP-450 from alkaliphiles bacteria. In order to address this problem, this thesis will aim to delineate the effect of different inducer and other parameter effects on expressed CYTP-450\textsubscript{alks} proteins. As a first step to addressing this, specific inducer (camphor) was added to culture broth media, (well studied environmental media in vitro) to force the alkaliphilic bacterial to express their monooxygenase mechanisms enzymes, such as Cytochrome C, b, and CYTP-450 monooxygenase, which will act and hydroxide this chemical and using it as carbon source for their growth sustain. Microorganisms are essentially ubiquitous and having potential to assimilate wide variety of chemicals. Genetic promiscuity, rapid growth rate and ability to use wide range of chemicals as a source of carbon and energy, permits them to degrade environmentally hazardous chemicals.

Microbial cytochrome P-450\textsubscript{cam} monooxygenase system plays an important role in oxidizing and detoxification of foreign substances of many toxic compounds. The prerequisite for complete mineralization of camphor, and other compounds are the combination of reductive and oxidative process. The involvement of cytochrome P450\textsubscript{cam} in the bioremediation of chemical hazards is well established. Several of the investigators have isolated and characterized Cytochrome P450\textsubscript{cam} from neutrophiles. No data is available on the cytochrome P-450\textsubscript{cam} monooxygenase from alkaliphiles. Therefore, the present study was planned to:

1. To isolate and identify the bacterial strain having the camphor, \(\alpha\)-naphthol, \(\beta\)-naphthol, aminopyrine, acetanilide and aniline degradation potential from alkaline pristine lake of Lonar, (MS) Buldana, India.
2. To isolate and identify the neutrophilic bacterial strain, from textile finishing industrial waste disposal site, Ichalkaranji, India.

3. Isolation, purification and characterization of cytochrome P-450\textsubscript{cam} monooxygenase system from alkaliphilic bacterial strain *Bacillus badius D1*, *Kocuria sp. DL* and *Aquiflexum sp. DL6*.

4. Isolation, purification and characterization of cytochrome P-450\textsubscript{cam} from neutrophilic bacterial strain *Lysinibacillus sphaericus DL8*.

5. In vitro oxidation of camphor, $\alpha$-naphthol, $\beta$-naphthol, aminopyrine, acetanilide and aniline compounds by purified cytochrome P-450\textsubscript{cam} monooxygenase.

6. To compare the degradation potential of alkaliphilic bacteria with the neutrophilic bacteria.