STUDIES ON CYTOCHROME P-450 DEPENDENT DETOXIFICATION OF XENOBIOTICS IN BACTERIA

A THESIS

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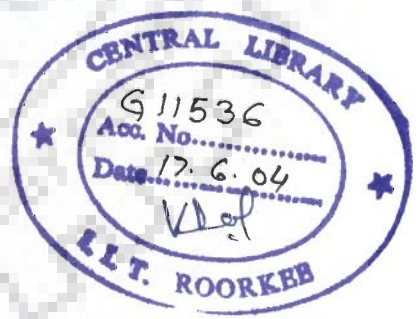
By

KAVITA SHAKTI SWAROOP RATTAN



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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"STUDIES ON CYTOCHROME P-450 DEPENDENT DETOXIFICATION OF XENOBIOTICS IN BACTERIA"** in fulfillment of the requirement for the award of the Degree of **Doctor of Philosophy** and submitted in the **Department of Biosciences and Biotechnology, Indian Institute of Technology - Roorkee** is an authentic record of my own work carried out during a period from January 1996 to January 2002 under the supervision of **Dr. P. Khanna & Dr. Rishi Shanker,** National Environmental Engineering Research Institute, Nagpur and **Dr. B.M.J. Pereira,** Department of Biosciences and Biotechnology, Indian Institute of Technology - Roorkee.

The matter presented in the thesis has not been submitted by me for the award of any other degree of this or any other University/Institute.

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Dated: Jan 21, 2002 **(Ms. Kavita Shakti Swaroop Rattan)**

This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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Abstract

Bioremediation has emerged as a potential tool for the conversion of hazardous organic chemicals and wastes into innocuous products. The treatment and disposal of hazardous wastes containing recalcitrant toxic compounds is not feasible based on our current knowledge of conventional waste treatment process engineering. Hence, cost-effective remediation of recalcitrant compounds in the environment requires novel bioremediation alternatives. Since many contaminated sites contain more than one pollutant it is often difficult for natural microorganisms to degrade, efficiently and simultaneously, a mixture of pollutants. Indigenous microorganisms when exposed to a mixture of pollutants may produce toxic intermediates that do not allow an overall reduction of toxicity of biotreated sample to a significant extent. In many cases, natural microorganisms have not evolved the genetic competence to mineralise certain synthetic compounds. The activity of natural pure cultures is often contaminant specific and is less applicable to complex mixtures of pollutants encountered in environmental situations. For this reason the search and isolation of naturally occurring bacterial strains, which rapidly metabolize recalcitrant pollutants, has been only partially successful. Genetically improved single cultures are considered more efficient than mixed cultures in degrading complex chemical wastes. Intense research effort has been directed at construction of bacteria with utility in bioremediation of hazardous wastes. Such genetically engineered organisms can be conferred an enhanced degradative capability for biotreatment of recalcitrant chemicals that confound natural degradation. Improved biocatalysts

possessing the desired catabolic or detoxification potential in environmentally robust hosts can be applied for the remediation of contaminated soils and sediments. The use of 'microbial inocula' is an attractive proposition provided that it could be cost-effective, with survival of organisms and rejuvenation of starting inocula.

The metabolic potential of microorganisms cannot be harnessed universally to develop effective biotechnological processes for remediation of hazardous wastes as the evolution of enzymes with wider substrate range proceeds very slowly in nature. Hence, the most desirable enzymes systems in biodegradation would be those that are involved in transforming and activating a wide variety of compounds rather than being specific. The ability of cytochrome P-450 enzymes to metabolize an extraordinary spectrum of diverse substrates renders them suitable for detoxification of hazardous wastes. Bacterial cytochrome P-450 cam and human cytochrome P-450 2E1 with their wide substrate range represent ideal catalysts for application in bioremediation.

The present study explored the feasibility of expression of native and *N*-terminus modified human cytochrome P-450 2E1 in *E. coli* and the environmentally robust host, *Pseudomonas putida*. Further, the role of luciferase to serve as electron transfer partner for the expressed P-450 2E1 was investigated. The study also evaluated the *in vivo* catalytic activity of *Pseudomonas putida* co-expressing *N*-terminus modified human cytochrome P-450 2E1 and luciferase.

Low yields of the native human cytochrome P-450 2E1 were observed in *E. coli* and *Pseudomonas putida*, necessitating *N*-terminus modification of the

hemoprotein to attain enhanced expression in bacterial hosts. The *N*-terminus modified human cytochrome P-450 2E1 subcloned on broad host range vector, exhibited improved expression in *E. coli* and *Pseudomonas putida* co-expressing luciferase. Successful co-expression of *N*-terminus modified P-450 2E1 and luciferase was obtained in *Pseudomonas putida* under the optimal growth conditions. The possibility of photo-reduction of modified P-450 2E1 by luciferase was assessed in *in vitro* assays containing the hemoprotein and luciferase. The generation of the characteristic P-450 peak in the absence of the chemical reductant validated the ability of luciferase to reduce the P-450 2E1 hemoprotein. This reduction was dependent on the concentration of luciferase that was comparable to the chemical reductant, dithionite. Resting cultures of *Pseudomonas putida* co-expressing cytochrome P-450 2E1 and luciferase incubated with carbon tetrachloride exhibited nearly 30% conversion to chloroform, as determined by GC-MS analysis. The *in vivo* reductive metabolism of carbon tetrachloride demonstrates that luciferase serves as an alternate electron transfer partner for P-450 2E1.

The present study also determined the metabolic competence of engineered *Pseudomonas* under nutrient stress conditions, utilizing a strain of *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase as a model. This organism provides both the reductive detoxification potential of the hemoprotein and a mechanism of its reduction in the absence of 'normal' P-450 redox partners. The ability of the organism to survive and remain metabolically

competent under nutrient stress was evaluated in soil slurries containing the halogenated substrates, viz. hexachloroethane and γ-hexachlorocyclohexane.

Genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase transformed hexachloroethane to tetrachloroethylene 8-10 fold faster than the uninoculated slurries. In addition, the engineerd *Pseudomonas* also mediated nearly 65% dehalogenation of γ-hexachlorocyclohexane to γ-3,4,5,6-tetrachlorocyclohexene in soil slurries under subatmospheric conditions, as confirmed by GC-MS.

The present study examined the response of engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase under single and multiple nutrient stress. In addition, the recovery and stability of the dual engineered functions under oligotrophic conditions in soil microcosm and soil slurry was also evaluated. The study also explored the feasibility of utilizing clay minerals as immobilization matrices for the generation of active microbial inocula for bioremediation applications.

More than 74% of the cells of the engineered *Pseudomonas* were culturable after 7 days of multiple nutrient (C,N,P) starvation. Diagnostic CO-difference spectra and luminescence exhibited by the cells validated the stability of the dual engineered traits. The engineered organism could be revived after repeated desiccation and starvation using Luria-Bertani medium, benzoate or citrate as nutrients. Significant survival and recovery of viable and culturable cells after slow desiccation observed even after 99 days in soil slurry validate the robust survival attributes of the engineered organism. Prolonged survival with full

retention of the engineered traits and recovery of 10-15% cells as active catalysts of lyophilized clay-matrix bound *Pseudomonas putida* even after 600 days establishes the potential of clay-minerals as effective microbial carriers.

The results obtained in the present study indicate that GEMs can be designed with broad substrate range detoxification catalysts such as cytochrome P-450 for the development of remediation processes for hazaradous wastes. The results also confirm that alternate electron transfer partners such as luciferase, may be utilized for cytochrome P-450-dependent bioremediation strategies.

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List of Presentations and Publications

I. Publications

1. ***Rattan, K.***, Shanker, R., Khanna, P. and Atkins W.M. (1999). Stress Survival of a Genetically Engineered *Pseudomonas* in soil slurries: Cytochrome P-450*cam*-Catalysed Dehalogenation of Chlorinated Hydrocarbons. *Biotechnology Progress* (ACS and AIChE) 15: 958-962.

II. Publications in Proceedings of International/National Conferences

1. ***Rattan, K.***, Shanker, R., Khanna, P. and Atkins,W.M. (1997). Bioremediation of halogenated compounds : Stress survival of genetically engineered *Pseudomonas putida*. In Proceedings of *International Symposium: Environmental Biotechnology*, April 21-23, Oostende, Belgium, pp. 487- 490.
2. Shanker, R., ***Rattan, K***. and Khanna, P. (1996). Bioremediation of Contaminated Ecosystems-Applicability of Genetically Engineered Microorganisms. In Proceedings of *Association for Science Cooperation in Asia Workshop on Capacity Building in Environmental Biotechnology* (Eds.) P. Khanna, T. Chakrabarti and R. Shanker, January 12-15, ASCA, Manila and NEERI, Nagpur, pp. 177-193.
3. Shanker, R., Wachasunder, S.D., ***Rattan, K***. and Khanna, P. (1996). Transformation of halogenated environmental pollutants by a genetically engineered *Pseudomonas*: Identification of metabolites by GC-MS. In Proceedings of 7th National Symposium on Mass Spectrometry, Nov. 26-28, DRDE, Gwalior, pp. 623 - 625.

III. Presentations in International / National Conferences

1. Padmanabhan, P., ***Rattan, K.***, and Shanker, R. (1999). Cytochrome P-450 cam and 2E1: bacterial expression of complementary biocatalysts for bioremediation. In Proceedings of the 68th Annual Meeting of Society of Biological Chemists (India), Dec. 27-29, IISc. Bangalore, India.
2. ***Rattan, K.***, Shanker, R., and Khanna, P. (1997). GEMs can serve as models for determination of survival and efficacy of "Microbial Inocula". In 38th Annual Meeting of Association of Microbiologists of India, Dec. 12-14, New Delhi.
3. ***Rattan, K.***, Padmanabhan, P., Shanker, R. and Khanna, P. (1997). Bioremediation: Strategies for engineering eco-friendly bacteria. In Indo-Swiss Collaboration in Biotechnology Symposium on *Bacterial Genetics and Pathway Engineering*, June 5-6, NEERI, Nagpur.
4. Padmanabhan, P., Ghule, J., ***Rattan, K.***, Shanker, R. and Khanna, P. (1997). Bioremediation: Detection of bacterial genotypes in hydrocarbon contaminated soils. In Indo-Swiss Collaboration in Biotechnology Symposium on *Bacterial Genetics and Pathway Engineering*, June 5-6, NEERI, Nagpur.
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6. Shanker, R., ***Rattan, K.***, Atkins, W. M. and Khanna, P. (1996). Bioremediation: Cytochrome P450 catalysed dehalogenation in genetically engineered bacteria. In

IUMS Congress: 8th *International Congress of Bacteriology & Applied Microbiology,* August 18-23, Jerusalem.

IV. Patent Application Filed

1 Shanker, R., ***Rattan, K.*** and Khanna, P. (1998). A process for preparation of synergistic composition useful for biological remediation of hazardous chemical wastes (NF/41/98).

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Abbreviations

BHRV:	Broad Host Range Vector
bp:	base pair(s)
CFU:	Colony Forming Units
CO:	Carbon monoxide
DMSO:	Dimethyl sulfoxide
DTT:	Dithiothreitol
EDTA:	Ethylenediaminetetraacetic acid
GC:	Gas Chromatography
GC-MS:	Gas Chromatography-Mass Spectrometry
GEM:	Genetically Engineered Microorganism
h:	hour
HCE:	Hexachloroethane
HCH:	γ-hexachlorocyclohexane
IPTG:	Isopropyl thio-β-D-galactoside
Kb:	Kilobase pair(s)
kDa:	kilodalton
LB:	Luria-Bertani Medium
min:	minute
mM:	millimolar
μM:	micromolar
MOPS:	3-[N-morpholino]propanesulfonoc acid

nm:	nanometer
PAGE:	Polyacrylamide gel electrophoresis
PCR:	Polymerase Chain Reaction
PIPES:	Piperazine-N,N'-bis [2-ethane-sulfonic acid]
RLU:	Relative Light Units
SDS:	Sodium dodecyl sulfate
TAE:	Tris-acetate/EDTA electrophoresis buffer
TE:	Tris-EDTA buffer
TCE:	Trichloroethylene
v/v:	volume/volume
w/v:	weight/volume
w/w:	weight/weight

Chapter 1

Introduction

The escalation in industrial activities, especially the chemical industry in the last century has led to the release of massive quantities of recalcitrant chemicals in the environment. The need to remediate soils and groundwater contaminated with hazardous chemicals causing public health and ecological concerns is well-recognised in view of the persistence and toxicity due to bioaccumulation and biomagnification of such compounds in living systems. Many of these anthropogenic compounds have been implicated in exerting teratogenic, carcinogenic and/or mutagenic effects. Biotechnological processes are necessary for the remediation of polluted soils and groundwater since conventional physico-chemical treatment methods or incineration are expensive and inadequate for detoxifying hazardous chemicals and wastes.

The use of microorganisms for the treatment of hazardous wastes has been regarded as a primary means of mineralisation of environmental pollution. Bioremediation, an important tool to alleviate environmental contamination, harnesses the catalytic activities of microorganisms to achieve pollutant destruction. Bioremediation, provides a safe, efficient and less expensive method for the decontamination of recalcitrant compounds including halogenated hydrocarbons. Unfortunately, natural microorganisms indigenous to contaminated sites often have a limited ability to degrade pollutants that possess novel chemical structures. Moreover, utilizing natural evolutionary processes to develop organisms with novel degradative abilities can be an extremely slow and unpredictable process particularly when the acquisition of multiple catalytic activities is necessary for contaminant decay or when toxic metabolites are

generated during pollutant destruction. Hence, genetically engineered microorganisms for enhanced degradative capabilities and wider substrate specificities could provide an effective option for decontamination of hazardous chemicals and wastes. Desirable catabolic traits can be selected and incorporated into robust organisms known to survive in target environments in an effort to engineer 'designer biocatalysts' for bioremediation. Though the use of genetically engineered organisms is regulated, this does not preclude their use in contained systems, viz. pump and treat systems where there is no release of bacteria into the environment.

The knowledge of enzymes that are useful in bioremediation is as important as the comprehension of factors like soil pH, temperature, moisture and the bioavailability of contaminants at a site for effective bioremediation. Knowledge of new and broad range metabolic capabilities, enzyme catalytic mechanisms and gene structure can then be integrated to construct organisms for the destruction of complex mixtures of environmentally persistent toxins. The most useful enzyme systems for bioremediation would be those that can metabolize a wide range of xenobiotics. Cytochrome P-450 enzymes are the most versatile biological catalysts known that catalyse metabolism of a wide variety of compounds including steroids, drugs, procarcinogens, antioxidants, solvents, pesticides, hydrocarbons and natural products. Thus, cytochrome P-450 enzymes with their inherent ability to transform xenobiotics combined with their wide range of vastly different substrate specificities are a logical choice for bioremediation.

The most well characterized naturally occurring microbial P-450 is P-450 cam monooxygenase from camphor utilizing *Pseudomonas putida*. The P-450 cam genes encoding camphor metabolism are clustered on a 240 Kbp transmissible plasmid, the 'CAM' plasmid. Cytochrome P-450 cam is known to catalyse both oxidative and reductive chemistry of a plethora of substrates ranging from hexachloroethane to pyrene. For example, hexachloroethane has the same dissociation constant (K_D of 0.6 μM), as the physiological substrate, camphor.

Mammalian cytochrome P-450 enzymes in mitochondria or endoplasmic reticulum are membrane-bound hemoproteins involved in a variety of biological processes including the synthesis and catabolism of steroid hormones and the detoxification of drugs and toxins. The participation of microsomal cytochrome P-450 enzymes in the transformation of a wide range of environmental xenobiotics, including halogenated hydrocarbons, encourages their prima facie use in bioremedial processes. Ethanol-inducible human cytochrome P-450 2E1 metabolizes an array of xenobiotic compounds including halothanes, trichloroethylene and vinyl chloride, and can serve as an important catalyst in bioremediation. Studies on mammalian cytochrome P-450 enzymes have been facilitated by the development of heterologous expression systems. Bacterial expression systems have been employed for the expression of mammalian cytochrome P-450 enzymes including P-450 2E1, 1A2 and 3A4. However, the heterologous expression of unmodified mammalian cytochrome P-450 enzymes, including human P-450 2E1, in *E. coli* has proved to be extremely difficult. Different approaches to achieve enhanced P-450 expression in bacteria have

included deletion/modification of the *N*-terminus of the protein, expression as enzymatically active fusion proteins with NADPH-cytochrome P-450 reductase, co-expression with NADPH-cytochrome P-450 reductase and the generation of NH_2-terminal translational fusions with bacterial leader sequences (*pel* B and *omp* A). These attempts have resulted in spectrally active functional proteins. Interestingly, different strategies evaluated for expression of cytochrome P-450 enzymes in *E. coli* demonstrated nearly similar hemoprotein yields.

The bacterial expression systems are convenient and inexpensive for the expression of mammalian cytochrome P-450 enzymes. However, their use is constrained because bacteria not only lack the endoplasmic reticulum (needed for binding mammalian P-450 enzymes to the microsomal membrane) but also the natural electron transfer protein, NADPH-dependent cytochrome P-450 oxidoreductase. Hence, recreating a functional microbial system capable of catalysing P-450 mediated reactions necessitates the co-expression of mammalian P-450 enzymes and their reductases in bacteria. Various approaches to resolve this issue have included the co-expression of cytochrome P-450 enzymes with NADPH-cytochrome P-450 oxidoreductase, expression of fusion proteins composed of a P-450 domain and NADPH-cytochrome P-450 reductase domain, and the use of bacterial flavoproteins. However, few reports are available on the activity of human cytochrome P-450 enzymes or thier reductase fusion constructs in live bacterial cultures. Therefore, generation of catabolically active, heterologous monooxygenase systems in live robust bacteria remains a challenging research task.

The successful application of engineered organisms for bioremediation relies largely on the survival attributes of the laboratory engineered strains under the non-optimal conditions prevalent in the natural environment. A key factor in the successful application of 'microbial inocula' is the ability of inoculants to survive, express their catabolic potential, and compete with the indigenous organisms in target habitats. An understanding of the fitness of genetically engineered microorganisms is critical because the engineered extra-metabolic potential often reduces the physiological potential to build up resistance against stress conditions. Monitoring the physiological response and adaptation of bacterial populations on introduction even in controlled treatment systems require the use of selective markers to ensure efficient and sensitive detection amidst indigenous microflora. Luminescence based marker systems enable sensitive, convenient and highly selective detection of introduced organisms. Moreover, there is no background luminescence among indigenous soil microflora which could mask the detection of light emitted by the engineered organisms.

A prerequisite in the use of 'microbial inocula' for the detoxification of hazardous wastes is the preservation regime and shelf-life of the natural or engineered organisms. Options for increasing the survival of microbial inoculants include delivering it in suitable formulations. Immobilization of bacteria in a variety of matrices including alginate, polyacrylamide, polyurethane, *k*-carrageenan, vermiculite, bentonite and kaolinite has been demonstrated to be more advantageous than free cells in the biodegradation of toxic compounds. Immobilization of bacteria is also reported to confer protection from high levels

of toxic compounds normally lethal to free cells. Thus, immobilization using suitable matrices may be a potential means for achieving enhanced survival and activity of microbial inocula to be deployed as bioremediative agents.

With this background the following objectives were delineated to evaluate a strategy for the detoxification of halogenated hydrocarbons:

1. To examine the feasibility of subcloning and expression of native and *N*-terminus modified human cytochrome P-450 2E1 in *E. coli*, and an environmentally robust host, *Pseudomonas putida*
2. To study the role of luciferase as an electron transfer protein for the expressed cytochrome P-450 2E1
3. To investigate the *in vivo* catalytic activity of *N*-terminus modified human cytochrome P-450 2E1 in *Pseudomonas putida* co-expressing luciferase
4. To elucidate the detoxification potential of cytochrome P-450 cam, utilizing the previously described *Pseudomonas putida* construct co-expressing cytochrome P-450 cam and luciferase
5. To determine the metabolic capability of engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase incubated with halogenated hydrocarbons in soil slurries
6. To utilize the engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase as a model organism to evaluate tolerance to nutrient stress, desiccation, and stability of engineered traits in terms of catalytic activity

7. To examine the response of genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase under single and multiple nutrient stress
8. To investigate the recovery and stability of the dual engineered functions under oligotrophic conditions in soil microcosm and soil slurry in *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase
9. To evaluate the feasibility of using clay minerals as immobilization matrices for the generation of active microbial inocula

Chapter 2

Review of Literature

2.1 Pollution

Industrial revolution has led to an unprecedented exploitation of the natural resources. Enormous quantities of pollutants are continuously entering the aquatic, terrestrial and subsurface environments due to inefficiencies of industrial, agricultural and other activities (Pye *et al.,* 1983; Ballantyne *et al.,* 1993; Bollag *et al.,* 1994; Rand, 1995). The relatively sudden introduction of xenobiotic chemicals, or the massive relocation of natural materials to different environmental compartments, can often overwhelm the self-cleansing capacity of recipient ecosystems. Environmental pollution can harm the flora and fauna due to uptake and accumulation of toxic chemicals in the food chains leading to instances of serious health problems (Richardson, 1996).

Over the last four decades, six billion tonnes of hazardous wastes have been released into terrestrial environments (Anderson and Coats, 1998). Global pesticide usage reached US $ 27,825 million during 1994, an increase of 10.1% over the previous year (Baloch and Haseeb, 1996). The generation of huge quantities of such toxic wastes is not confined to the industrialized nations alone. Developing countries, like India, also contribute to the growing problems associated with toxic waste generation and disposal. In the state of Maharashtra alone, the total waste generation from 362 industries was computed at a million tonnes per year (Shanker *et al.,* 1998).

Recalcitrant environmental pollutants are anthropogenic compounds that are characterized by structures or substituents not commonly encountered in unpolluted natural environments that make them resistant to biotransformation

and biodegradation (Holliger *et al.,* 1988; Hardman, 1991; Bewley, 1992; Baker and Herson, 1994). Diverse and ever-increasing range of recalcitrant compounds are aliphatic halogenated hydrocarbons, polynuclear aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), chlorophenols, and chlorinated pesticides (Neilson *et al.*, 1985; Heitkamp *et al.*, 1988; van Schooten *et al.,* 1995).

Heavily halogenated hydrocarbons are one of the most prevalent classes of man-made recalcitrant environmental contaminants and are often encountered in subsurface environments (Ornstein, 1993). These pollutants have been identified as 'priority pollutants' by the US Environmental Protection Agency (Li and Wackett, 1993; Ornstein, 1993). Other pollutants listed in this category include pesticides, halogenated aliphatics and aromatics, nitro-aromatics, chloroaromatics, polychlorinated biphenyls, phthalate esters, polycyclic aromatic hydrocarbons and nitrosoamines. An EPA listing of 129 Priority pollutants contains 62 halogenated organic compounds, and 27 out of 65 compound classes on a list of toxic pollutants were halogenated hydrocarbons (Faison, 1992; Ornstein, 1993).

The halogenated organics are widely employed as solvents, herbicides, pesticides and degreasers, and additionally occur as by-products of industrial processes, for instance, chloroanilines or chlorolignins (Chaudhry and Chapalamadugu, 1991). Chlorinated ethenes such as vinyl chloride, trichloroethylene and tetrachloroethylene are frequent contaminants of drinking water resources (Love and Eilers, 1982; Ornstein, 1993). Persistent organic pollutants (PoPs) including chlorinated pesticides, like hexachlorocyclohexane, are also implicated in causing contamination of air, water and subsurface

environments (Peyton, 1984; Bachman *et al.*, 1988; Singh *et al.*, 2000). Though the use of such organochlorine pesticides has been banned in most countries, once introduced into the environment these persist for many years (Baloch and Haseeb, 1996). The presence of the halogenated compounds in drinking water aquifers is a cause for concern due to their toxicity and/or carcinogenicity (Jones, 1989). Many of these compounds are known or potential threats to human health (Shales *et al.*, 1989; Jain *et al.*, 1992; Bouwer *et al.*, 1994) and the environment, thereby necessitating stringent regulatory control.

Permissible limits in surface and groundwaters and in soil have been delineated by regulatory authorities worldwide for many of these contaminants. For instance, the maximum containment levels pertaining to drinking water have been specified by the US EPA for a variety of pollutants (Buonicore, 1995). These include the halogenated organic compounds including atrazine (0.003 mg/L), lindane (0.0002 mg/L), polychlorinated biphenyls (0.0005 mg/L) and tetrachloroethylene (0.005 mg/L). In accordance with the growing perception on the negative impacts of even trace levels of xenobiotic pollutants, regulations concerning the discharge of treated wastewater have been made more stringent by agencies like the US EPA (The Hazardous Waste Consultant, 1993). For example, the discharge standard for hexachlorobenzene in treated wastewaters has been reduced from the earlier level of 55 μg/L to 15 μg/L. Similarly, hexachloroethane, terachloroethylene and trichloroethylene concentrations in the treated effluent for discharge have been restricted to 54 μg/L, 22 μg/L and 21 μg/L, respectively (The Hazardous Waste Consultant, 1993). Guidelines for the management of hazardous

wastes have also been established in India. These guidelines encompass discharge norms for treated effluents of hazardous and toxic wastes. For instance, the discharge limit for benzene hexachloride and DDT in treated effluents has been restricted to 10 μg/L. Likewise, the concentration of lindane and endosulfan in treated wastewaters for discharge has been restricted to 100 μg/L (CPCB-India, 2000).

Many of the halogenated compounds are carcinogenic and/or mutagenic and tend to bioaccumulate. Hence, their presence in the environment poses a significant hazard to human health (Hutzinger and Veerkamp, 1981; Shales *et al.*, 1989; Chen and Mulchandani, 1998). Poorly metabolized pollutants such as many PCB congeners accumulate to highest tissue concentrations in vertebrates at the top of the food chains (van Schooten *et al.*, 1995). Human intake of toxic compounds such as hexachlorocyclohexane, is largely through food consumption (Baloch and Haseeb, 1996). Lindane, the gamma isomer of hexachlorocyclohexane, is toxic to animals, humans and aquatic species. Lindane has been shown to cause liver, lung, endocrine gland and certain other types of cancer in animals (Singh *et al.*, 2000). Acute toxic effects may include the death of animals, birds or fish and death or low growth rate in plants. Human ailments resulting from the intake of food material contaminated with pesticide residues include cancer, liver diseases and hypertension (Baloch and Haseeb, 1996). Thus, due to the acute toxicity perpetrated by recalcitrant compounds such as the halogenated hydrocarbons, their remediation has become a global priority (Atlas, 1995).

2.2 Conventional Techniques for Pollutant Mitigation

Conventional treatment approaches deployed to remove or destroy contaminants include physical, chemical and biological techniques. Physical processes of waste treatment include adsorption, air- or vapor-stripping, distillation, and floatation in addition to the thermal processes such as evaporation and incineration (Shailubhai, 1986). Chemical methods of waste treatment include hydrolysis, neutralization, oxidation-reduction, and solvent extraction amongst others (Faison, 1992; Bouwer *et al.*, 1994). However, these methods merely transfer the pollutant from one environmental medium to another.

Conventional biological treatment methods have relied on the degradative abilities of microorganisms for decades to achieve pollutant mitigation (Atlas and Pramer, 1990). Municipal and industrial wastewaters have been treated in bioreactors (as activated sludges), in waste ponds or lagoons, in fixed media reactors using trickling filters, or in fluidized bed reactors containing an active biofilm of microorganisms (Lewandowski *et al.*, 1988; Faison, 1992).

The conventional treatment techniques are in wide use and have been instrumental in achieving the required treatment norms. However, these methods possess certain limitations, which necessitate alternate waste treatment strategies (Mackay and Cherry, 1989). Many of the existing techniques simply dilute or sequester the contaminants or transfer them to another environmental medium, and thus fail to achieve complete pollutant destruction (Bouwer, 1992; Bouwer *et al.*, 1994). For instance, processes like air- or vapor-stripping result in only a phase transfer of pollutants (Ornstein, 1993). Similarly, processes like

incineration lead to the formation of toxic by-products like dioxins which are proven carcinogens and bioaccumulate in higher organisms. The utility of the aerobic biological treatment methods, such as the activated sludge process, is hampered due to the limitations associated with the treatment and disposal of biomass sludge. Disposal techniques for the sludge formed during the aerobic treatment process usually involve transfer to sludge-drying beds. However, this process may in-turn lead to groundwater contamination due to percolation into the vadose and subsurface zones. Energy-intensive treatment processes, like incineration, increase the operational cost of the conventional treatment techniques thereby reducing their applicability particularly in less-developed-countries like India. Additionally, conventional secondary treatment processes need to be supplemented with the tertiary treatment methods including activated carbon treatment to meet the regulatory requirements, which further leads to the burgeoning costs of these methods.

2.3 Bioremediation

Growing perception on the toxic effects of xenobiotic chemicals have resulted in highly stringent legislation and complex waste management practices (Piotrowski, 1991; Bewley, 1992). This no-win situation demands remedial technologies which are efficient, cost-effective, reduce health risks, and help in restoring natural resources (Bollag *et al.,* 1994). Complex regulatory requirements warrant the application of amelioration techniques that are environmentally benign and cost-effective in resolving growing and past environmental problems in developing nations with limited fiscal resources.

Microbial treatment of contaminated soils has been regarded as a primary means of ameliorating environmental pollution (Ghisalba, 1983). The removal of organic contaminants from waters and soils is almost always a consequence of microbial activity (Harayama and Timmis, 1992; Singleton, 1994). The microbial world is characterized by an incredible metabolic and physiological versatility that permits microorganisms to degrade an amazing spectrum of organic chemicals (Leisinger *et al.*, 1981; Chakrabarty, 1982; Rochkind *et al.*, 1987; Sayler, 1991). Since cost-effective engineering solutions yet not exist for dealing with disperse and recalcitrant pollutants in the deep subsurface, i.e. ground-water, soils and sediments, the biological degradation of toxic, xenobiotic compounds can be considered as a viable option (Ornstein, 1993). Biological remediation, as opposed to physical or chemical processes, is considered a safe, efficient and less expensive method of removing harmful pollutants (Morgan and Watkinson, 1989; Atlas and Pramer, 1990). Moreover, this approach may result in a permanent solution rather than transferring the substances from one phase to another, as often happens with the conventional 'clean-up' methods.

Bioremediation is the use of biological methods to remove or reduce the concentration of organic wastes at contaminated sites (Hicks and Caplan, 1993). Bioremediation harnesses the ability of microorganisms to reduce pollutant concentrations to levels that no longer represent a risk to human health and the environment (Gibson and Sayler, 1992; Thomas *et al.*, 1992; Atlas and Bartha, 1993; Hamer, 1993; Bouwer *et al.*, 1994).

The biological agents used to degrade or transform pollutants in bioremediation are most frequently microorganisms, commonly bacteria and fungi (Shanker *et al.*, 1985; 1990; 1991; 1998; Shanker and Bollag, 1990; Shanker and Robinson, 1991; Muller, 1992; Snyder, 1993). The natural capacities of microorganisms to degrade even complex chemical pollutants on a large scale makes bioremediation possible (Atlas and Pramer, 1990). Bioremediation can be applied to improve environmental quality as part of a balanced program that includes measures to enhance natural cleaning processess. Though current state of knowledge in bioremediation has its limitations, these should be obviated with research and development, as bioremediation is expected to have large-scale application for removing pollutants from contaminated soils and waters (Liu and Suflita, 1993).

Bioremediation has emerged as a cost-effective and environmentally-benign treatment option (Liu and Suflita, 1993). The advantages of bioremediation application for pollution mitigation include destruction of contaminants on-site, accelerated cleanup time, minimal disruption to operations, lower remediation costs, and a reduction of future liability (Hicks and Caplan, 1993). A principal factor responsible for the emergence of bioremediation as a potential tool for site clean-up is its capability for achieving significant cost reduction (Bollag *et al.,* 1994). Costs as low as $75 to $200 per cubic yard are reported, compared with conventional technology costs for incineration or secured land-filling of $200 to $800 per cubic yard (Gabriel, 1991). Bioremediation has

also been demonstrated to be atleast six times less expensive than incineration, and three times more economical than confinement (Alper, 1993).

There is a global market for bioremediation. Increasing interest of bioremediation companies on a world-wide scale is mainly attributed to the fact that this technology is economically feasible (Caplan, 1993). The US Environmental Protection Agency, recognizing the potential of bioremediation as a mitigation tool, has promoted the use of this technique through a number of demonstration sites and sponsored programs (EPA, 1993). Although, bioremediation accounted for only 10% in the waste treatment technologies utilized in the US Superfund Program, it was however listed second among the innovative technologies after vapor extraction (19%). *In-situ* bioremediation accounted for 4%, and *ex-situ* bioremediation the remaining 6%, of the novel options utilized for waste treatment (Olguin *et al.*, 2000). In India, bioremediation is fast becoming a feasible means to combat a wide variety of hazardous waste problems (Khanna *et al.*, 1995).

2.3.1 Bioremediation Categories

Bioremediation treatment technologies have been categorised on the basis of *in-situ* or *ex-situ* biodegradation (Blackburn and Hafker, 1993; Baker and Herson, 1994). *In-situ* techniques involve the enhancement of the biodegradation rate of organic contaminants within the affected soil and groundwater environments (Morgan and Watkinson, 1989a, 1989b; Compeau *et al.*, 1991; St. John and Sykes, 1998). *In-situ* bioremediation is particularly feasible at sites where excavation is not possible. The *ex-situ* techniques require the physical

removal of the contaminated material followed by treatment under contained conditions in bioreactors, compost heaps or lagoons (Blackburn and Hafker, 1993).

2.3.1.1. In-situ Bioremediation

In-situ bioremediation utilizes the metabolic versatility of indigenous microorganisms in achieving pollutant mitigation. The degradative capabilities of natural microbes may be enhanced by the provision of nutrients and electron acceptors (Morgan and Watkinson, 1989b; Liu and Suflita, 1993).

Both *in-situ* and *ex-situ* bioremediation techniques include the stimulation of indigenous microbial populations through nutrient amendment, addition of co-metabolic substrates, and inoculation with indigenously isolated organisms known to degrade target pollutants (Pardick *et al.*, 1992; Providenti *et al.*, 1993).

2.3.1.2 Ex-situ Bioremediation

Ex-situ bioremediation techniques are usually aerobic and involve the treatment of contaminated soils or sediments using solid or slurry-phase systems (Litchfield, 1991; Blackburn and Hafker, 1993). Slurry-phase bioremediation is a batch treatment technique wherein excavated soils or sediments are mixed with water and treated biochemically in reactor vessels or in contained ponds or lagoons (Blackburn and Hafker, 1993; Troy, 1994). In addition to the provision of adequate aeration and mixing, nutrients are universally added. Microbial inocula is also added to effect contaminant removal. Effective bioremediation has been performed using slurry-phase systems for soils and sediments contaminated with a

wide range of organic compounds including pesticides and polychlorinated biphenyls (Yare, 1991; Troy, 1994).

Bioremediation processes for waste treatment are also categorised as either 'forced' or 'programmed' (Shanker *et al.,* 1998). Forced bioremediation involves the remediation of pre-existing contaminated sites by enhancing desired microbial activity. Programmed bioremediation envisages the development of an appropriately designed site for the disposal of specific wastes. These processes involve the stimulation and maintenance of catabolically active indigenous bacterial populations either by nutrient amendment or catabolic inducers and substrates to enhance the activity of the indigenous or inoculated organisms (Shanker *et al.,* 1998).

2.3.2 Biostimulation

The stimulation of endogenous microbial populations by the amendment of nutrients and/or external electron acceptors is another option in bioremediation (Thomas *et al.*, 1992). Most commonly, stimulation is achieved by the establishment of optimal growth conditions, usually nitrogen or phosphorus concentrations, molecular oxygen, redox potential and moisture levels (Atlas, 1992). Many soils are nutrient-limited, and nitrogen and/or phosphate need to be supplied to ensure that the microorganisms are in an active metabolic state. The addition of nutrients to contaminated soils to stimulate microbial destruction of contaminants has been demonstrated for halogenated organic compounds (Morgan and Watkinson, 1989; Leahy and Colwell, 1990; Mercer *et al.*, 1990; Prince, 1992; Atlas and Bartha, 1992; Pritchard *et al.*, 1992).

2.3.3 Co-metabolism

Co-metabolism is defined as the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound (Dalton and Stirling, 1982). The process usually involves the transformation of hazardous chemical(s) as an indirect consequence of the metabolism of another chemical utilized as carbon and energy source (Alexander, 1984; Pritchard *et al.*, 1996). Co-metabolic transformation is an important mechanism in the transformation of chlorinated aliphatic and aromatic contaminants (Cohen and McCarty, 1991; Bouwer and Zehnder, 1993; Liu and Suflita, 1993). Co-metabolic transformation has been demonstrated both aerobically and anaerobically for trichloroethylene, 1,2-dichloroethylene, DDT and polychlorinated biphenyls (Quensen *et al.*, 1988; Little *et al.*, 1988; Bouwer and Zehnder, 1993). Polynuclear aromatic hydrocarbon biodegradation has been shown to be associated with co-metabolic reactions. A study evaluating PAH degradation by *Pseudomonas paucimoblis* utilizing flouranthene as a sole carbon and energy source has demonstrated the biotransformation of many other related compounds like naphthalene, biphenyl, etc. (Mueller *et al.*, 1990).

2.3.4 Bioaugmentation

Bioaugmentation is the process of introducing microorganisms (relative to the existing microflora) into a site (Pritchard *et al.*, 1996; Colleran, 1997). Microbial inoculants may be added in instances where a competent degradative population either does not exist or longer time-frames are required for the adaptation of the existing microflora (Bouwer and Zehnder, 1993; Liu and Suflita,

1993; Singleton, 1994). Bioaugmentation may involve the addition of natural isolates or genetically engineered microorganisms. The process requires the monitoring of the inoculated organisms to evaluate their survival and ability to significantly effect transformation of the target chemical (Pritchard *et al.*, 1996).

Bioaugmentation trials using natural isolates have met with varying degrees of success. For example, pentachlorophenol degradation in soil was reported to be enhanced by the inoculation of *Rhodococcus chlorophenolicus* (Middledorp *et al.*, 1990), *Flavobacterium* species (Crawford and Mohn, 1985) and *Arthrobacter* species (Edgehill and Finn, 1983). Similarly, transformation of polychlorinated biphenyls in soils was found to be enhanced by the inoculation of an *Acinetobacter* species (Brunner *et al.*, 1985) and by chlorobenzoate degrading *Pseudomonas* species (Hickey *et al.*, 1993).

2.3.5 Natural Organisms

Natural communities of microorganisms in various habitats have an amazing versatility to metabolise and often mineralise an enormous number of organic molecules. The degradation of natural organic substituents in the environment is primarily mediated by bacteria, actinomycetes and fungi (Shanker *et al.*, 1985, 1990, 1991; Shanker and Bollag, 1990; Shanker and Robinson, 1991; Muller, 1992; Snyder, 1993). Communities of bacteria and fungi also metabolise a multitude of synthetic chemicals. Among the microorganisms that are frequently identified as active members of microbial consortia effecting bioremediation are *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Flavobacterium*, *Pseudomonas*, *Xanthobacter* etc. (Cookson, 1995; Harwood and Parales, 1996). The genus

Pseudomonas constitutes a very important and versatile group of bacteria in the rhizosphere (Shanker *et al.,* 1990; Timmis *et al.,* 1994; Chattre *et al.,* 1996; Chugani *et al.*, 1997). The genus *Pseudomonas* with the demonstrated inherent robustness is an useful tool for application in bioremediation processes.

Successful bioremediation depends on the availability of appropriate microorganisms (Fewson, 1988). The effectiveness of bioremediation is often a function of the extent to which a microbial population or consortium can be enriched and maintained in an environment. Microorganisms survive in a habitat because they are metabolically able to exploit its resources and occupy a suitable niche. Contaminants are often potential energy sources for microorganisms, yet the resident microflora may be incapable of harnessing such compounds. Under such conditions, non-indigenous organisms able to use the pollutant chemical can establish themselves more readily.

Microorganisms have not evolved appropriate metabolic pathways for novel xenobiotic compounds. Certain xenobiotic compounds may be inefficiently transformed while no degradative routes may be known for some compounds. In addition, complex mixtures of contaminants prevent degradation by existing pathways (Pieper and Rieneke, 2000). In most waste treatment situations, the contaminated soils or waters contain more than one toxicant to be degraded. Complex waste mixtures present formidable challenges in the engineering of biological treatment systems (Logan *et al.*, 1993). These include the toxicity of one or more compounds to the biodegrading organism that can prevent degradation of other compounds. In addition, different pollutants with similar

chemical structures may compete for uptake and enzyme binding sites which profoundly affects the biodegradation of individual waste components (Logan *et al.,* 1993). Although microorganisms collectively may be almost infallible, the accumulation of highly toxic and recalcitrant pollutants in the environment underscores the fact that microbial infallibility is not adequate to protect the biosphere from the residues of industrial and agricultural activities. Furthermore, natural microorganisms require a nutritional boost to enhance the populations capable of detoxifying pollutants in a prescribed time frame (Aust *et al.,* 1994; Howard and Fox, 1994) for cost-effectivity.

The likelihood of developing novel or enhanced degradative abilities by genetic rearrangement or exchange is related to the frequency of gene replication and inter-organism interactions. However the high growth rates and dense microbial populations favoring such interactions are not usually encountered under field conditions. The exchange of genetic material between phylogenetically distant organisms, while sometimes possible, can represent a biological barrier to evolution. Harnessing natural evolutionary processes to develop organisms with novel degradative abilities can be an extremely slow and unpredictable process, particularly when the acquisition of multiple catalytic activities is necessary for contaminants decay, or when toxic metabolites are generated during pollutants destruction. Thus, the construction of suitable organisms by genetic manipulation presents a feasible alternative for environmental clean-up (Reineke, 1986; Rojo *et al.*, 1987; Lindow *et al.*, 1989).

In comparison to more conventional engineering remediation solutions, the application of "designer" microbes is relatively cost-effective (Ornstein, 1993).

2.3.6 Genetically Engineered Microorganisms

Genetically engineered microorganisms can be employed to overcome the limitations imposed by the use of indigenous bacteria (Sayler and Day, 1991; Lau and Lorenzo, 1999). For instance, the presence of complex waste mixtures requires the functioning of several pathways to achieve contaminants destruction. Apart from the lower efficiencies and hence catabolic rates anticipated when intermediates are cycled between different organisms in complex environmental matrices, released intermediates can be mis-routed by unproductive microorganisms into dead-end pathways, or even transformed into toxic products that destabilise the community and inhibit the biodegradative process. The application of recombinant organisms, able to mineralize pollutants through efficient metabolic routes, minimizes these problems (Timmis *et al.*, 1994).

It has been suggested that different functions could be engineered into a single species for the degradation of such complex compounds (Abramowicz, 1990; Sayler *et al.*, 1990). It has been shown that a single culture with the appropriate genetic competence to degrade a mixture of chemicals is more efficient than a mixture of cultures with equal genetic capability (Haugland *et al.*, 1990). A useful approach for bioremediation could also be the modification of metabolic activities of indigenous microorganisms and the creation of consortia (Colwell *et al.*, 1996). It has been observed that mixtures of toxic wastes discharged into waste streams may be best treated with mixtures of

microorganisms working together in a consortium (Colwell *et al.,* 1996). While single organisms have been demonstrated to effect the degradation of single or groups of compounds, usually a consortium of bacteria is required to mediate the degradation of a mixed waste stream. Under many circumstances, consortia have been shown to be more effective for the degradation of hazardous wastes (Dolfing and Tiedje, 1987; Genthner *et al.,* 1989).

2.3.6.1 Regulatory Scenario

Various regulatory constraints limit the release of genetically engineered microorganisms (GEMs) in the environment (Wilson and Lindow, 1993; Ryder, 1994; Ramos *et al.*, 1995; Wackett and Bruce; 2000). Though the release of GEMs is regulated, this does not preclude their use in contained systems like soil slurry reactors (Chaudhry and Chapalamadugu, 1991; Bewley, 1992). Laboratory-contained microcosms can serve an important function in the study of GEMs (Dwyer *et al.*, 1988). Regulatory standards strongly recommend research with recombinant bacteria within contained settings (Awong *et al.,* 1990).

Several factors need to be considered before the release of GEMs to the environment (Dwyer and Timmis, 1990). The potential of recombinant organisms to compete, increase and displace certain members of natural populations in the environment at the site of release or following transport may present unique regulatory problems (Cairns and Pratt, 1986). The survival and recombinant gene expression or transfer of the recombinant genes to other microorganisms provides insight into whether potential risks are associated with an intended or unintended environmental release of GEMs (Levin and Strauss, 1991; Orvos, 1992; Siedler,

1992; Smit *et al.,* 1992). Genetically engineered strains can also help to understand the fate of the released microorganisms in general, because their recombinant gene is a perfect target of detection against the high background of natural microorganisms (Vahjen *et al.,* 1997).

The number of cases of deliberate release of genetically engineered bacteria into the environment are relatively few (Halvorson *et al.*, 1985; Awong *et al.,* 1990; Wackett and Bruce, 2000). Field trials have been carried out involving genetically engineered strain of *Pseudomonas syringae*, the first to be approved for release into the environment, *Pseudomonas fluorescens* carrying a lac ZY marker gene, *Rhizobium meliloti* carrying a Tn5 marker gene, genetically engineered *Baculovirus* and genetically engineered *Pseudomonas putida* WCS358r (De Leij *et al.,* 1995; Scott-Angle *et al.,* 1995; Prosser *et al.,* 1996; Glandorf *et al.*, 2001). These field trials have revealed the behaviour of *Pseudomonas fluorescens* lac ZY strains and the presence of *Pseudomonas syringae* could be closely predicted by the results of experiments done in contained systems before release into environment.

Although none of the potential adverse effects of GEMs have been observed so far (Hirsch, 1988; US Congress Office of Technology Assessment, 1988; Drahos, 1992; Possee, 1992; Ryder, 1994), however a detailed understanding of the behaviour of GEMs in different representative ecosystems is necessary. Seven authorized GEM releases (Lindow *et al.,* 1989) have produced no unanticipated effects. Infact the recombinant organisms all declined in concentration after release. The use of engineered microorganisms to assist in the removal of anthropogenic environmental contaminants may thus represent a

viable alternative due to economic, regulatory and health reasons (Ornstein, 1993).

There has been only a single field release of a GEM for bioremediation purposes (Ford *et al.*, 1999). A genetically engineered strain of *Pseudomonas fluorescens* HK44 was released into a contained soil environment. The strain possessed the naphthalene catabolic plasmid, pUTK21, in addition to a transposon-based lux gene fused within a promoter for the naphthalene catabolic genes. Exposure of the engineered strain to naphthalene or the metabolic intermediate salicylate resulted in increased catabolic gene expression, naphthalene degradation and concomitant bioluminescence. The study demonstrated the survival of the engineered *Pseudomonas fluorescens* for a year.

2.3.6.2 Strategies for Engineering Biocatalysts

The restricted degradative ability of natural microbes for complex wastes warrant the design of improved biocatalysts by genetic manipulation (Lau and Lorenzo, 1999; Timmis and Pieper, 1999; Pieper and Reineke, 2000; Diaz and Prieto, 2000). Various approaches have been developed for the design of improved strains and enzymes. Desirable catabolic traits have been selected from different organisms in an effort to engineer 'designer biocatalysts' (Chen *et al.*, 1999). Robust organisms with known survival attributes on introduction into natural oligotrophic environments can be engineered with the desired catabolic potential (Pieper and Reineke, 2000).

The rate of degradation of environmental pollutants by natural microorganisms may not be optimal for bioremediation. In addition, entire

pathways for the complete degradation of some pollutants do not exist in natural microorganisms and may lead to the accumulation of toxic products. For example, most microorganisms able to degrade polychlorinated biphenyls (PCB) metabolise only one aromatic ring and accumulate the other as the corresponding chlorobenzoate. Even when a PCB degrading organism can mineralize chlorobenzoates, these compounds or their metabolic derivatives can inhibit PCB-degrading activity as observed with biphenyl induced oxygenase activities involved in chlorobenzoate and catechol transformation in *Pseudomonas testosteroni* B356 (Timmis *et al.,* 1994). In such instances, engineering the biodegradative pathway offers the potential of expanding the natural metabolic capacity (Johnston and Robinson, 1984; Sayler, 1991). The design of improved catalysts involves different approaches including the creation of novel metabolic pathways or the modification of existing ones (Brim *et al.*, 2000). Biodegradation reactions are multistep pathways controlled by multicomponent regulatory networks that co-ordinate pollutant degradation with other metabolic processes. Hence, strategies to improve catalyst performance need to consider the enzymatic or regulatory steps that are rate limiting under process conditions (Timmis *et al.,* 1994).

Design of superior biocatalysts may also involve the rational combination of catabolic segments from different organisms within one recipient strain (Haro and Lorenzo, 2001). Several degradative activities can be combined within a single host organism to aid the generation of complete metabolic routes for xenobiotics that are only co-metabolised. This would avoid the formation of dead-

end products or even toxic metabolites. If a single strain is constructed to perform several related or un-related metabolic activities, the efficiency and predictability of the process may be significantly enhanced. This strategy has been successfully applied for the degradation of highly toxic trihalopropanes, for which mineralization has not been described (Timmis *et al.*, 1994).

Expression of biocatalytic pathways in foreign microorganisms, with robust survival attributes, can also significantly enhance the efficiency of the biodegradation process (Chen *et al.*, 1999). The feasibility of this approach has been demonstrated in engineered *Pseudomonas* strains. The catabolic genes responsible for desulphurization in *Rhodococcus erythropolis*, were inserted into the chromosome of the *Pseudomonas* strains (Gallardo *et al.*, 1997). These recombinant microbes were demonstrated to desulfurise dibenzothiophene more efficiently than the native organism, *R. erythropolis* IGTS8.

Protein engineering can also be employed to improve the stability and substrate-specificity of enzymes useful for bioremediation purposes (Timmis and Pieper, 1999). The properties of native enzymes may be modified in an attempt to increase their substrate range and stability (Timmis *et al.*, 1994; Haro and Lorenzo, 2001). Rational design may be applied to enzymes capable of metabolizing a wide range of substrates. Such enzymes that perform well can be incorporated into robust hosts for environmental applications (Trombly *et al.*, 1995).

The environmental relevance of cytochrome P-450 enzymes is due to the wide range of reactions that it can perform and also the broad substrate

range of these enzymes. Cytochrome P-450 enzymes are instrumental in effecting the activation of hydrophobic molecules and rendering them amenable to further degradation by the incorporation of an oxygen atom. Cytochrome P-450 enzymes thus catalyze the initial steps in both the activation and the detoxification of xenobiotic compounds, such as the halogenated hydrocarbons (Raucy *et al.*, 1993).

In view of the natural diversity of cytochromes P-450s, it appears that the intentional redesign of additional cytochrome P-450 enzymes for incorporation into microorganisms for environmental bioremediation purposes could be a potentially acceptable risk, especially in cases where no other acceptable means exist for degrading known hazardous contaminants. Considering that the technology already exists for the heterologous expression of foreign proteins, it may be plausible to express specific bacterial and mammalian cytochrome P-450 enzymes in suitable microorganisms for the mineralization of toxic chemicals in the environment in contained systems, like soil slurry bioreactors.

2.3.7 Inoculant Survival

A key concern for the safety, efficacy and commercial potential of any environmental application of microbial inocula, is the ability of the organism to survive in target habitats. In applications, such as the bioremediation of contaminated soils, it may be desirable for introduced GEMs to be self-sustaining (Thompson *et al.*, 1995).

The potential use of bacteria with chromosomally encoded, or with natural or chimeric-plasmid encoded, catabolic pathways to augment or initiate *in situ*

biodegradation depends on the stability of the genetic information harbored and the successful survival of the introduced organisms under specific conditions (Ramos *et al.,* 1991). Such information is also crucial in providing safety assurances relative to the environmental risk of such applications. By modifying bacteria to express multiple unique phenotypes, it is possible to determine the fate of inocula reintroduced to its natural environment. Prospects for the routine release of genetically engineered organisms into the environment are becoming increasingly feasible. Information is therefore needed to properly evaluate and assess the potential impact of GEMs on representative ecosystems (Awong *et al.,* 1990; Fox, 1994). Numerous studies investigating bacterial survival in the natural environment have mainly targeted naturally occurring pathogens and other microorganisms of public health significance. Such studies have also been extended to include other species, but to date few reports have examined the fate of recombinant bacteria and their " novel" genes in natural ecosystems.

With the increasing amounts and varieties of pollutants that are being released into the environment, it is important to understand how genetically engineered strains behave under the added stress. In addition to having to deal with the toxicant, engineered organisms will also have to compete with indigenous organisms, including those that have already been adapted to the environment and are capable of degrading existing toxicants. Laboratory microcosm is an important tool for investigating, separately or simultaneously, the impact of several physical, chemical or biological factors on the fate of a genetically engineered organism (Siedler, 1992). The use of simple laboratory

microcosms can provide a useful means of evaluating the fate of bacteria introduced to soil (Thompson *et al.,* 1992).

The ability of bacteria to survive and proliferate in soil is an important factor in their success as inoculants for bioremediation (Young and Burns, 1993). In order to become established in the field, inoculants must co-exist with competitors and predators and maintain themselves during periods of low nutrient availability (Alexander, 1986). Many soil inoculants, shown to be beneficial in the laboratory experiments, fail when used in the field and this is probably due to a combination of physical, chemical and biological stresses encountered by the introduced species. It may prove more successful to isolate bacteria from the target soil and screen for beneficial species which can then be reintroduced in much larger numbers (Young *et al.,* 1995). Such bacteria are more likely to survive and express their properties because they are adapted to the recipient soil environment and should compete effectively with the indigenous microorganisms.

Natural conditions may present a number of problems for laboratory designed bacteria reintroduced into the environment. Low substrate concentrations may not support the growth of these specialized strains, or the introduced organisms may abandon metabolism of the contaminant in favour of other substrates (Goldstein *et al.,* 1985). Naturally-occurring toxins, predators and interspecific competition may all reduce the survival of the introduced strain (Liang *et al.,* 1982; Klein and Alexander, 1986). On the other hand, alternate carbon sources available in natural ecosystems may enhance the survival of

introduced strains and hence the degradation of the contaminants (Schmidt and Alexander, 1985; Ramos *et al.,* 1991).

The physiological responses to carbon, nitrogen or phosphorus starvation have been investigated in detail in *E. coli* but only limited information is available for *Pseudomonas fl*uorescens and *P. putida* (Kragelund and Nybroe, 1994). The bacterial response to carbon starvation has been studied under defined conditions in *in vitro* systems with *E. coli*, *Vibrio* species and *P. putida* (van Overbeek *et al.,* 1995). Upon starvation, *P. putida* cells survived extremely well in liquid medium, as measured by viable counts, which indicated that the bacterial adaptation to starvation conditions did not interfere with cellular culturability. There are reports that starved cells of *P. fluorescens* R2f also survived in high numbers in liquid medium without substantial loss of CFU numbers.

The survival and activity of non-indigenous bacteria released into the environment are governed by physical, chemical and biological parameters. To colonize a new environment, a released bacterial population must be able to withstand the stress induced by restricted nutrient availability, predation and suboptimal physical conditions (Leser *et al.,* 1995).

Soil moisture and temperature regimes, pH and texture as well as oxygen and nutrient availability have been suggested as the major abiotic factors that govern the survival of the introduced bacteria in the soil (Dupler and Baker, 1984; van Elsas *et al.,* 1986; Davies and Whitbread, 1989). Abiotic factors greatly alter the survival of a bacterial species, either promoting its decline or favouring its persistence. It seems likely that several parameters that define the physico-

chemical characteristics of a soil determine bacterial survival, and hence the survival of a given strain will vary depending on the soil (Ramos *et al.,* 1991). Postma *et al.* (1989) showed that survival of *Rhizobium leguminosarum* biovar *trifolii* cells was better in soils with a lower initial moisture content than in soils of higher moisture content. However, if the soils are very dry introduced bacteria die rapidly (Moffett *et al.,* 1983). Reports have also demonstrated that lower pH soils had a stressful effect and that the decline of introduced *E. coli* (Sjogren, 1994) or *Pseudomonas putida* (Hartel *et al.,* 1994) was higher in soils with a pH lower than 6.0 than in soils of a neutral pH.

Studies on the effect of matric potential on survival of *P. fluorescens* chromosomally encoded with the lux *AB* genes, measured as viable cell concentration indicated no significant reduction in viable cell counts due to changes in the matric potential of soil (Meikle *et al.,* 1995). This contrasts with studies on the effect of matric stress on survival of *E. coli* in soil (Rattray *et. al.*, 1990), in which significant loss in cell viability was observed. This may be due to differences in soil properties, as the soil used in the former study had a greater clay content, which has been implicated in the increased survival of *P. fluorescens*. Tolerance to matric stress is known to vary between microorganisms and *E. coli*, whose natural habitat is not the soil, may be more susceptible to matric stress than *P. fluorescens*. In other investigations it was shown that the clay fraction stabilized a population of inoculated microorganisms probably by protecting their ingestion by protozoa (van Elsas *et al.,* 1986, 1989; Postma *et al.,*

1990). Thus, increased survival has been demonstrated in soils of a heavier texture containing a higher clay fraction.

The influence of the clay-mineral bentonite in improving the survival of *Rhizobium* cultures in loamy sand has also been demonstrated (Heijnen *et al.*, 1988, 1992). The influence of bentonite amendment was attributed to the creation of protective microhabitats against predating protozoa. The presence of bentonite was found to effect a greater influence on rhizobial survival than the physiological state of the cells prior to the introduction into soil (Heijnen *et al.*, 1988, 1992). These results were also in accordance with those obtained by Wessendorf and Lingens (1989) who reported that soil conditions such as the soil texture had a greater influence on the survival of *P. fluorescens* introduced into soil, in comparison to bacterial culture conditions.

Predation by protozoa (van Elsas *et al.*, 1986; Acea *et al.*, 1988; Acea and Alexander, 1988), microbial antagonism and competition (Dupler and Baker, 1984; Acea *et al.*, 1988) and the physiological status of the introduced bacteria (Sinclair and Alexander, 1984; Tal and Okon, 1985) have been suggested as the main biological factors that determine the fate of bacterial species introduced into soil. For example, the survival studies of *A. tumefaciens* and several species of *Rhizobium* indicated the decline in numbers of these bacteria in non-sterile soil, but not in sterile soil. It is likely that predation by protozoa plays an important role in the decrease in numbers of bacteria in soil (Acea, Moore and Alexander, 1988).

The effect of physiological differences of a *P. fluorescens* inoculum on its survival on introduction into soil was evaluated by Vandenhove *et al.* (1991). The physiological state of the inoculum was demonstrated to produce a marked influence on the survival of the strain, with cells from the late exponential phase exhibiting better survival than early exponential and stationary phase bacteria.

The nutrient status of the soil also influences the survival of introduced organisms. The addition of readily available carbon sources has a major effect on the growth of bacteria in soil and such amendments also result in a significant increase in the size of individual bacterial populations introduced into soil. However, this increase is often followed by an abrupt decline. Little information is available on the reasons why bacterial populations decline after they multiply and reach large populations in soil (Acea and Alexander, 1988).

A progressive decline in bacterial cell numbers after introduction into soil has often been observed with various strains, including *Rhizobium*, *Pseudomonas*, *Bacillus* and *Arthrobacter*, all of which are considered to be typical soil-inhabiting bacteria. In most reported cases, inoculant numbers decrease soon after introduction. For example, when persistence of *Rhizobium japonicum* in a field soil was monitored for 56 weeks following inoculation (Ellis *et al.*, 1984), significantly greater numbers of the inoculant species were detected within the first 7 weeks compared with indigenous *Rhizobium* counts. Thereafter, the inoculant population decreased to the background level. The same pattern of survival has been observed for *Pseudomonas* strains in the field (Dupler and Baker, 1984).

The capacity of bacteria to resist starvation may also influence their survival and Sinclair and Alexander (1984) provided evidence that starvation susceptible bacteria do not persist in environments that are nutrient-deficient. The relationship between starvation resistance and the ability of bacteria to survive in soil has also been demonstrated by Acea *et al.* (1988). The bacterial strains evaluated in the study shown to be starvation resistant in buffer survived at higher densities in soil. This study also indicated that bacteria like *Pseudomonas* sp. introduced into sterile soils grew and survived at the same or even higher densities than the initial inoculum size. Caldwell *et al.* (1989) investigated the long-term starvation survival of *Pseudomonas cepacia* (pRO101) in sterile well water and reported only slight loss of viability and no change in plasmid expression for over 250 days, validating the robust survival attributes of *Pseudomonads*.

2.3.8 Molecular Marker Systems for Tracking

Assessment of the efficiency of microbial inocula, and determination of potential risks to the target ecosystem, requires knowledge of the effects of environmental factors on microbial survival, activity and dispersal in the soil (Meikle *et al.*, 1995). The majority of risk assessment studies have involved the determination of microbial survival by enumeration of viable populations following cell extraction and cultivation on laboratory media. Such enumerations are unable to detect non-culturable cells and are also limited by their inability to distinguish between indigenous and recombinant organisms.

The tracking of bacteria under laboratory culture conditions and in soil and aquatic microcosms is facilitated by the use of molecular marker systems.

These involve the intentional introduction of genes conferring distinctive phenotypic properties that enable tracking of the marked organism after introduction into the environment (Prosser, 1994; Prosser *et al.*, 1996).

The most commonly employed system is the antibiotic resistance marker. Genes encoding antibiotic resistance introduced into host strains provide enumeration against the indigenous population. However, this technique is limited due to the requirement of cultivation of the marked organisms under laboratory conditions and the presence of antibiotic-resistant populations in the natural environments (Prosser, 1994). Other marker systems employed for the detection of organisms include those based on the *lac* ZY or the *xyl* E genes. The *lac* ZY genes have been exploited as a marker system for fluorescent soil *Pseudomonads* (Drahos *et al.*, 1986; Hofte *et al.*, 1990; Ryder *et al.*, 1994). Though this technique allows sensitive detection to the tune of 10 cells $(g\ soil)^{-1}$ (de Leij *et al.*, 1993), it suffers from the disadvantage of laboratory cultivation and can only be used for marking of lac^- strains (Prosser, 1994). The *xyl* E gene from the TOL plasmid pWWO of *P. putida* encoding for catechol 2,3-dioxygenase, has been utilized to mark a number of Gram negative bacteria (Winstanley *et al.*, 1989). This marker system was evaluated in lake water microcosms (Morgan *et al.*, 1989) inoculated with marked strains of *E. coli* and *P. putida*. Catechol 2, 3-dioxygenase activity was demonstrated to be lower for *E. coli* than for *P. putida* with minimum detection limit of $5x10^3$ CFU/ml. The utility of this marker system in monitoring the survival of marked *P. putida* in sterile and non-sterile lake water has also been reported by Winstanley *et al.* (1991). The use of these marker

systems enables the selective enumeration and tracking of microbial inocula. However, they do not provide information on the activity as also the influence of environmental factors on this activity, of the introduced organisms.

2.3.8.1 Bioluminescence-based Markers

Bioluminescence-based marker systems involve the introduction of genes for light emission originally cloned from the naturally luminescent marine bacteria *Vibrio fischeri* or *Vibrio harveyi* (Hastings *et al.,* 1985; Meighen, 1988; 1991; Silverman *et al.,* 1989). Luminescence marker systems have been used for the environmental detection of a wide range of microorganisms (Prosser, 1994; Jansson, 1995). This technique is useful in terrestrial and freshwater environments where luminescent activity is negligible, although luminescent fungi are known and a luminescent symbiont of nematodes, *Xenorhabdus luminescens*, has been isolated from soil (Campbell, 1989; Schmidt *et al.,* 1989).

Bacterial luciferase (lux) from *V. fischeri* or *Vibrio harveyi* has been widely employed for the detection of recombinant organisms (Prosser, 1994; Prosser *et al.,* 1996). Eukaryotic firefly luciferase (luc) has also been used to detect *Rhizobium meliloti* in root nodules (Palomares *et al.,* 1989).

The genes encoding bacterial luminescence have been identified for both *V. harveyi* and *V. fischeri* (Meighen, 1991). The lux CDABEGH operon of *V. harveyi* encodes the enzymatic functions necessary for light production. The lux A and lux B genes encode the functional subunits of luciferase, while luxC, luxD and luxE encode the components of the fatty acid reductase complex responsible for the synthesis and recycling of the aldehyde substrate (Engebrecht and

Silverman, 1984). The lux G and lux H genes may encode proteins involved in the synthesis of the reduced flavin (Bassler *et al.*, 1993). The lux AB genes have been sequenced from both *V. fischeri* (Foran and Brown, 1988) and *Vibrio harveyi* (Cohn *et al.*, 1985; Johnston *et al.*, 1986).

In strains containing the full lux cassette, luciferase production is autoinducible (Eberhard *et al.*, 1981) and luminescence per cell consequently increases with cell concentration. The presence of only the luciferase structural genes (lux A and B) provides constitutive enzyme production and yields a direct proportionality between light output and biomass concentration (Rattray *et al.*, 1990). The bacterial luciferase genes have been incorporated into the chromosome using modified transposon systems. The luciferase lux AB genes have been introduced onto the chromosome of Gram negative bacteria using a mini Tn5 lux AB transposon (DeLorenzo *et al.*, 1990). A novel transposon system, Tn 4431, has been similarly employed for the introduction of the complete lux operon containing the lux CDABE genes (Shaw *et al.*, 1988).

The luminescence-based systems allow selective detection of the marked organisms amidst high numbers of indigenous background populations, as demonstrated for *V. fischeri* lux-marked *Erwinia carotovora* cells (Grant *et al.*, 1991). The effect of the introduction of the luciferase marker genes on host fitness has been investigated (Rattray *et al.*, 1993). The determination of the effect of the additional 'metabolic burden' due to the presence of the luciferase lux AB genes was examined in chromosomally marked lux strains of *Pseudomonas flourescens* 10586. The viable cell numbers of parent and lux-marked *P. fluorescens* strains

inoculated separately and together in unsterile soil exhibited no significant difference even 14 days after inoculation. In addition, other investigations have evaluated the stability of the luciferase marker in liquid culture and in soil (Amin-Hanjani *et al.,* 1993). The study compared the properties of chromosomal and plasmid markers in *P. fluorescens* and reported the stability of the chromosomally marked *P. fluorescens* for over 200 generations. The study also detected a half-life of 11.5 generations for the plasmid marked strain on repeated subculture in liquid medium. The loss of the plasmid was observed to be greater in soil than in liquid medium suggesting the negative influence of soil-associated stress factors.

Luminometry has been demonstrated to be a highly sensitive and rapid technique in the *in-situ* monitoring of chromosomally *V. harveyi* lux-marked *P. fluorescens* inoculated into sterile soil (Meikle *et al.,* 1992). In comparison to monitoring by dehydrogenase activity, luminometry was observed to be more sensitive by three orders of magnitude and yielded results within 5 minutes of sampling compared with the 6-hour incubation period required for the dehydrogenase assay. Luminescence-based techniques also serve as tools for the detection of potential activity of the marked organisms, following amendment with nutrients. Meikle *et al.* (1994) employed potential luminescence to assess the recovery of lux-marked *P. fluorescens* following starvation in soil and in liquid culture. Luminescence was measured after incubation with complex organic medium for 2 hours, to evaluate the size of the starved population capable of recovering activity. Such enumerations of the ability of marked inocula to recover

from periods of nutrient deprivation are significant with respect to risk assessment of GEM inocula.

Luciferase has also been utilized as an environmental reporter gene for naphthalene catabolism, enabling detection of naphthalene in soil (King *et al.*, 1990) and detection of marked bacteria in the rhizosphere (de Weger *et al.*, 1991).

Luminometry, in conjunction with charge-couple device (CCD) imaging was employed in the determination of the survival and dispersal of lux-marked *Xanthomonas campestris* inoculated onto cabbage plants and into surrounding soil (Shaw *et al.*, 1992). The study reported a direct proportionality between light intensity and the cell number, yielding minimum detection level of $1.5x10^4$ CFU/leaf.

2.3.9 Immobilization of Bacteria for Enhanced Survival and Efficacy as 'Microbial Inocula'

The pre-requisite to the use of microorganisms for the detoxification of hazardous wastes is the ability to retain viability in storage for extended periods i.e. possess an extended shelf-life. Immobilization of bacteria in a variety of matrices such as calcium alginate, *k*-carrageenan and polyurethane has been shown to provide advantages over the use of free cells or enzymes in various biotechnological applications (Stormo *et al.*, 1992; Mulchandani *et al.*, 1999). Microorganisms have been immobilized using techniques such as encapsulation in numerous cases (Paau, 1988). However, there are few instances of the application of such immobilized organisms for application in bioremediation (O'Reilly and Crawford, 1989; Lin and Wang, 1991).

Most of the marketed bacterial inoculants in current use are peat-based formulations (Bashan and Gonzalez, 1999). Other formulations have also been utilized including polymer-based carriers. The polymers-based formulations can be easily manipulated according to species requirement and may also be amended with nutrients to improve short-term survival upon inoculation (Bashan and Holguin, 1998). However, the use of these polymeric materials is cumbersome in comparison to the peat-based inoculants and hence has limited application.

Peat has been the preferred carrier for species like *Rhizobium* (Pritchard *et al.*, 1996). However, peat-based formulations require the gamma-irradiation of the peat samples prior to use and hence are not very cost-effective. In addition, other sterilization procedures may cause the formation of toxic by-products harmful to microbial growth. Alternate carriers like gum arabic, methyl cellulose, clay minerals and humus have been employed for the preparation of microbial inoculants (Jawson *et al.*, 1989).

The clay-minerals including vermiculite, bentonite and kaolinite have been used as effective microbial carriers. The advantages of such clay-based carriers include their cost-effectiveness, compatibility for environmental applications and good moisture-holding properties (Graham-Weiss *et al.*, 1987).

Storage properties and the convenience of distribution also determine the suitability of any carrier (Jawson *et al.*, 1989). Lyophilization with the ability to concentrate cells may be employed for achieving long-term storage of bacterial cells. Natural and non-toxic carriers may be preferable for environmental applications (Bashan and Gonzalez, 1999). A longer shelf-life in addition to full

retention of strain characteristics is also a desirable feature for bacterial inoculants (Fages, 1990; 1992; Smith, 1992).

Carriers can be amended with nutrients to increase the chances of microbial survival (Pritchard *et al.*, 1996). It has been demonstrated that carriers amended with nutrients like skim milk in combination with clay resulted in the enhanced survival of bacteria including *Rhizobium* and *Pseudomonas* in soil (van Elsas *et al.*, 1992).

Genetically engineered *P. fluorescens*, encapsulated and later released into soil microcosms showed significantly longer survival rates than those of unencapsulated cells after three months (van Elsas *et al.*, 1992). Encapsulation of *Pseudomonas* sp. UG30 has been shown to be effective in achieving mineralization of pentachlorophenol.

The potential of encapsulation in the bioremediation of polynuclear aromatic hydrocarbons was demonstrated in a study conducted by the US EPA Gulf Breeze Laboratory. A model system was developed wherein a pure culture capable of degrading phenanthrene was successfully encapsulated in the polymeric material, polyvinyl alcohol (Baker and Herson, 1994). The encapsulated cells were stored at 4°C for several months with minimal loss of viability and a phenanthrene mineralization rate similar to fresh bacterial cells.

Thus, the use of binding matrices for attaining an increased survival and long shelf-life with a concomitant retention of strain characteristics of 'microbila inocula' is desirable for bioremediation purposes. In addition, viable

immobilization procedures are required for the proper packaging of unique metabolic or genetically engineered traits.

2.4 Cytochrome P-450 Enzymes

Cytochrome P-450 enzymes belong to a highly versatile superfamily of more than 750 heme containing isozymes found in all eukaryotes, prokaryotes and archaebacteria, which catalyse the monooxygenation of a wide variety of organic molecules (Anders, 1985; Ortiz de Montellano, 1986; Guengerich, 1992; Wiseman, 1993). These hemoprotein monooxygenases play a critical role in the synthesis and degradation of many physiologically important compounds and xenobiotics (Guengerich, 1990; Gonzalez, 1996; Nelson, 1996). The enzymes exist in various living organisms ranging from bacteria and yeast to plants and higher animals (Snyder, 2000). This superfamily metabolizing a plethora of substrates mediates an amazing variety of chemical reactions including hydroxylation of carbon and heteroatoms, dealkylation of amines and ethers, epoxidation of double bonds and reductive dehalogenation. Cytochrome P-450 enzymes also catalyse stereo- and regioselective hydroxylation of hydrocarbons. Many of these reactions are central to bioremediation chemistry and cytochrome P-450 enzymes can thus serve as potentially attractive catalysts for the degradation of environmental pollutants.

Among biological catalysts, P-450 is unmatched in its multiplicity of isoforms, substrates and catalytic and regulatory mechanisms. Also unexpected is the very large list of xenobiotic substrates including drugs, procarcinogens, antioxidants, solvents, anaesthetics, dyes, pesticides, petroleum products,

alcohols, etc. attacked by these enzymes (Porter and Coon, 1991, 1992; Coon *et al.*, 1996). Recent progress has revealed the immense diversity of cytochrome P-450s and their functions, with about 750 isozymes known (Nelson *et al.*, 1996; Nelson, 1998) and by a cautious estimate about a thousand individual substrates now recognized (Coon *et al.*, 1996). Thus, cytochrome P-450 enzymes, with their inherent ability to degrade xenobiotics, combined with their wide range of vastly different substrate specificities are ideal catalysts for application in bioremediation processes (Kellner *et al.*, 1997).

2.4.1 Reactions Catalyzed

Cytochrome P-450 enzymes act upon a wide spectrum of substrates and catalyse a range of oxidative and reductive reactions (Guengerich and Macdonald, 1990; Guengerich, 1992; Coon *et al.*, 1996). This diversity of substrates and reactions is due not only to the multiplicity of P-450 isoforms, but also to the fact that single isoforms are able to catalyse the entire range of P-450-dependent transformations with a broad range of substrates. The knowledge of the scope of P-450 catalyzed reactions is still incomplete, as this enzyme is widespread in nature and many new forms are yet to be fully characterized or even identified. Animals, plants and microorganisms contain cytochrome P-450 enzymes and in mammals the enzyme system has been found in all tissues examined. In mammals, cytochrome P-450 is found predominantly in the endoplasmic reticulum and mitochondria, and in greatest abundance in the liver. The substrates for cytochrome P-450 encompass a host of xenobiotics, including substances that occur biologically but are foreign to animals, such as antibiotics and unusual

compounds in plants, as well as synthetic organic chemicals and a variety of steroids and other physiologically occurring lipids. Given the possibilities for synthetic modification of new and existing drugs and xenobiotics, an upper limit on the number of compounds acted on by this enzyme family cannot be predicted.

2.4.2 Mechanism of P-450 Action

Most of the cytochrome P-450 catalysed reactions begin with the transfer of electrons from NAD(P)H to either NADH-cytochrome P-450 reductase in the microsomal system or a ferredoxin reductase and a non-heme iron protein in the mitochondrial and bacterial systems, and then to cytochrome P-450. This leads to the reductive activation of molecular oxygen followed by the insertion of one oxygen atom into the substrate (Porter and Coon, 1991). Many of the cytochrome P-450 catalysed reactions proceed as indicated below:

$$2NAD(P)H + O_2 + RH \rightarrow 2NAD(P)^+ + H_2O + ROH$$

In a reaction of this type, hydroxylation usually occurs at a carbon atom, but is also seen at heteroatoms such as N, S, and I. Dealkylation of amines and ethers, and epoxidation as well as reduction reactions are also catalysed by P-450s.

The first step in the reaction cycle is substrate binding, which perturbs the spin state equilibrium of the cytochrome and facilitates uptake of the first electron. Substrates that undergo reduction rather than oxygenation, such as epoxides, *N*-oxides, nitro and azo compounds and lipid hydroperoxides, accept two electrons in a step-wise fashion to give $RH(H)_2$. To initiate the oxidative reactions, O_2 is bound to the ferrous P-450 with co-ordination to iron trans to thiolate. This intermediate is denoted as the resonance form, $Fe^{3+}(O_2)^-$, with

substrate still present. Transfer of the second electron then occurs, with the possible involvement of cytochrome b_5 as an additional electron donor in mammalian microsomal systems. The next step involves splitting of the oxygen-oxygen bond with the uptake of two protons and the generation of an activated oxygen and the release of H_2O.

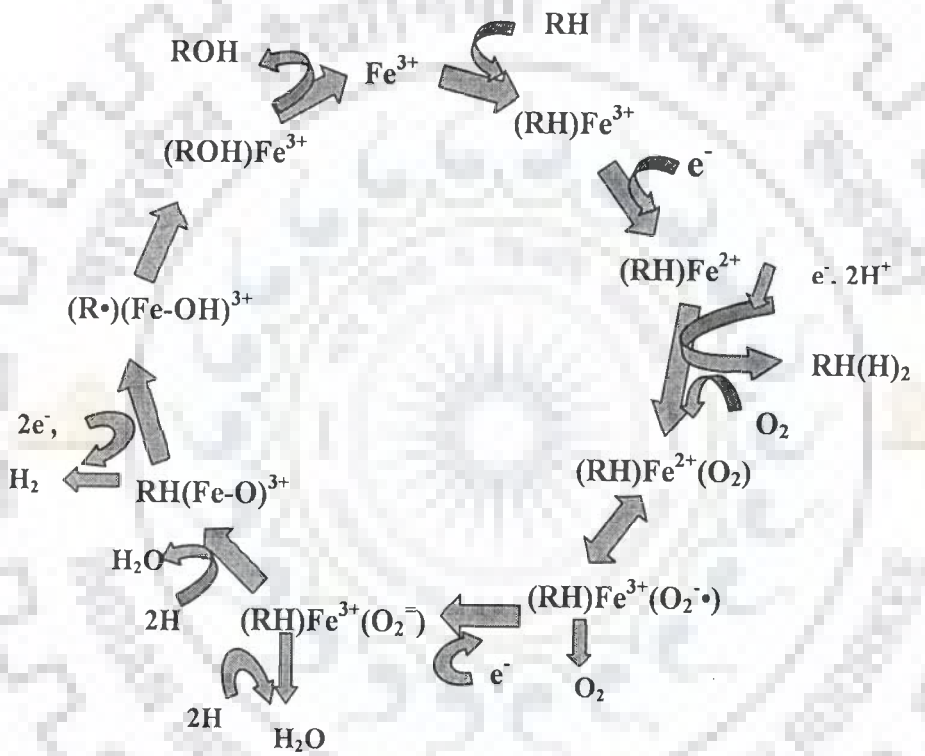


Figure 1. Cytochrome P-450 Reaction cycle*

[R and ROH represent substrate and hydroxylated substrate respectively, and $RH(H)_2$ represents a reduction product]

(*modified from Porter and Coon, 1991)

2.4.3 Cytochrome P-450 cam

Among the cytochrome P-450 enzymes, soluble bacterial camphor metabolizing P-450 cam is the most well characterized P-450 enzyme. Camphor-hydroxylating cytochrome P-450 cam has been purified from the soil bacterium *Pseudomonas putida*, ATCC 17453 (Gunsalus and Wagner, 1978). This enzyme uses *d*-camphor as the sole carbon source and catalyzes the stereospecific methylene hydroxylation of *d*-camphor to form 5-exohydroxycamphor as the first step in camphor metabolism (Nakamura *et al.,* 1994). The P-450 cam genes encoding camphor metabolism are clustered on a 240 Kb transmissible plasmid, the 'CAM' plasmid. Cytochrome P-450 cam functions as the terminal monooxygenase in the camphor hydroxylating multienzyme system (Unger *et al.*, 1986) in which the reducing equivalents of NADH are transferred from putidaredoxin reductase (a flavoprotein encoded by *cam*A gene) to P-450 cam via putidaredoxin (an iron-sulfur protein, encoded by *cam* B gene). Cytochrome P-450 cam is the first P-450 for which crystallographic structures have been solved. The physiological oxidative reaction cycle has been extensively investigated and an X-ray structure has been solved to a resolution of 1.7°A (Raag and Poulos, 1991). Cytochrome P-450 cam has served as the paradigm for understanding structure and mechanism for the broad class of cytochrome P-450 monooxygenases.

2.4.3.1 Cytochrome P-450 cam reaction cycle

The reaction cycle of P-450 cam consists of the following steps:

1. substrate binding to the oxidized form of P-450 cam followed by production of the substrate-bound oxidized form,
2. formation of the reduced form by receiving the first electron from putidarredoxin,
3. formation of substrate-bound oxygenated form by introduction of molecular oxygen and
4. reverting to the initial substrate-free oxidized form by receiving the second electron from putidaredoxin and releasing a water molecule and the hydroxylated substrate (Poulos and Raag, 1992).

2.4.3.2 Substrates for Cytochrome P-450 cam

The most extensively characterized naturally occurring microbial P-450, cytochrome P-450 cam from *P. putida* has been implicated in the metabolism of a wide variety of compounds (Table-1). Cytochrome P-450 cam is known to metabolize a plethora of substrates ranging from chloroalkanes like hexachloroethane, pentachloroethane, styrenes, ethylbenzene to polyhalomethanes and polynuclear aromatic hydrocarbons like naphthalene and pyrene. In addition to normal alkane hydroxylation activity, this enzyme is capable of reductively dehalogenating halogenated methane and ethane substrates (Vilker and Khan, 1989; Kulisch and Vilker, 1991; Filipovic *et al.*, 1992; Koe and Vilker, 1993; Li and Wackett, 1993; Lefever and Wackett, 1994; England *et al.*, 1996; Kellner *et al.*, 1997; Sibbesen *et al.*, 1998).

The reductive dehalogenation of hexachloroethane and pentachloroethane by P-450 cam has been demonstrated to produce tetrachloroethylene and

trichloroethylene, respectively (Lefever and Wackett, 1994). In addition, P-450 cam is able to oxidize trichloroethane and 1,2-dichloropropane generating 1,1,1-trichloroethanol and chloroacetone, respectively (Lefever and Wackett, 1994). Further, it has been shown that hexachloroethane binds to the active site of cytochrome P-450 cam with a dissociation constant of 0.6 μM, an affinity comparable to the binding of physiological substrate, camphor (Fisher and Sligar, 1985; Logan *et al.*, 1993).

Table-1 Certain Substrates* Metabolised by Cytochrome P-450 cam

Substrate	Metabolite formed
Camphor	6-hydroxycamphor
Naphthalene	6-hydroxy naphthalene
Hexachloroethane	Tetrachloroethylene
Pentachloroethane	Trichloroethylene
1,1,1,2-Tetrachloroethane	2,2,2-Trichloroacetaldehyde
1,1,2,2-Tetrachloroethane	Dichloroacetic acid
1,1,2-Trichloroethane	Chloroacetic acid
1,1,1-Trichloroethane	2,2,2-Trichloroethanol
1,2-Dichloropropane	Chloroacetone

[* Logan *et al.*, 1993; Lefever and Wackett, 1994; Kellner *et al.*, 1997]

Genetic variants of cytochrome P-450 enzymes, including cytochrome P450cam, possessing a wider substrate range have been constructed (Miles *et al.*, 2000). The cytochrome P-450 cam active site has been engineered by site-directed

mutagenesis to achieve enhanced degradation of substrates that are poorly attacked by the native enzyme (Loida and Sligar, 1993; Manchester and Ornstein, 1996; Sligar *et al.*, 1996; Walsh *et al.*, 2000). For instance, the mutations at active site residues F87 and Y96 of cytochrome P-450 cam yielded enhanced activity for the oxidation of polynuclear aromatic hydrocarbons like phenanthrene, fluoranthene, pyrene and benzo-pyrene (Harford-Cross *et al.*, 2000).

2.4.4 Electron Transport in Cytochrome P-450 Enzymes

2.4.4.1 Electron Transport in Cytochrome P-450 cam

The cytochrome P-450 cam monooxygenase system consists of three proteins, two of which shuttle two electrons to a terminal enzyme that catalyzes substrate decomposition. NADH supplies the reducing power to the first subunit, putidaredoxin reductase, which in turn reduces putidaredoxin, and finally the cytochrome P-450 monooxygenase (Raag and Poulos, 1991).

2.4.4.2 Electron Transport in Mammalian Cytochrome P-450 Enzymes

Microsomal electron transport is mediated by a multicomponent monooxygenase system in which reducing equivalents from NADPH ultimately are transferred to molecular oxygen. In its simplest form, the monooxygenase system consists of NADPH cytochrome P-450 reductase and one of the many cytochrome P-450 isozymes.

The flavoprotein oxidoreductase, NADPH-cytochrome P-450 reductase required for the P-450s to function catalytically is also membrane-bound and serves to supply P-450s with reducing equivalents from NADPH (Wang *et al.*,

1997). P-450 reductase contains 1 mol each of the flavin cofactors FAD and FMN per mol enzyme, and electron transfer has been shown to occur via the following general pathway (Jenkins and Waterman, 1994):

$$NADPH\text{-}[e^-] \rightarrow FAD\text{-}[e^-] \rightarrow FMN\text{-}[e^-] \rightarrow P\text{-}450$$

(P-450 reductase)

2.4.5 Other Bacterial Cytochrome P-450 Enzymes

The other bacterial cytochrome P-450 enzymes of interest include cytochrome P-450 BM3, P-450terp and P-450eryF. Cytochrome P-450terp and cytochrome P-450 BM_3 have been isolated from *Pseudomonas* spp. and *Bacillus megaterium*, respectively. In addition to the crystal structure of cytochrome P-450 cam, crystal structures for these enzymes have also been resolved. Hasemann *et al.* (1995) reported a comparison of P-450 cam, P-450 BM3 and P-450terp crystal structures, greatly increasing the understanding of structural similarities between the P-450 enzymes. The overall tertiary structure of these enzymes appears to be remarkably similar. The bacterial fatty acid ω-hydroxylase P-450 system, P-450BM_3, is functionally analogous to the microsomal P-450 system, including the transfer of electrons to the heme from NADPH via FAD and FMN.

2.4.6 Mammalian Cytochrome P-450 Enzymes

Mammalian cytochrome P-450 enzymes, in mitochondria or the endoplasmic reticulum, are membrane-bound hemoproteins that catalyse the transformation of a diverse range of endogenous and xenobiotic compounds including halogenated hydrocarbons (Guengerich, 1992; Coon *et al.*, 1996). The participation of microsomal cytochrome P-450 enzymes in a wide range of

biological processes that include the synthesis and catabolism of steroid hormones, the detoxification of drugs and toxins, as well as the transformation of xenobiotics provides an impetus to their application as catalysts for bioremediation (Coon *et al.*, 1996).

Mammalian cytochrome P-450 enzymes of environmental relevance include cytochrome P-450 2E1, 1A1 and 2D6 amongst numerous others. The P-450s involved in xenobiotic metabolism are most highly expressed in the liver, but at lower levels in the extrahepatic tissues such as the gastro-intestinal tract, lung and kidney. There is no known crystal structure available for the mammalian membrane-bound enzymes (Dai *et al.*, 2000; Szklarz *et al.*, 2000), and cytochrome P-450 cam has served as the paradigm for understanding the structure-function relationships of the mammalian isozymes.

Studies on structure-function relationships have been limited by the lack of a three-dimensional structure for a mammalian isoform. Most of the models for mammalian P-450s are based on the known structure of cytochrome P-450 cam from *Pseudomonas putida* that exhibits less than 20% sequence identity with the mammalian isoforms. Cytochrome P-450 cam is a cytosolic protein, whereas mammalian P-450s, whether found in the mitochondria or endoplasmic reticulum, are integral membrane proteins. The microsomal isoforms are inserted into the membrane of the endoplasmic reticulum in a signal-recognition-particle-dependent manner in which the NH_2-terminal segment acts as a noncleavable signal sequence (Neve and Ingelman-Sundberg, 2000). It is generally accepted that this signal peptide serves as either all or part of the anchor to the membrane.

Thus, the two currently accepted possibilities for P-450 membrane topology are a P-450 cam-like cytosolic domain anchored by either a single NH_2-terminal segment or by a hairpin loop composed of this segment and the following hydrophobic segment.

The microsomal forms are targeted to the endoplasmic reticulum by a hydrophobic *N*-terminal sequence. This sequence also acts as a stop-transfer signal and anchors the protein to the membrane so that the catalytic domain of the protein is on the cytoplasmic side of the membrane. The signal anchor sequence is required for the expression of functional proteins in eukaryotic cells but is not required for expression of fully functional mammalian P-450s in bacteria (Chen *et al.*, 1997).

2.4.6.1 Human Cytochrome P-450 2E1

Ethanol-inducible human cytochrome P-450 2E1 is implicated in the metabolism of a wide range of substrates. The ethanol-inducible cytochrome P-450 2E1, plays an important role in the bioactivation of many relatively low molecular weight substrates, many of which are suspected carcinogens (Table-2). Cytochrome P-450 2E1 is readily induced by many structurally diverse chemicals such as ethanol, benzene, isoniacid and acetone. Cytochrome P-450 2E1 enzymes oxidize ethanol and other alcohols, a number of *N*-nitrosoamines, small halogenated hydrocarbons and vinyl monomers and a few drugs such as acetaminophen and chlorzoxazone (Koop, 1992; Gillam *et al.*, 1994; Urban *et al.*, 1994; Wang *et al.*, 1996). Cytochrome P-450 2E1 is present both in animals and in man, and is found not only in the liver, but also in several extrahepatic tissues.

Regulation of 2E1 enzyme levels involves both endogenous and exogenous factors, and the extent to which the enzyme is expressed may play an important role in the outcome of human exposure to halogenated hydrocarbons. Endogenous regulation of 2E1 expression entails physiological conditions such as fasting, diabetes and obesity. Exogenous factors include exposure to xenobiotics such as alcohol, which can result in the marked induction of 2E1 enzyme levels (Raucy *et al.*, 1993; Smith *et al.*, 2000).

Table-2 Certain Substrates* Metabolised by Cytochrome P-450 2E1

Substrate	Metabolite formed
p-Nitrophenol	4-Nitrocatechol
Chlorzoxazone	6-hydroxychlorzoxazone
Benzene	Phenol
Phenol	Hydroquinone
Aniline	*p*-Aminophenol
Ethanol	Acetaldehyde
N,N-Dimethylnitrosoamine	Formaldehyde/nitrite
Azoxymethane	Azoxymethanol
Trichloroethylene	Chloral
Chloromethane	Formaldehyde
1,1,1-Trichloroethane	1,1,1-Trichloro-2- hydroxyethane
Vinyl chloride	1,N-Ethenoadenosine
Halothane	Trifluoroacetic acid
Carbon Tetrachloride	Chloroform

[* Koop, 1992]

2.4.7 Heterologous Expression of Mammalian Cytochrome P-450s

Structure-function analysis of the cytochrome P-450 enzymes has been impeded by the difficulty of purifying these enzymes from tissue in quantities sufficient for such studies. While a number of P-450 enzymes have been isolated from mammalian microsomes, purifications have been complicated by limited yield and low activity. Heterologous expression systems have been developed in order to gain a greater understanding of the molecular-mechanisms involved in P-450-mediated catalysis (Guengerich and Parikh, 1997; Guengerich *et al.*, 1997). Studies of cytochrome P-450 enzymes have been greatly facilitated by the successful application of heterologous expression techniques. The expression systems most commonly utilized include mammalian cells, yeast, insect cells and bacteria. Study of eukaryotic P-450s enzymes, most of which are present at relatively low levels *in vivo*, have been enhanced by the development of heterologous P-450 expression systems including yeast, Sf9 cells and COS cells and *E. coli* (Lehnerer *et al.*, 1995).

Yeast expression systems have been explored and have certain advantages including the capacity for expression of recombinant mutants and the presence of an endogenous NADPH P-450 reductase which functions as an electron donor for heterologously expressed P-450. However their use is hindered due to low yields of the recombinant protein, slow growth of organisms and high cost.

Although not totally devoid of drawbacks, bacterial expression systems have been employed successfully for the expression of a number of microsomal

P-450 enzymes. In many instances, they have provided an ideal combination of low cost and facility of handling (Guengerich *et al.*, 1996a, 1996b).

2.4.7.1 Constraints to the Heterologous Expression of Mammalian Cytochrome P-450 enzymes

Expression of mammalian cytochrome P-450s in *E. coli* has proven to be a valuable means to characterize these enzymes (Chen *et al.*, 1996). While bacterial expression of these enzymes is relatively easy and inexpensive, bacteria lack the endoplasmic reticulum to which mammalian microsomal P-450s bind, and they lack cytochrome P-450 reductase, the requisite electron transfer partner for microsomal cytochromes P-450. Cytochrome P-450 enzymes expressed in *E. coli* bind to the inner bacterial membrane (Dong and Porter, 1996). The expression of cytochrome P-450 in *E. coli* suffers a serious limitation in comparison to several other widely used expression systems as activity cannot be observed in cell lysates without the addition of NADPH-dependent cytochrome P-450 reductase. In contrast, with expression in yeast or mammalian cells in culture, the endogenous P-450 reductase can often couple with the heterologous P-450, generating a catalytically active monooxygenase that can be easily monitored in a cell lysate or subcellular fraction.

The heterologous expression of unmodified human cytochrome P-450 enzymes including cytochrome P-450 2E1 in *E. coli* has proved to be extremely difficult (Gillam *et al.*, 1994; Dong and Porter, 1996). Important approaches include modifying the *N*-terminus of the protein for optimal expression. To date, high level expression has only been achieved after altering the 5'-end of the native

cDNA, resulting in amino acid changes within the P-450 protein chain (Pritchard *et al.*, 1997).

2.4.8 Strategies for Expression of Mammalian Cytochrome P-450s in E. coli

Various strategies have been evaluated to achieve high yields of the recombinant P-450 enzymes in *E. coli*. The most common approach has been to replace the natural P-450 NH_2-terminus with a sequence (MALLLAVF) derived from the bovine steroid 17α-hydroxylase, CYP 17A, the first P-450 to be expressed in *E. coli* (Barnes *et al.*, 1991). The results obtained in this study, wherein the native cDNA encoding bovine microsomal 17α-hydroxylase cytochrome P-450 was modified within the first seven codons to favour expression in *E. coli*, indicated high expression yields of the recombinant enzyme.

Pritchard *et al.* (1997) explored the expression of human P-450s by generating NH_2-terminal fusion to a bacterial signal peptide. The results demonstrated the successful high level expression of unmodified human P-450s in *E. coli*, by generating NH_2-terminal translational fusions to bacterial leader sequences. Two leader sequences *omp* A and *pel* B were fused to cytochrome P-450s 3A4, 2E1 or 2A6 and produced spectrally active, functional proteins.

2.4.8.1 N-Terminus Modification of Mammalian Cytochrome P-450s for Heterologous Expression in E. coli

Different strategies have been employed to achieve high expression yields of mammalian cytochrome P-450 enzymes in bacteria. All previous successful strategies have necessitated making modifications to the 5'-end of the P-450 cDNA, in order to reflect *E. coli* codon preferences and to minimize the potential

for secondary structure formation. This has almost always resulted in changes to the amino acid sequence within the P-450 NH_2-terminus. The various approaches utilized have involved the modification of the *N*-terminus either by truncation of the *N*-terminal hydrophobic sequence (Larson *et al.,* 1991) or by modification of specific residues at the *N*-terminus of the native enzyme (Gillam *et al.,* 1994).

2.4.8.1.1 N-terminus Modification of Cytochrome P-450 2E1

Human cytochrome P-450 2E1 is poorly expressed in *E. coli* (Dong and Porter, 1996). The reason for this low content is not known, but is generally attributed to poor transcription and/or translation. As demonstrated by Winters and Cederbaum (1992) and Gillam *et al.* (1994), modifications of the NH_2-terminal sequence of human P-450 2E1 can increase the level of expression. Winters and Cederbaum (1992) demonstrated high yields of the recombinant cytochrome P-450 2E1 containing an altered nucleotide sequence without changing the amino-acid sequence. Gillam *et al.* (1994) reported high expression levels of P-450 2E1 by the deletion of 21 amino acids from the NH_2 terminus.

Gillam *et al.* (1994) explored the expression of several modified variants of human cytochrome P-450 2E1. The construction of these variants was based on different successful strategies reported earlier for P-450 2E1 expression. In addition to the expression of native unmodified P-450 2E1 in *E. coli*, the other constructs generated in this study contained truncated hydrophobic amino acid sequences (Larson *et al.,* 1991) or the bovine 17A modified sequence shown earlier to be effective in obtaining high yields in *E. coli* (Barnes *et al.,* 1991). In addition, variants possessing modifications in the native sequence wherein the

first 21 amino acids were deleted were also constructed. The analysis of the different variants indicated the highest P-450 2E1 yields in the construct wherein 21 amino acids were deleted from the *N*-terminus of the protein and the resulting *N*-terminal methionine was changed to alanine, to facilitate expression in *E. coli.*

Larson *et al.* (1991) expressed human cytochrome P-450 2E1 lacking the hydrophobic NH_2-terminal segment in *E. coli.* The truncated P-450 lacking the residues 3-29 was demonstrated to be catalytically active in reconstituted system. Thus, *N*-terminus deletion/modification has been demonstrated as a successful strategy to obtain enhanced P-450 yields in bacteria.

The various strategies employed to facilitate P-450 expression in *E. coli* yielded spectrally active functional proteins, however the membrane P-450 content of the resulting constructs was not significantly different. The membrane cytochrome P-450 2E1 content reported by Dong and Porter (1996) co-expressing P-450 2E1 and rat NADPH P-450 reductase, was nearly similar to the cytochrome P-450 2E1 levels reported by Gillam *et al.* (1994), wherein the expression in *E. coli* entailed modifications of the *N*-terminal sequence of the native P-450 2E1. In addition, the membrane P-450 content of 0.15 to 0.20 nmol/mg protein reported by Pritchard *et al.* (1997) for the construct possessing P-450 2E1 fused with *omp* A was no higher than the 0.19 nmol/mg protein level reported previously for a 17α-type CYP 2E1 construct in which the NH_2-terminal segment of P-450 2E1 (21 amino acids) had been removed (Gillam *et al*, 1994); and only slightly better than the 0.11 nmol/mg protein level obtained when unmodified P-450 2E1 was co-expressed with NADPH-cytochrome P-450 reductase (Dong and Porter, 1996).

Thus, the different approaches for high level of expression of P-450 2E1 in *E. coli* have attained almost similar yields, irrespective of the strategy employed for the heterologous expression of mammalian P-450 enzymes.

2.4.9 Alternate Electron Transfer Partners for Bacterial and Mammalian P-450 enzymes

The expression and activity of native or engineered cytochrome P-450 enzymes in non-native hosts requires an efficient electron transfer system. Both eukaryotic and prokaryotic cytochrome P-450 enzymes have been demonstrated to undergo anaerobic photo-reduction *in vitro* in the presence of small-molecule redox mediators. These photo-reduced enzymes were demonstrated to be metabolically competent (Greenbaum *et al.*, 1972; Lipscomb *et al.*, 1976).

The issue of providing a source of electrons for the heterologously expressed cytochrome P-450 enzyme has been addressed by a variety of approaches including coexpression of the P-450 with mammalian cytochrome NADPH P-450 reductase, the expression of fusion proteins composed of a cytochrome P-450 domain and a truncated cytochrome NADPH reductase domain and the use of bacterial flavoproteins.

The co-expression of cytochrome P-450 reductase with cytochrome P-450 2E1 from the same plasmid vector in *E. coli* to generate a fully active monooxygenase system has been utilized to address the limitation imposed on the expression of human P-450s in bacteria (Dong and Porter, 1996). The cDNA used in this study was not altered in the NH_2-terminal region. The other strategy applied successfully for improving the level of expression of mammalian P-450

enzymes in bacteria has been the generation of fusion proteins composed of a cytochrome P-450 domain and a truncated NADPH cytochrome P-450 reductase domain as part of a single polypeptide chain (Parikh and Guengerich, 1997). The results obtained in this study demonstrate the enzymatic activity of this construct possessing human P-450 1A1 and NADPH-P-450 reductase expressed in *E. coli.*

Fisher *et al.* (1992) reported the construction of two fusion proteins containing the functional domains of different microsomal P-450s linked to the microsomal flavoprotein domain of NADPH-dependent cytochrome P-450 reductase. These fusion proteins were prepared by fusing the cDNAs for the steroid-metabolizing bovine adrenal P-450 17A with the cDNA for rat liver NADPH-P-450 reductase. Similarly, cytochrome P-450 4A1 was linked with the cDNA for rat liver NADPH-cytochrome P-450 reductase to yield a catalytically active enzyme. Expression of human cytochrome P-450 2E1 and NADPH-cytochrome P-450 oxidoreductase in dual expression and co-infection systems with baculovirus in insect cells has also been demonstrated (Wang *et al.*, 1996). In this instance, human P-450 2E1 was coexpressed with human oxidoreductase cDNA in Sf9 cells with a dual expression virus which carried both h2E1 and hOR.

Parikh *et al.* (1997) demonstrated that the hepatic P-450 monooxygenase complex, comprising a chosen form of P-450 and its cognate flavoprotein reductase, NADPH-P-450 reductase can be replicated by expression of the recombinant enzymes in *E. coli.*

Bacterial flavoproteins have been implicated as electron transfer proteins for heterologously-expressed mammalian cytochrome P-450. Flavodoxin and NADPH-flavodoxin reductase from *E. coli* in combination, were demonstrated to support the 17α-hydroxylase activities of heterologously expressed bovine 17α-hydroxlase cytochrome P-450 (P-450c17) (Jenkins and Waterman, 1994). The ability of the *E.coli* flavodoxin-flavodoxin reductase system to support microsomal P-450 reductase dependent enzymes other than bovine P-450c17 has also been evidenced for other heterologous expression systems. Two different mammalian microsomal P-450s, P-4502 C4 and P-450 2C5, have been reported to be enzymatically active within *E. coli* in the absence of added NADPH-cytochrome P-450 reductase. Thus, these studies demonstrate that certain microsomal P-450s are capable of being reduced by this system in *E. coli.*

Novel electron transfer partners explored for heterologous expression systems have also included luciferase, a bacterial flavin monooxygenase, which has been implicated in reduction of the bacterial cytochrome P-450 cam (Shanker and Atkins, 1996). Reductive dehalogenation of pentachloroethane and hexachloroethane was demonstrated in the engineered *Pseudomonas putida* construct co-expressing cytochrome P-450 cam and luciferase lux AB genes and required the expression of both the proteins. Such studies demonstrate the utility of alternate electron transfer partners to achieve cytochrome P-450 mediated catalysis in bacterial hosts.

Sibbessen *et al.* (1996) reported the first heterologous self-sufficient catalytic system for the oxidation of camphor and other substrates by P-450 cam.

In this study, a putidaredoxin reductase-putidaredoxin-cytochrome P-450 cam triple fusion protein was generated using short peptide linkers. These and other strategies may thus be explored to fulfill the requirement of efficient electron systems for the expressed P-450 isozymes.

2.4.10 Heterologously-Expressed Mammalian Cytochrome P-450 enzymes: Catalytic Activity in vivo

The various strategies employed to achieve high P-450 expression in bacteria have yielded spectrally active, functional proteins. However, attempts to demonstrate P-450 monooxygenase activity *in vivo* with whole cells have not yielded successful results (Dong and Porter, 1996). Strategies involving reductase P-450 fusion constructs generally have not encompassed evaluation of the catalytic activity in live bacterial cultures, although modest was observed with a P-45017 fusion protein *in vivo* (Shet *et al.*, 1994). The generation of active, heterologous monooxygenase in live bacterial cultures thus remains one of the challenges of contemporary biotechnology. Despite years of success in the heterologous expression of individual recombinant enzymes, the task of engineering bacteria to carry out multi-step metabolic pathways remains challenging. With improved knowledge of the active site of P-450 and of the role of specific amino acid residues, model catalysts may be devised that will mimic the cytochrome P-450 enzymes in function and may have greater stability. The problem of providing electrons to achieve an active enzyme may be circumvented if alternative electron transfer partners can be exploited.

Novel metabolic pathways utilizing cytochrome P-450 enzymes have been engineered to achieve complete mineralization of target compounds. Wackett *et al.* (1994) reported the construction of engineered *P. putida* expressing cytochrome P-450 cam and toluene dioxygenase, capable of complete dehalogenation of pentachloroethane. The mineralization of pentachloroethane to carbon dioxide was effected by this engineered strain which utilized a combination of the reductive and oxidative pathways mediated by cytochrome P-450 cam and toluene dioxygenase, respectively. Such approaches may thus be explored to generate effective biocatalysts for contaminant removal in bioremediation processes.

Chapter 3

Subcloning of Native and N-terminus Modified Human Cytochrome P-450 2E1 in Broad Host Range Vectors

3.1 Introduction

Ethanol-inducible human cytochrome P-450 2E1 is an environmentally important member of the microsomal P-450 superfamily. The wide substrate-range of this enzyme attests its potential as an effective biocatalyst for hazardous waste treatment.

The microsomal cytochrome P-450 enzymes are anchored to the endoplasmic reticulum by a single transmembrane segment present in the *N*-terminal region of the protein (Chen *et al.*, 1997; Wachenfeldt *et al.*, 1997). Heterologous expression systems have been employed for the expression of a number of microsomal P-450 enzymes (Larson *et al.*, 1991; Gillam *et al.*, 1994; Guengerich and Parikh, 1997; Guengerich *et al.*, 1997). Though the expression of microsomal P-450 enzymes in bacteria is desirable due to low cost and convenience in handling (Guengerich *et al.*, 1996a; 1996b), their utility is however hampered due to the absence of the endoplasmic reticulum required for binding these enzymes.

Attempts at the heterologous expression of native microsomal cytochrome P-450 enzymes in *E. coli* have been largely unsuccessful (Dong and Porter, 1996; Guengerich *et al.*, 1997; Pritchard *et al.*, 1997). This has necessitated the evaluation of different strategies to achieve enhanced P-450 yields in bacteria such as *E. coli*. Many of these have targeted the *N*-terminus region of the protein in order to facilitate cytochrome P-450 expression in bacteria. Such approaches have incorporated modifications to reflect *E. coli* codon preferences and to minimize the potential for secondary structure formation. In addition, changes

have also been made to counter the presence of any rare codons at the *N*-terminus of the P-450 cDNA. For example, the most common strategy has been to replace the natural P-450 NH_2-terminus with a sequence MALLLAVF derived from the bovine steroid 17α-hydroxylase, CYP17A, the first cytochrome P-450 enzyme to be expressed in *E. coli* (Barnes *et al.*, 1991).

Successful approaches involving manipulation of the *N*-terminus of cytochrome P-450 2E1 for achieving high enzyme yields in *E. coli* include the deletion and modification of the *N*-terminal hydrophobic signal anchor segment (Larson *et al.*, 1991; Gillam *et al.*, 1994). Larson *et al.*, (1991) demonstrated high expression levels in *E. coli* of recombinant rabbit cytochrome P-450 2E1 upon deletion of the NH_2-terminal residues 3-29. The truncated P-450 2E1 enzyme lacking the hydrophobic NH_2-terminal segment exhibited identical catalytic activity as the native cytochrome P-450 2E1 isolated from rabbit liver microsomes. Gillam *et al.* (1994) employed *N*-terminus modification at the second amino acid in conjunction with deletion of the hydrophobic segment to attain high yields of human cytochrome P-450 2E1 in *E. coli*. The study involved the construction of several *N*-terminal variants of human cytochrome P-450 2E1 and a comparison of the expression levels of each in *E. coli*.

The present study describes the subcloning of native and *N*-terminus modified human cytochrome P-450 2E1 gene in *E. coli* and *Pseudomonas putida* co-expressing *V. harveyi* luciferase luxAB genes. In the present study the strategy adopted for the construction of *N*-terminus modified human cytochrome P-450 2E1 was similar to that reported by Gillam *et al.* (1994).

3.2 Materials and Methods

3.2.1 Chemicals and Glassware

Bacto-tryptone, yeast extract and Noble agar were purchased from DIFCO Laboratories (Detroit, USA). The antibiotics chloramphenicol, ampicillin and piperacillin were obtained from SIGMA Chemical Company (St. Louis, USA). Restriction enzymes were purchased from Boehringer-Mannheim, Germany. Plasmid DNA purification and Qiaquick gel extraction kits were procured from Qiagen Inc., USA. Agarose was purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, USA). Primers were obtained from Integrated DNA Technologies, USA. All other chemicals were of the highest purity commercially available. Glassware utilized was borosilicate glass of Borosil or Vensil make.

3.2.2 Culture Media

3.2.2.1 Luria Bertani Medium

Luria Bertani medium contained per litre: 10g Bacto-tryptone, 5g Bactro yeast extract and 10g NaCl. The culture medium was sterilized by autoclaving at 121°C for 15 min (Sambrook *et al.*, 1989).

3.2.2.2 SOB and SOC Media

SOB medium contained per litre: 20g Bacto-tryptone, 5g Bacto-yeast extract, 0.5g NaCl, 0.186g KCl and 0.95g $MgCl_2$ The culture medium was sterilized by autoclaving at 121°C for 15 min. The composition of SOC medium is similar except that the medium contained 20 mM glucose, added prior to use (Sambrook *et al.*, 1989).

3.2.3 Plasmid Vectors

The broad host range plasmid vectors pMMB66HE and pMMB 206 (Morales *et al.,* 1990; 1991) were utilized for the expression of native and *N*-terminus modified cytochrome P-450 2E1. The vectors pMMB66HE (8.807 Kb size) and pMMB 206 (9.311 Kb size) possess ampicillin and chloramphenicol as the drug resistance markers, respectively (Figure 2). The vectors were obtained from Dr. M Bagdasarian, University of Michigan, East Lansing.

3.2.4 Cytochrome P-450 2E1 gene

The cDNA encoding human cytochrome P-450 2E1 cloned into the *EcoRI* restriction site (1.6 Kb fragment) of the Bluescript plasmid SK^- (Stratagene) was obtained from Department of Medicinal Chemistry, University of Washington, Seattle, USA.

3.2.5 Bacterial Strains

Escherichia coli DH5α was utilized as host organism for expression of native and *N*-terminus modified human cytochrome P-450 2E1. Genetically engineered *Pseudomonas putida* expressing luciferase lux AB genes downstream from the aromatic hydrocarbon-inducible *xyl* S and *Pm* promoter system and containing the minitransposon *Tn*5 was employed as the host organism for expression of human cytochrome P-450 2E1. The construction of genetically engineered *Pseudomonas putida* expressing luciferase has been described earlier (DeLorenzo *et al.,* 1993; Shanker and Atkins, 1996). In addition, the wild-type strain *Pseudomonas putida* MTCC 102 was also employed as host for the expression of *N*-terminus modified human cytochrome P-450 2E1.

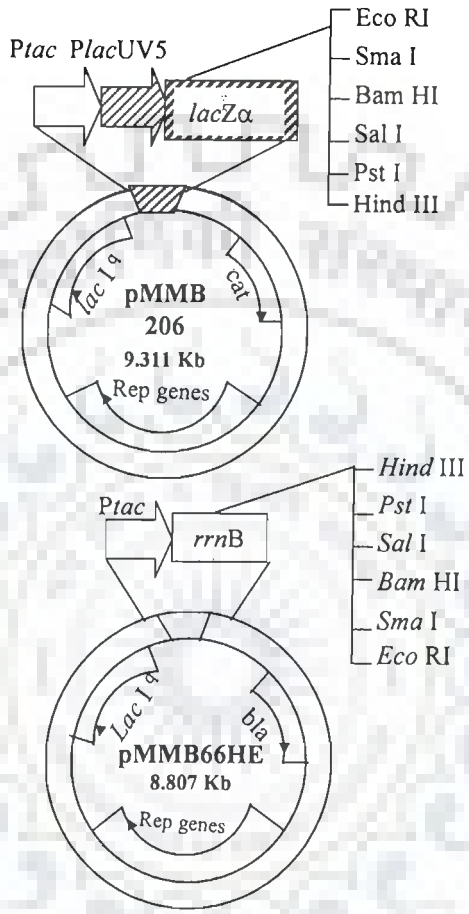


Figure 2. Diagram representing broad host range plasmid vectors pMMB 206 and pMMB66HE (Abbreviations: P*tac*: *tac* promoter; P*lac*UV5: *lac* promoter; lacZα: α-peptide of β-galactosidase; *lac*I^q: lac repressor gene; *rrn*B: transcriptional terminator;cat: chloramphenicol acetyltransferase; bla: β-lactamase; Rep genes: replication genes)

3.2.6 Plasmid DNA Isolation

Plasmid DNA was isolated using the Qiagen plasmid preparation kit along with the appropriate buffer solutions, according to the protocol recommended by the manufacturer (Qiagen Inc., USA).

- The bacterial pellet washed with TE buffer (10 mM Tris-Cl, 1 mM EDTA [pH 8.0]) was taken in a sterile tube and resuspended in 4 ml of the supplied resuspension buffer P1 (50 mM Tris-Cl, 10mM EDTA, 100 μg/ml RNase A)
- 4 ml of the lysis buffer P2 (200 mM NaOH, 1% SDS) was then added and the contents of the tube mixed thoroughly and incubated at room temperature for 5 min
- Subsequently, 4 ml of chilled neutralization buffer P3 (3.0 M potassium acetate, pH 5.5) was added to the tube. The contents were mixed, incubated on ice for 15 min and centrifuged at 20,000 xg for 30 min at 4^0C
- The supernatant was applied to a Qiagen-tip 100, previously equilibrated with 4 ml of the supplied equilibration buffer QBT (750 mM NaCl, 50 mM MOPS, 15%isopropanol, 0.15% Triton X-100). The resin was then washed twice with 10 ml of wash buffer QC (1.0 M NaCl, 50 mM MOPS, 15%isopropanol)
- DNA was eluted from the resin by applying 4 ml of the elution buffer QF (1.6 M NaCl, 50 mM MOPS, 15%isopropanol) to the Qiagen column. DNA was precipitated with 0.7 volumes of isopropanol and recovered by centrifugation at 15,000 xg for 30 min at 4^0C. The precipitated DNA was washed with 2 ml of ice-cold 70% ethanol, air-dried for 5 min and redissolved in 100 μl of TE buffer (pH 8.0).

3.2.6.1 Preparation of vector SK⁻ DNA containing human cytochrome P-450 2E1

Bluescript SK⁻ vector containing the 1.6 Kb human cytochrome P-450 2E1 gene cloned in the *EcoRI* site was isolated using the Qiagen plasmid preparation kit. *E. coli* DH5α cells were grown in 5ml Luria-Bertani medium supplemented with 50 μg/ml ampicillin and incubated at 37^{0}C on a rotary shaker at 175 rpm for 18h. An aliquot (0.5%, v/v) from this overnight culture was used to inoculate 100 ml Luria-Bertani medium supplemented with 50 μg/ml ampicillin and incubated at 37^{0}C and 175 rpm. Cells were harvested after 18h by centrifugation at 8000 xg and 4^{0}C and washed with ice-cold sterile TE buffer (pH 8.0). Plasmid DNA was extracted using the Qiagen Q-100 DNA columns, according to the protocol described earlier (section 3.2.6).

3.2.6.2 Preparation of broad host range vector DNA

E. coli DH5α cells harboring either the plasmid pMMB66HE or pMMB 206 were inoculated in 5 ml Luria-Bertani medium supplemented with either 50 μg/ml ampicillin or 20 μg/ml chloramphenicol and incubated at 37^{0}C on a rotary shaker at 175 rpm for 18h. An aliquot (0.5%, v/v) from this culture was transferred to 100 ml Luria-Bertani medium supplemented with the appropriate antibiotic and incubated at 37°C on a rotary shaker at 175 rpm. Cells were harvested after 18h by centrifugation at 8000 xg and 4^{0}C and washed with ice-cold sterile TE buffer (pH 8.0). Plasmid DNA was isolated using the Qiagen Q-100 DNA columns as described earlier (section 3.2.6).

3.2.7 Agarose gel electrophoresis

The extracted plasmid DNA was visualized by gel electrophoresis using a 1% horizontal agarose gel containing 0.5 μg/ml ethidium bromide with 1X TAE buffer (40 mM Tris-acetate, 2mM EDTA [pH 8.0]) as the tank buffer, according to the method described by Sambrook *et al.* (1989). The agarose gel containing the DNA was examined by UV light on a Vilber-Lourmat Kaiser RA-1 transilluminator.

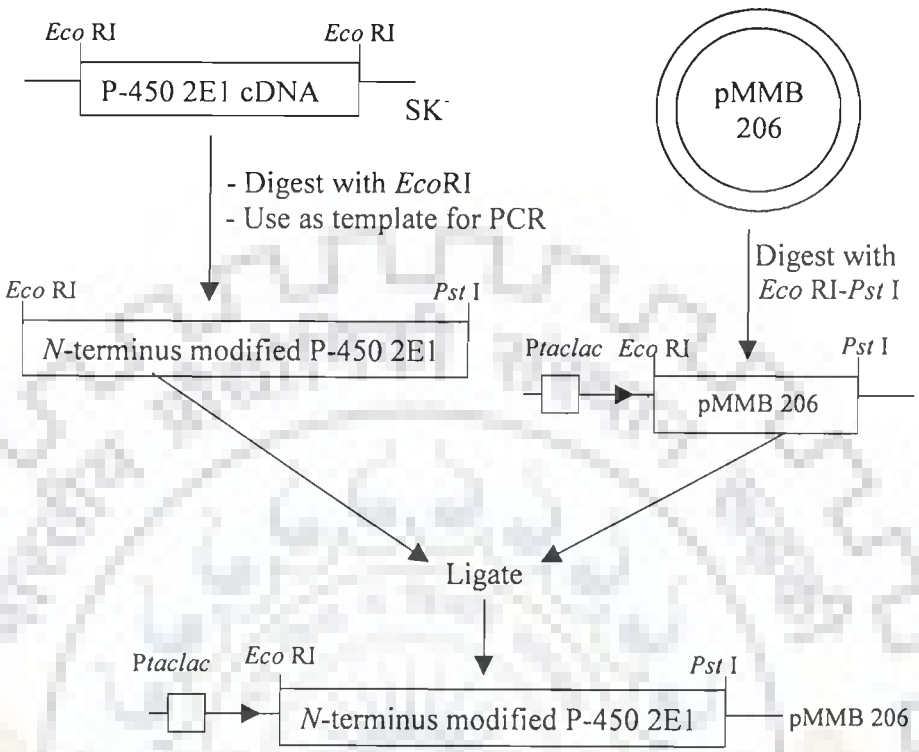
3.2.8 N-terminus modification of human cytochrome P-450 2E1

3.2.8.1 Strategy and designing of PCR primers

Polymerase chain reaction (PCR) was employed for the *N*-terminus modification of cytochrome P-450 2E1 using designed oligonucleotides to improve expression in host bacteria. The PCR mutagenesis strategy involved the deletion of the first 21 amino acids at the *N*-terminus and the long template amplification of 1.461 Kb cytochrome P-450 2E1 gene product based on the strategy described by Gillam *et al.* (1994). A schematic illustration of the construction of *N*-terminus modified P-450 2E1 is outlined in figure 3.

The oligonucleotides were designed based on the published human cytochrome P-450 2E1 gene sequence (Umeno *et al.*, 1988; Genbank accession number J02843). The native cytochrome P-450 gene sequence after deletion of introns served as the template for primer design using the biocomputing software "Lasergene" (DNASTAR).

Primers were designed with the aid of the "Primer Select" option available in the software. The option requires an input of various parameters pertaining to



N-terminal DNA sequence

Native*: 5'- ATG TCT GCC CTC GGA GTC ACC GTG GCC CTG CTG GTG TGG GCG GCC TTC CTC CTG CTG GTG TCC ATG **TGG AGG CAG GTG CAC AGC AGC** TGG AAT CTG CCC - 3'

Modified: 5'- ATG GCT CGT CAA GTT CAA GTT CAT GTT CAT TCT TCT TGG AAT CTG CCC –3'

(*Underlined sequence indicates deletion and bold-face type indicates modification of the native cytochrome P-450 2E1 gene)

N-terminal amino acid sequence

```
                1                   20        25
Native:     MSALGVTVALLVWAAFLLLVSMWRQVHSS
Modified:   ---------------------------------- M ARQVHSS
```

Figure 3. Strategy for construction of *N*-terminus modified human cytochrome P-450 2E1

primer design including stringency requirements and product size. In addition, a complete restriction map of the human cytochrome P-450 2E1 gene was obtained using the "Map Draw" option, to aid in the choice of restriction enzyme sites for subcloning in the broad host range plasmid vector, pMMB 206.

Modifications at the *N*-terminus of the native gene sequence were introduced using the designed 5'-PCR primer designated 2E1P1. An *EcoRI* restriction enzyme site, not existing in the native cytochrome P-450 2E1 sequence was also incorporated in the primer to aid in cloning in BHRV, pMMB 206 (Table 3).

The C-terminus primer was similarly selected using the "Primer Select" option. This primer did not involve any modifications, other than the incorporation of a *Pst I* restriction enzyme site, and is complementary to the 3'-end of the native cytochrome P-450 2E1 gene sequence. The primer was designated 2E1 LP2 (Table 3).

In addition, a pair of internal primers was also designed to aid in the selection of clones. This primer pair utilized the same 5'-end primer as described above (2E1P1). The 3'-end primer selected was located at 462-433 bp and yielded an amplification of a 403 bp region in the 5'-end of the gene. This primer was designated 2E1P2 (Table 3).

Table - 3 Primers and PCR amplification conditions

Primer	Sequence and Location (5'-3')	PCR conditions	Product size
2E1P1	C GGG CTG CAG GAA TTC ATG GCT CGT CAA GTT CAT TCT TCT **TGG AAT CTG CCC** (highlighted sequence: 88-99)	*Primers:2E1P1-2E1LP2* Cycles 1-35: Denaturation for 75s at 94°C Annealing for 35s at 82°C Extension for 165s at 72°C Cycle 36: Elongation for 420s at 72°C	1461 bp
2E1LP2	AGG GCT GCA GGG TGT CCT CCA CAC ACT CAT GAG CGG (1520-1485)		
2E1P2	CTC CCT CTG GAT CCG GCT CTC ATT GCC CTG (462-433)	*Primers: 2E1P1-2E1P2* Cycles 1-35: Denaturation for 75s at 94°C Annealing for 60s at 79°C Extension for 105s at 72°C	403 bp

3.2.8.2 Molecular modeling

Computer-derived homology model of the *N*-terminus modified human cytochrome P-450 2E1 was generated to determine the influence of deletion of 21amino acids on the substrate and heme-binding domains. Homology modeling

was carried out using Ex-PDB templates of P-450 101, BM-3, 2C17A and 1DT6A. The initial model obtained was fine-tuned using SWISS PDB viewer v3.51 and final model was made by using the optimize mode of SWISS-MODEL. Output files were generated in PDB format and visualized using RASMOL windows version 2.6.

3.2.8.3 Long Template PCR Amplification

Long template PCR amplification was performed using the Expand™ High Fidelity PCR system (Boehringer-Mannheim, Germany), according to the manufacturers instructions. This PCR system is composed of a mix of *Taq* DNA polymerase and *Pwo* DNA polymerase. The optimal reaction conditions, including thermal cycling times and temperature, concentration of the enzyme mix, template DNA and magnesium concentration were determined to achieve the long template amplification of 1.461 Kb cytochrome P-450 2E1 gene.

The long template amplification involved the preparation of two master mixes, according to the manufacturer's instructions. Reaction mix 1 was prepared by aliquoting 200 μM (each) dNTP, 200 nM (each) primer, 0.1 μg template DNA and sterile deionized water to yield a final volume of 50 μl. Reaction mix 2 was similarly prepared by aliquoting 1X PCR buffer, 2.6 units of the enzyme mix, *Taq* and P_{wo} DNA polymerase and sterile deionized water to yield a final volume of 50 μl. The above components were mixed together in thin-walled PCR tubes and the samples overlaid with 70-100 μl of mineral oil to prevent evaporation. PCR was carried out on a Perkin Elmer GenAmp 9600 Thermocycler for 35 cycles (Table 3).

3.2.8.4 Agarose Gel Electrophoresis and Purification of the PCR Product

The PCR product obtained (1.461 Kb) was electrophoresed on a 1% horizontal agarose gel in 1X TAE buffer (pH 8.0) as described earlier (section 3.2.7) and visualized on a UV transilluminator. The PCR product was purified using the Qiaquick PCR purification kit according to the manufacturer's instructions (Qiagen Inc, USA). In brief, the PCR reaction mix was mixed with 5 volumes of supplied buffer PB and the sample applied to the Qiaquick PCR purification column and centrifuged for 1 min at 10,000 xg at room temperature. The column was washed with 0.75 ml of buffer PE and centrifuged again for 1min at room temperature. The PCR product was eluted with 50 µl of buffer EB and stored at -20 °C till further use.

3.2.9 Restriction enzyme digestions

The Bluescript SK$^-$ vector containing human cytochrome P-450 2E1 cDNA and the plasmid vectors, pMMB66HE and pMMB 206, were subjected to restriction enzyme digestion with the appropriate enzymes. The purified PCR product was also digested with the appropriate enzymes. Restriction enzyme digestion was performed as described by Sambrook *et al.* (1989).

The cytochrome P-450 2E1 cDNA (1.6 Kb fragment) was excised from the SK$^-$ vector by restriction digestion with *EcoRI* enzyme. To analyse the vector SK$^-$ DNA by cleavage with restriction enzymes, 5 µl (approximately 1µg) of the DNA resuspended in TE buffer was taken in a fresh microfuge tube that contained 12 µl of sterile deionized water. The appropriate 10X restriction enzyme buffer (2 µl) was added to the DNA solution. The contents of the tube were mixed by

vortexing and 1 unit of the restriction enzyme, *EcoRI*, was added to the reaction mix that was incubated for 3h at 37^{0}C. The DNA fragments in the restriction digest were analysed by agarose gel electrophoresis and purified as described in section 3.2.10.

The broad host range plasmid vectors pMMB66HE and pMMB 206 were also subjected to restriction enzyme digestion in a similar manner. The vector pMMB66HE was incubated with *EcoRI* restriction enzyme while pMMB 206 was subjected to restriction enzyme digestion with *EcoRI* and *PstI* enzymes. Each restriction digestion mix contained approximately 1μg DNA and 1X restriction enzyme buffer in a final volume of 250 μl. The appropriate enzymes (1 unit each) were added and the reaction mix incubated at 37°C for 3h.

The PCR product obtained with primers 2E1P1 and 2E1LP2 was also subjected to restriction digestion with *EcoRI* and *PstI* enzymes. The reaction mix contained approximately 1 μg DNA and 1X restriction enzyme buffer in a volume of 20 μl. The restriction enzymes, *EcoRI* and *PstI* (1 unit each) were added to the reaction mix that was incubated at 37°C for 3h.

3.2.10 Purification of restriction enzyme digested DNA

The restriction digestion reaction mixes of the plasmid vectors and the PCR product were loaded on 1% low-melting agarose gels containing 0.5 μg/ml ethidium bromide and electrophoresed in ice-cold 1X TAE buffer (pH 8.0) as described by Sambrook *et al.* (1989). The DNA was visualized on a UV transilluminator and required DNA fragments were carefully excised from the gel. The 1.6 Kb cytochrome P-450 2E1 cDNA and the digested plasmid vectors

pMMB66HE and pMMB 206 were purified using the Qiaquick gel extraction kit along with the supplied buffer solutions according to the manufacturer's instructions (Qiagen Inc., USA).

The required DNA fragment was excised from the agarose gel and weighed. Three volumes of buffer QG was added to the gel slice (100mg ≈100µl). The tube containing the agarose slice was then incubated at 50°C for 10 min. After dissolution of the gel slice one gel volume of isopropanol was added and the contents applied to the Qiaquick gel extraction column. The column was centrifuged for 1min at 10000 xg at room temperature. The column was washed with 0.75 ml of buffer PE and centrifuged again for 1min. DNA was eluted with 50 µl of buffer EB and stored at -20 °C till further use.

3.2.11 Cohesive Ligations

Cohesive-end ligations to generate the different constructs were performed using the Rapid DNA Ligation Kit (Boehringer-Mannheim, Germany) according to the protocol provided by the manufacturer but with certain changes incorporated to optimize the yield of the ligation reactions. The vector DNA to insert ratios were also varied in separate reactions to achieve optimal yields of ligated products.

The *EcoRI*-digested fragment (1.6 Kb) encoding the native cytochrome P-450 2E1 gene was ligated into the *EcoRI* site of the broad host range vector pMMB66HE. The purified PCR fragment (1.461 Kb) encoding *N*-terminus modified cytochrome P-450 2E1, digested with *EcoRI* and *PstI* restriction enzymes was similarly ligated into the cognate sites of broad host range plasmid

vector pMMB 206. Each ligation reaction contained 3 µl of either vector pMMB66HE or pMMB 206 DNA; 6 µl of the native 2E1 DNA or the PCR product, 1X DNA dilution buffer and 1X T4 Ligase buffer. The final reaction volume was adjusted to 20 µl with sterile deionized water and 1 unit of T4 DNA Ligase was added to the mix. The ligation reaction mixes were incubated at 20°C for 1h.

3.2.12 Transformation of E. coli DH5α

Competent *E. coli* DH5α cells were transformed with the ligation reaction mix either by electroporation or chemical transformation. Electroporation was carried out in a GIBCO-BRL Cell Porator as recommended by the manufacturer (GIBCO-BRL Inc., USA). Chemical transformation was performed according to Inoue *et al.*, (1990).

3.2.12.1 Electroporation

Electrocompetent cells of *E. coli* DH5α were prepared as described by Sambrook *et al.* (1989). *E. coli* DH5α cells were grown in 5 ml SOB medium for 18h at 37°C on a rotary shaker at 175 rpm. An aliquot (5%, v/v) was transferred to 50 ml SOB medium and grown for 3h at 37°C and 175 rpm. Cells were recovered by centrifugation at 4000 xg for 10 min at 4°C and the cell pellet resuspended by gentle vortexing in 20 ml of ice-cold transformation buffer (10%, v/v glycerol). After storage on ice for 10 min the cells were centrifuged again at 4000 xg for 10 min at 4°C. The cell pellet obtained was resuspended by gentle vortexing in 4 ml of ice-cold transformation buffer.

E. coli DH5α was transformed by mixing 0.1 volume of the ligation mix with the electrocompetent cells. The mix was placed in the electroporation cuvettes and electroporation performed using the GIBCO-BRL Cell Porator under the following conditions: 2.5 kV; 25 μF and 200 ohm. After electroporation, 1.0 ml of SOC medium was added to the cuvettes, the contents transferred to 5 ml polypropylene tubes and incubated at 37^{0}C for 1 h on a rotary shaker at 100 rpm. Aliquots (0.1-0.2 ml) of transformed *E. coli* DH5α cells were plated on LB-agar supplemented with either 50 μg/ml ampicillin or 20 μg/ml of chloramphenicol and incubated at 30^{0}C for 1-7 days.

3.2.12.2 Chemical transformation

A single colony of *E. coli* DH5α was grown overnight in 5 ml SOB medium and incubated at 37^{0}C on an orbital shaker at 175 rpm. Overnight grown cells (1ml) were transferred to 250 ml SOB medium and grown for 4h at 37^{0}C, with vigorous shaking at 200 rpm. The flask was subsequently removed from the incubator and placed immediately on ice for 10 min. The culture was then harvested by centrifugation at 2500 xg for 10 min at 4^{0}C and the pellet resuspended in 80 ml of ice-cold transformation buffer (10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl). Cells were incubated in an ice-bath for 10 min and centrifuged again as described above. The resulting cell pellet was gently resuspended in 20 ml of transformation buffer and DMSO added with gentle swirling to yield a final concentration of 10%. The cell suspension was dispensed into small aliquots, frozen in liquid nitrogen and stored at -70^{0}C until use.

E. coli DH5α cells were transformed by the chemical transformation protocol (Inoue *et al.*, 1990). Competent *E. coli* DH5α cells were quick-thawed at room temperature and dispensed as 0.2 ml aliquots into 15 ml polypropylene tubes and immediately placed on ice. 0.2 ml of the ligation mix was added to the tubes which were incubated on ice for 30 min. The tubes were subsequently heat-pulsed without agitation for 30s at 42^{0}C and placed immediately on ice. 0.8 ml of SOC medium was added and the tubes incubated at 37^{0}C for 1 h on a rotary shaker at 100 rpm. Aliquots (0.1-0.2 ml) of *E. coli* DH5α cells were plated on LB-agar supplemented with either 50 μg/ml ampicillin or 20 μg/ml of chloramphenicol and incubated at 30^{0}C for 1-7 days.

3.2.13 Screening of clones

Single colonies of transformed *E. coli* DH5α cells were aseptically transferred to 5 ml Luria-Bertani medium supplemented with either 50 μg/ml ampicillin or 20 μg/ml chloramphenicol and grown for 18h at 37^{0}C on an orbital shaker at 175 rpm. Clones were screened by mini plasmid preparations using the boiling lysis protocol as described by Sambrook *et al.* (1989). In addition, plasmid DNA was isolated from the clones using Qiagen DNA columns as described earlier (section 3.2.6).

The presence of the native or *N*-terminus modified cytochrome P-450 2E1 gene in the transformants was confirmed by PCR using DNA extracted from the clones as template for the PCR amplification.

3.2.13.1. Plasmid DNA isolation by boiling lysis minipreparation

- Overnight grown cells (1.5 ml) were taken in a fresh microfuge tube and harvested by centrifugation at 12,000 xg for 30s at 4^0C. The bacterial pellet was resuspended in 350 µl of STET(0.1 M NaCl, 10 mM Tris.Cl, 1mM EDTA (pH 8.0), 5% Triton-X-100)
- 25 µl of a freshly prepared solution of lysozyme (10 mg/ml) was added and the contents mixed by vortexing for 1 min
- The tube was placed in boiling water bath for 45 seconds. The bacterial lysate was centrifuged at 12,000 xg for 10 min at room temperature and the pellet of bacterial debris was removed from the microfuge tube
- 40 µl of 2.5M sodium acetate (pH 5.2) and 420 µl of isopropanol were added to the tube, mixed by vortexing and stored at room temperature for 5 min
- The pellet of nucleic acids was recovered by centrifugation at 12,000 xg for 15 min at 4^0C in a microfuge and the supernatant removed gently. The precipitated DNA was washed with 1ml of ice-cold 70% ethanol, air-dried for 5 min and redissolved in 25 µl of TE buffer (pH 8.0).

The plasmid DNA isolated from the clones possessing either native or *N*-terminus modified cytochrome P-450 2E1 was visualized on a 1% horizontal agarose gel as described earlier (section 3.2.7).

3.2.13.2 Screening by PCR

PCR amplification targeting a 403 bp region at the 5'-end of the gene was employed for verifying the presence of the 2E1 gene in the clones using the primers 2E1P1 and 2E1P2 (Table 3). Plasmid DNA isolated from the clones

served as the template for PCR amplifications. The reaction conditions were optimized with respect to the thermal cycling and incubation times and temperature. The PCR reaction contained 200 μM (each) dNTP, 200 nM (each) primer, 1X PCR buffer and sterile deionized water in a final volume of 100 μl. 2 units of *Taq* DNA polymerase were added to the reaction mix, which was overlaid with 30-50 μl of mineral oil. Amplification was performed on a Perkin Elmer GenAmp 9600 Thermocycler for 35 cycles (Table 3).

3.2.14 Subcloning of Native and N-terminus modified human cytochrome P-450 2E1 in Pseudomonas putida expressing luciferase

Pseudomonas putida expressing luciferase lux AB genes was subcloned with the native and *N*-terminus modified human cytochrome P-450 2E1. Plasmid DNA was isolated using the Qiagen DNA columns (section 3.2.6) from the selected *E. coli* clones screened by PCR. Competent cells of *Pseudomonas putida* expressing luciferase were prepared and transformed with the isolated plasmid DNA by the chemical transformation protocol described earlier (section 3.2.12.2). Similarly, *Pseudomonas putida* MTCC 102, the wild-type strain, was also subcloned with native and *N*-terminus modified human cytochrome P-450 2E1. Competent cells of *Pseudomonas putida* were transformed with the plasmid DNA isolated from the *E. coli* clones by the chemical transformation method. The transformed cells were plated on LB-agar supplemented with either 50 μg/ml ampicillin or 20 μg/ml chloramphenicol and incubated at 28^0C.

3.2.15 Screening of Pseudomonas putida clones

3.2.15.1 Screening of Pseudomonas putida clones possessing native cytochrome P-450 2E1

The transformed colonies of *Pseudomonas putida* expressing luciferase and subcloned with the plasmid pMMB66HE containing the native cytochrome P-450 2E1 gene were selected on LB-agar plates containing 50 μg/ml amipicillin. Single colonies observed after 3-4 d of incubation at 30^0C were transferred to 5 ml Luria-Bertani medium supplemented with 50 μg/ml ampicillin, 1mM IPTG and 200 μM of the luciferase-inducer, *m*-toluate. Colonies were screened by PCR and luminescence detection. PCR was performed using the primers 2E1P1 and 2E1P2 for amplification of the 403 bp product of cytochrome P-450 2E1 (Table 3). Luminescence detection was performed in a Luminometer by withdrawing 1ml aliquot of culture and monitoring luminescence as RLU, after the addition of 1μl *n*-decanal, the luciferase substrate.

3.2.15.2 Screening of Pseudomonas putida clones possessing N-terminus modified cytochrome P-450 2E1

Clones of *Pseudomonas putida* co-expressing luciferase and *N*-terminus modified human cytochrome P-450 2E1 were selected on LB-agar supplemented with 20 μg/ml chloramphenicol and 30 μg/ml piperacillin. The colonies exhibited slow growth and appeared between 10-15d incubation at 28^0C. Clones were screened by PCR and luminescence detection. PCR was performed using primers 2E1P1 and 2E1P2 for amplification of 403 bp product of cytochrome P-450 2E1 (Table 3). For the determination of luminescence, single colonies were inoculated

in 5 ml Luria-Bertani medium supplemented with 20 μg/ml chloramphenicol, 30 μg/ml Piperacillin, 1 mM IPTG and 200 μM *m*-toluate and incubated at 28^0C for 36h. Luminescence detection was performed in a Luminometer by withdrawing 1ml aliquot of culture and monitoring luminescence after the addition of 1 μl *n*-decanal.

Colonies of *Pseudomonas putida* MTCC 102 were visible after 24h incubation and were grown for 18h in Luria-Bertani medium supplemented with 20 μg/ml chloramphenicol and 1 mM IPTG and incubated at 26^0C on an orbital shaker at 175 rpm. These clones were also screened by PCR using primers 2E1P1 and 2E1P2 (Table 3).

3.3 Results

3.3.1 Isolation of plasmid vector DNA and restriction digestion

Bluescript vector SK⁻ containing the native human cytochrome P-450 2E1 gene extracted from *E. coli* DH5α cells on being restricted digested with *EcoRI* yielded a 1.6 Kb fragment of the native cytochrome P-450 2E1 cDNA (Figure 4A and 4B).

The vector pMMB66HE DNA digested with restriction enzyme *EcoRI* exhibited a DNA band at 8.8 Kb, consistent with the linearized plasmid DNA (Figure 5A and 5B). Similarly, vector pMMB 206 DNA on restriction enzyme digestion with *EcoRI* and *PstI* enzymes yielded the expected DNA band at 9.3 Kb (Figure 5C).

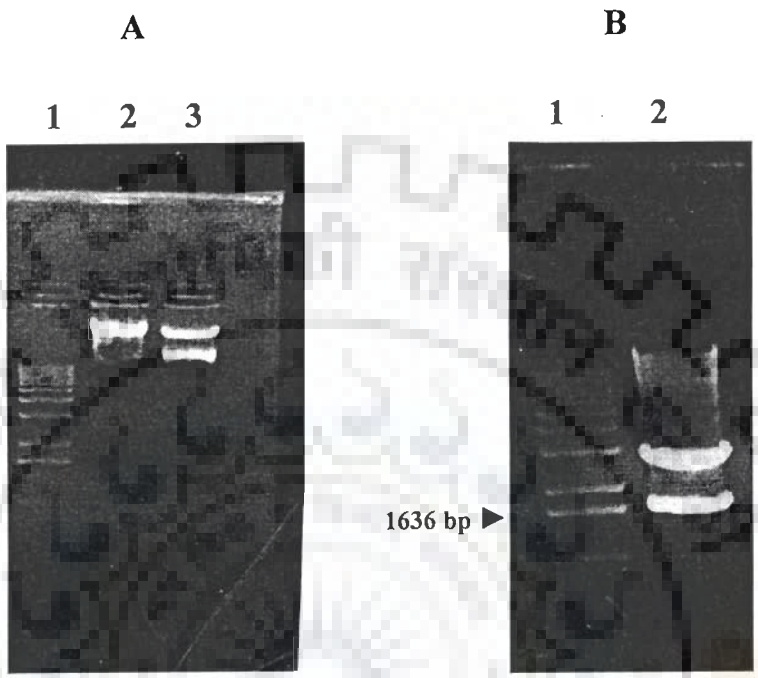


Figure 4. DNA preparation and restriction digestion of SK⁻ plasmid vector containing human cytochrome P-450 2E1 gene

A. Vector SK⁻ DNA containing cytochrome P-450 2E1 gene
Lanes:
1 - 1 Kb ladder (GIBCO-BRL)
2-3 - Vector SK⁻ containing cytochrome P-450 2E1 gene

B. Restriction digestion with *EcoRI* of plasmid vector SK⁻ containing native cytochrome P-450 2E1 gene
Lanes:
1 - 1 Kb ladder (GIBCO-BRL)
2 - 1.6 Kb fragment of cytochrome P-450 2E1 gene obtained after restriction digestion of SK⁻ vector with *EcoRI*

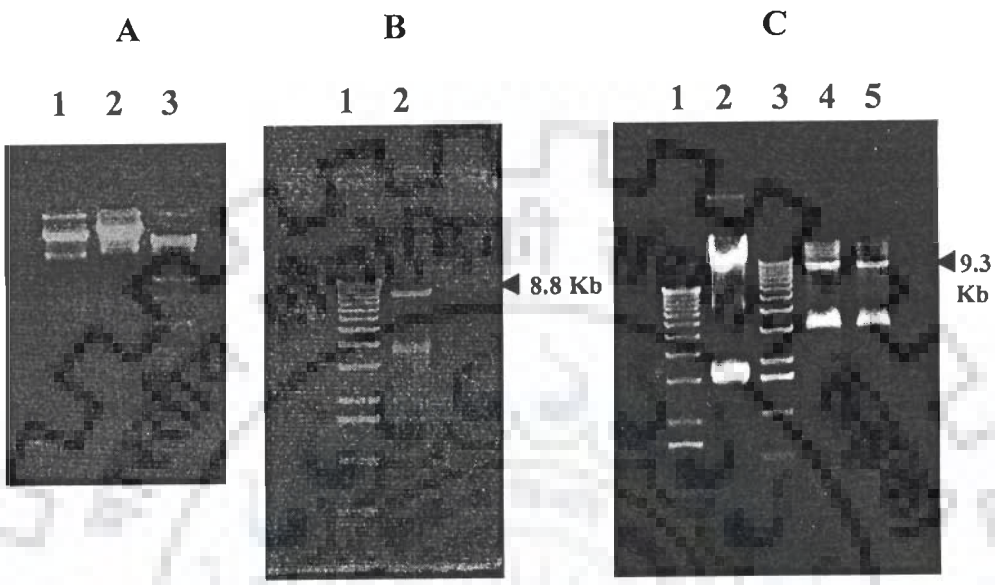


Figure 5. DNA preparation and restriction digestion of broad host range vectors pMMB66HE and pMMB 206

A. BHRV pMMB66HE

Lanes:

1-2 - pMMB66HE DNA prepared using Qiagen column

3 - 1 Kb ladder (GIBCO-BRL)

B. Restriction digestion of pMMB66HE

Lanes:

1 - 1 Kb ladder (GIBCO-BRL)

2 - pMMB66HE DNA digested with *EcoRI*

C. BHRV pMMB 206

Lanes:

1 - 1 Kb ladder (GIBCO-BRL)

2 - pMMB 206 DNA prepared using Qiagen column

3 - 1 Kb ladder (GIBCO-BRL)

4-5 - pMMB 206 DNA subjected to double digestion with *EcoRI* and *Pst I* enzymes

3.3.2 N-terminus modification of human cytochrome P-450 2E1

Long template PCR amplification using primers 2E1P1 and 2E1LP2 yielded a 1.461 Kb PCR product of the *N*-terminus modified cytochrome P-450 2E1 gene (Figure 6A). The amplified DNA subjected to digestion with the enzyme *BamHI*, a unique restriction site in human cytochrome P-450 2E1 gene, confirmed the PCR product as P-450 2E1 DNA fragment (Figure 6B).

3.3.3 Cohesive ligations and screening of clones

The 1.6 Kb cDNA of native cytochrome P-450 2E1 was ligated into the *EcoRI* site of the vector pMMB66HE. The PCR product (1.461 Kb) obtained after long template amplification of *N*-terminus modified human cytochrome P-450 2E1, digested with *EcoRI* and *PstI* was ligated into the cognate sites of the vector pMMB 206 (Figure 7A and 7B).

Ampicillin-resistant clones of *E. coli* containing the native P-450 2E1 gene after screening by plasmid DNA isolation and PCR amplification of a 403 bp target in the P-450 2E1 gene (Figure 8A and 8B) were grown in Luria-Bertani medium containing IPTG. Clones exhibiting pink cell pellets were selected for diagnostic CO-difference spectral screening.

Similarly, PCR amplification using plasmid DNA extracted from the *E. coli* clones containing the *N*-terminus modified P-450 2E1 as template yielded the desired product (Figures 9A, 9B and 10). Clones cultured in Luria-Bertani medium containing IPTG exhibiting pink cell pellets, chloramphenicol resistance as well as the desired 403 bp PCR product were selected for the diagnostic CO-difference spectral screening.

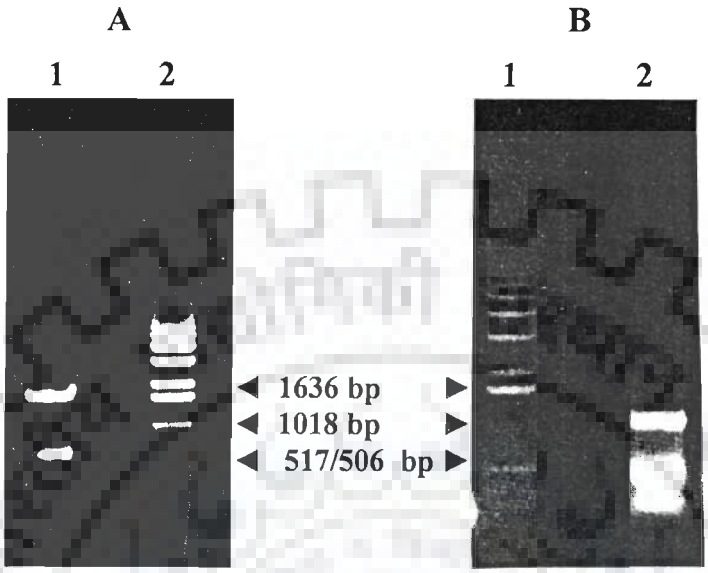


Figure 6. Long Template PCR amplification and product confirmation of *N*-terminus modified human cytochrome P-450 2E1 gene

A. Cytochrome P-450 2E1 DNA obtained after PCR amplification

Lanes:

1 - PCR product (1.461 Kb)* obtained with primers 2E1P1/ 2E1LP2

2 - 1 Kb ladder (GIBCO-BRL)

B. PCR product confirmation by digestion of amplified DNA with *Bam HI*

Lanes:

1 - 1 Kb ladder (GIBCO-BRL)

2 - DNA fragments (1071 and 390 bp) on digestion of PCR product (1.461Kb)* obtained with primers 2E1P1/2E1LP2

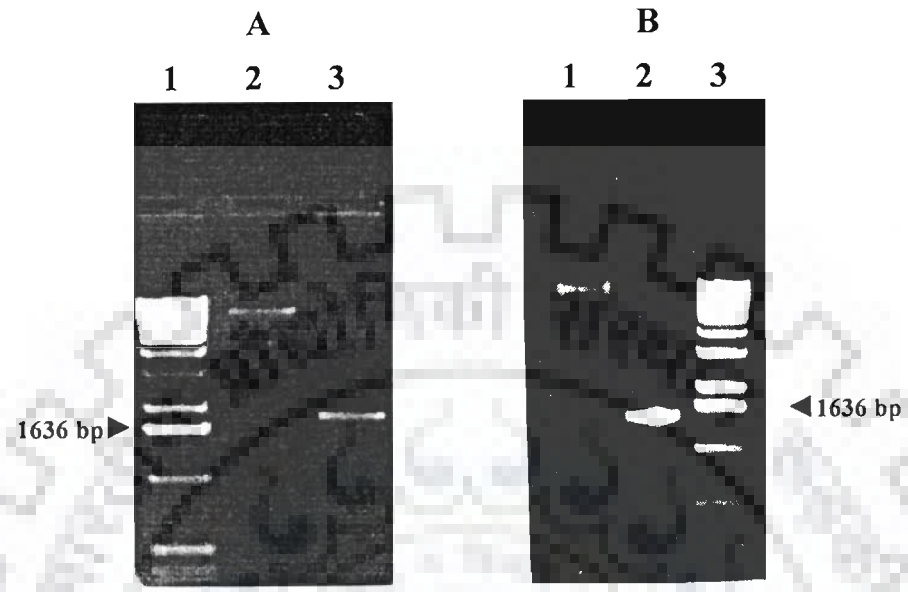


Figure 7. Low-melt agarose gel purification of BHRVs (pMMB66HE and pMMB 206) and human cytochrome P-450 2E1 gene (native and *N*-terminus modified)

A. Low-melt agarose gel purified products of native human cytochrome P-450 2E1 gene and pMMB66HE DNA after restriction digestion with *EcoRI*

Lanes:

1 - 1 Kb ladder (GIBCO-BRL)

2 - *EcoRI* digested and purified pMMB66HE DNA

3 - *EcoRI* digested and purified native human P-450 2E1 gene

B. Low-melt agarose gel purified products of *N*-terminus modified human cytochrome P-450 2E1 gene and pMMB 206 DNA after double digestion with *Eco RI* and *Pst I* enzymes

Lanes:

1 - Purified pMMB 206 DNA digested with *Eco RI* and *Pst I* enzymes

2 -*N*-terminus modified human cytochrome P-450 2E1 PCR product after restriction digestion with *EcoRI* and *Pst I*

3 - 1 Kb ladder (GIBCO-BRL)

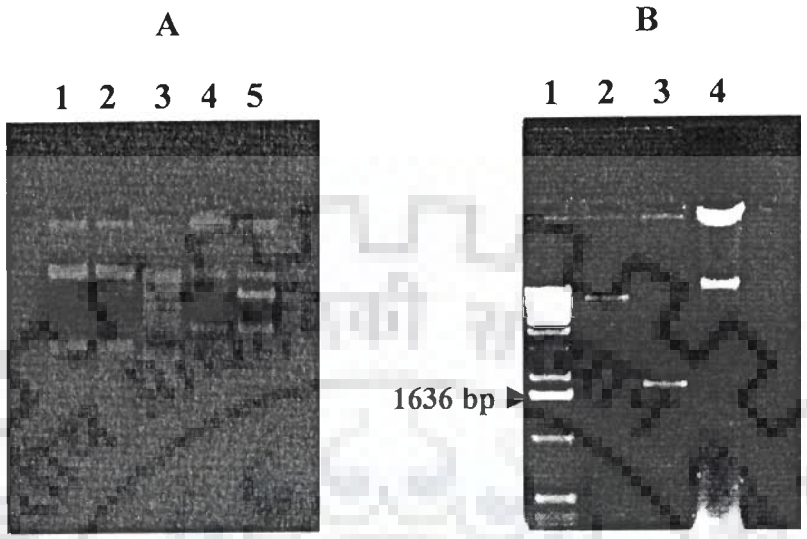


Figure 8. DNA preparations of BHRV pMMB66HE subcloned with native human cytochrome P-450 2E1 gene from *E. coli* and *Pseudomonas putida* clones

A. Plasmid DNA isolated from *E. coli* and *Pseudomonas putida* clones

Lanes:

1-2 - Plasmid DNA from *E. coli* DH5α clones containing native cytochrome P-450 2E1 gene

3 - 1 Kb ladder (GIBCO-BRL)

4-5 - Plasmid DNA from *P. putida* clones containing native cytochrome P-450 2E1 gene

B. DNA preparation of pMMB66HE subcloned with native human cytochrome P-450 2E1 from selected *E. coli* clone

Lanes

1 - 1 Kb ladder (GIBCO-BRL)

2 - Purified pMMB66HE DNA used for subcloning of native cytochrome P-450 2E1

3 - Gel purified native human cytochrome P-450 2E1 gene

4 - BHRV pMMB66HE DNA extracted from selected *E. coli* clone possessing native human cytochrome P-450 2E1(Clone designated as HE-2E1)

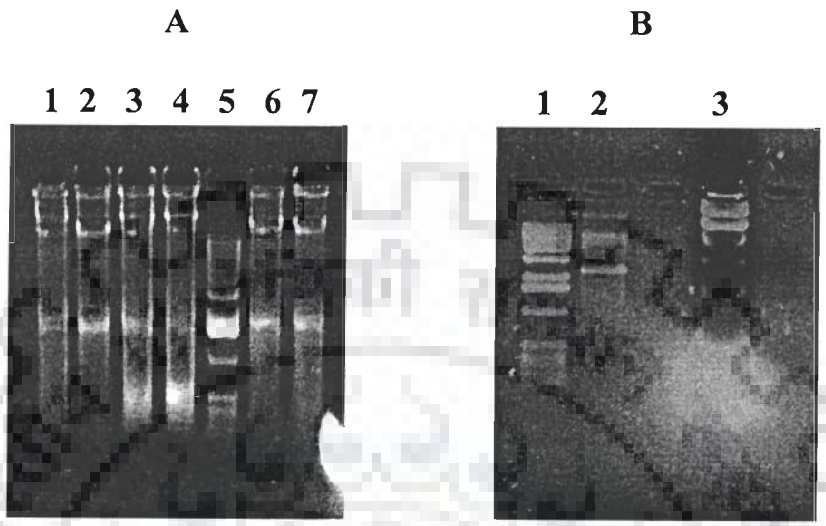


Figure 9. BHRV pMMB 206 subcloned with *N*-terminus modified human cytochrome P-450 2E1 isolated from *E. coli* and *Pseudomonas putida* clones

A. Plasmid DNA isolated from *E. coli* clones
Lanes:
1-4 - Plasmid DNA from *E. coli* clones
5 - 1 Kb ladder (GIBCO-BRL)
6-7 - Plasmid DNA from *Pseudomonas putida* clones

B. Plasmid pMMB 206 isolated from selected *E. coli* and *Pseudomonas putida* clones showing pink cell pellets
Lanes:
1 - 1 Kb ladder (GIBCO-BRL)
2 - Plasmid DNA from selected *E. coli* clone (Clone designated as C4-2E1)
3 - Plasmid DNA from selected *Pseudomonas putida* clone (Clone designated as PpW-lux-2E1)

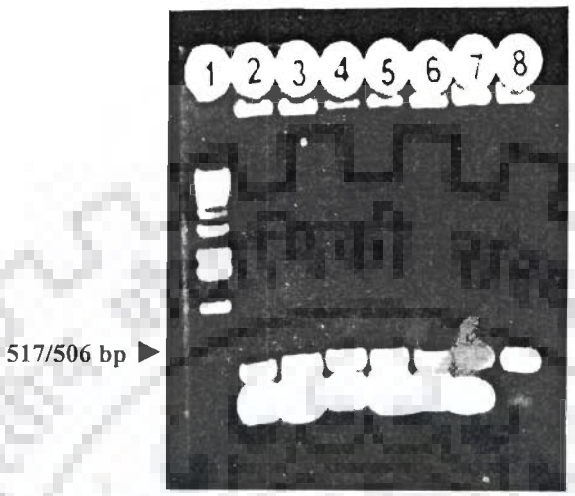


Figure 10. Screening of *E. coli* clones possessing native and *N*-terminus modified human cytochrome P-450 2E1 gene by PCR amplification

(DNA extracted from overnight culture of suspect colonies of transformants was used as template for PCR amplification of target 403 bp at 5'-end of cytochrome P-450 2E1 gene using primers 2E1P1 and 2E1P2)

Lanes:
1 - 1 Kb ladder (GIBCO-BRL)
2-6 *E. coli* clones possessing *N*-terminus modified human cytochrome P-450 2E1 gene
7-8 *E. coli* clones containing native cytochrome P-450 2E1 gene

PCR screening of *Pseudomonas putida* clones expressing luciferase and subcloned with the native 2E1 gene exhibited the required product at 403 bp (Figure 11). The PCR-positive clones cultured in Luria-Bertani medium containing the cytochrome P-450 (IPTG) and luciferase (*m*-toluate) inducers exhibited pink cell pellets, indicating the production of hemoprotein.. However, no light emission via induction of luciferase and reaction in the presence of n-decanal could be observed with these clones.

Pseudomonas putida clones expressing luciferase and subcloned with *N*-terminus modified cytochrome P-450 2E1 exhibited red cell pellets and luminescence when cultured in Luria-Bertani medium containing IPTG and *m*-toluate. In addition, PCR amplification using plasmid DNA extracted from these clones yielded the required product at 403 bp, confirming the presence of the P-450 2E1 gene (Figures 9B and 11B). Clones of *Pseudomonas putida* MTCC 102 containing *N*-terminus modified P-450 2E1 also exhibited red cell pellets on growth in Luria-Bertani medium supplemented with IPTG and the required PCR product at 403 bp (Figure 11). These *Pseudomonas putida* clones were selected for diagnostic CO-difference spectral screening.

3.4 Discussion

Previous reports have highlighted the difficulties in achieving high yields of human cytochrome P-450 2E1 in *E. coli* (Winters and Cederbaum, 1992; Gillam *et al.*, 1994; Dong and Porter, 1996). Several strategies have been developed to overcome this limitation. Enhanced enzyme yields have been

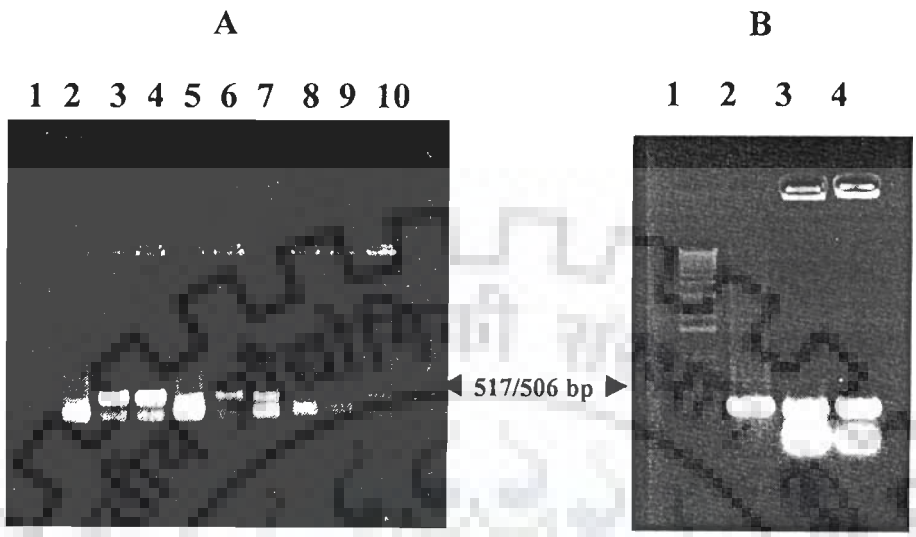


Figure 11. Screening of *Pseudomonas putida clones possessing native or *N*-terminus modified human cytochrome P-450 2E1 gene by PCR amplification using primers 2E1P1 and 2E1P2**

*(co-expressing chromosomally encoded luciferase)

A. PCR-based selection of *Pseudomonas putida* clones containing native or *N*-terminus modified cytochrome P-450 2E1

Lanes:

1 - 1 Kb ladder (GIBCO-BRL)

2-10 Suspect *Pseudomonas putida*-lux transformants

B. PCR amplification of target 403 bp region in selected *Pseudomonas putida* clones

Lanes:

1 - 1 Kb ladder (GIBCO-BRL)

2 - Plasmid DNA of SK^- vector containing native human cytochrome P-450 2E1 gene used as template for PCR (positive control)

3 - *Pseudomonas putida* clone (PpW-lux-2E1) plasmid DNA used as template for PCR

4 - *Pseudomonas putida* clone (PpW-2E1) plasmid DNA used as template for PCR

demonstrated by altering the NH_2-terminal nucleotide (Winters and Cederbaum, 1992) or amino acid sequence (Gillam *et al.,* 1994). Attempts have also included the functional expression of unmodified human cytochrome P-450s 2E1, 3A4 and 2A6 in *E. coli* by generating NH_2-terminal translational fusions to bacterial leader sequences, *pel* B and *omp* A (Pritchard *et al.,* 1997). The successful co-expression of native human P-450 2E1 with rat NADPH-cytochrome P-450 reductase has also been demonstrated earlier (Dong and Porter, 1996). Interestingly, these varied approaches to attain improved cytochrome P-450 2E1 expression in *E. coli* have resulted in nearly similar enzyme yields. The membrane P-450 content obtained by Dong and Porter (1996) with the native human 2E1 gene (0.11 nmol/mg protein) was not significantly different from that reported by Pritchard *et al.,* (1997) with the *omp* A-2E1 construct (0.14 nmol/mg protein). Though the membrane P-450 content obtained by Winters and Cederbaum (1992) with human P-450 2E1 containing silent mutations at the *N*-terminus was slightly lower (0.06 nmol/mg protein), the highest membrane P-450 content was obtained by Gillam *et al.* (1994) for *N*-terminus deleted human P-450 2E1 (0.19 nmol/mg protein). Since this latter construct with the altered *N*-terminus sequence MARQVSS was demonstrated to be the most successful in terms of cytochrome P-450 2E1 expression, the present study focussed on this sequence for the generation of modified P-450 2E1.

In the present study, the *N*-terminus modification of human cytochrome P-450 2E1 was achieved by the removal of the first 21 codons and the replacement of tryptophan with alanine as the second codon (Figure 3). It has been reported

that blocks to translation, such as 'rare codons' are of major importance when present within the first 25-30 codons of a sequence (Pritchard *et al.*, 1997). Hence, the removal of the first 21 codons from the native P-450 2E1 sequence would thus facilitate expression in host bacteria. Moreover, the presence of alanine as the second codon is desirable since it is a strongly preferred second codon after methionine in bacterial genes (Pritchard *et al.*, 1997) and is also considered essential for optimal P-450 expression in *E. coli* (Barnes *et al.*, 1991; Gillam *et al.*, 1994).

In the absence of a known crystal structure for human cytochrome P-450 2E1, computer-derived homology model of the *N*-terminus modified cytochrome P-450 2E1 was generated to determine the effect of the amino acid deletion on the substrate and heme-binding domains. The homology model indicates that the modifications incorporated at the *N*-terminus do not perturb the heme-binding domain or the active-site region of cytochrome P-450 2E1 (Figure 12).

Vector systems utilized for the expression of native or *N*-terminus modified cytochrome P-450 2E1 have been restricted to *E. coli* as host. For instance, the plasmid vectors pCW and pJL have been employed for cytochrome P-450 expression in *E. coli* (Gillam *et al.*, 1994; Dong and Porter, 1996). These plasmid vectors are based on narrow host-range replicons specific for *E. coli* and cannot be stably maintained in other bacterial species. Since the intention of the present investigation was to examine the utility of the environmentally robust host, *Pseudomonas putida* for expression of cytochrome P-450 2E1, the use of broad host range plasmid vectors was necessary. The wide-host range vectors,

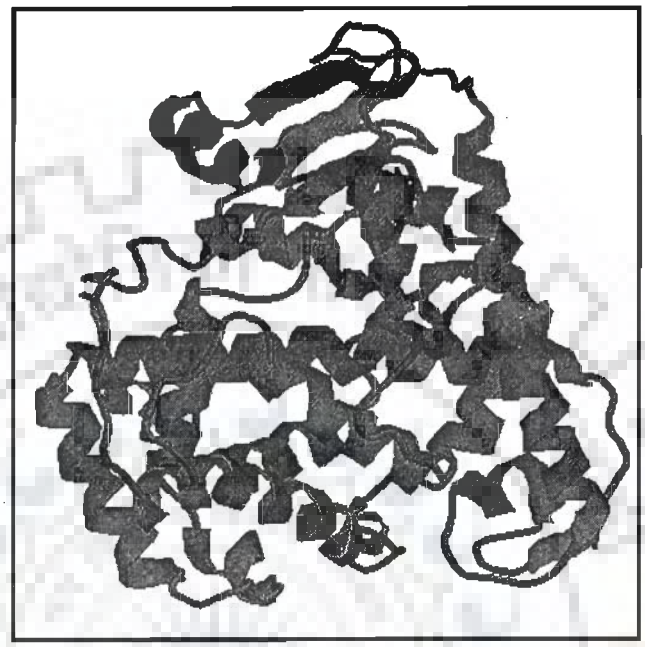


Figure 12. Homology Model of human cytochrome P-450 2E1 protein
(Highlighted region depicts the *N*-terminus deleted from native protein)

pMMB66HE and pMMB 206, are useful for expression in a range of Gram-negative bacteria (Morales *et al.*, 1990; 1991). These controlled-expression vectors are constructed from the same parent plasmid, pMMB66. The vector pMMB66HE encoding ampicillin resistance possesses the P*tac* promoter, while pMMB 206 encoding chloramphenicol resistance possesses the P*taclac* promoter. The vector pMMB 206 also incorporates a lacZα fragment and both vectors contain the *rrn*B transcriptional stop-point after the polylinker (Figure 2).

The DNA of the *N*-terminus modified cytochrome P-450 2E1 subcloned into the *Eco*RI-*Pst*I sites of BHRV pMMB 206 was characterised earlier. To rule out the possible fusion of amino acids (of β-galactosidase from pMMB 206 vector), open-reading frames (ORF) were checked using EditSeq program of DNASTAR. The ORF in the cloned gene differs from the native gene due to *N*-terminus deletion of 21 amino acids. In addition, the second amino acid tryptophan is replaced with alanine which is known to help expression in bacteria.

The over-expression of cytochrome P-450 enzyme has been reported to be toxic to *E. coli*, resulting in plasmid loss and instability (Porter and Larson, 1991). It is therefore desirable to use vectors containing regulated promoters for cytochrome P-450 expression. The vectors pMMB66HE and pMMB 206 were chosen in the present study since both P*tac* and P*taclac* promoters are useful for the expression of genes deleterious to the cell when over-expressed (Morales *et al.*, 1991). Both these vectors also code for the *lacI*^{q} repressor of the P*tac* and P*taclac* promoters, ensuring that the protein is constitutively suppressed but can be induced when appropriate. In the absence of induction with IPTG a low level

of expression of the cloned gene is observed with these vectors. Moreover, induction with IPTG concentrations as low as 0.05 mM (Morales *et al.,* 1991) results in significant increase in the expression levels, thereby demonstrating the usefulness of these vectors in the stable and regulated expression of catabolic gene(s).

The number of *E. coli* and *Pseudomonas putida* clones possessing cytochrome P-450 2E1 gene obtained after transformation was quite low in comparison to cells transformed with only the vector DNA, included as controls. A few discrete colonies of *E. coli* clones were visible on LB-agar plates supplemented with the appropriate antibiotics after an incubation of 2 days at 30°C. However, the small size of these colonies required a prolonged incubation period of 7 days to obtain sufficient growth and pink cell pellets, indicative of hemoprotein production. Discrete red colonies of the *Pseudomonas putida* clones co-expressing cytochrome P-450 2E1 and luciferase developed on LB-agar plates between 10-15 days incubation at 26°C. These colonies exhibiting slow growth required an incubation of 20 days for sufficient growth before transfer to Luria-Bertani broth. In addition, the temperature of incubation also significantly influenced the number and growth of clones. A higher number of colonies could be observed on LB-agar plates incubated at 30°C and 26°C for *E. coli* and *Pseudomonas putida*, respectively. Moreover, the colonies arising at the lower temperatures exhibited red cell pellets not observed with incubations at the higher temperatures of 37°C and 30°C for *E. coli* and *Pseudomonas putida*, respectively. The clones exhibiting red cells consistent with the production of hemoprotein and

yielding the 403 bp P-450 2E1 gene product on PCR amplification (Figures 10 and 11) were selected for diagnostic CO-difference spectral screening.

3.5 Concluding Remarks

PCR mutagenesis was employed to modify the *N*-terminus of human cytochrome P-450 2E1 with the intent to enhance expression in the bacterial hosts, *E. coli* and *Pseudomonas putida* expressing luciferase. Native and *N*-terminus modified human cytochrome P-450 2E1 were subcloned on broad host range vectors, and clones screened by PCR selected for expression studies. The present study thus demonstrates the utility of broad host range vector systems for engineering cytochrome P-450 enzymes like P-450 2E1 for expression in environmentally robust hosts.

Chapter 4

Expression of Native and N-terminus Modified Human Cytochrome P-450 2E1 in E. coli and Pseudomonas putida

4.1 Introduction

The successful expression of human cytochrome P-450 2E1 in *E. coli* has been achieved by a variety of approaches including modification of the NH_2-terminus of the protein (Winters and Cederbaum, 1992; Gillam *et al.*, 1994; Dong and Porter, 1996; Pritchard *et al.*, 1997). However, these attempts have been constrained by poor yields as well as the labile nature of the recombinant protein. The reasons for the low content of cytochrome P-450 2E1 in *E. coli* relative to other mammalian P-450 enzymes are not known, but have been attributed to poor translation as also a rapid degradation of this enzyme in *E. coli* cells (Dong and Porter, 1996). The expression of catalytic activity of microsomal cytochrome P-450 enzymes in bacteria is also hindered because bacteria lack NADPH-cytochrome P-450 oxido-reductase, the natural electron transfer partner for these enzymes. Hence, recreating a functional microbial system capable of catalyzing P-450 mediated reactions presents the difficult task of providing a source of electrons for reduction of the heterologously expressed enzyme.

Different approaches to resolve this issue include the co-expression of cytochrome P-450 enzyme with NADPH-cytochrome P-450 reductase in *E. coli* (Dong and Porter, 1996). The co-expression of human cytochrome P-450 3A4, NADPH-cytochrome P-450 reductase and cytochrome b5 in *E. coli* has been demonstrated and resulted in enhanced *in vivo* enzymatic activity and stability of the hemoprotein in *E. coli* (Voice *et al.*, 1999). Fusion proteins composed of a cytochrome P-450 domain and the NADPH-cytochrome P-450 reductase domain have also been generated in an attempt to achieve catalytically self-sufficient

monooxygenase systems (Fisher *et al.*, 1992; 1993; Shet *et al.*, 1993; 1994; Blake *et al.*, 1996; Chun *et al.*, 1996; 1997; Parikh and Guengerich, 1997). In addition, bacterial flavoproteins have been utilized as alternate electron transfer partners for the cytochrome P-450 enzymes (Yamazaki *et al.*, 1995; Dong *et al.*, 1996). It has been demonstrated that flavodoxin and NADPH-flavodoxin reductase from *E. coli* were in combination able to support the catalytic activity of heterologously expressed bovine 17α-hydoxylase cytochrome P-450 (Jenkins and Waterman, 1994). Luciferase, a flavin monooxygenase has also been implicated in the *in vivo* reduction of cytochrome P-450 cam. The luciferase-reduced enzyme was demonstrated to be metabolically competent and mediated the transformation of a number of halogenated substrates (Shanker and Atkins, 1996).

The present study evaluates the expression of native and *N*-terminus modified cytochrome P-450 2E1 in *E. coli* and *Pseudomonas putida* co-expressing luciferase. The study also explores the possibility of luciferase to serve as an alternate electron transfer partner for the expressed P-450 2E1 enzyme.

4.2 Materials and Methods

4.2.1 Chemicals and Glassware

Bacto-tryptone, yeast extract and Noble agar were purchased from DIFCO Laboratories (Detroit, USA). The antibiotics chloramphenicol, ampicillin and piperacillin were obtained from SIGMA Chemical Company (St. Louis, USA). Centricon protein purification columns were obtained from Amicon Inc. (Beverly, MA, USA). Coomassie Plus Protein Assay kit was obtained from Pierce Chemical Company (Illinois, USA). All other chemicals were of the highest purity

commercially available. Glassware utilized was borosilicate glass of Borosil or Vensil make.

4.2.2 Culture Media

4.2.2.1 Terrific Broth

The culture medium contained per litre: 12g Bacto-tryptone, 24g Bacto-yeast extract and 4 ml glycerol. The medium was sterilized by autoclaving at 121°C for 15 min. 100 ml of a sterile solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 was added to the medium prior to use (Sambrook *et al.*, 1989).

4.2.3 Bacterial Constructs

The *E. coli* constructs possessing the native or *N*-terminus modified cytochrome P-450 2E1 gene on broad host range vectors were utilized in the study. Genetically engineered *Pseudomonas putida* expressing luciferase lux AB genes and possessing native or *N*-terminus modified cytochrome P-450 2E1 were also employed in the study. In addition, the *P. putida* construct possessing only the *N*-terminus modified cytochrome P-450 on a BHRV pMMB 206 was also used. The construction of all these strains has been described in Chapter 3. The bacterial hosts viz. *E. coli* DH5α and genetically engineered *Pseudomonas putida* expressing luciferase lux AB genes (Shanker and Atkins, 1996) were utilized as the control strains for the expression studies.

4.2.4 Optimization of Growth Conditions of Bacterial Hosts

The expression of cytochrome P-450 enzymes in bacteria is greatly influenced by the culture conditions employed. Culture conditions such as the time and temperature of incubation and the initial inoculum levels are important

determinants in obtaining high enzyme yields. There is hence a need for establishing the optimal growth conditions for each P-450 expressing strain. The expression levels of the enzyme were examined in cells grown in different media such as Terrific broth or Luria-Bertani medium, inoculated with varying initial inoculum levels ranging from 0.1% to 10% of the culture media volume. In addition, the cultures were grown at temperatures ranging between 30°C to 37°C for the *E. coli* constructs and between 26-30°C for the *Pseudomonas putida* constructs. To determine the optimal incubation period, the time-course of cytochrome P-450 2E1 expression was monitored for all the constructs by varying the incubation time from 16 to 42h. Diagnostic CO-difference spectra were recorded for all the constructs cultivated under the various growth conditions and used as a measure to establish optimal growth conditions for P-450 expression.

4.2.4.1 Optimization of Growth Conditions for the E. coli constructs

Single colonies of the selected *E. coli* clones possessing either the native or *N*-terminus modified cytochrome P-450 2E1 were grown for 18h at 30°C in 5ml Luria-Bertani medium supplemented with 50 μg/ml ampicillin or 20 μg/ml chloramphenicol and 1mM IPTG. 100 ml of Terrific broth or Luria-Bertani medium was inoculated with various volumes (0.1% to 1.0% (v/v) of the overnight culture. The culture media was supplemented with 50 μg/ml ampicillin or 20 μg/ml chloramphenicol, 1 mM IPTG and 5 μM 4-methyl pyrazole, a high-affinity ligand for cytochrome P-450 2E1. Cells were incubated at 30°C and 37°C and harvested at various time intervals between 16-42h by centrifugation at

8000 xg for 10 min at 4°C and fractionated for the determination of diagnostic CO-difference spectrum (as described in section 4.2.6)

4.2.4.2 Optimization of growth conditions for Pseudomonas putida constructs

Single colonies of the selected *Pseudomonas putida* clones co-expressing luciferase and native or *N*-terminus modified human cytochrome P-450 2E1 were grown for 36h in 5 ml Luria-Bertani medium supplemented with 50 μg/ml ampicillin or 20 μg/ml chloramphenicol, 1 mM IPTG and 200 μM of the luciferase inducer, *m*-toluate. Various aliquots from this culture (0.1% to 2.0%, v/v) were inoculated in either 100 ml Terrific broth or Luria-Bertani medium. The culture medium was amended with 50 μg/ml ampicillin or 20 μg/ml chloramphenicol, 1 mM IPTG, 200 μM *m*-toluate and 5 μM 4-methylpyrazole and incubated at either 26°C or 28°C on an orbital shaker at 175 rpm. Cells were harvested at various time intervals between 16-42h by centrifugation at 8000 xg for 10 min at 4°C and fractionated for the determination of CO-difference spectra as described in sections 4.2.5 and 4.2.6. In addition, luciferase expression in the cells was also monitored periodically by measuring luminescence directly in an aliquot of culture as described in section 4.2.7.

A single colony of the *Pseudomonas putida* clone possessing *N*-terminus modified human cytochrome P-450 2E1 was grown for 18h in 5 ml Luria-Bertani medium supplemented with 20 μg/ml chloramphenicol and 1 mM IPTG. Different aliquots of the overnight culture (0.1% to 1.0%, v/v) were used to inoculate 100 ml of either Terrific broth or Luria-Bertani medium amended with 20 μg/ml chloramphenicol and 1mM IPTG. Cells were incubated at 26°C or 28°C on an

orbital shaker at 175 rpm and harvested between 16-42h by centrifugation at 8000 xg and 4°C for 10 min. The harvested cells were fractionated and utilized for recording the CO-difference spectra as described in sections 4.2.5 and 4.2.6.

4.2.5 Subcellular Fractionation

Cell pellets of the desired clones obtained after centrifugation of the cultures at 8000 xg and 4°C for 10 min were resuspended in 5 volumes (w/v) of ice-cold 100 mM sodium phosphate buffer, pH 7.4, containing 50 mM KCl and 50 μM 4-methylpyrazole. All subsequent steps were performed at 0-4°C. The cell suspension was subjected to sonication for 5 min in cycles of 20s pulse with a 15s interval after each pulse. Sonication was performed at 50W in a '*Torbeo*' ultrasonic processor. The disrupted cells were centrifuged at 9000 xg for 10 min at 4°C to remove residual whole cells and cell debris. The cell lysate was then centrifuged at 1,50,000 xg for 1h at 4°C in TLA-100.4 Rotor in a Beckman Optima TLX ultracentrifuge (Beckman, USA). The resulting supernatant was utilized for the determination of diagnostic CO-difference spectra. The 1,50,000 xg pellet was resuspended in ice-cold 100 mM sodium phosphate buffer, pH 7.4, containing 50 mM KCl and 50 μM 4-methylpyrazole and utilized for the determination of diagnostic CO-difference spectra as described in section 4.2.6.

4.2.6 CO-difference Spectroscopy

The expression of native and *N*-terminus modified human cytochrome P-450 2E1 in the *E. coli* and *Pseudomonas putida* constructs was evaluated by recording the diagnostic CO-difference spectra in the cell-fractions of each construct. CO-difference spectra were recorded as described by Omura and

Sato (1964). Difference spectra were obtained after reduction of the subcellular fractions with sodium dithionite crystals (approximately 100mg). Aliquots of the dithionite-reduced 1,50,000 xg supernatant (1ml each) were placed in the sample and reference cuvettes and utilized for recording a baseline from 550 nm to 350 nm on a dual beam Perkin-Elmer *lambda* 900 UV-Vis spectrophotometer (Perkin-Elmer, USA). Carbon monoxide gas was subsequently bubbled slowly through only the sample cuvette for 1-2 min and the resulting CO-difference recorded from 550 nm to 350 nm. Similarly, the 1,50,000 xg pellet resuspended in sodium phosphate buffer, was reduced with a pinch of sodium dithionite crystals and utilized for determination of the CO-difference spectra.

The cytochrome P-450 content was calculated from the reduced CO-difference spectra obtained for the individual constructs using an absorbance coefficient of $91 mM^{-1}cm^{-1}$, for the difference in absorbance between 490 nm and 450 nm (Omura and Sato, 1964).

4.2.7 Bioluminesescence Measurement

The expression of luciferase lux AB genes in the *Pseudomonas putida* constructs co-expressing native or *N*-terminus modified cytochrome P-450 2E1 and luciferase was evaluated by monitoring light emission in cells grown in the presence of the luciferase inducer, *m*-toluate. Cells were grown in 100 ml Terrific broth amended with 50 μg/ml ampicillin or 20 μg/ml chloramphenicol, 1mM IPTG and 200 μM *m*-toluate and incubated at 28°C on an orbital shaker at 175 rpm for 16-42h. Aliquots of culture (1 ml) were withdrawn at various time

intervals and luminescence monitored in a luminometer after the addition of 1µl *n*-decanal, the luciferase substrate.

4.2.8 SDS-PAGE of N-terminus modified human cytochrome P-450 2E1

SDS-Polyacrylamide gel electrophoresis of the 1,50,000 xg supernatant and pellet fractions of the *E. coli* construct possessing *N*-terminus modified cytochrome P-450 2E1 was performed as described by Sambrook *et al.* (1989). Electrophoresis was performed using the discontinous buffer system on 10% (w/v) polyacrylamide gels and the gels stained with Coomassie Brilliant Blue. The protein content in the bacterial fractions was estimated using the Coomassie Plus Protein Assay reagent (Pierce Chemical Co., Illinois, USA) with bovine serum albumin as standard.

4.2.8.1 Preparation of Samples for SDS-PAGE

The 1,50,000 xg supernatant and pellet fractions containing the *N*-terminus modified cytochrome P-450 2E1 were prepared as described earlier from the *E. coli* cells expressing the hemoprotein. Cell fractions were concentrated by selective ultrafiltration using Centricon concentrators (Amicon Inc., USA) possessing different cut-off values (C-30, C-50 and C-100). The 1,50,000 xg supernatant of the host organism *E. coli* DH5α was also included as control.

4.2.8.2 Preparation of SDS-Polyacrylamide gels

- The appropriate volumes of solutions containing the desired concentration of acrylamide for 10% (w/v) resolving gel were taken in an Erlenmeyer flask and mixed well. The acrylamide solution was poured between the glass plates with sufficient space remaining for the stacking gel and carefully overlaid with

isobutanol. After polymerization was complete (approximately 30 min), overlay was poured off and the gel washed several times with deionized water to remove any unpolymerized acrylamide

- The stacking gel was similarly prepared by placing the appropriate volumes of solutions containing the desired concentration of acrylamide in a disposable plastic tube. The stacking gel solution was poured directly onto the surface of the polymerized resolving gel and a clean teflon comb immediately inserted into the stacking gel solution. After polymerization, the gel was washed thoroughly with deionized water
- The protein samples were denatured by heating to 100^0C for 3 min in 1X SDS gel loading buffer (50 mM Tris-Cl [pH 6.8]; 10 mM DTT; 2% SDS; 0.1 % bromophenol blue; 10% glycerol) and loaded into the gel slots
- Electrophoresis was performed in Tris-glycine buffer (25 mM Tris; 250 mM glycine, pH 8.3; 0.1% SDS) with an initial voltage of 8 V/cm. After the dye moved into the resolving gel, the voltage was increased to 15 V/cm and the gel run until the bromophenol blue reached the bottom

4.2.8.3 Staining SDS Polyacrylamide Gels with Coomassie Brilliant Blue

The gel was immersed in atleast 5 volumes of the staining solution (0.25g Coomassie Brilliant Blue R250 dissolved in 90 ml of methanol:water [1:1, v/v] and 10 ml of glacial acetic acid) and placed on a slowly rotating platform at room temperature. After 4h the stain was removed and the gel destained by soaking in methanol/acetic acid (90 ml of methanol and 10 ml of acetic acid). The destaining

solution was changed 3-4 times and the gel stored in water containing 20% glycerol in a sealed plastic bag.

4.2.9 Co-expression of N-terminus modified cytochrome P-450 2E1 and luciferase in Pseudomonas putida

4.2.9.1 Time-dependence of cytochrome P-450 2E1 and luciferase expression

The time-course of expression of the engineered traits was monitored in growing cultures of the *Pseudomonas* construct co-expressing *N*-terminus modified cytochrome P-450 2E1 and luciferase (PpW-lux-2E1). Cytochrome P-450 2E1 content was evaluated in the cell fractions by reduced CO-difference spectra and luciferase expression was monitored by luminescence detection.

Pseudomonas putida possessing *N*-terminus modified P-450 2E1 and luciferase (PpW-lux-2E1) was grown in 100 ml Terrific broth inoculated with 2 ml aliquot of a 36h grown culture. The medium was supplemented with 20 μg/ml chloramphenicol, 1 mM IPTG, 200 μM *m*-toluate and 5 μM 4-methylpyrazole and incubated at 26°C on an orbital shaker at 175 rpm. Cells were harvested at different intervals for the determination of the CO-difference spectra and luminescence detection as described in sections 4.2.6 and 4.2.7

4.2.9.2 Effect of chloramphenicol on cytochrome P-450 2E1 and luciferase expression in Pseudomonas putida

The influence of the antibiotic chloramphenicol on cytochrome P-450 2E1 and luciferase expression in *Pseudomonas putida* was determined in cells grown in the presence or absence of the antibiotic chloramphenicol.

PpW-lux-2E1 cells were grown in 5 ml Luria-Bertani medium supplemented with 200 μM *m*-toluate and 5 μM 4-methylpyrazole and incubated at 26°C for 36h. An aliquot (2 ml) from this culture was transferred to 100 ml Terrific broth amended with 1 mM IPTG, 200 μM *m*-toluate and 5 μM 4-methyl pyrazole but lacking the antibiotic chloramphenicol. Cells were incubated at 26°C on an orbital shaker at 175 rpm. In addition, PpW-lux-2E1 cells were grown under identical conditions in 100 ml Terrific broth containing 20 μg/ml chloramphenicol, 1 mM IPTG, 200 μM *m*-toluate and 5 μM 4-methylpyrazole. Cells grown in the absence and presence of chloramphenicol were harvested at 26h for recording the reduced CO-difference spectra and monitoring luminescence as described earlier (sections 4.2.6 and 4.2.7).

4.2.9.3 Effect of variable inducer (m-toluate) concentration on luciferase expression in Pseudomonas putida

The effect of varying concentrations of the inducer, *m*-toluate on luciferase expression was evaluated in *Pseudomonas putida* co-expressing cytochrome P-450 2E1 and luciferase (PpW-lux-2E1).

PpW-lux-2E1 cells were grown for 36h in 5ml Luria-Bertani medium supplemented with 20 μg/ml chloramphenicol and 1 mM IPTG, but lacking *m*-toluate. An aliquot from this culture (0.1ml) was transferred to 25 ml Terrific broth taken in 125 ml Erlenmeyer flasks and supplemented with 20 μg/ml chloramphenicol, 1 mM IPTG and a range of *m*-toluate concentrations (0-200 μM). A similar set of flasks containing 25 ml Terrific broth supplemented with 20 μg/ml chloramphenicol and a range of *m*-toluate concentrations (0-200 μM) but

lacking IPTG, was also included. All flasks were incubated at 26°C on an orbital shaker at 175 rpm. After 26h, 1ml aliquot of culture was withdrawn from the different flasks for luminescence detection, as described earlier (section 4.2.7).

4.2.10 Spectral determination of luciferase mediated reduction of N-terminus modified cytochrome P-450 2E1

In-vitro assays were performed to investigate the possibility of luciferase mediated reduction of the *N*-terminus modified cytochrome P-450 2E1 in the absence of the reductant, sodium dithionite. The reduction of the hemoprotein by luciferase was assessed by recording the CO-difference spectra in the 1,50,000 xg supernatants of *P. putida* expressing only modified cytochrome P-450 2E1 (construct PpW-2E1) and *P. putida* expressing only luciferase lux AB (PpW-lux).

To determine whether luciferase reduced the hemoprotein in a light dependent (photoreduction) or light-independent mechanism, experiments were performed both in the presence and the absence of decanal-induced luminescence (termed as light and dark reactions, respectively)

4.2.10.1 Growth and Subcellular fractionation of Pseudomonas putida variants

Pseudomonas putida expressing *N*-terminus modified cytochrome P-450 2E1 (construct PpW-2E1) was grown in 100 ml Luria Bertani medium supplemented with 20 μg/ml chloramphenicol, 1 mM IPTG and 5 μM 4-methylpyrazole. Cells were incubated at 26°C on an orbital shaker at 175 rpm for 28h and harvested by centrifugation at 8000 xg for 10 min at 4°C. The cell pellet was resuspended in 5 volumes (w/v) of 100 mM sodium phosphate buffer (pH 7.4) containing 50 mM KCl and 50 μM 4-methylpyrazole For the preparation of

the 1,50,000 xg supernatant, the cells were subjected to fractionation as described earlier (section 4.2.5). The cytochrome P-450 2E1 content in the resulting 1,50,000 xg supernatant was evaluated by recording the CO-difference spectra in an aliquot of the supernatant, after reduction with sodium dithionite according to the methodology described earlier (section 4.2.6).

A single colony of *Pseudomonas putida* expressing luciferase lux AB (PpW-lux) was grown in 5 mL Luria-Bertani medium supplemented with 30 μg/ml piperacillin and incubated at 30°C for 23h. An aliquot (0.1ml) was used to inoculate 100 ml of Luria-Bertani medium amended with 30 μg/ml piperacillin and 200 μM *m*-toluate and incubated at 30°C on an orbital shaker at 175 rpm. Cells were harvested after 23h by centrifugation at 8000 xg and 4°C for 10 min and resuspended in 10 volumes (w/v) of 100 mM sodium phosphate buffer pH 7.4, containing 50 mM KCl. Cells were lysed by sonication at 50W for 5 min using cycles of 20s pulse and 15s interval in a *Torbeo* Ultrasonic Processor. The crude extract was centrifuged at 9000 xg at 4°C for 10 min to remove unbroken cells and the cell debris. The supernatant obtained was then centrifuged at 1,50,000 xg for 1h at 4°C TLA-100.4 Rotor in Beckman Optima TLX ultracentrifuge (Beckman, USA). An aliqout from the resulting supernatant was used for luminescence detection in a luminometer. For *in-vitro* assays, the 1,50,000 xg supernatant was either utilized as such or after concentration using a Centricon protein concentration device. The 1,50,000 xg supernatant was concentrated approximately 13-fold by ultrafiltration using Centricon C-50 columns (Amicon Inc., USA) and stored at -70°C until use.

4.2.10.2 Luciferase-dependent photo-reduction of cytochrome P-450 2E1: light reaction

For the determination of luciferase and light dependent reduction of cytochrome P-450 2E1, 1 ml of 1,50,000 xg supernatant of PpW-2E1 cells was taken in a 5ml serum vial and mixed with an equal volume of 1,50,000 xg supernatant of PpW-lux cells. Luminescence corresponding to 1 ml of PpW-lux 1,50,000 xg supernatant was determined in a separate aliquot after the addition of 1µl *n*-decanal. Luminescence was detected as RLU in a luminometer and was equivalent to 7.4 x 10^6 RLU. The contents of the serum vial were mixed gently and the vial sealed with a butyl rubber stopper. The vial was evacuated on ice for 12 min at 15 inches of Hg vacuum. After evacuation 1µl *n*-decanal was injected into the vial and the resulting luminescence allowed to stabilize for 15 min at room temperature. The serum vial was subsequently opened and the contents of the vial transferred rapidly in 1ml aliquots to spectrophotometer cuvettes. A baseline scan was obtained between 550 nm to 350 nm on a Perkin-Elmer *lambda* 900 UV-vis spectrophotometer. Difference spectra were then recorded after repeated bubbling of the sample cuvette with carbon monoxide according to the methodology described earlier (section 4.2.6).

4.2.10.3 Effect of varying luciferase concentrations on photo-reduction of cytochrome P-450 2E1

In an attempt to determine the minimum luciferase concentration required to achieve photo-reduction of cytochrome P-450 2E1 judged by the formation of the characteristic Soret peak at 452 nm, different volumes of the concentrated

1,50,000 xg luciferase supernatant were employed for the *in-vitro* assays. Luciferase concentration was monitored as RLU by luminescence detection in a luminometer.

The assays with variable luciferase concentrations were identical to those described earlier. 1 ml of 1,50,000 xg PpW-2E1 supernatant taken in a 5 ml serum vial was mixed with an aliquot of the concentrated PpW-lux supernatant. The final reaction volume was adjusted to 2 ml by the addition of 100 mM sodium phosphate buffer pH 7.4, containing 50 mM KCl. Luminescence was determined separately in an identical volume of the concentrated 1,50,000 xg supernatant and corresponded to 8.0×10^6 RLU. The contents of the vial were mixed gently and the vial sealed with butyl rubber stopper. The vial was evacuated on ice for 12 min at 15 inches Hg vacuum. Subsequently 1µl *n*-decanal was injected into the vial and the resulting luminescence allowed to decay for 15 min before transferring the contents of the vial to spectrophotometer cuvettes as described earlier. Difference spectra were then obtained on a dual beam Perkin-Elmer *lambda* 900 UV-vis spectrophotometer as described earlier.

Similar assays were performed with progressively lower luciferase concentrations evaluated for their ability to reduce the hemoprotein and yield the characteristic 452 nm peak. The concentrations employed were one-half and one-fifth of the initial concentration and corresponded to luminescence of 4×10^6 RLU and 1.6×10^6 RLU, respectively.

4.2.10.4 Luciferase-dependent reduction of cytochrome P-450 2E1 in the absence of luminescence: dark reaction

Luciferase-mediated reduction of cytochrome P-450 2E1 in the absence of luminescence was determined by mixing 1 ml of 1,50,000 xg PpW 2E1 supernatant with 0.1 ml of the concentrated 1,50,000 xg PpW-lux supernatant taken in a 5 ml serum vial. 100 mM sodium phosphate buffer pH 7.4 containing 50 mM KCl was added to the vial to a final reaction volume of 2 ml and the vial sealed with a butyl rubber stopper. The luciferase concentration utilized in the assay was determined by measuring luminescence separately in a volume of the concentrated PpW-lux supernatant identical to that used for the assay and corresponded to 3.7 x 10^7 RLU. The serum vial was then vacuumed on ice for 15 min at 15 inches of Hg vacuum and allowed to stabilize for 5 min at room temperature. The contents of the vial were subsequently transferred to spectrophotometer cuvettes for recording the CO-difference spectra as described earlier. A baseline was recorded between 550 nm to 350 nm. Carbon monoxide gas was bubbled through the sample cuvette and the difference spectra obtained in the absence of the reductant, sodium dithionite.

CO-difference spectra were also recorded utilizing only the 1,50,000 xg PpW-lux supernatant to negate the possibility of any interference contributed by the high luciferase concentrations employed in the light and the dark reactions. The spectra were monitored in the concentrated 1,50,000 xg PpW-lux supernatant with the luciferase concentration employed similar to that utilized in the luciferase-mediated reduction of modified 2E1 in the absence of luminescence:

dark reaction (5.1 x 10^7 RLU). CO-difference spectra were recorded with the concentrated luciferase supernatant reduced with sodium dithionite crystals. In addition, CO-difference spectra were also recorded separately in the presence of both dithionite and *n*-decanal. Difference spectra were recorded after the addition of dithionite and *n*-decanal and allowing the light emission to decay for 15 min. All spectra were obtained in a manner similar to that described earlier for the light and dark reactions.

4.3 Results

4.3.1 Expression of native human cytochrome P-450 2E1 in E. coli and Pseudomonas putida

The optimum P-450 expression monitored by diagnostic CO-difference spectra in *E. coli* subcloned with native human cytochrome P-450 2E1 (Construct HE-2E1) yielded the best results with cells cultivated in Terrific broth using an initial inoculum of 0.5% (v/v), and incubated at 30^0C for 22h. However, the diagnostic CO-difference spectra obtained exhibited reduced levels of functional P-450. The difference spectrum recorded also failed to yield an absorbance maximum at 452 nm, which is characteristic for P-450 2E1, instead yielding a maximum at 455 nm (Figure 13). Moreover, the *Pseudomonas putida* construct possessing luciferase and native cytochrome P-450 2E1 subcloned in plasmid pMMB66HE did not yield any detectable luminescence or a defined spectral maximum at 452 nm.

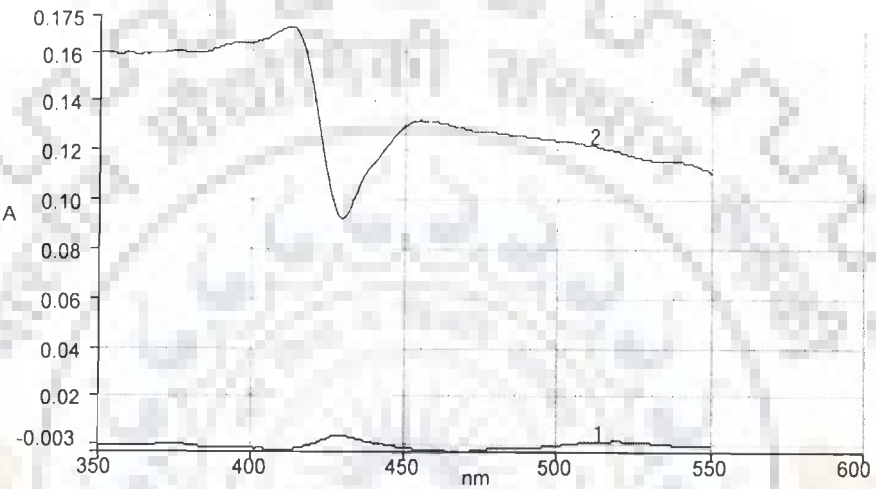


Figure 13. Expression of native human cytochrome P-450 2E1 in *E. coli* monitored by CO-difference spectra

(Spectra monitored in 1,50,000 xg supernatant of *E. coli* cells subcloned with native cytochrome P-450 2E1 grown for 18h at 30°C)

1- Baseline recorded after the addition of dithionite as reductant

2- Difference spectrum recorded after saturation of sample cuvette with carbon monoxide

4.3.2 Expression of N-terminus modified cytochrome P-450 2E1 in E. coli and Pseudomonas putida

Diagnostic CO-difference spectra were recorded in cell fractions of the *E. coli* construct possessing modified cytochrome P-450 2E1 (C4-2E1). The maximum P-450 2E1 yield was obtained in cells grown for 25h in Terrific broth with an initial inoculum of 1% (v/v) and incubated at 30^0C (Figure 14A). The spectra recorded in the 1,50,000 xg supernatant displayed the defined spectral maximum of this cytochrome P-450 at 452 nm under the expression conditions. The 1,50,000 xg supernatant of the *E. coli* host did not indicate any absorbance at 450 nm (Figure 14B). Subsequent analysis indicated that the presence of 4-methylpyrazole, both during growth and cell fractionation, increased the stability of the protein evidenced by the defined Soret peak at 452 nm in difference spectra recorded in the 1,50,000 xg supernatant (Figure 15A). CO-difference spectra recorded in the 1,50,000 xg pellet did not exhibit a defined absorbance peak at 452 nm. In addition high absorbance in the 420 nm region was observed indicating negligible P-450 activity in this fraction (Figure 15B). Optimum yields of spectrally detectable cytochrome P-450 2E1 in the *E. coli* construct, C4-2E1, as produced at 30^0C and a 25h-incubation time routinely averaged 35-40 nmol of P-450/liter culture.

Diagnostic CO-difference spectra recorded in the 1,50,000 xg supernatant of *Pseudomonas putida* co-expressing *N*-terminus modified cytochrome P-450 2E1 and luciferase (PpW-lux-2E1) indicated optimum P-450 and luciferase expression at 26h. The characteristic Soret peak at 452 nm was evidenced in cells

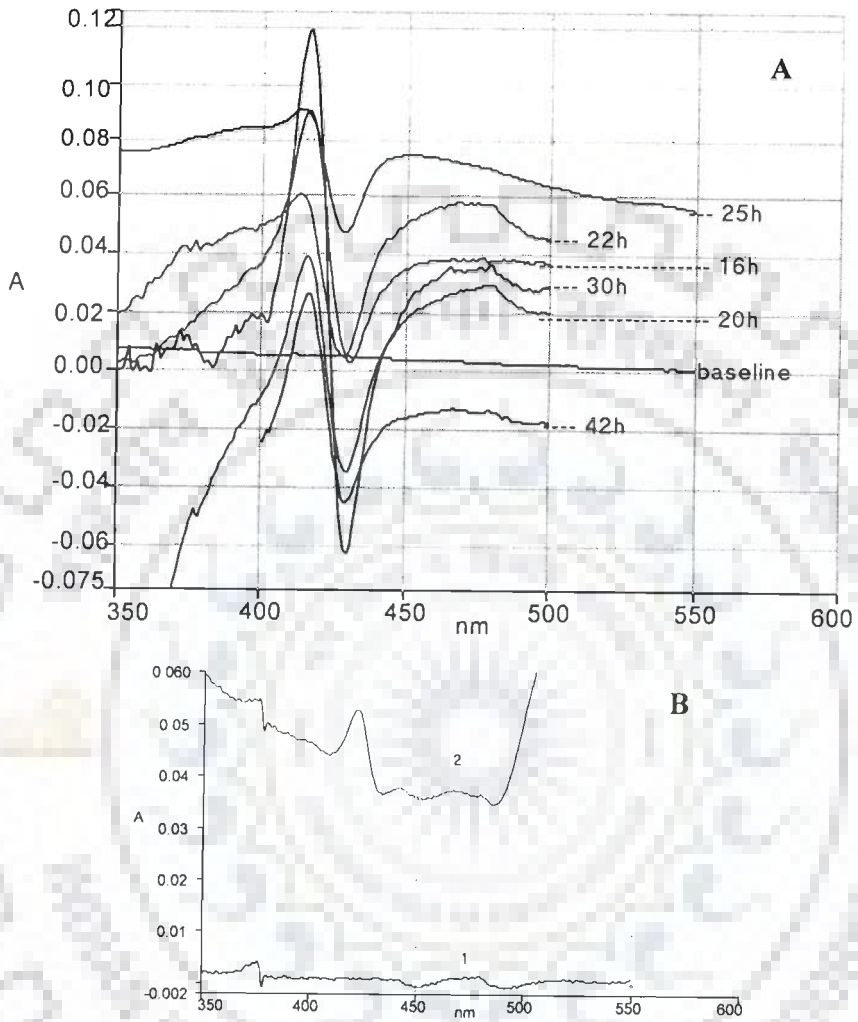


Figure 14. Time-course of cytochrome P-450 2E1 expression in *E. coli* subcloned with *N*-terminus modified human cytochrome P-450 2E1 monitored by carbon monoxide difference spectra

A. CO-difference spectra recorded in 1,50,000 xg supernatant of *E. coli* cells expressing *N*-terminus modified P-450 2E1 grown for different periods (16-42h) at 30°C

B. CO-difference spectra recorded in 1,50,000 xg supernatant of host strain *E. coli* DH5α

1 - Baseline recorded after the addition of dithionite as reductant

2 - Difference spectra recorded after saturation of sample cuvette with carbon monoxide

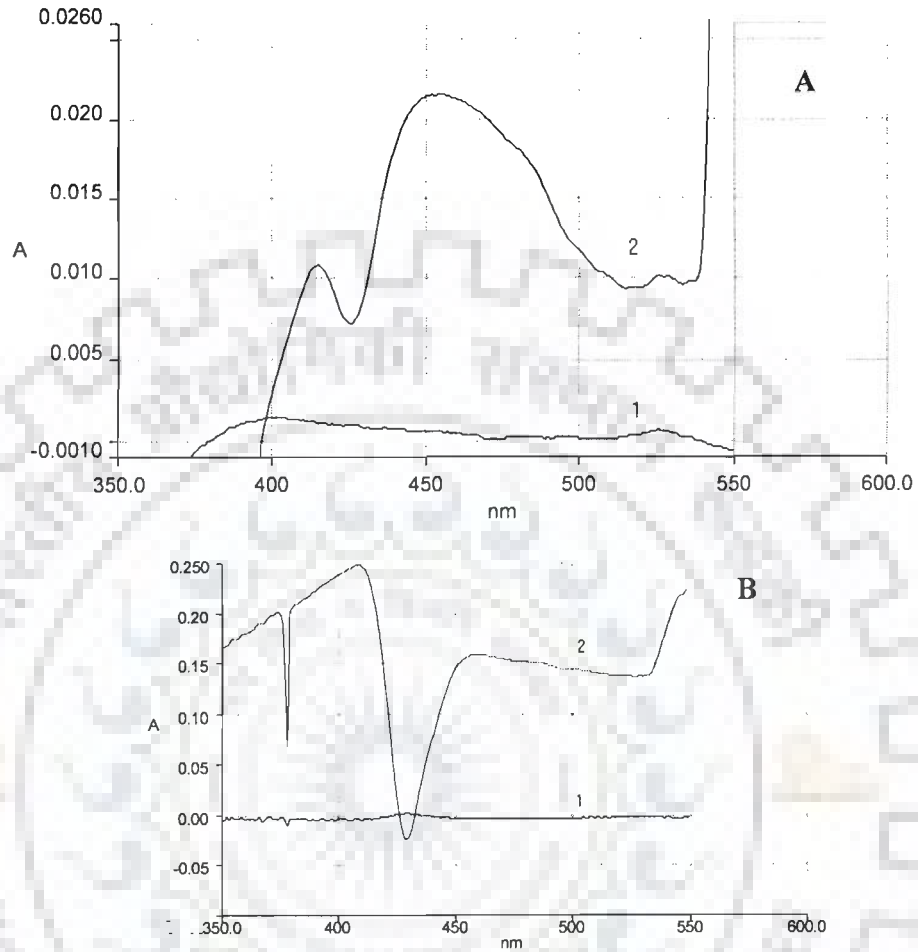


Figure 15. Expression of *N*-terminus modified human cytochrome P-450 2E1 in *E. coli* monitored by carbon monoxide difference spectra

(Spectra recorded in 1,50,000 xg supernatant and pellet fractions of cells grown for 25h at 30°C in presence of 4-methyl pyrazole)

1- Baseline recorded after the addition of dithionite as reductant

2- Difference spectra recorded after saturation of sample cuvette with carbon monoxide

A. Carbon monoxide difference spectrum recorded in 1,50,000 xg supernatant

B. Carbon monoxide difference spectrum recorded in 1,50,000 xg pellet

grown in Terrific broth with an initial inoculum of 2% (v/v) and incubated at 26^0C for 26h (Figure 16A and 16B). Cytochrome P-450 2E1 yields in this construct (PpW-lux-2E1) routinely averaged 40-50 nmol of P-450/liter culture, under the afore-mentioned growth conditions.

Similarly, cytochrome P-450 2E1 expression in *Pseudomonas putida* possessing *N*-terminus modified cytochrome P-450 2E1 (PpW-2E1) evaluated under different growth conditions indicated the highest yields of the hemoprotein at 28h. The CO-difference spectra recorded in the 1,50,000 xg supernatant exhibited maximum P-450 levels in cells grown in Luria-Bertani medium with an initial inoculum of 0.15% (v/v) and incubated at 26°C for 28h. (Figure 17). The optimum cytochrome P-450 2E1 content obtained for this construct routinely averaged 55-65 nmol P-450/liter culture.

The electrophoretic mobility of selectively ultrafiltered 1,50,000 xg fraction proteins determined by qualitative SDS polyacrylamide gel electrophoresis indicated a band around 56.9 kDa for the target P-450 2E1 protein (Figure 18). The cell fractions of the host strain, *E. coli* DH5α did not exhibit any band in this region.

4.3.3 Co-expression of N-terminus modified cytochrome P-450 2E1 and luciferase in Pseudomonas putida

The time-course of expression of cytochrome P-450 2E1 in the 1,50,000 xg supernatant of *Pseudomonas putida* co-expressing luciferase revealed that some absorbance in the 450 nm region was observed around 20h. However, the spectra did not exhibit a defined maximum at 452 nm that was observed in cells

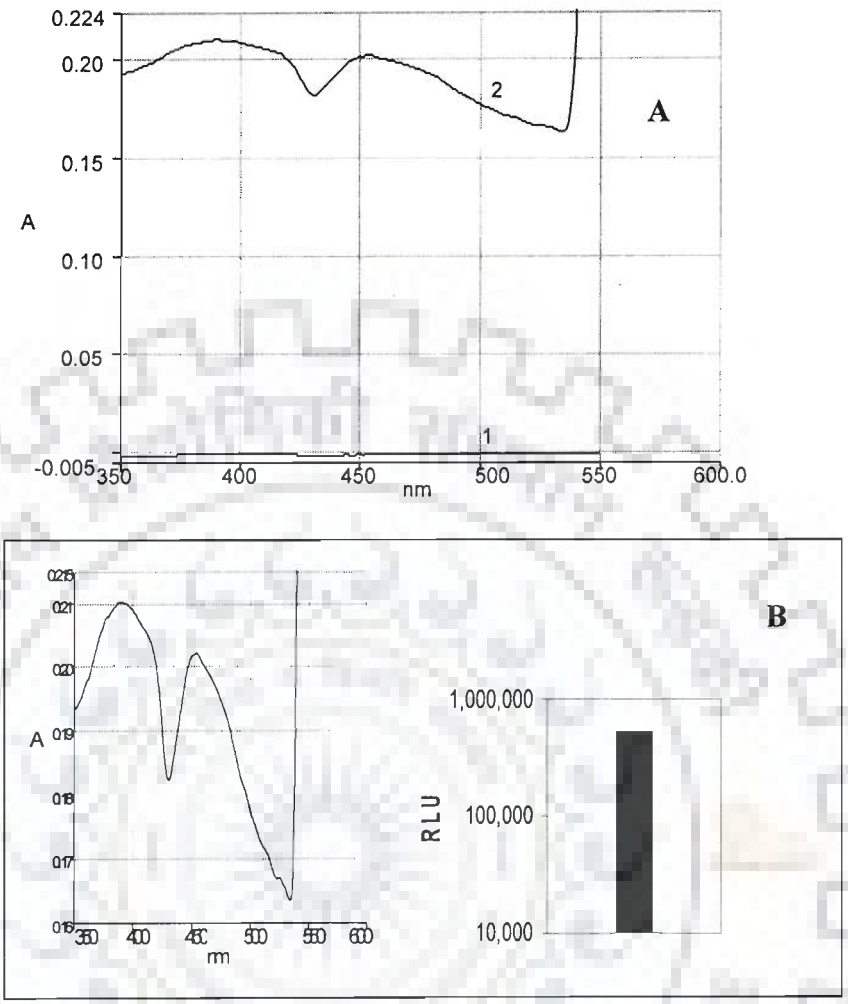


Figure 16. Expression of *N*-terminus modified human cytochrome P-450 2E1 in *Pseudomonas putida* co-expressing luciferase lux AB

A. CO-difference spectra recorded in 1,50,000 xg supernatant of cells grown for 26h at 26°C

1-Baseline recorded after the addition of dithionite as reductant

2-Difference spectra recorded after saturation of sample cuvette with carbon monoxide

B. Co-expression of cytochrome P-450 2E1 and luciferase monitored by CO-difference spectra and luminescence detection

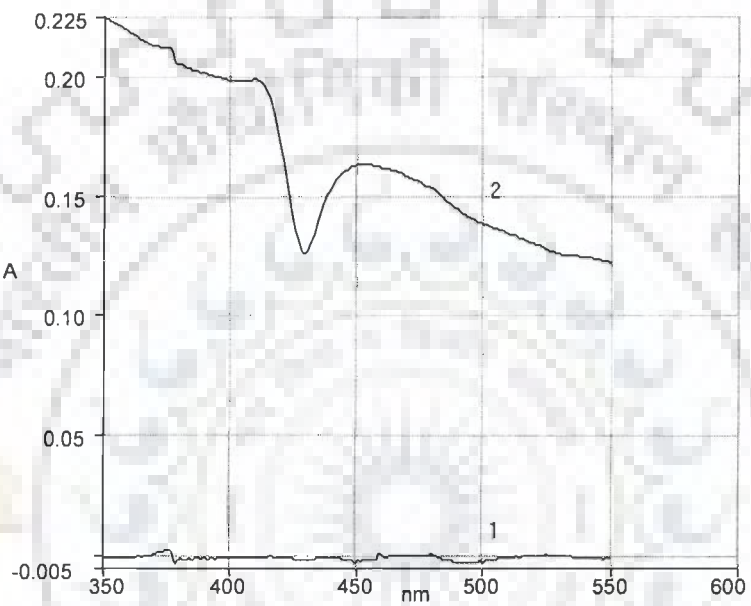


Figure 17. Expression of *N*-terminus modified human cytochrome P-450 2E1 in *Pseudomonas putida* MTCC 102 monitored by carbon monoxide difference spectra

(Spectra recorded in 1,50,000 xg supernatant of cells grown for 28h at 26°C)

1-Baseline recorded after the addition of dithionite as reductant

2-Difference spectra recorded after saturation of sample cuvette with carbon monoxide

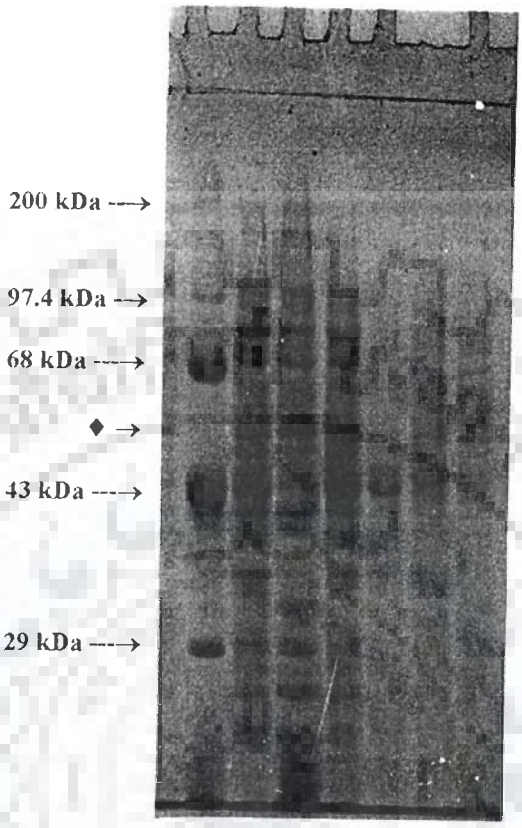


Figure 18. Electrophoretic mobility of proteins in cytosolic and membrane fractions of *E. coli* DH5α expressing *N*-terminus modified human cytochrome P-450 2E1 in 10% polyacrylamide gel

L→R

Lane 1: Protein molecular weight standards - GIBCOBRL (15,000 - 200,000 kDa)
Lane 2: Supernatant* from centricon 30
Lane 3: Membrane* from centricon 30
Lane 4: Supernatant* from centricon 50
Lane 5: Supernatant* from centricon 100
Lane 6: Supernatant fraction
Lane 7: Membrane fraction

* Fractions obtained after centrifugation of S9 fraction at 1,50,000 xg for 1h in ultracentrifuge

◆ Arrow indicates suspect band for cytochrome P-450 2E1 protein

grown for 26h. Concomitant luminescence detection to monitor the time-course of luciferase expression indicated detectable luminescence in cells grown for 16h with a maximum luciferase activity detected as RLU obtained at 22h of growth (Figure 19).

Pseudomonas putida cells co-expressing *N*-terminus modified cytochrome P-450 2E1 and luciferase grown in the absence of chloramphenicol yielded better expression of both the engineered traits. CO-difference spectra recorded in 1,50,000 xg supernatant indicated an enhanced P-450 expression with the characteristic Soret peak at 450-452 nm. The cytochrome P-450 2E1 yield observed in the absence of chloramphenicol exhibited a significant boost to about 160-200 nmol P-450/liter culture, in comparison to the routine yields of approximately 50 nmol P-450/liter culture, observed in cells grown in the presence of chloramphenicol. Luciferase expression in the absence of chloramphenicol was also observed to be higher with respect to the routine levels detected in cells grown with chloramphenicol and exhibited an increase of about 25% in the luminescence signal detected as RLU (Figure 20). The influence of varying concentrations of *m*-toluate on luciferase induction examined in growing cultures of *Pseudomonas putida* co-expressing cytochrome P-450 2E1 indicated negligible luminescence in cells grown at concentrations below 10 μM. Lowering the *m*-toluate concentration from the routinely employed 200 μM to 25 μM led to a significant decline in luciferase activity evident from the decreased

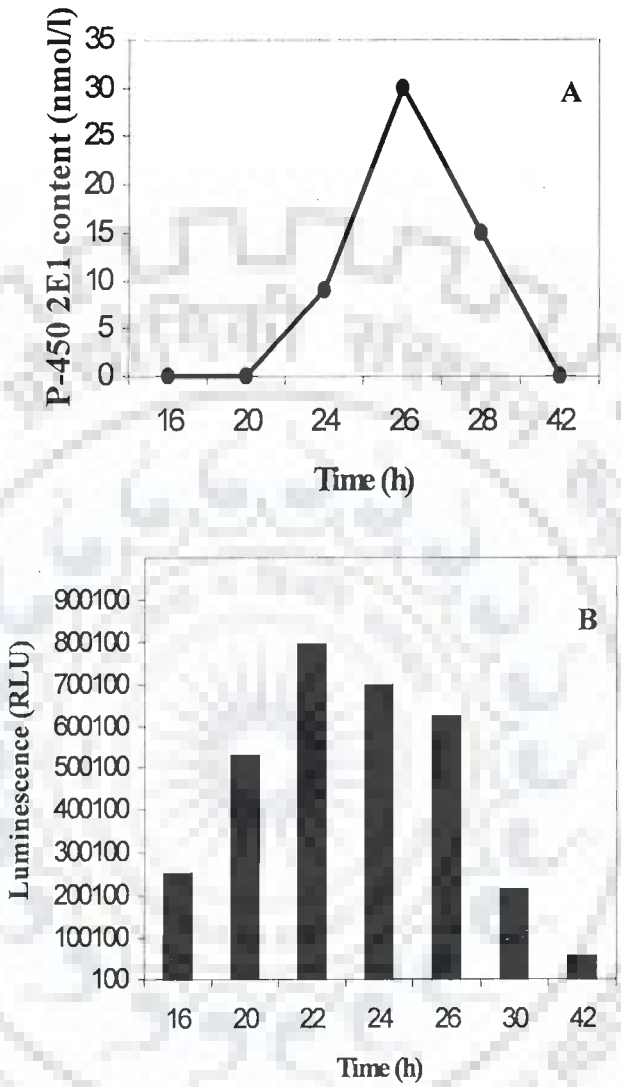


Figure 19. Time-course profile of *N*-terminus modified human cytochrome P-450 2E1 and luciferase expression in engineered *Pseudomonas putida*

A. Cytochrome P-450 2E1 expression monitored by CO-difference spectra in cells grown for different periods (16-42h)
B. Luciferase expression monitored as RLU in cells grown for different periods (16-42h)

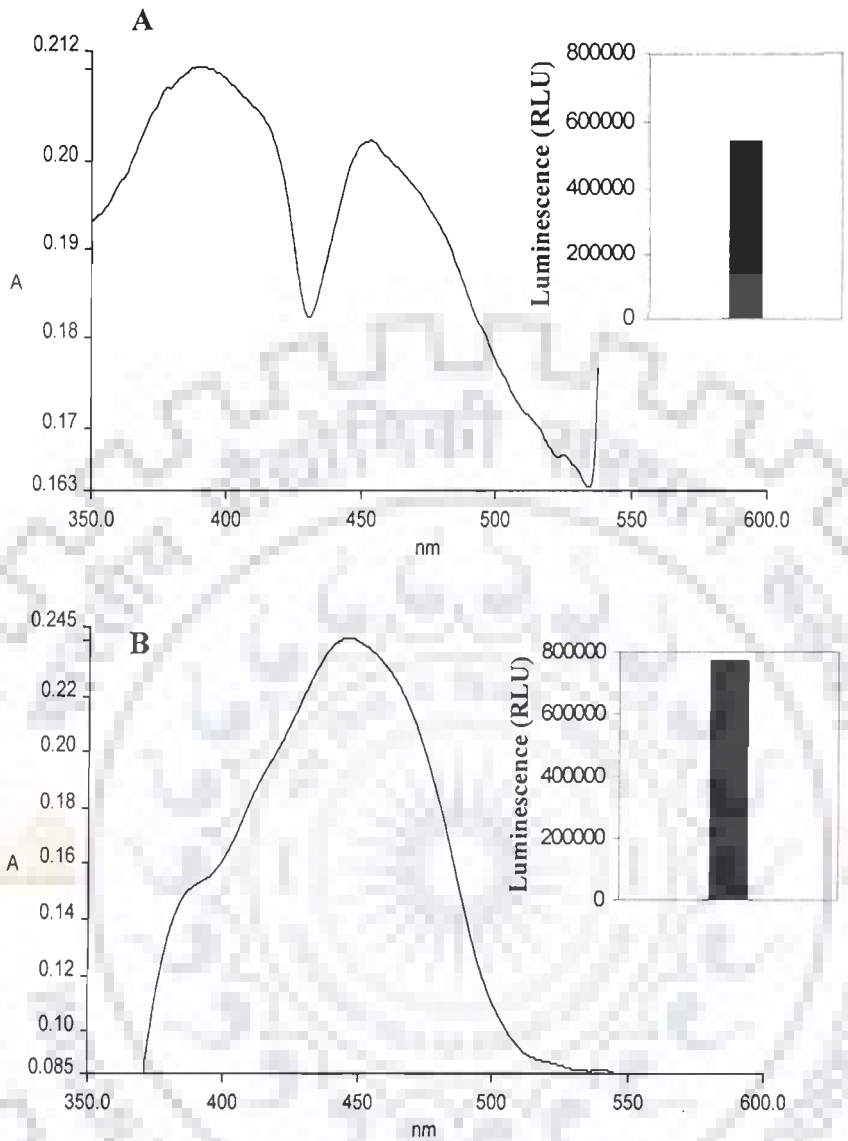


Figure 20. Influence of chloramphenicol on cytochrome P-450 2E1 and luciferase expression in engineered *Pseudomonas putida* (PpW-lux-2E1)

(Difference spectra recorded in 1,50,000 xg supernatant after the addition of the reductant sodium dithionite)

A - PpW-lux-2E1cells grown with chloramphenicol (20 mg/L)

B - PpW-lux-2E1 cells grown in the absence of chloramphenicol

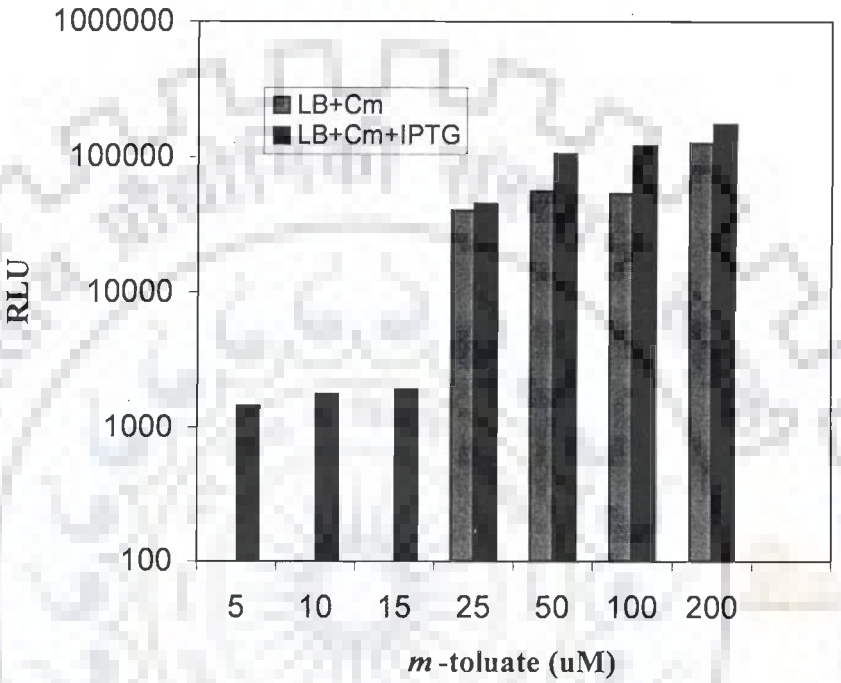


Figure 21. Effect of *m*-toluate concentration on luciferase induction in *Pseudomonas putida* co-expressing *N*-terminus modified human cytochrome P-450 2E1 and luciferase

(PpW-lux-2E1 cells were grown in Luria-Bertani medium in presence of chloramphenicol (20 mg/L) and cytochrome P-450 inducer, IPTG [1mM])

luminescence observed in such cultures. However, cells grown with the higher concentrations of 50-200 μM exhibited nearly similar luminescence values. In addition PpW-lux-2E1 cells grown in the presence of both IPTG and *m*-toluate exhibited enhanced luminescence in comparison to cells grown with only *m*-toluate and without IPTG (Figure 21).

4.3.4 Luciferase-mediated reduction of N-terminus modified cytochrome P-450 2E1

In-vitro assays investigating the ability of luciferase to mediate reduction of cytochrome P-450 2E1 yielded the characteristic Soret peak at 452 nm, consistent with the reduced hemoprotein. This reduction was evidenced both in the presence and absence of decanal-dependent luminescence providing that luciferase reduced the hemoprotein in a light-independent mechanism.

The diagnostic CO-difference spectra obtained in the presence of luminescence induced by the addition of *n*-decanal (7.4 x 10^6RLU; light reaction) indicated the characteristic Soret peak at 452 nm and did not require the addition of the reductant, sodium dithionite (Figure 22). Relatively higher concentrations of luciferase (8 x10^6 and 4 x 10^6 RLU) were required to reduce the hemoprotein as perceived by the failure to generate the characteristic absorbance peak in assays possessing lower luciferase concentrations. Spectra recorded with lower amounts of luciferase (1.6 x 10^6 RLU) failed to yield the desired absorbance at 452 nm, instead indicating a maximum at 445-446 nm (Figure 23).

In-vitro assays exploring luciferase-mediated reduction of cytochrome P-450 2E1 in the absence of decanal-dependent luminescence (dark reaction)

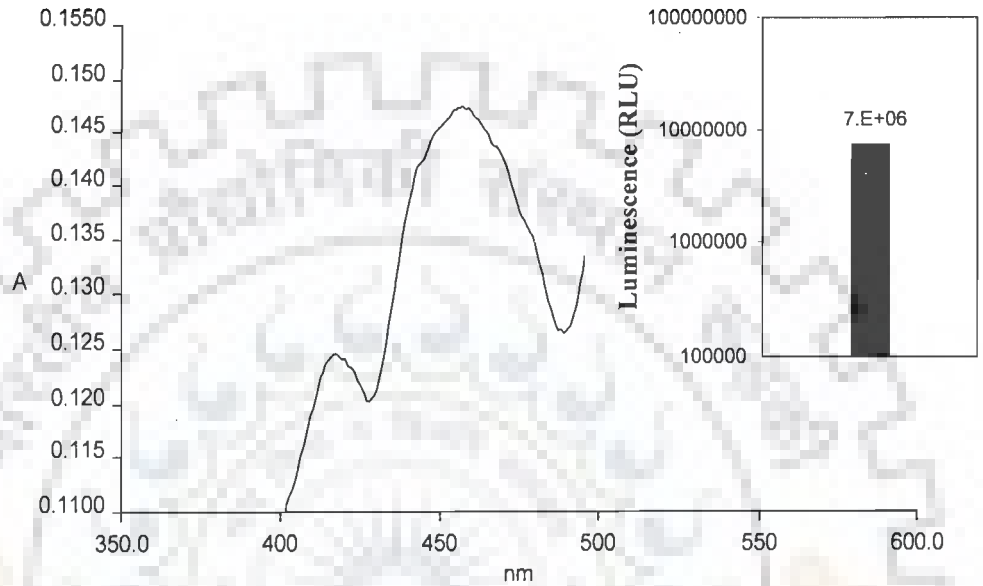


Figure 22. LIGHT REACTION: Luciferase-dependent photo-reduction of *N*-terminus modified human cytochrome P-450 2E1 expressed in *Pseudomonas putida* (PpW-2E1)

[*In vitro* reaction assay containing 1,50,000 xg supernatants of PpW-2E1 and PpW-lux (luminescence equivalent: 7.4×10^6 RLU) and *n*-decanal was purged with carbon monoxide to obtain diagnostic CO-difference spectrum. Chemical reductant sodium dithionite was not added]

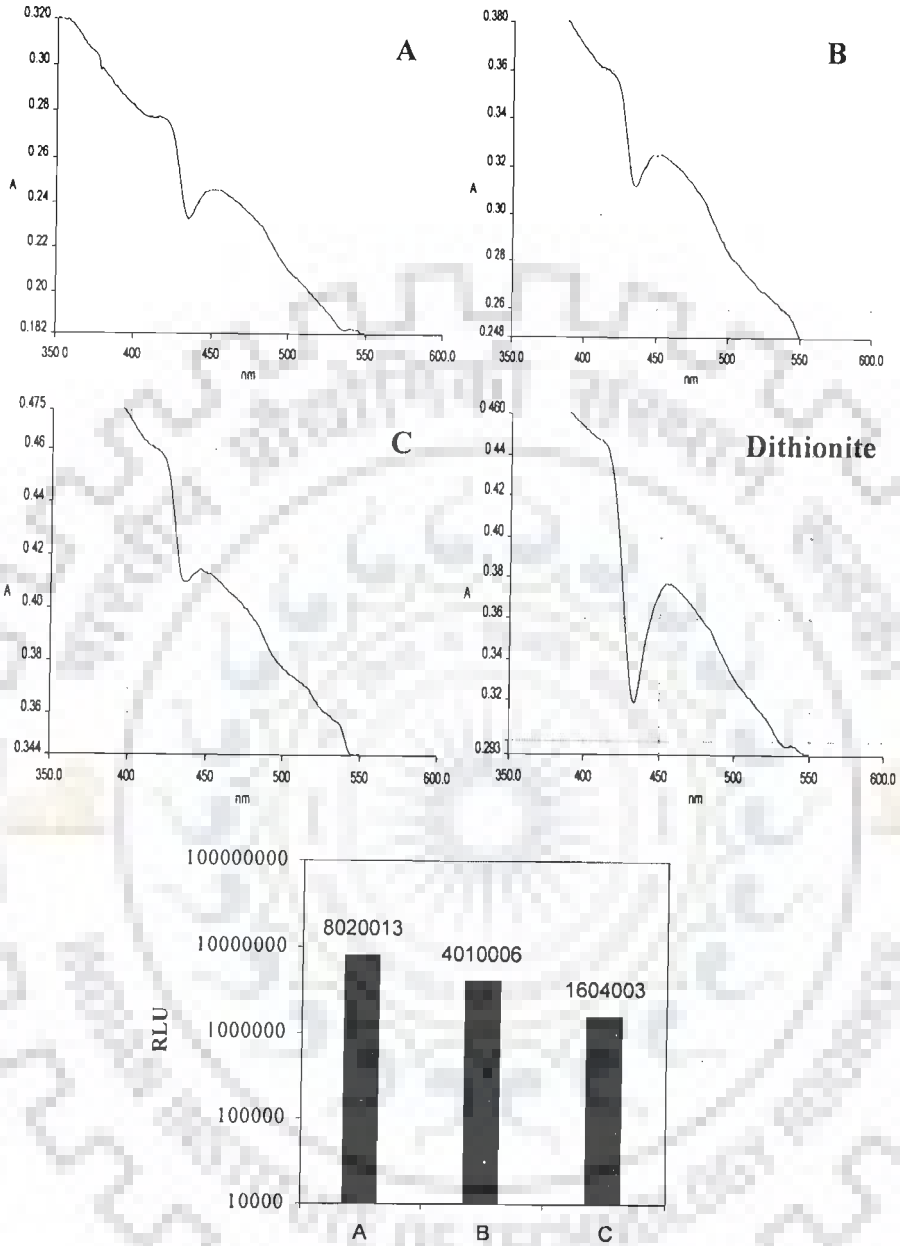


Figure 23. LIGHT REACTION: Luciferase-dependent photo-reduction of *N*-terminus modified human cytochrome P-450 2E1 expressed in *Pseudomonas putida* (PpW-2E1)

[*In vitro* reaction assay containing 1,50,000 xg supernatants of PpW-2E1 and PpW-lux (luminescence equivalent: **A** - $8x10^6$ RLU; **B** - $4x10^6$ RLU; **C**- $1.6x10^6$ RLU) and *n*-decanal was purged with carbon monoxide to obtain diagnostic CO-difference spectrum. Chemical reductant sodium dithionite was not added]

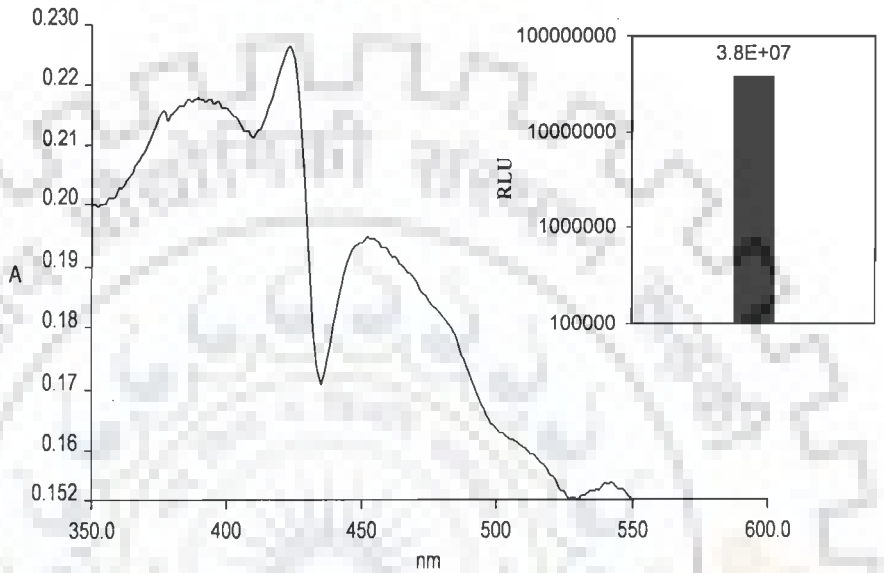


Figure 24. DARK REACTION: Luciferase-dependent reduction of *N*-terminus modified human cytochrome P-450 2E1 expressed in *Pseudomonas putida* (PpW-2E1)

[*In vitro* reaction assay containing 1,50,000 xg supernatants of PpW-2E1 and PpW-lux (luminescence equivalent: 3.8×10^7 RLU) was purged with carbon monoxide to obtain diagnostic CO-difference spectrum. Chemical reductant, sodium dithionite and luciferase substrate, *n*-decanal were not added]

required higher concentrations of luciferase to obtain the absorbance peak at 452 nm. The luciferase concentration (3.7×10^7 RLU) required to achieve reduction of the hemoprotein in the absence of luminescence was nearly double of that employed in the light dependent reaction. The diagnostic CO-difference spectra recorded in the absence of luminescence and the reductant sodium dithionite yielded the absorbance maximum at 452 nm, thereby establishing the luciferase-mediated reduction of P-450 2E1 (Figure 24). Moreover, CO-difference spectra recorded with the 1,50,000 xg supernatant from PpW-lux cells, with and without reduction with sodium dithionite and in the presence of *n*-decanal did not yield any absorbance in the 450 nm region. In the presence of *n*-decanal, some absorbance around 480 nm was observed, presumably contributed by the flavin domain of luciferase.

4.4 Discussion

The expression of unmodified cytochrome P-450 2E1 in *E. coli* and *Pseudomonas putida* observed in the present study was quite low. The *E. coli* construct possessing native cytochrome P-450 2E1 subcloned on vector pMMB66HE yielded low levels of the protein (Figure 13), corroborating earlier reports on poor expression levels of the unmodified enzyme in *E. coli* (Gillam *et al.*, 1993; 1994; Dong and Porter, 1996). The *Pseudomonas putida* construct possessing native cytochrome P-450 2E1 and luciferase also yielded low P-450 2E1 content. In addition, these cells did not exhibit any detectable luminescence. In an attempt to determine the reasons for the absence of luminescence in this

construct, competent cells of the host strain, *Pseudomonas putida* expressing luciferase, were transformed with only the vector pMMB66HE DNA, without the native 2E1 gene. These transformed cells also did not exhibit any luminescence. However, the same competent cells when transformed with the plasmid vector pMMB 206 exhibited high luminescence values. It may thus be assumed that the ampicillin resistance encoded by the vector pMMB66HE may presumably be hindering luciferase expression from the mini-Tn5 transposon encoding the kanamycin-resistance marker gene, thereby leading to the absence of luciferase activity in the cells. To avoid this incompatibility, the broad-host range plasmid vector pMMB 206 coding for chloramphenicol resistance was selected for subcloning of the *N*-terminus modified P-450 2E1. The low expression levels obtained with the native enzyme thus necessitated the generation of an *N*-terminally modified variant of cytochrome P-450 2E1 to facilitate the expression of this enzyme in *E. coli* and *Pseudomonas putida.*

Growth factors like time and temperature of incubation, culture media and initial inoculum levels influence the yield of cytochrome P-450 enzymes. Modifications of the optimal growth parameters may result in a rapid conversion of the active form of the protein to the denatured form, known as P-420 characterized by the absorbance maximum at 420 nm of the ferrous-CO complex of the protein. The labile nature of the cytochrome P-450 2E1 protein thus necessitated the optimization of growth conditions for all the constructs.

Diagnostic CO-difference spectra was used as a measure to evaluate optimal P-450 2E1 expression. The Fe^{2+}.CO spectra characteristics of endogenous

E. coli hemoproteins obscure the 450 nm peak confounding observations of P-450 CO-difference spectra in whole cells (Porter and Larson, 1991; Gillam *et al.*, 1994). Diagnostic CO-difference spectra were hence monitored in the cell-fractions of *E. coli* and *Pseudomonas putida*.

To optimize P-450 2E1 expression from the various constructs, different culture media, incubation temperatures and incubation times were systematically evaluated (Figure 14). It has been reported that the variation in conditions for optimal expression of different P-450 enzymes is greater than for similar constructs of the same P-450 (Gillam *et al.*, 1993; 1994). Therefore, the variations in the growth parameters evaluated for the *Pseudomonas putida* constructs possessing *N*-terminus modified P-450 2E1 were narrowed to encompass the optimal factors determined for the *E. coli* construct. For each construct, expression was allowed to continue between 25-28h (Figures 15-17), longer incubation times being associated with a reduced recovery of hemoprotein.

The culture medium and the initial inoculum also influenced P-450 2E1 expression from each construct. Terrific broth medium elucidated higher yields in the *E. coli* construct and *Pseudomonas putida* possessing *N*-terminus modified P-450 2E1 and co-expressing luciferase, with an initial inoculum of 1% and 2% (w/v), respectively. However, the use of the more nutrient-rich Terrific broth medium for cultivation of the *Pseudomonas putida* strain possessing only *N*-terminus modified P-450 2E1 failed to yield the characteristic P-450 peak at 452 nm. Optimal P-450 expression for this construct required cultivation in Luria-Bertani medium with a lower initial inoculum (0.15%, w/v).

The role of incubation temperatures in growing cultures of bacteria expressing P-450 enzymes is an important determinant in achieving high enzyme yields. Even slight elevation of the optimum growth temperature results in reduced levels of cytochrome P-450 expression (Parikh and Guengerich, 1997). Lower temperatures boost P-450 expression with enhanced P-450 expression usually obtained when incubation temperature has been lowered to about 30^0C for *E. coli* cells expressing cytochrome P-450 enzymes. Similar results were obtained in this study with the *E. coli* construct wherein the P-450 yield obtained with cells grown at 30^0C was much higher than incubations at 37^0C with all growth conditions unchanged. Incubation at the higher temperatures of 37^0C instead of 30^0C reduced the P-450 2E1 protein to spectroscopically undetectable levels. As observed with the *E. coli* cells, P-450 expression in *Pseudomonas putida* expressing modified 2E1 with and without luciferase (constructs PpW-lux-2E1 and PpW-2E1, respectively) required a lower incubation temperature with optimal P-450 expression and high luminescence emission observed with an incubation at 26°C (Figures 16 and 17). Higher incubation temperatures yielded much lower luminescence and P-450 levels. The co-expression of *N*-terminus modified human cytochrome P-450 2E1 and luciferase was evidenced in *Pseudomonas putida* grown at 26°C in the presence of inducers of cytochrome P-450 (IPTG) and luciferase (*m*-toluate) (Figure 19). *Pseudomonas putida* co-expressing *N*-terminus modified cytochrome P-450 2E1 and luciferase cultured in the absence of the antibiotic chloramphenicol demonstrated a significant increase in P-450 expression (4-5 fold) compared to cultures grown in the presence of

chloramphenicol. The cultures also demonstrated higher luciferase expression in the absence of chloramphenicol (Figure 20).

Previous reports have indicated that the addition of ligands may help stabilize the modified cytochrome P-450 2E1 protein both *in vivo* and *in vitro* (Larson *et al.*, 1991). Other studies with bacterial systems have demonstrated the stabilizing effect of 4-methylpyrazole to be more important *in vitro* than *in vivo* (Pritchard *et al.*, 1997). Results obtained in this study indicate that the presence of the ligand 4-methylpyrazole in the culture medium of the *E coli* construct possessing modified cytochrome P-450 2E1 yielded a higher P-450 content (Figure 15). However the presence of this ligand in the culture medium for the *Pseudomonas putida* constructs possessing modified cytochrome P-450 2E1 did not produce any significant increase in expression levels. Although the *in vivo* effect of 4-methyl pyrazole was not very pronounced, the presence of this ligand in the buffers during subcellular fractionation allowed for stabilization of the P-450 2E1 protein *in vitro*.

The *E. coli* and *P. putida* constructs produced P-450 that was spectrally active, elucidated by the diagnostic CO-difference spectra recorded in the cell-fractions (Figures 15-17). A direct comparison of the expression yields obtained in this study with other reports on cytochrome P-450 2E1 expression in *E. coli* is difficult since different vector systems have been utilized in various studies. For instance, the high-copy number plasmid vectors, pCW and pJL have been employed for cytochrome P-450 2E1 expression (Gillam *et al.*, 1994; Dong and Porter, 1996) whereas the present study employed vectors having a low copy

number, thereby affecting the yield of the hemoprotein. Moreover, a comparison of the yield is further constrained since there are few reports on the expression of human cytochrome P-450 2E1 in *Pseudomonas putida*.

Reports on the subcellular localization of recombinant microsomal cytochrome P-450 enzymes expressed in *E. coli* have varied widely (Gillam *et al.*, 1994; Pernecky *et al.*, 1995; von Wachenfeldt *et al.*, 1997). The removal of the putative membrane-spanning domain from microsomal P-450 enzymes prevented the integration of the modified proteins into *E. coli* membranes (von Wachenfeldt *et al.*, 1997). Removal of the *N*-terminus of P-450 7A led to a high proportion of the enzyme being expressed in *E. coli* as a cytosolic protein (Li and Chiang, 1991). Other reports on the removal of the putative membrane spanning sequences of several microsomal P-450 enzymes indicate the localization of a substantial amount of the P-450 in the membrane fraction, although the relative amounts of the expressed protein found in the cytosolic and membrane fractions of *E. coli* have differed widely (von Wachenfeldt *et al.*, 1997). Pernecky *et al.* (1995) constructed chimeras utilizing the *N*-terminal 19 codons of P-450 102 to replace the first 43 and 44 codons of P-450 2B4 and P-450 2E1, respectively. The cytochrome P-450 hemoprotein in these chimeras was distributed in both the membrane and cytosolic fractions of *E. coli*. Such observations corroborate the results obtained in the present study wherein the CO-difference spectra recorded in the 1,50,000 xg supernatant yielded a defined spectral maximum at 452 nm. However, though the 1,50,000 xg pellet failed to exhibit a defined Soret peak at 452 nm, some absorbance was witnessed at 454-455 nm (Figure 15B). These

results indicate that a higher proportion of the *N*-terminus modified human cytochrome P-450 2E1 was present in the 1,50,000 xg supernatant fraction of *E. coli* and *Pseudomonas putida* cells.

Although the heterologous expression of cytochrome P-450 enzymes in bacteria such as *E coli* is convenient and inexpensive, recombinant protein activity is not observed in cell lysates without the exogenous addition of NADPH cytochrome P-450 oxido-reductase. An electron transfer partner is therefore required to reduce the expressed P-450 and to generate an enzymatically active protein. The present study attempted to circumvent this limitation by utilizing luciferase as an electron transfer partner for the expressed P-450 2E1 to yield a catalytically active protein. The *in vitro* anaerobic photo-reduction of eukaryotic and prokaryotic cytochrome P-450 enzymes in the presence of small molecule redox mediatiors has been demonstrated and the photoreduced enzymes were shown to be metabolically competent (Greenbaum *et al.*, 1972; Lipscomb *et al.*, 1976). A direct reduction of cytochrome P-450 cam by luciferase in the absence of molecular oxygen has also been demonstrated earlier. The reduced enzyme was shown to be catalytically active effecting the dehalogenation of pentachloroethane and hexachloroethane (Shanker and Atkins, 1996).

The present study explored the possibility of luciferase-mediated reduction of human cytochrome P-450 2E1 and the results obtained confirm that luciferase was effectively able to reduce the hemoprotein in oxygen-depleted samples (Figures 22 and 23). A direct reduction of cytochrome P-450 2E1 by luciferase was further supported by the fact that this reduction was not light-dependent and

occurred both in the presence and absence of decanal induced luminescence (Figure 24). These results establish that luciferase can serve as a substitute for the normal electron transfer partner of cytochrome P-450 2E1.

4.5 Concluding Remarks

The present study demonstrates successful co-expression of human cytochrome P-450 2E1 and luciferase in an environmentally robust host, *Pseudomonas putida*. In addition, the results obtained herein provide conclusive evidence of luciferase-mediated reduction of human cytochrome P-450 2E1.

Chapter 5

Cytochrome P-450 Dependent Transformation of Halogenated Hydrocarbons by Genetically Engineered Pseudomonas putida

5.1 Introduction

Halogenated organic chemicals constitute the largest single group of compounds on the list of priority pollutants compiled by the US EPA (Keith and Telliard, 1979; Li and Wackett, 1993; Liu and Jones, 1995). A list of 129 *Priority Pollutants* compiled by US EPA included 62 halogenated organic compounds, while 27 out of 65 compound classes on a list of toxic pollutants were halogenated hydrocarbons (Ornstein, 1993). Halogenated hydrocarbons, widely employed as herbicides, pesticides and solvents, are recalcitrant to biodegradation, and thus of considerable public health and ecological concern due to their persistence, bioaccumulation and toxicity to a wide range of biological systems (Shailubhai, 1986; Shales *et al.,* 1989; Jain *et al.,* 1992). Hence there is a need to remediate soils and waters contaminated with such compounds.

The persistence of halogenated hydrocarbons in the environment is indicative of the limited capability of naturally occurring microorganisms to effect their degradation. Reductive dehalogenation is the most important biochemical mechanism for the transformation and detoxification of highly halogenated compounds including chlorinated hydrocarbons such as hexachlorobenzene, tetrachloroethylene and carbon tetrachloride (Li and Wackett, 1993, Ornstein, 1993). However, only a small number of bacterial pure cultures capable of catalyzing reductive dehalogenation reactions have been isolated (DeWeerd and Suflita, 1990; Mohn and Tiedje, 1992). In addition, attempts to obtain pure cultures from anaerobic consortia showing reductive dehalogenation activity have also been only moderately successful (Tiedje *et al.,* 1987; Logan *et al.,* 1993).

Although bacteria capable of anaerobically metabolizing several common chlorinated solvents are known, they have not been commonly used for bioremediation as it is difficult to ensure anaerobic conditions at most remediation sites.

The construction and introduction into the environment of bacterial strains with improved degradative abilities may be a feasible alternative to alleviate environmental pollution (Pemberton, 1981; Jones *et al.*, 1984; Harwood *et al.*, 1990; Nublein *et al.*, 1992; Pipke *et al.*, 1992). The success of such an approach depends not only on the improved catabolic abilities of the constructed strains, but also on their ability to compete with indigenous microflora. Though the use of genetically engineered organisms is regulated, this does not preclude the commercial use of these organisms, especially in "pump-and-treat" systems where there would be no release of bacteria to the environment. With the enforcement of more stringent discharge norms (The Hazardous Waste Consultant, 1993), bioslurry and compost treatment of contaminated solids may offer the optimum technology for soil bioremediation (Glaser and Potter, 1996). Engineered bioreactors can be designed and equipped with various process-control instruments to create ideal conditions for biodegradation of recalcitrant compounds. Slurry bioreactors often considered as off-the-shelf technology for the treatment of conventional non-hazardous materials are now being evaluated for the decontamination of hazardous wastes (Glaser and Potter, 1996).

Cytochrome P-450 enzymes are a logical enzyme choice for application in bioremediation due to their enormous catalytic versatility (Trombly, 1995). The

dehalogenation potential of cytochrome P-450 enzymes including the environmentally relevant members of this enzyme superfamily, cytochrome P-450 cam and cytochrome P-450 2E1, has been explored. Polyhalogenated hydrocarbons have been reported to undergo one and two-electron reductions mediated by these enzymes (Ornstein, 1993). Cytochrome P-450 cam monooxygenase has been shown to mediate the transformation of various halogenated substrates including hexachloroethane, pentachloroethane, 1,1,1,2-tetrachloroethane, 1,1,1- and 1,1,2-trichloroethane and 1,2-dichloroethane (Castro *et al.,* 1985, 1989; Vilker and Khan, 1989; Li and Wackett, 1993; Logan *et al.,* 1993; Ornstein, 1993; Lefever and Wackett, 1994). Human cytochrome P-450 2E1 has also been implicated in mediating the transformation of a range of xenobiotics including trichloroethylene, halothanes, diethyl ether and carbon tetrachloride (Ahr *et al.*, 1982; Koop, 1992; Gillam *et al.,* 1994; Urban *et al.*, 1994; Wang *et al.,* 1996; Smith *et al.*, 2000).

Mammalian cytochrome P-450 enzymes including human cytochrome P-450 2E1 required modifications in the *N*-terminus to be successfully expressed in *E. coli* (Larson *et al.,* 1991; Gillam *et al.,* 1994; Pritchard *et al.,* 1997). The catalytic activity of various mammalian P-450 enzymes, including human P-450 2E1, expressed in *E. coli* has been demonstrated in reconstituted membrane preparations supplemented with NADPH-cytochrome P-450 reductase (Larson *et al.*, 1991; Gillam *et al.*, 1994; Dong and Porter, 1996; Pritchard *et al.*, 1997). However, attempts to determine the catalytic activity of the heterologously-expressed enzymes in live bacterial cultures have met with little success (Dong

and Porter, 1996). The *in vivo* metabolism of steroids evaluated in *E. coli* cultures expressing modified cytochrome P-450 17α-hydroxylase exhibited poor transformation yields (Barnes *et al.*, 1991; Shet *et al.*, 1994). Thus, the generation of active functional microbial systems capable of P-450 mediated detoxification reactions needs to be explored.

The present study determines the metabolic capability of genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase to effect dehalogenation of hexachloroethane and the widely used insecticide, γ-hexachlorocyclohexane, in resting cells and in soil slurry. The study also investigates the *in vivo* catalytic activity of genetically engineered *Pseudomonas putida* co-expressing *N*-terminus modified human cytochrome P-450 2E1 and luciferase.

5.2 Materials and Methods

5.2.1 Chemicals and Glassware

Bacto-tryptone, yeast extract and Noble agar were purchased from DIFCO Laboratories (Detroit, USA). The antibiotics chloramphenicol and kanamycin were obtained from SIGMA Chemical Company (St. Louis, USA). High purity hexachloroethane and γ-hexachlorocyclohexane were also obtained from SIGMA Chemical Company. High purity carbon tetrachloride was obtained from Supelco Inc., USA. High purity *n*-hexane was purchased from E-Merck (Germany). All other chemicals were of the highest purity commercially available. Glassware utilized was borosilicate glass of Borosil or Vensil make.

5.2.2 Culture Media

5.2.2.1 M9 Medium

M9 medium contained per litre: 12.8g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 1g NH_4Cl, 0.492g $MgSO_4$, 0.015g $CaCl_2$, 0.005g $FeSO_4$. The medium was sterilized by autoclaving at 121°C for 15 min (Sambrook *et al.,* 1989) and supplemented with 6.8 mM citrate as carbon source after sterilization.

5.2.3 Bacterial Constructs

Genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase was utilized in the study. The construction of this strain possessing cytochrome P-450 cam C gene on a broad host range plasmid vector (pMMB 206) and *Vibrio harveyi* luciferase lux AB genes on the chromosome, downstream from the aromatic hydrocarbon-inducible *xyl* S and *Pm* promoter system and containing the minitransposon *Tn*5 has been described earlier (Shanker and Atkins, 1996).

Genetically engineered *Pseudomonas putida* possessing the complete cam monooxygenase system (cam CAB genes) was also utilized in the study. Competent cells of *Pseudomonas putida* were transformed with the broad host range vector pJP 43 possessing 5.7 Kb *Xho* I-*Hind* III fragment encoding the cam CAB genes of the cam monooxygenase system (Koga *et al.,* 1989) by electroporation using a GIBCO-BRL Cell Porator. Kanamycin-resistant transformants were screened by PCR and CO-difference spectroscopy. Genetically engineered *Pseudomonas putida* co-expressing *N*-terminus modified

human cytochrome P-450 2E1 and luciferase was also employed in the study. The construction of this strain is described in chapter 3.

5.2.4 Soil

Soil utilized in the study was black cotton agricultural soil. Soil was collected from the top 15 cm of the soil profile, passed through a coarse sieve and stored moist at 4°C for future use. Soil analysis was performed according to standard protocols (Page *et al.*, 1982).

5.2.5 Optimization of Growth conditions of Bacterial Hosts

The optimum growth conditions facilitating the expression of both the engineered traits, viz. cytochrome P-450 cam and luciferase in engineered *Pseudomonas putida* were evaluated by CO-difference spectroscopy and luminescence measurement, respectively. Single colony of *Pseudomonas putida* co-expressing cytochrome P-450 cam C and luciferase lux AB genes was grown for 18h at 30°C in 5 ml Luria-Bertani medium supplemented with 20 μg/ml chloramphenicol, 1mM IPTG and 200 μM *m*-toluate. 100 ml of Luria-Bertani medium supplemented with 20 μg/ml chloramphenicol, 1mM IPTG and 200 μM *m*-toluate was inoculated with 0.15 ml (v/v) of the overnight culture. Cells were incubated at 28°C on an orbital shaker at 175 rpm and harvested at 20h by centrifugation at 8000 xg for 10 min at 4°C. The cell pellet was resuspended in either 100 mM sodium phosphate buffer, pH 7.4 containing 50 mM KCl for the determination of diagnostic CO-difference spectrum or in M9 medium (1:50, w/v) for substrate transformation assays.

Genetically engineered *Pseudomonas putida* possessing the complete cam monooxygenase system (cam CAB genes) was similarly grown in 100 ml Luria-Bertani medium supplemented with 50 μg/ml kanamycin. Cells were harvested at 20h and resuspended in either 100 mM sodium phosphate buffer, pH 7.4, for determination of CO-difference spectra or in M9 medium (1:50, w/v) for substrate transformation assays.

Genetically engineered *Pseudomonas putida* co-expressing *N*-terminus modified cytochrome P-450 2E1 and luciferase lux AB genes was grown in Terrific broth as described earlier (section 4.2.4.2). Cells were harvested at 26h and resuspended in either 100 mM sodium phosphate buffer, pH 7.4 for determination of CO-difference spectra or in M9 medium (1:25, w/v) for metabolism studies.

5.2.6 CO-difference Spectra

The expression of cytochrome P-450 cam C or cam CAB genes in genetically engineered *Pseudomonas putida* was confirmed by determination of diagnostic CO-difference spectra in the cell-free extracts. Cell pellets were resuspended in 10 volumes (w/v) of ice-cold 100 mM sodium phosphate buffer, pH 7.4, containing 50 mM KCl. The cell suspension was subjected to sonication for 5 min in cycles of 20s pulse with a 15s interval after each pulse. Sonication was performed at 50W in a '*Torbeo*' ultrasonic processor. The cell suspension was centrifuged at 12,000 xg for 15 min to remove the cell debris and the supernatant utilized for the determination of CO-difference spectra as described earlier (section 4.2.6).

The expression of *N*-terminus modified human cytochrome P-450 2E1 in engineered *Pseudomonas putida* was determined by recording the CO-difference spectra in the 1,50,000 xg supernatant as described earlier (section 4.2.6).

5.2.7 Bioluminescence Measurement

The expression of luciferase lux AB genes in genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam or *N*-terminus modified human cytochrome P-450 2E1 was evaluated by monitoring luminescence in cells grown in the presence of the luciferase inducer, *m*-toluate, as described earlier (section 4.2.7). Luminescence measurement was performed in a luminometer after the addition of *n*-decanal.

Luminescence measurement in soil slurries was performed by placing 1 ml or 3 ml slurry in a luminometer cuvette or scintillation vial, respectively. Luminescence was measured after the addition of 1 µl *n*-decanal, either as relative light units (RLU) in a luminometer or as photon counts in a Packard Tri-carb liquid scintillation counter model 2100 TR operated in photon detection mode.

5.2.8 Dehalogenation of Hexachloroethane by Pseudomonas putida co-expressing cytochrome P-450 cam and luciferase

Transformation of hexachloroethane by genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase was investigated in pure culture and in soil slurries.

5.2.8.1 Whole cell assays

The efficacy of *Pseudomons putida* co-expressing cytochrome P-450 cam and luciferase to dehalogenate hexachloroethane under low oxygen tension was

evaluated in resting cultures. Whole cell assays were carried out in duplicate, by placing 3 ml cell suspension prepared in M9 medium, in 5 ml serum vials. The vials were secured with teflon coated stoppers and aluminium crimp seals and evacuated for 5 min (15" Hg vacuum). After evacuation, 8.45 μM hexachloroethane was introduced into the reaction vials by a gas-tight syringe (Hamilton, USA). The contents of the vials were mixed thoroughly and vials incubated stationary at 30°C for 3 hours. Appropriate chemical and boiled cell controls were also incubated concurrently. *Pseudomonas putida* possessing the complete cam monoxygenase system (cam CAB genes) was also incubated with hexachloroethane under identical conditions.

Dehalogenation of hexachloroethane was monitored by analysis of headspace gas by capillary gas chromatography (GC) and mass spectrometry (GC-MS), as described in section 5.2.11.

5.2.8.2 Transformation of hexachloroethane in soil slurries

The ability of *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase to mediate the dehalogenation of hexachloroethane and survive under *in situ* conditions was determined in soil slurries. Duplicate 15 ml serum vials containing 4g unsterile soil were amended with sterile deionized water to obtain 1:1 (w/w) soil:water mixture. A separate set of serum vials was amended with 6.8 mM citrate and 200 μM benzoate. The slurry was inoculated with *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase at $1.4x10^{10}$ CFU/g soil. The vials were secured with teflon coated stoppers and aluminium crimp seals and evacuated (15" Hg vacuum) for 5 min. After

evacuation, all vials including an uninoculated control, were amended with 8.45 μM hexachloroethane and incubated at 30°C on a rotary shaker at 100 rpm.

Dehalogenation of hexachloroethane was estimated at 0, 1, 4 and 7 days by analysis of the headspace gas by capillary GC and GC-MS as described later (section 5.2.11). Luminescence was determined concomitantly in soil slurry as photon counts in a scintillation counter at 0, 1, 4 and 7 days of incubation.

In a separate assay, the complete disappearance of hexachloroethane in soil slurries was similarly evaluated by placing duplicate samples of 10g unsterile soil in 40 ml serum bottles. Sterile deionized water was added to the soil to obtain a 1:1 (w/w) soil/water mixture and slurry amended with 6.8 mM citrate and 200 μM benzoate. *Pseudomonas putida* cells co-expressing cytochrome P-450 cam and luciferase were added at 2.8 x 10^{12} CFU/g soil, the serum bottles sealed and evacuated as described earlier, amended with 8.45 μM hexachloroethane and incubated at 30°C on an orbital shaker at 100 rpm. Uninoculated soil slurry served as control.

The disappearance of parent compound and formation of metabolites was monitored in duplicate samples at 0, 7 and 21 days incubation by analysis of headspace gas by capillary GC and GC-MS.

5.2.9 Transformation of γ-hexachlorocyclohexane by Pseudomonas putida co-expressing cytochrome P-450 cam and luciferase

Transformation of γ-hexachlorocyclohexane by genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase was investigated in pure culture and in soil slurries.

5.2.9.1 Whole cell assays

Whole cell assays with engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase were performed by placing 3 ml cell suspension prepared in M9 medium in 5 ml serum vials. The vials were secured with teflon coated stoppers and aluminium crimp seals and evacuated on ice for 5 min (15" Hg vacuum). After evacuation, 100 μM of γ-hexachlorocyclohexane was injected into the vials using a gas tight syringe. Appropriate chemical and boiled cell controls were included and the vials incubated stationary at 30°C for 18 hours. *Pseudomonas putida* possessing the complete cam monoxygenase system (cam CAB genes) was also incubated with hexachlorocyclohexane under identical conditions.

Metabolism of γ-hexachlorocyclohexane was estimated in triplicate samples after extraction with *n*-hexane (1:1, v/v) by capillary GC and GC-MS as described in section 5.2.11.

5.2.9.2 Transformation of γ-hexachlorocyclohexane in soil slurries

Transformation of γ-hexachlorocyclohexane by engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase under sub-atmospheric situation was investigated in soil slurry by placing 10g unsterile soil in 40 ml serum bottles. Sterile deionized water was added to the soil to obtain 1:1 (w/w) soil:water mixture. A separate set of serum bottles was amended with 6.8 mM citrate and 200 μM benzoate. The soil slurries were inoculated with *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase to yield $3.5x10^8$ CFU/g soil. The bottles were secured with teflon coated stoppers and

aluminium crimp seals, evacuated and amended with 350 μM γ-hexachlorocyclohexane. Serum bottles were incubated at 30°C on an orbital shaker at 100 rpm. Control bottles not amended with engineered *Pseudomonas* were run concurrently.

Disappearance of parent compound and formation of metabolites was estimated by capillary GC and GC-MS in triplicate samples extracted with *n*-hexane (1:1, v/v) at 0, 1, 3 and 4 weeks as described in section 5.2.11. Luminescence was determined in soil slurry concomitantly.

In a separate assay, the influence of surfactant addition on the metabolism of γ-hexachlorocyclohexane was monitored in soil slurries. Duplicate samples of 10g unsterile soil were placed in 40 ml serum bottles and sterile deionized water added to yield a 1:1 (w/w) soil:water mixture. The bottles were amended with an aqueous solution of the surfactant, Tween-80 (0.1%, v/v) and the contents mixed thoroughly. Soil slurry was inoculated with *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase at 2.6×10^{10} CFU/g soil. The serum bottles were secured with teflon coated stoppers and aluminium crimp seals. After evacuation, 350 μM γ-hexachlorocyclohexane was injected into all bottles. Soil slurry not amended with Tween-80 and uninoculated slurry served as controls. The serum bottles were incubated at 30°C on a rotary shaker at 100 rpm.

γ-hexachlorocyclohexane metabolism was monitored in triplicate samples extracted with *n*-hexane (1:1, v/v) at 0, 10 and 21 days by capillary GC and GC-MS as described in section 5.2.11.

5.2.10 Catalytic activity of N-terminus modified human cytochrome P-450 2E1

The ability of *N*-terminus modified human cytochrome P-450 2E1 to effect reductive dehalogenation of a known P-450 2E1 substrate, carbon tetrachloride, was investigated '*in vivo*' in resting cultures of *Pseudomonas putida* co-expressing cytochrome P-450 2E1 and luciferase.

Pseudomonas putida cells co-expressing *N*-terminus modified human cytochrome P-450 2E1 and luciferase were grown according to the conditions described earlier (section 4.2.4.2) to achieve maximal P-450 and luciferase expression, confirmed by CO-difference spectra and luminescence measurement, respectively. The cell pellet was washed with and resuspended in 25 volumes (w/v) of M9 medium supplemented with 6.8 mM citrate. Carbon tetrachloride metabolism was assayed under low oxygen conditions, by placing 3 ml cell suspension in 5 ml serum vials that were secured with teflon-coated stoppers and aluminium crimp seals. After evacuation for 5 min, 50 μM of high purity carbon tetrachloride was introduced into the vials using a gas-tight syringe. The vials were incubated at 26°C for 18h. Appropriate chemical and boiled-cell controls were run concurrently. The host strain, *Pseudomonas putida* expressing luciferase was also incubated with carbon tetrachloride under similar conditions, in order to determine the transformation of the substrate by the host organism. High purity carbon tetrachloride standard utilized for the metabolism studies was evaluated by GC-MS to confirm the absence of even trace concentrations of chloroform.

Reductive dehalogenation and formation of chloroform was monitored by analysis of head space gas by capillary GC and GC-MS as described in section 5.2.11.

5.2.11 Analysis

Hexachloroethane (HCE) and tetrachloroethylene (TCE) concentrations were determined by analysis of the headspace gas. To sample, 50 µl of the headspace gas was removed from the serum vials using a gas-tight syringe (Pressure-Lok series A-2, Dynatech Precision Sampling Corp., USA) and injected into a capillary gas chromatograph. Carbon tetrachloride metabolism and formation of chloroform was similarly monitored by analysis of 50 µl of the headspace gas by capillary gas chromatography and GC-MS.

To determine γ-hexachlorocyclohexane metabolism, samples were amended with *n*-hexane (1:1, v/v) and vortexed thoroughly. The mixture was then centrifuged at 3500 xg for 2-3 min. The supernatant hexane extract was recovered for gas chromatographic analysis. Depletion of parent compound and formation of metabolites was determined by capillary gas chromatography.

Capillary gas chromatography was performed on a Perkin-Elmer 'Autosystem' 1022 Plus gas chromatograph equipped with a flame ionization detector and a 30 m x 0.25 µm DB-5 capillary column. The chromatographic conditions for the determination of hexachloroethane and tetrachloroethylene were 40°C hold for 0.5 min, followed by a ramp to 200°C at 10°C/min and for assaying γ-hexachlorocyclohexane metabolism were 80°C hold for 0.5 min, followed by a ramp to 280°C at 10°C/min. Chromatographic conditions for

monitoring carbon tetrachloride depletion were 30°C hold for 2 min, followed by a ramp to 150°C at 5°C/min.

The identity of the metabolites was established by GC-MS. Mass spectra were obtained with a Varian Saturn 3 GC-MS in Ion trap configuration equipped with a 30 m x 0.25 μm DB-5 capillary column and CI/GP/NIST 98 libraries.

5.3 Results

5.3.1 Expression of engineeered traits in Pseudomonas putida

The expression of cytochrome P-450 cam and luciferase in engineered *Pseudomonas putida* was evident from the reddish color of the cell cultures, the formation of reduced CO-difference spectra and decanal-dependent luminescence exhibited by cells grown in presence of IPTG and *m*-toluate (Figure 25A). The expression of cytochrome P-450 cam CAB genes in engineered *Pseudomonas putida* was confirmed by the diagnostic CO-difference spectra (Figure 25B). The expression of *N*-terminus modified human cytochrome P-450 2E1 and luciferase in engineered *Pseudomonas putida* was similarly validated by the formation of reduced CO-difference spectra and decanal-dependent luminescence in cultures supplemented with IPTG and *m*-toluate (Figure 16). Cytochrome P-450 yields in the *Pseudomonas putida* constructs routinely averaged 120-150 nmol of cytochrome P-450 cam/liter culture and 40-50 nmol of cytochrome P-450 2E1/liter culture, under the mentioned growth conditions.

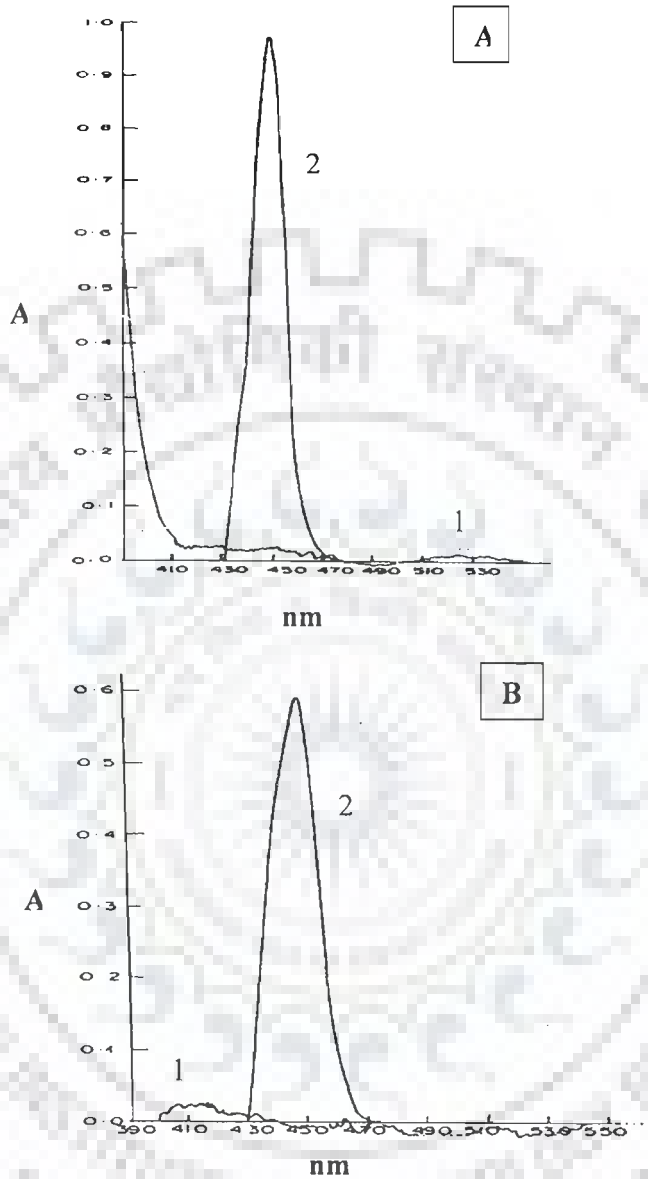


Figure 25. Expression of cytochrome P-450 cam in genetically engineered *Pseudomonas putida* monitored by CO-difference spectra

A. CO-difference spectra of *P. putida* expressing cytochrome P-450 cam CAB genes

B. CO-difference spectra of *P. putida* co-expressing cytochrome P-450 cam C and luciferase

1 - Baseline recorded after the addition of dithionite as reductant

2 - Difference spectra recorded after saturation of sample cuvette with dithionite

5.3.2 Dehalogenation of hexachloroethane by genetically engineered Pseudomonas putida

Pseudomonas putida cells co-expressing cytochrome P-450 cam and luciferase converted hexachloroethane to tetrachloroethylene at low oxygen tension as confirmed by GC-MS. The formation of tetrachloroethylene was dependent on the expression of both the hemoprotein and luciferase. Control incubations with boiled cells did not yield any detectable metabolites, confirming a biologically catalysed reaction. Under the assay conditions, nearly 15% of hexachloroethane initially present was converted to tetrachloroethylene in 3 hours validated by capillary gas chromatography and GC-MS (Figure 26). The rate of metabolism was observed to be comparable to that exhibited by *Pseudomonas putida* containing the complete cam monooxygenase system (cam CAB genes) incubated under similar conditions.

The functional stability of the engineered traits as well as the ability to compete with natural microorganisms of engineered *Pseudomonas* co-expressing cytochrome P-450 cam and luciferase evaluated in soil slurries (soil characteristics: pH 8.3, 56.11% clay, 23.2% silt, 20.69% sand, 62.45% water holding capacity, 0.6% organic carbon and 0.054% total nitrogen) yielded a rapid conversion of hexachloroethane to tetrachloroethylene. The soil slurries inoculated with the GEM transformed hexachloroethane to tetrachloroethylene 8-10 fold more rapidly as compared to uninoculated controls under sub-atmospheric conditions with a substantial decline in light emission and concurrent loss of more

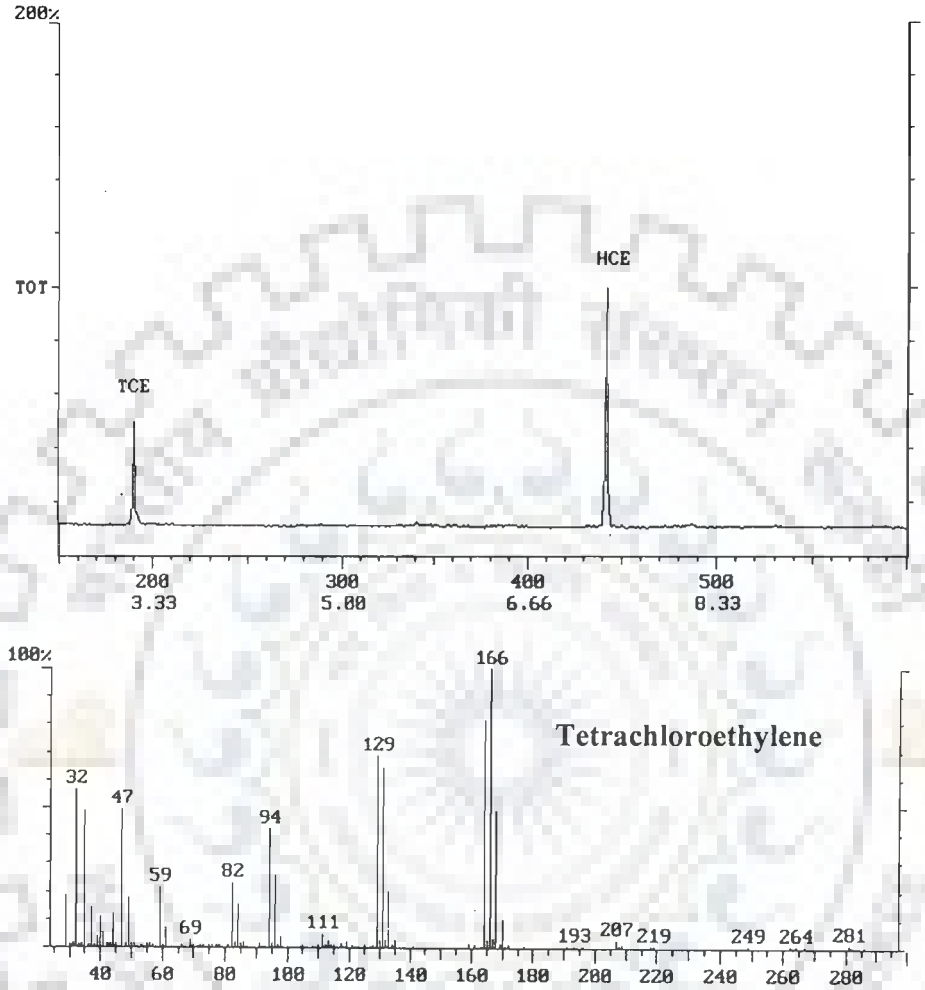


Figure 26. **Gas chromatogram and mass spectrum of tetrachloroethylene detected on transformation of hexachloroethane by *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase**
[HCE: Hexachloroethane; TCE: Tetrachloroethylene]

than 60% HCE after 7 days. Control slurry not amended with GEM cells exhibited a loss of only 7-9% HCE during the same period (Figure 27).

Longer incubations to determine the complete loss of hexachloroethane in soil slurries amended with the GEM exhibited a decline of 44% in the HCE concentration at 7 days with only trace concentrations of hexachloroethane detected after 14 days incubation. After 21 days, hexachloroethane as well as tetrachloroethylene were observed to be completely metabolized in slurries inoculated with the GEM whereas the uninoculated controls exhibited a persistence of 68% of the initial hexachloroethane concentration, as determined by GC and GC-MS analysis (Figure 28).

5.3.3 Dehalogenation of γ-hexachlorocyclohexane by genetically engineered Pseudomonas putida

Pseudomonas putida cells co-expressing cytochrome P-450 cam and luciferase reductively dehalogenated γ-hexachlorocyclohexane to γ-3,4,5,6-tetrachlorocyclohexene, under low oxygen tension. Resting cultures of genetically engineered *Pseudomonas* demonstrated a conversion of 10% γ-hexachlorocyclohexane in 3 hours, compared to the controls that exhibited no detectable metabolites. Products were unambiguously identified by their mass spectra. *Pseudomonas putida* containing the complete cam monooxygenase system (cam CAB genes) incubated with γ-hexachlorocyclohexane under identical conditions exhibited similar dehalogenation rates.

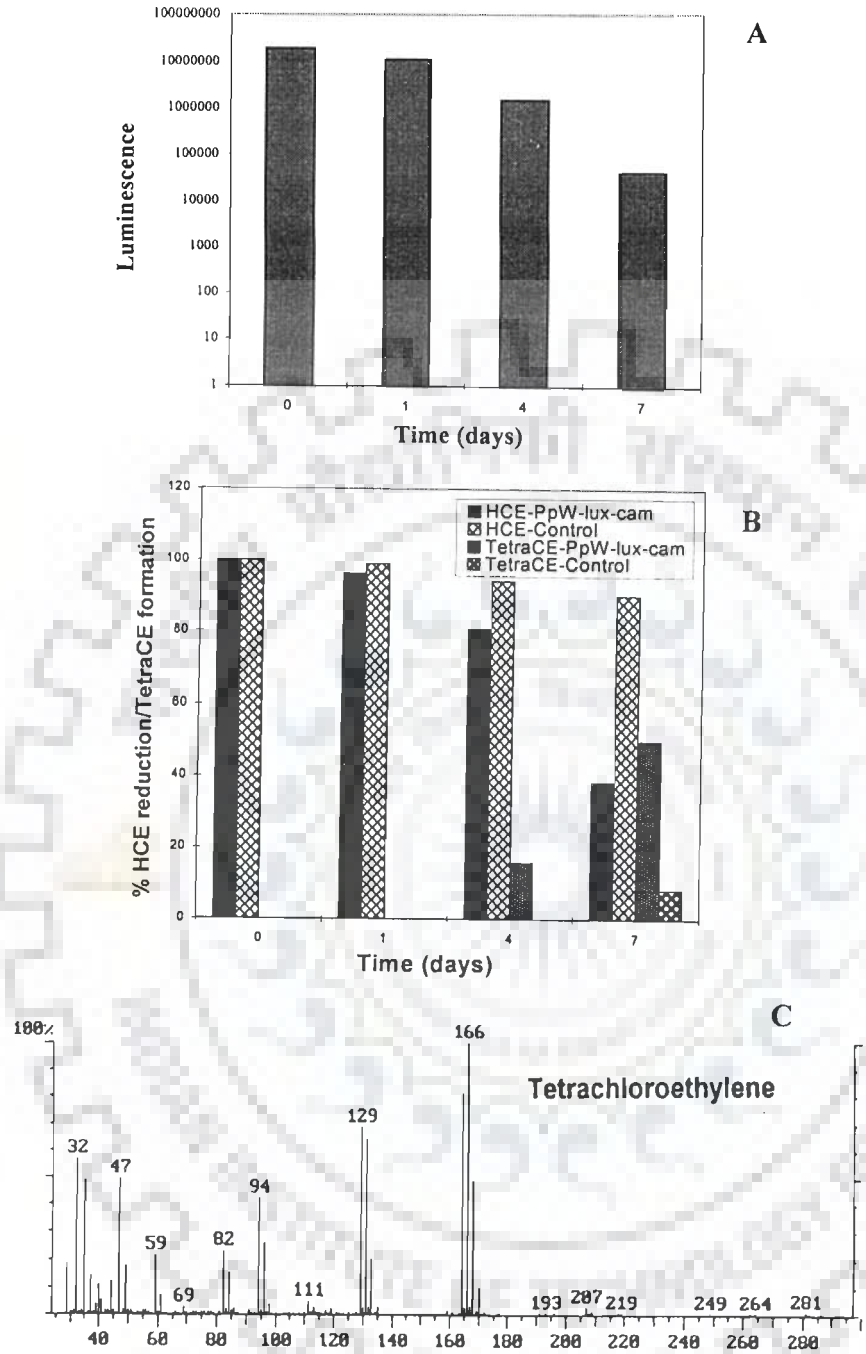


Figure 27. Dehalogenation of hexachloroethane in soil slurry by engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase

A. Luminescence signal of engineered *Pseudomonas* cells detected in soil slurry contaminated with hexachloroethane

B. Transformation of hexachloroethane to tetrachloroethylene by engineered *Pseudomonas* in soil slurry microcosm

C. Mass Spectrum of tetrachloroethylene

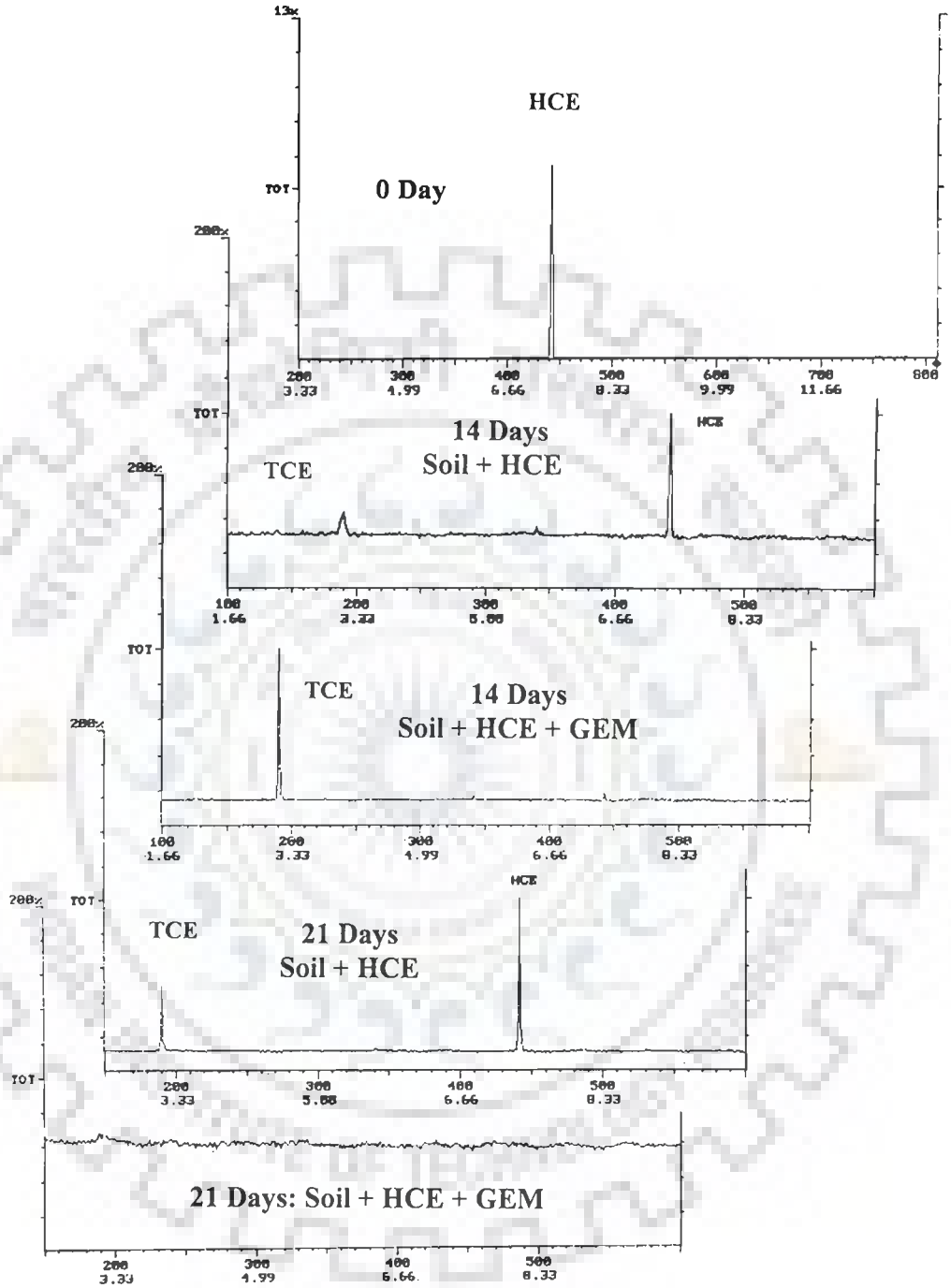


Figure 28. Dehalogenation and disappearance of Hexachloroethane by genetically engineered *Pseudomonas putida* in soil slurry microcosm

[HCE: Hexachloroethane; TCE: Tetrachloroethylene]

Metabolism of γ-hexachlorocyclohexane in soil slurries similarly yielded γ-3,4,5,6-tetrachlorocyclohexene as confirmed by GC-MS analysis (Figure 29). In addition, the incubations in the soil slurries also yielded other metabolites, γ-1,2,3,4,5-pentachlorocyclo-hex-1-ene, 1,2,4-trichlorobenzene and 1,2-dichlorobenzene confirmed by GC-MS analysis (Figure 30). After 4 weeks more than 65% decline in γ-hexachlorocyclohexane concentration was observed in slurries inoculated with the GEM as compared to a reduction of 8% in the uninoculated controls (Figure 29). Luminescence recorded concomitantly in soil slurries amended with the GEM although exhibiting a substantial decline, could be detected even after an incubation of 4 weeks.

Metabolism of γ-hexachlorocyclohexane monitored in slurries amended with the GEM and supplemented with Tween-80 exhibited an enhanced transformation of the compound compared to slurries that did not receive the surfactant as also the uninoculated controls. Soil slurries containing Tween-80 yielded more than 58% decline in γ-hexachlorocyclohexane concentration after 10 days compared to a 33% decline evidenced in slurries without Tween-80 (Figure 29C). After 20 days, only trace concentrations of γ-hexachlorocyclohexane were evidenced in slurries amended with the surfactant, Tween-80 whereas soil slurries not amended with Tween-80 exhibited 70% transformation of γ-hexachlorocyclohexane. Control uninoculated soil slurry with and without amendment with Tween-80 exhibited a loss of only 11% and 7% during the same period.

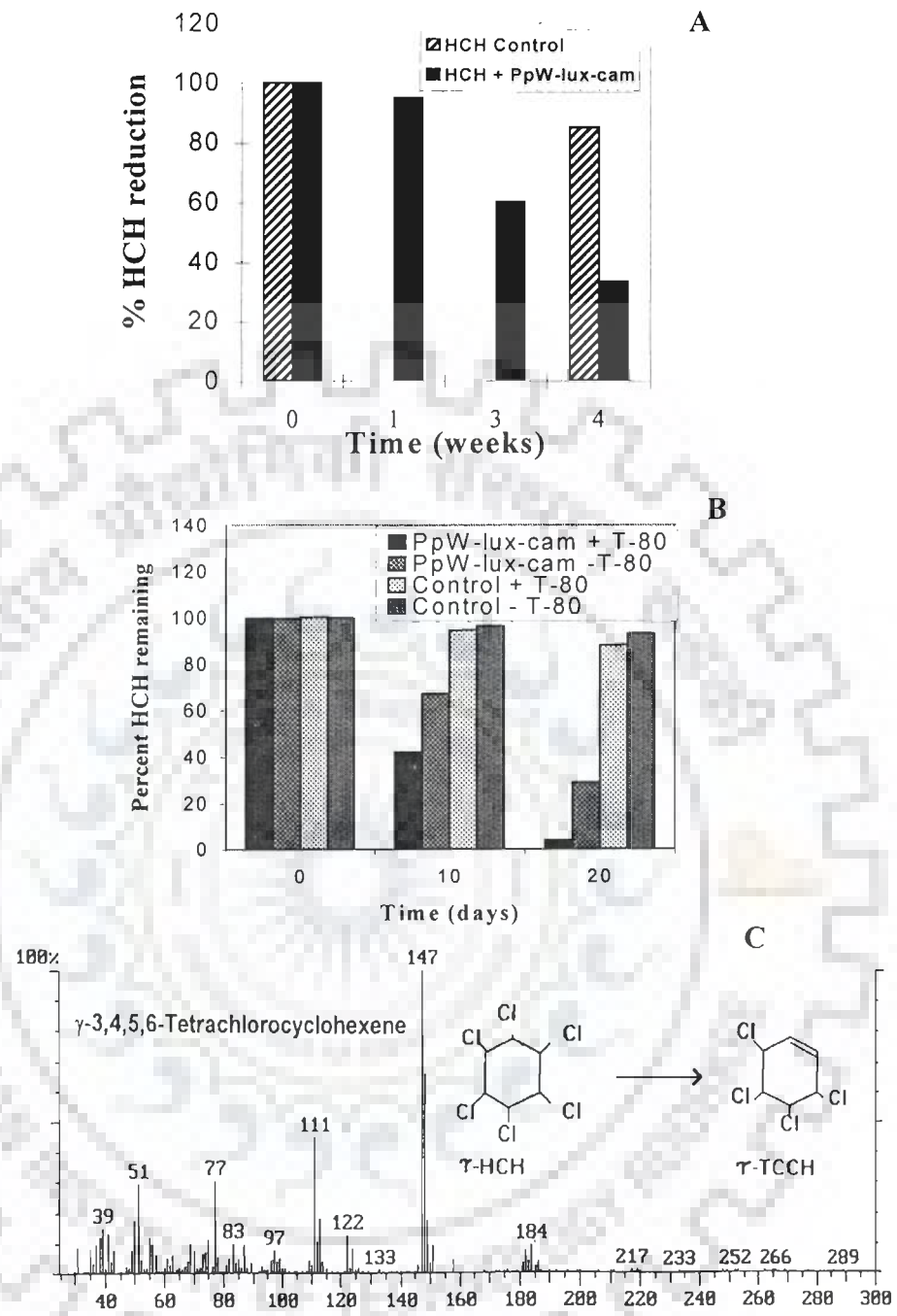


Figure 29. Dehalogenation of γ-hexachlorocyclohexane in soil slurry by genetically engineered *Pseudomonas putida*

A - Disappearance of γ-hexachlorocyclohexane in soil slurry
B - Influence of surfactant (Tween-80) addition on dehalogenation of γ-hexachlorocyclohexane in soil slurry
C - Mass spectrum of γ-3,4,5,6-tetrachlorocyclohexene

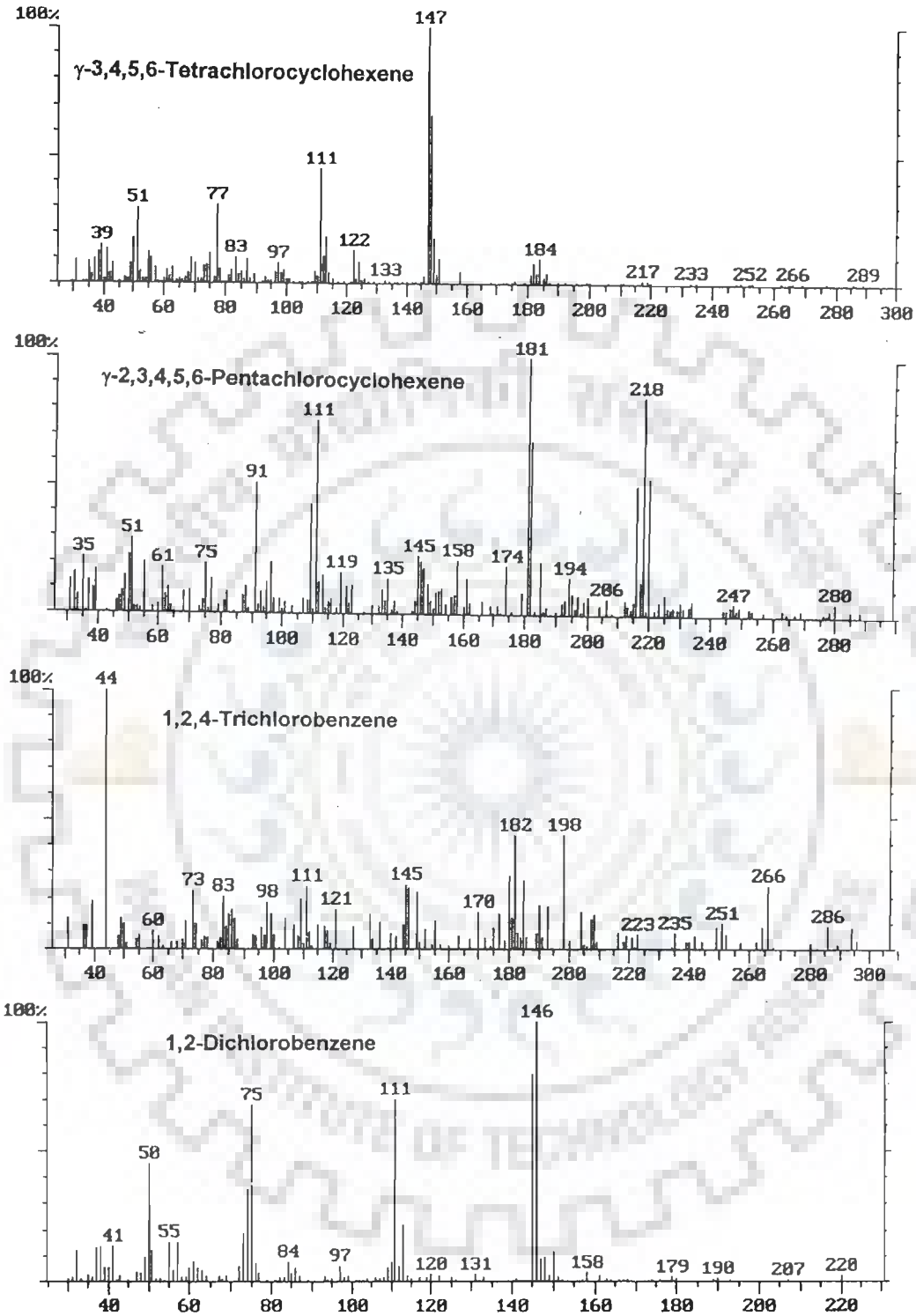


Figure 30. Certain metabolites detected by GC-MS on transformation of γ-hexachlorocyclohexane by engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase in soil slurry microcosm

5.3.4 Catalytic activity of Pseudomonas putida co-expressing cytochrome P-450 2E1 and luciferase

Resting cultures of *Pseudomonas putida* co-expressing cytochrome P-450 2E1 and luciferase under low oxygen tension converted carbon tetrachloride to chloroform, as determined by GC-MS analysis (Figure 31). Control incubations with boiled cells and the chemical control did not yield any detectable chloroform, indicating that the conversion observed was due to biotic process. The host strain, *Pseudomonas putida* expressing luciferase incubated with carbon tetrachloride under similar conditions also did not exhibit transformation of carbon tetrachloride. Thus, the *in vivo* reductive metabolism of carbon tetrachloride by *Pseudomonas putida* co-expressing cytochrome P-450 2E1 and luciferase (in the absence of any reducing equivalents) demonstrates that luciferase serves as an alternate electron transfer partner for P-450 2E1 and the enzyme is capable of catalysing reductive reactions *in vivo*.

5.4 Discussion

The use of genetically engineered microorganisms for bioremediation applications requires that the organisms survive and express their catabolic potential in the natural environment (Ramos *et al.*, 1991; Nublein *et al.*, 1992). Earlier reports on the degradation of halogenated and aromatic hydrocarbons by engineered organisms demonstrate that it is possible to design and engineer novel catabolic pathways for the *in situ* degradation of toxic chemicals (Ramos *et al.*, 1987; Rojo *et al.*, 1987; Nublein *et al.*, 1992; Pipke *et al.*, 1992). The bioremediation of chloroaromatics has also been demonstrated on a laboratory

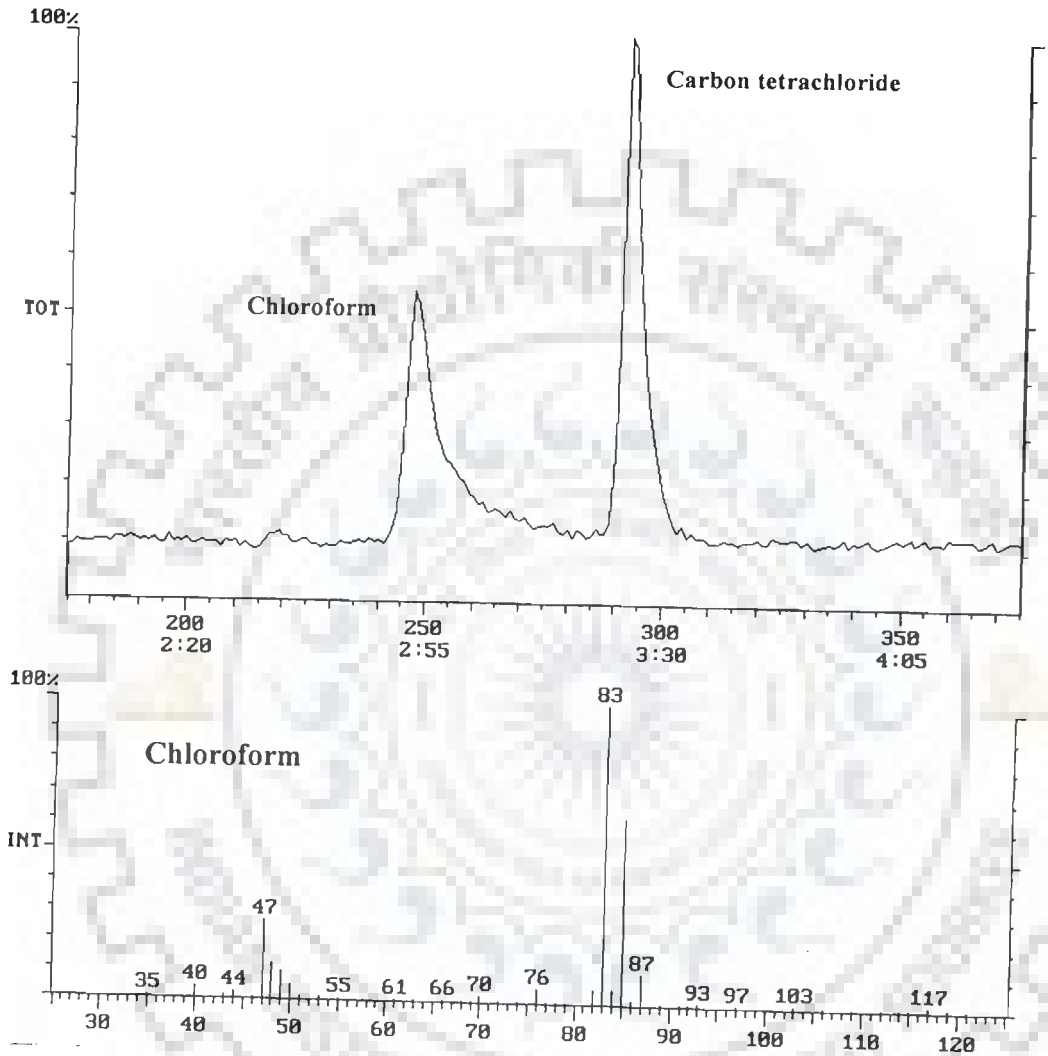


Figure 31. Transformation of Carbon Tetrachloride to Chloroform by *Pseudomonas putida* co-expressing cytochrome P-450 2E1 and luciferase as determined by GC-MS analysis

scale with soil columns (Kuhn *et al.,* 1985; van der Meer *et al.*, 1987; Pfarl *et al.,* 1990) or with soil slurry systems (Rosenberg and Alexander, 1980; Oldenhuis *et al.,* 1989; Mueller *et al.,* 1991; Fava and Bertin, 1999). The application of organisms in the field has also been described for soils contaminated with oil or pentachlorophenol (Lamar and Dietrich, 1990; Brunsbach and Reineke, 1993). Such studies are required to determine the expression and stability of the catabolic traits under oligotrophic situations of microorganisms targeted for use as bioremediative agents.

The present study evaluated the expression and stability of the engineered catabolic functions of genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase in soil slurries. This organism has previously been shown to reductively dehalogenate several chlorohydrocarbons using luciferase as an electron transfer protein for cytochrome P-450 cam (Shanker and Atkins, 1996). Cytochrome P-450 cam in this construct is subcloned on the broad host range vector pMMB 206 under the control of the P*taclac* promoter, while the expression of chromosomally-encoded luciferase is under the control of the *Pm* promoter. Since higher expression and substrate transformation yields of cytochrome P-450 cam are evidenced in *Pseudomonas putida* in comparison to the expression yields of human cytochrome P-450 2E1, this organism was utilized as a model to assess the stability of engineered functions in terms of catalytic activity under oligotrophic situations.

In the present study, *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase transformed the target compounds under nutrient stress

conditions, as evidenced by the dehalogenation of hexachloroethane and γ-hexachlorocyclohexane in soil slurries (Figures 27-29). The study also validated the survival of engineered organism in sufficient numbers to effect the dehalogenation of the target chemicals, without any selective pressure of antibiotics in the soil slurries. Engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase catalysed a rapid conversion of hexachloroethane to tetrachloroethylene in soil slurry with significant reduction evidenced after 7 days (Figure 27). The presence of only trace concentrations of hexachloroethane as well as tetrachloroethylene detected after 14 and 21 days, respectively in soil slurry amended with the engineered strain is noteworthy. In comparison, control soil slurry without the GEM exhibited high concentrations of both the compounds after the same period of incubation (Figure 28). The disappearance of both hexachloroethane and tetrachloroethylene evidenced in soil slurry amended with the GEM may possibly be due to synergistic activity of the engineered organism and the indigenous microorganisms present in the soil. Such results confirm the potential of genetically engineered organisms to enhance the detoxification of persistent compounds and render them amenable for transformation by the autochthonous microflora.

Engineered *Pseudomonas putida* also mediated the dehalogenation of a widely used insecticide, γ-hexachlorocyclohexane in soil slurries (Figure 29). The presence of the engineered *Pseudomonas* effected the removal of substantial amounts of γ-hexachlorocyclohexane from the slurry with the detection of other downstream metabolites (Figure 30). The addition of the surfactant, Tween-80,

lead to enhanced rates of metabolism in comparison to incubations without the surfactant. The addition of surfactants has been shown to be beneficial for the degradation of hydrophobic compounds by altering the interfacial tension and phase partitioning of the compounds (Laha and Luthy, 1992; Fu and Alexander, 1995). The addition of surfactants, such as Tween-80, increases the accessibility of the parent molecule and metabolites thereby enhancing the activity of the engineered as well as the indigenous organisms. The presence of Tween-80 caused a two-fold increase in the observed metabolism of γ-hexachlorocyclohexane in comparison to slurries not supplemented with the surfactant (Figure 29C). Slurrying of the soil has been demonstrated to promote the microbial transformation of aromatic hydrocarbons (Fu and Alexander, 1995). Slurry treatment in combination with the use of surfactants may thus represent a viable means for the degradation of persistent halogenated compounds.

Several factors markedly influence the success of biodegradation in soil (Goldstein *et al.,* 1985; Nublein *et al.,* 1992; Havel and Reineke, 1992; Brunsbach and Reineke, 1993). The inoculant populations may not increase in size to cause extensive biodegradation due to competition from other species inhabiting the soil environment for the limited supply of nutrients (Havel and Reineke, 1992). In addition, the introduced bacterial population may be adversely affected by predation before it becomes sufficiently large to bring about appreciable destruction of the polluting chemical (Ramadan *et al.,* 1990; Havel and Reineke, 1992). These factors possibly did not impede the transformation of hexachloroethane and γ-hexachlorocyclohexane in soil slurries, and the

engineered *Pseudomonas* was effectively able to sustain a small population in soil slurries for a prolonged duration evidenced by the luminescence signal obtained even after a period of 4 weeks. The chromosomally encoded luciferase in the engineered organism not only has a role as electron transfer protein for cytochrome P-450 cam but also as a bioluminescence reporter system with sufficient robustness to allow detection of the organism under chemical stress even at sub-lethal concentrations as observed with hexachloroethane and γ-hexachlorocyclohexane.

The present study explored the catalytic activity of *N*-terminus modified human cytochrome P-450 2E1 expressed in *Pseudomonas putida* co-expressing luciferase. It was anticipated that the luciferase-reduced hemoprotein would be capable of catalyzing reductive reactions *in vivo*. To investigate this hypothesis, the reductive metabolism of carbon tetrachloride was evaluated in resting cultures. The demonstration of reductive dehalogenation activity effectively validated the utility of luciferase to serve as an alternate electron transfer protein for the heterologously expressed P-450 2E1 enzyme (Figure 31). Moreover, the ability of luciferase to generate a catalytically active enzyme overcomes the need of exogenous addition of NADPH-dependent cytochrome P-450 reductase which severely limits its utility in bacteria.

There have been conflicting reports on the effect of *N*-terminus truncation on the catalytic activity of microsomal cytochrome P-450 enzymes. Previous studies with certain shortened forms of cytochrome P-450 have shown that truncation of the *N*-terminal sequences, albeit favorable to expression on one

hand, can severely perturb the catalytic properties of these hemoproteins on the other (Lehnerer *et al.,* 1995). However, Larson *et al.,* (1991) demonstrated that the NH_2-terminus, though important as a membrane anchor signal '*in vivo*', has no function in the catalytic activity of cytochrome P-450 2E1. In the present study, the deletion of 21 amino acids from the NH_2-terminus of human cytochrome P-450 2E1 did not perturb atleast the catalytic activity for reductive reaction as evidenced by the metabolism of carbon tetrachloride by the luciferase-reduced hemoprotein.

5.5 Concluding Remarks

The present study demonstrates the *in situ* expression and stability of engineered catabolic functions in *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase under oligotrophic conditions. The demonstration of carbon tetrachloride metabolism in resting cultures of *Pseudomonas putida* co-expressing *N*-terminus modified human cytochrome P-450 2E1 and luciferase is significant since few observations are available on the activity of mammalian P-450 enzymes in live bacterial cultures. These results validate the viability of utilizing cytochrome P-450 isozymes as bioremediative agents for detoxification of halogenated hydrocarbons.

Chapter 6

Stress Survival and Generation of 'Microbial Inocula' of Genetically Engineered Pseudomonas putida

6.1 Introduction

The ability of bacteria to survive and proliferate in soil is an important determinant in their success as inoculants for bioremediation (Alexander, 1985; Acea and Alexander, 1988; Young and Burns, 1993; Young *et al.,* 1995). An engineered strain must compete successfully with indigenous microbes in an oligotrophic environment. The assessment of the efficiency of microbial inocula requires knowledge of the effects of environmental factors on microbial survival and activity in soil (Meikle *et al.,* 1995). Though the physiological response of bacterial populations following introduction into the natural soil environment is poorly understood, information on the physiology of inoculants regarding their resistance to soil factors is required to obtain a better insight into their survival strategies (van Overbeek *et al.,* 1995).

Genetically engineered organisms introduced into the environment have to deal with a number of stress factors. Low concentrations of the xenobiotic compound may not support the growth of these specialized strains (Goldstein *et al.,* 1985; Ramos *et al.,* 1991). In addition, naturally occurring toxins, predators and interspecific competition for nutrients may also reduce the survival of the introduced strains (Liang *et al.,* 1982; Dupler and Baker, 1984; Klein and Alexander, 1986; Acea *et al.,* 1988; Ramos *et al.,* 1991; Vandenhove *et al.,* 1991). Changes in behavior of many bacteria introduced into oligotrophic environments, in terms of adaptation to low nutrient conditions and alteration in pattern of gene expression and nonculturability, have been reported (Roszak and Colwell, 1987; Kjelleberg, 1993). In specific cases the success of inoculants generally decreased in

presence of indigenous microflora, as there is a marked domination of gram-negative bacteria in the rhizosphere and bulk soil (Kozdroj, 1996). Genetically engineered strains with marker or 'signal' genes can serve as model organisms to assess and predict both survival and function of 'microbial inocula' of natural organisms under *ex-situ* conditions in bioreactors as well as *in* situ conditions in soil environment.

Luminescence-based techniques enable efficient and sensitive detection and assessment of activity of microbial inocula in soil (Rattray *et al.,* 1990; 1992). In addition, luminescence-based marker systems also provide information on the metabolic activity of viable and non-culturable cells without the need for the extraction of cells (Meikle *et al.,* 1995; Amin-Hanjani *et al.,* 1993). Moreover, there is no significant background luminescence among indigenous soil microflora which could mask the detection of light emitted by the engineered organisms (Duncan *et al.,* 1994). Thus, luminescence based marker systems being more sensitive, convenient and highly selective can serve as potential reporter genes to monitor activity of engineered organisms released in the environment.

The genus *Pseudomonas* constitutes an important group of bacteria from an environmental biotechnology viewpoint (Shanker *et al.,* 1990; Timmis *et al.,* 1994; Chattre *et al.,* 1996; Chugani *et al.*, 1997). However, little information is available on the growth conditions faced by these bacteria in the soil and rhizosphere and their genetic/physiological responses to these fluctuating conditions (Givskov *et al.,* 1994). In addition, there is a paucity of data on recombinant bacteria with dual, viz. both chromosomally and plasmid engineered, traits in the same cells.

A prerequisite to use of 'microbial inocula' is the preservation regime and shelf life of material. The preservation of both natural and genetically engineered microorganisms is becoming increasingly important due to their potential as remediative agents. The use of solid carriers for soil applications of microbial inoculants has been explored as a potential means for achieving enhanced survival and activity of microbial inocula (Cassidy *et al.,* 1997). Biodegradation of toxic compounds using microbial encapsulation technology has been studied using various carriers including peat (Graham-Weiss *et al.,* 1987), polyacrylamide (Jawson *et al.,* 1989; Thomas and White, 1990), polyurethane (Hu *et al.,* 1994), alginate (Weir *et al.,* 1995) and *k*-carrageenan (Chapatwala *et al.,* 1993). Clay-minerals including vermiculite have also been successfully employed as inoculant carriers (Jawson *et al.,* 1989). Immobilization in a polymer matrix has been reported to confer protection from high levels of toxic compounds normally lethal to free cells (Cassidy *et al.,* 1997). Enhanced survival of encapsulated genetically engineered *Pseudomonas fluorescens* released into soil microcosms has been demonstrated, in comparison to the unencapsulated cells, after 3 months (van Elsas *et al.,* 1992).

Natural, non-toxic and biodegradable carriers like peat, alginate and clay minerals may be preferable for environmental applications (Cassidy *et al.,* 1997). Though powdered peat is the most common carrier for inoculant production, it may contain inhibitors for bacterial growth as also sterilization procedures may produce toxic by-products hindering bacterial growth and survival (Graham-Weiss, 1987; Parker and Vincent, 1981). Thus, alternate binding matrices including clay minerals

like vermiculite, bentonite and lignite may be employed as carriers for microbial inocula.

The present study evaluates the nutrient stress survival of genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase in clay-based matrices for the generation of active microbial inocula.

6.2 Materials and Methods

6.2.1 Chemicals and Glassware

Bacto-tryptone, yeast extract and Noble agar were purchased from DIFCO Laboratories (Detroit, USA). The antibiotics chloramphenicol and piperacillin were obtained from SIGMA Chemical Company (St. Louis, USA). All other chemicals were of the highest purity commercially available. Glassware utilized was borosilicate glass of Borosil or Vensil make.

6.2.2 Culture Media

6.2.2.1 Luria-Bertani Medium

The composition of Luria-Bertani medium is described in section 3.2.2.1

6.2.2.2 M9 Medium

The composition of M9 medium is described in section 5.2.2.1

6.2.3 Bacterial Constructs

Genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam C and *Vibrio harveyi* luciferase lux AB was used for the studies. The construction of this organism has been described earlier (Shanker and Atkins, 1996). The wild-type parent strain *Pseudomonas putida* MTCC 102 was also utilized for nutrient stress survival studies.

6.2.4 Soil

A black cotton agricultural soil was employed for the survival studies and for the isolation of the clay fraction utilized as binding matrix. Soil was collected from the top 15 cm of the soil profile, passed through a coarse sieve to remove gravel and other debris and stored moist at 4°C for future use. For the determination of stress survival in sterile soil, the soil was sterilized three times at 121°C for 15 min, on alternate days and stored at 30°C between autoclaving, to ensure sterility. Soil analysis was performed according to the standard protocols (Page *et al.*, 1982).

6.2.5 Binding Matrix/Carrier Material

Clay or the clay minerals viz. kaolin and vermiculite were used as carriers/binding matrices for the cells. Crude clay utilized as carrier was isolated from black cotton soil collected from the top 15 cm of the soil profile, according to standard protocols (Black, 1965). Clay-minerals vermiculite and kaolin utilized as binding matrices were of horticultural and pharmaceutical grade, respectively. Prior to use, exfoliated vermiculite was ground to a fine powder in a blender and sieved through U.S. Standard Sieve No. 45 (354 micron).

6.2.6 Optimization of growth conditions of genetically engineered Pseudomonas putida

Pseudomonas putida co-expressing cytochrome P-450 cam and luciferase was routinely cultivated in Luria-Bertani medium supplemented with 20 μg/ml chloramphenicol, 1mM IPTG and 200 μM *m*-toluate (as described in section 5.2.5). The expression of both the engineered traits, viz. cytochrome P-450 cam

and luciferase was evaluated by CO-difference spectroscopy and luminescence measurement, respectively, as described earlier (sections 5.2.6 and 5.2.7).

6.2.7 Detection of engineered Pseudomonas putida in liquid culture and in soil slurry

The sensitivity of luminescence detection in liquid culture and soil slurry was assessed for *Pseudomonas putida* cells co-expressing cytochrome P-450 cam and luciferase. Known CFU were added to liquid medium and to 1:1 (w/w) soil/water slurry and correlated with the luminescence signal recorded therein. Standard curves were plotted for luminescence signal against a range of viable cell counts.

Cell pellet of *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase grown in Luria-Bertani medium (section 5.2.5) was resuspended in 10 volumes of Luria-Bertani medium (w/v) and served as the inoculum for serial dilutions. The viable cell concentration of the inoculum was determined by plating appropriate dilutions on LB-agar supplemented with 20 μg/ml chloramphenicol and 30 μg/ml piperacillin.

6.2.7.1 Detection in liquid culture

Minimum detection levels in liquid culture were assessed by monitoring light output of different concentrations of cells in a luminometer and a scintillation counter. Serial dilutions were prepared in triplicate, in Luria-Bertani medium and utilized directly for luminescence determination. Luminescence was measured in 3 ml or 1 ml triplicate samples of each dilution, either in a Packard-

Tricarb 1600 TR scintillation counter or luminometer respectively, after the addition of 1 µl *n*-decanal.

6.2.7.2 Detection in soil slurry

The ability to enumerate luminescent GEM cells in the presence of indigenous soil population was determined in 1:1 (w/w) soil/water slurry. The quantification of minimum detection limit was performed by the addition of a range of cell concentrations to triplicate samples of 3 ml or 1 ml unsterile soil slurry taken in scintillation vials or luminometer cuvettes. The soil suspension was mixed thoroughly by vortexing and luminescence was determined in a scintillation counter or a luminometer after the addition of 1µl *n*-decanal.

6.2.8 Nutrient stress survival of engineered Pseudomonas putida

Genetically engineered *Pseudomonas putida* and wild-type parent strain *P. putida* MTCC 102 were subjected to carbon and multiple nutrient starvation using resuspension technique (Kolter *et al.,* 1993) to evaluate stress responsiveness of the organism. For *in vitro* carbon starvation studies, log phase cells of engineered *Pseudomonas* grown in Luria-Bertani medium were washed three times with and inoculated in 100 mL M9 minimal medium taken in 250 mL Erlenmeyer flasks and incubated stationary at 30°C. Multiple nutrient starvation was carried out similarly in 0.9% NaCl. The parent strain, *Pseudomonas putida* MTCC 102 was similarly incubated in M9 minimal medium and 0.9% NaCl. Viable counts were determined by plating appropriate dilutions of the incubated samples on LB-agar at 0 and 7 days of incubation. The effect of inducers of *lux* AB and cytochrome P-450 *cam* C on the survival of engineereed *Pseudomonas* and consequent recovery of the engineered

traits was elucidated by inoculating cells in M9 medium supplemented with either 200 μM *m*-toluate or 1mM IPTG or both. For this purpose, log phase cells washed with and resuspended in M9 medium were inoculated in 100 mL medium amended with the requisite inducer. Flasks were incubated stationary at 30°C. Appropriate dilutions were plated on LB-agar for enumeration of viable counts at 0 and 7 days of incubation. The expression of *lux* AB and cytochrome P-450 cam C was assayed after 7 days by addition of 0.5 volumes of Luria Bertani medium containing either 200 μM *m*-toluate and/or 1 mM IPTG to the M9 culture medium with incubation at 30°C on a shaker at 200 rpm. The cells were harvested after 20h by centrifugation at 8,000 xg for 10 min and resuspended in Luria-Bertani medium (1:50, w/v) to determine CO-difference spectra and luminescence emission.

6.2.9 Stress survival of genetically engineered Pseudomonas putida in soil microcosms

The stress survival of genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase was determined in a high clay content black cotton soil. Cells grown in Luria-Bertani medium (section 5.2.5) and harvested by centrifugation at 8000 xg and 4°C for 10 min were washed with and resuspended in M9 medium (1:10 w/v suspension) to yield the inoculum for addition to the soil.

Stress survival in soil microcosms was monitored by placing triplicate samples of 5 g unsterile soil in 25 ml Erlenmeyer flasks. The soil was amended with sterile distilled water. The water content of the soil was adjusted prior to inoculation such that after the addition of the cell suspension the soil was at 60% moisture

holding capacity. In a parallel experiment to assess the effect of selective nutrient amendment, a similar set of flasks containing 5 g unsterile soil was amended with selective nutrients viz. 6.8 mM citrate and 200 μM benzoate, at the completion of 6, 12 and 16 days. To determine inoculant survival in the absence of competition from the indigenous microflora, 5 g of sterile soil was similarly taken in 25 ml Erlenmeyer flasks. The moisture content was adjusted with sterile distilled water to attain 60% moisture holding capacity after inoculum amendment. All flasks were inoculated with *Pseudomonas putida* cells co-expressing cytochrome P-450 cam and luciferase to achieve final cell concentrations of $1.4x10^{10}$ CFU/g soil. The inocula and other additions were applied evenly over the soil surface by adding small volumes of liquid and gently rotating the flasks to assure mixing of the inoculum and nutrients with the soil. The soil samples including three replicates were incubated at 30°C, periodically weighed and water, if required, added to maintain the moisture levels.

At regular intervals, triplicate microcosms were sampled destructively. For analysis, 5 ml Luria-Bertani medium was added to each Erlenmeyer flask and mixed thoroughly by voretxing. An aliquot (1ml) of the resulting soil suspension was then placed in a luminometer cuvette for monitoring luminescence. Luminescence was monitored as RLU in a luminometer, after the addition of 1μl *n*-decanal.

6.2.10 Survival and reactivation of genetically engineered Pseudomonas in soil slurry

Survival of *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase was evaluated in soil slurry by placing duplicate 10 g samples of unsterile

soil in 125 mL Erlenmeyer flasks. Sterile distilled water was added to each flask to yield 1:1 (w/w) soil/water slurry. *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase was grown as described earlier (section 5.2.5), washed with and resuspended in M9 minimal medium. Cells were added to the soil slurry at final concentration of 1.8 x 10^6 CFU/g of soil. Soil slurry was amended with 6.8 mM citrate and 200 μM benzoate and incubated at 30°C on a rotary shaker at 100 rpm. Selective substrates viz. 200 μM benzoate, 6.8 mM citrate and 1% (v/v) Luria-Bertani medium were added to the soil slurry at various periods of incubation. Samples were maintained as 1:1 (w/w) soil/water slurry for 15 days and then allowed to desiccate slowly to 10% moisture content. Subsequently 1:1 (w/w) soil/water slurry was reconstituted and amended with citrate, benzoate or Luria-Bertani medium as required. Incubation was continued further up to 99 days without readjustment of water content.

Samples in duplicate were analyzed at various intervals up to 99 days. 1 mL aliquot soil slurry was added to 2 mL of Luria-Bertani medium and mixed thoroughly by vortexing for 1 minute to obtain a homogenous suspension. Luminescence was determined after the addition of 1 μl *n*-decanal in scintillation counter. Viable cell counts were enumerated by plating appropriate dilutions of the homogenous soil suspensions on LB-agar containing 20 μg/ml chloramphenicol and 30 μg/ml piperacillin.

6.2.11 Survival of matrix-bound genetically engineered Pseudomonas putida

Studies were conducted to elucidate the survival attributes of genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and

luciferase immobilized on clay matrices. The survival characteristics and stability of the engineered traits in the matrix-bound cells on prolonged storage were ascertained by two methods. Short-term stability was assessed in cells added to clay or the clay-minerals kaolin and vermiculite and stored as a wet paste. For long term survival studies, lyophilization was used in conjunction with the clay minerals to evaluate the effectiveness of the immobilization technique.

Genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase was grown in Luria-Bertani medium (section 5.2.5). Harvested cells were washed with and resuspended in 10 volumes (w/v) of M9 minimal medium supplemented with 0.1% (w/v) magnesium sulfate, 6.8 mM citrate and 200 μM benzoate.

The binding matrices, 5 g each of clay, kaolin and vermiculite were placed in sterile 15 ml serum vials and inoculated with 1 x 10^{10} CFU/g binding matrix. To examine the effect of sterility of the clay matrix on survival, pre-sterilized clay was placed in 15 ml serum vials and aseptically inoculated with the cell suspension at 1 x 10^{10} CFU/g binding matrix. The contents were mixed to ensure the absorption of the liquid into the clay matrix with the final product yielding a wet paste at moisture content of 60%. The clay-matrix bound cells were stored at room temperature and at 4°C and sampled for luminescence enumeration at regular intervals. Luminescence measurement was performed in a scintillation counter with 0.1g paste suspended in 3 ml Luria-Bertani medium, after the addition of 1 μl *n*-decanal.

Lyophilization was employed as an alternative strategy to immobilize *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase on the clay matrices. For such studies, 4 g exfoliated vermiculite and kaolin were taken separately in 125 ml Erlenmeyer flasks and inoculated with the cell suspension applied evenly to both the matrices, at 1.5 x 10^{15} CFU/g matrix. The samples were subjected to lyophilization in a Christ ALPHA 2-4 Lyophilizer. Different parameters were evaluated to optimize the lyophilization protocol including the temperature and time of lyophilization, and the concomitant vacuum applied. The temperature was varied between –25°C to –35°C in combination with a vacuum applied of 0.60 to 1.032 mbar. Lyophilization was performed for various periods between 4 to 10 hours. The freeze-dried samples were stored desiccated at room temperature in serum bottles sealed with butyl rubber stoppers.

Luciferase stability and survival of clay-mineral bound lyophilized cells on storage at room temperature was assessed by monitoring luminescence and viable cell counts in triplicate 0.1g samples. Lyophilized samples were enumerated after resuspension in 3 ml Luria-Bertani medium and incubation at 4°C for 30-60 minutes. Luminescence was monitored directly in the rehydrated samples in a scintillation counter after addition of 1 µl *n*-decanal. Viable cell counts were enumerated by plating serial dilutions on LB-agar supplemented with 20 µg/ml chloramphenicol and 30 µg/ml piperacillin with incubation at 28°C for 2-3 days.

6.2.11.1 Determination of dehalogenation activity of matrix-bound engineered Pseudomonas putida

The survival and stability of the engineered traits on prolonged storage of the lyophilized clay-mineral bound cells was evaluated by the determination of the ability of stored samples to dehalogenate hexachloroethane. Duplicate 15 ml serum vials containing 4g unsterile soil were amended with sterile deionized water to obtain 1:1 (w/w) soil:water mixture. The slurry was inoculated with kaolin-bound engineered *Pseudomonas putida* cells stored at 4°C for 14 weeks to yield $3.1x10^{10}$ CFU/g soil. The vials were secured with teflon coated stoppers and aluminium crimp seals and evacuated (15" Hg vacuum) for 5 min. After evacuation, all vials including an uninoculated control, were amended with 8.45 μM hexachloroethane and incubated at 30°C on a rotary shaker at 100 rpm.

Hexachloroethane conversion to tetrachloroethylene was monitored by analysis of the headspace gas by capillary GC and GC-MS as described earlier (section 5.2.11). Luminescence was determined concomitantly in 1 ml soil slurry in a scintillation counter after addition of 1 μl *n*-decanal.

6.2.12 Proliferation and survival of engineered Pseudomonas putida on nutrient-supplemented binding matrices

Clay-minerals, kaolin and vermiculite supplemented with a nutrient source and moisture were evaluated as support for the growth and survival of engineered *Pseudomonas putida.*

Kaolin and exfoliated vermiculite, 5g, were taken separately in 125 ml screw-cap bottles and amended with 2.5 ml of Luria-Bertani medium. The

contents were sterilized by autoclaving at 121°C for 15 min. *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase was grown in Luria-Bertani medium (section 5.2.5), harvested and resuspended in the same medium (1:100, w/v). Cells were added to both kaolin and vermiculite to achieve 10^7CFU/g matrix such that final carrier/nutrient ratios of 1:1 (w/v) and 1:1.5 (w/v) were obtained. In addition, vermiculite was also amended with the same inoculum of cells to obtain a final carrier/nutrient ratios of 1:2 (w/v). The carrier/nutrient ratios of 1:1 (w/v) and 1:1.5 (w/v) for the clay-mineral kaolin yielded 50% and 40% slurry (w/v), respectively, whereas vermiculite at similar ratios yielded a wet paste with the 1:2 ratio resulting in 33% (w/v) slurry. Samples were stored at room temperature for 30 days without any amendments for maintenance of the moisture content during the incubation.

Proliferation and survival of cells was monitored periodically by withdrawing 0.02g to 0.1g of the inoculated matrix for luminescence assay and viable count enumeration. The inoculated matrix was resuspended in 3 ml Luria-Bertani medium and luminescence monitored in a scintillation counter after the addition of 1 µl *n*-decanal. The viable cell counts were enumerated by plating serial dilutions on LB-agar supplemented with 20 µg/ml chloramphenicol and 30 µg/ml piperacillin and incubated at 28°C for 2-3 days.

6.3 Results

Genetically engineered *Pseudomonas putida* co-expressed cytochrome P-450 cam C and luciferase lux AB when cultures were supplemented with 1mM

IPTG and 200 μM *m*-toluate, respectively. This was confirmed by the diagnostic CO-difference spectra and luminescence recorded in such cultures (Figure 25).

6.3.1 Sensitivity of luminescence detection

Luciferase-based detection of *Pseudomonas putida* cells co-expressing cytochrome P-450 cam and luciferase assessed in liquid culture exhibited a linear relationship between the viable cell concentration and light output. The minimum detection limits for luminescence evaluated in scintillation counter and luminometer were 1.4×10^2 CFU/ml and 3.5×10^4 CFU/ml, respectively (Figure 32).

Luminescence measurement for quantification of cells in 1:1 soil/water slurry indicated that light output was proportional to the cell concentration using either method of measurement viz. scintillation counter or luminometer. However, the sensitivity of luminescence detection attained with the scintillation counter was higher in comparison to that obtained with the luminometer with minimum detection limits being 2.3×10^4 CFU/g soil and 4.5×10^6 CFU/g soil, respectively (Figure 32).

6.3.2 Nutrient stress survival of engineered Pseudomonas putida co-expressing cytochrome P-450 cam and luciferase

Pseudomonas putida cells co-expressing cytochrome P-450 cam and luciferase exhibited the ability to survive under nutrient stress including carbon or multiple nutrient starvation (C,N,P). Engineered *Pseudomonas* cells starved of a carbon source at 30°C for 7 days yielded only 40% of the population as culturable cells. In contrast, the culturable cells of the wild-type parent strain were approximately the same as at 0 day. Cells of engineered *Pseudomonas* incubated in

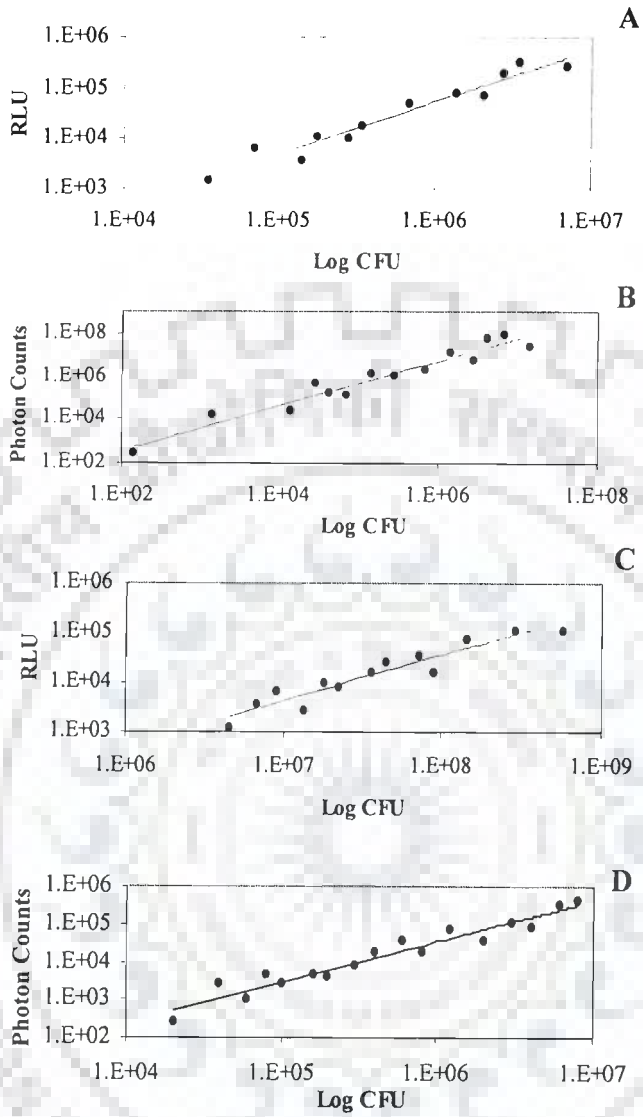


Figure 32. Sensitivity of luciferase based detection of genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase in liquid culture and soil slurry

A. Luminescence of cells added to Luria-Bertani medium enumerated as RLU in a luminometer

B. Luminescence of cells added to Luria-Bertani medium enumerated as photon counts in scintillation counter

C. Luminescence of cells added to soil slurry enumerated as RLU in a luminometer

D. Luminescence of cells added to soil slurry enumerated as photon counts in scintillation counter

absence of C, N and P in 0.9% NaCl, yielded 74% culturable cells after 7 days in comparison to 35% culturable cells of the parent strain (Table 4). In presence of glucose as carbon source only 50% of the engineered *Pseudomonas putida* cells were culturable while cells of the parent strain *Pseudomonas putida* increased by an order of magnitude. In another experiment, the effect of inducers, *m*-toluate for luciferase and IPTG for cytochrome P-450 cam, on the culturable population of the engineered *Pseudomonas* was determined. In the presence of toluate or IPTG there was a 16-17 fold increase in the population of culturable cells. This appears to be due to utilization of *m*-toluate or IPTG as carbon source. This was confirmed by a 55-fold increase in the population of culturable cells of engineered *Pseudomonas* in 7 days, when both inducers were present in M9 medium (Table 4).

Engineered *Pseudomonas putida* cells incubated under the aforementioned conditions, for 7 days, were simultaneously used as inoculum to determine both the availability and stability of engineered traits in culturable cells. The diagnostic CO-difference spectra indicating the presence of cytochrome P-450 cam could be detected in cells exposed to the nutrient starvation conditions as well as to inducers (Table 4). Similar observations were made with luminescence emitted from the same cells indicating the expression of luciferase (Table 4). The diagnostic spectra, although qualitative, reflect the stability of the engineered traits in a significant fraction of surviving population of engineered *Pseudomonas putida.*

Table- 4 Effect of Nutrient Stress and Inducers of Lux AB and Cytochrome P-450cam on Survival of Genetically Engineered *Pseudomonas putida* (PpW-lux-cam)

A. Single and Multiple Nutrient Starvation

Nutrient Condition	Colony forming units/ ml of medium				GEM: Detection of Engineered Trait	
	Incubation period (days)				Luciferase: Luminescence Intensity	Cytochrome: P-450 cam (Absorbance 450nm)
	GEM		WILD			
	0	7	0	7		
M9 medium (+) Glucose	$19x10^9$	$9.6x10^9$	$2x10^{10}$	$6.3x10^{13}$	++	+++
M9 medium (-) Glucose	$18x10^9$	$7.3x10^9$	$2x10^{10}$	$2.5x10^{10}$	+++	++++
0.9% NaCl	$19x10^9$	$14x10^9$	$2.2x10^{10}$	$7.5x10^9$	++++	++

B. Effect of Inducers on PpW-lux-cam

Nutrient Condition	Colony forming units/ ml of medium		Detection of Engineered Trait (7 days)	
	Incubation period (days)		Luciferase: Luminescence Intensity	Cytochrome P-450 cam (Absorbance 450 nm)
	0	7		
M9 medium	$29x10^9$	$44x10^{10}$	Not Detected	Not Detected
M9 medium {+ *m*-toluate}	$30x10^9$	$49x10^{10}$	++	+++
M9 medium {+ IPTG}	$27x10^9$	$47x10^{10}$	++++	++++
M9 medium {+ *m*-toluate} {+ IPTG}	$31x10^9$	$171x10^{10}$	+	++

6.3.3 Survival of engineered Pseudomonas in soil microcosms

A significant population of engineered *Pseudomonas* could be detected in unsterile soil (soil characteristics: pH 8.3, clay 56.11%, silt 23.2%, sand 20.69%, water holding capacity 62.45%, organic carbon 0.6 % and total nitrogen 0.054%) even after 21 days by using luminescence based detection of luciferase encoded cells. Enhanced survival was observed in soil amended with the carbon sources citrate and benzoate with an average of 42% cells detected on completion of 21 days in triplicate assays. Survival evidenced in sterile soil was higher with nearly 60% cells detected after the same period, in triplicate samples (Figure 33).

6.3.4 Reactivation of engineered Pseudomonas in soil slurry

Genetically engineered *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase exhibiting low luminescence signal when inoculated into soil slurry and provided with citrate and benzoate showed almost 17-fold increase in luminescence after 9 days. The luminescence signal declined rather rapidly to the 0 day level, on the 10th day. Amendment with citrate and benzoate on the 55th day after desiccation or intermittently during the course of incubation (Figure 34) did not enhance the luminescence signal to the extent observed on addition of LB, LB-benzoate or LB-citrate-benzoate at 70, 74 and even after second desiccation upto 98 days (Figure 34). The recovery of the luminescence signal was observed to be better in the presence of rich nutrients viz. LB compared to citrate, which is indicative of the rapid increase in cell population. A 100-fold increase in the luminescence signal was observed at 99 days, as compared to 0 day, in spite of two complete drying steps between 15-55 days and 77-98 days.

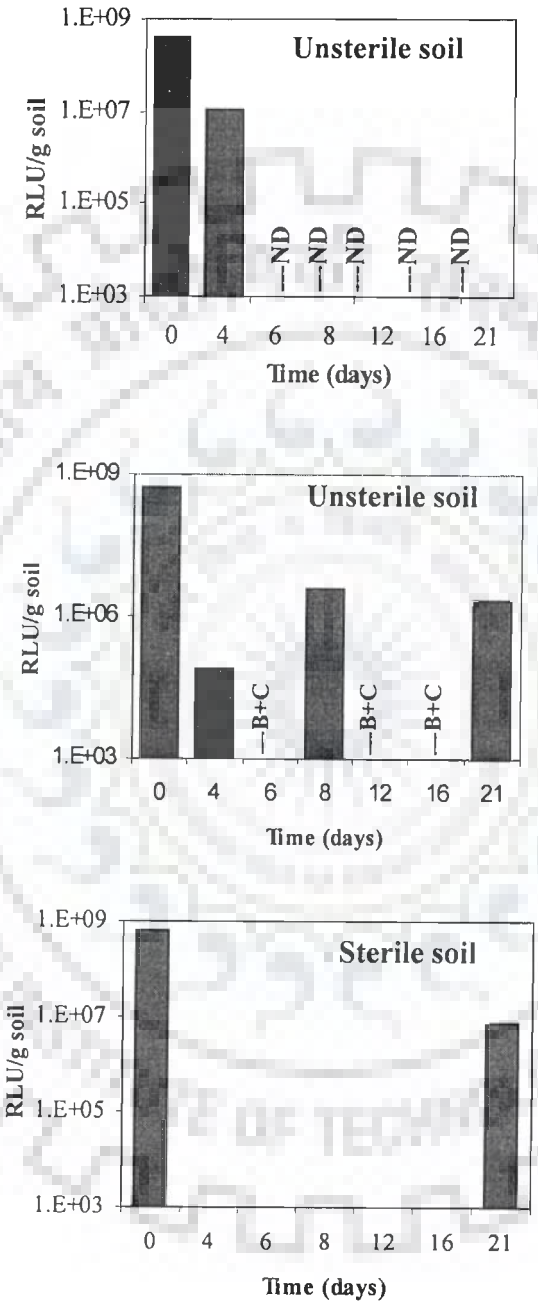


Figure 33. Role of selective nutrients on survival and recovery in soil of genetically engineered *Pseudomonas putida* as determined luciferase tracking system

[Symbols: C - Citrate, B - Benzoate, ND - Not Detected]

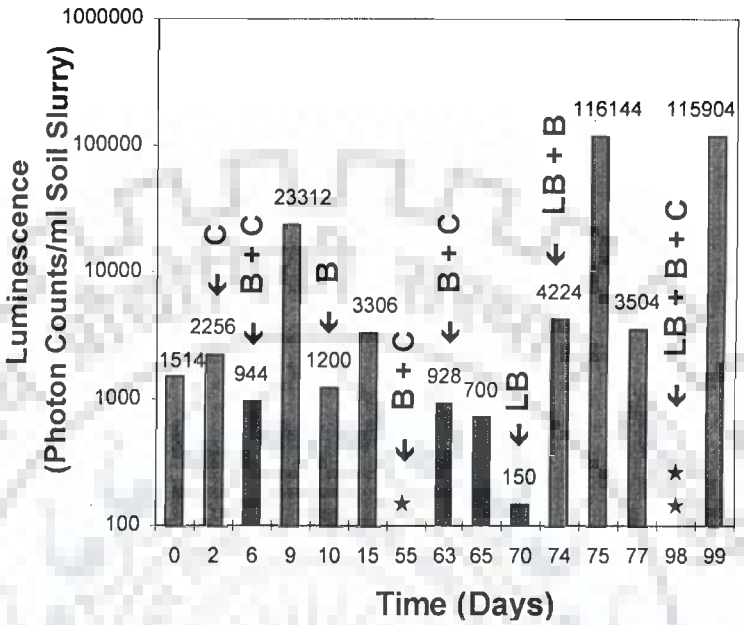


Figure 34. Long term survival and selective nutrient mediated reactivation of genetically engineered *Pseudomonas* in soil slurry as determined by luciferase luminescence

[Symbols: C - Citrate, B - Benzoate, LB - Luria Bertani medium

★ 50% soil slurry reconstituted after slow drying to 10% moisture content at room temperature

★★ Soil slurry incubated at room temperature for 20 days without nutrient and moisture amendments]

6.3.5 Survival of matrix bound Pseudomonas

Evaluation of short-term survival of cells of engineered *Pseudomonas* bound to clay matrix and stored as a wet paste indicated significant luminescence even after storage for 26 days at room temperature. In addition, sterilization of the clay-matrix prior to use as inoculant carrier yielded higher survival evidenced by the increased luminescence of the immobilized cells stored at room temperature (Figure 35). Incubations performed at the lower temperature of 4°C showed better survival monitored by light emission with more than 50% viable cells detected after 26 days, in a typical experiment (Figure 35). Similarly, engineered *Pseudomonas* bound to the clay-minerals kaolin and vermiculite exhibited significant luminescence upto 38 days (Figure 36). In this instance also, luciferase stability was evident and nearly 40-45% of the initial viable cell counts could be detected on storage at 4°C on completion of 38 days. Storage at room temperature yielded lower viable cell counts with 25-30% of kaolin and vermiculite-bound cells detected at 38 days, in a typical assay (Figure 36).

Prolonged survival and the stability of the engineered function monitored by viable cell counts and luminescence assays in the lyophilized matrix bound cells indicated significant survival in all the lyophilized inoculants (Figure 37). Cell viability was evident even after 600 days, irrespective of the conditions employed for the freeze-drying procedure (Figure 38). The lyophilization efficiency was highest when freeze-drying was performed at -25°C and 1.032 mbar for 10 hours. Though other freeze-drying conditions exhibited lower viable cell counts, a

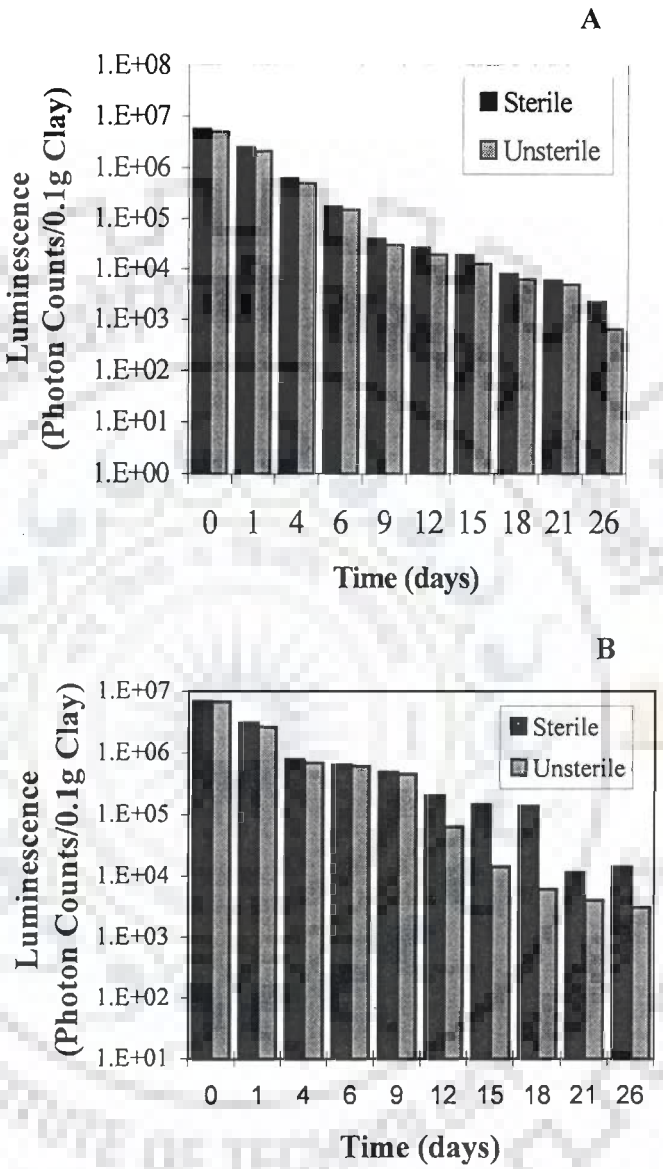


Figure 35. Bioluminescence pattern indicating survival of clay-bound genetically engineered *Pseudomonas putida*

A. Luminescence of cells bound to clay paste on storage at room temperature

B. Luminescence of cells bound to clay paste on storage at 4°C

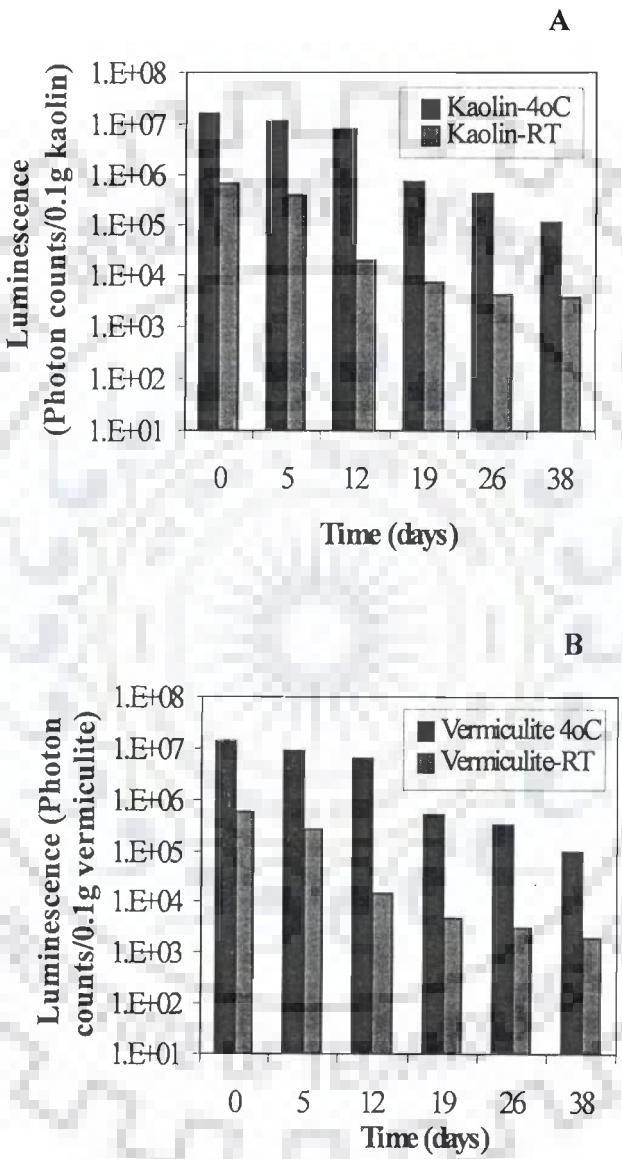


Figure 36. Bioluminescence pattern indicating survival of clay-mineral bound genetically engineered *Pseudomonas putida* stored as wet paste at room temperature and 4°C

A. Luminescence of cells bound to kaolin
B. Luminescence of cells bound to vermiculite

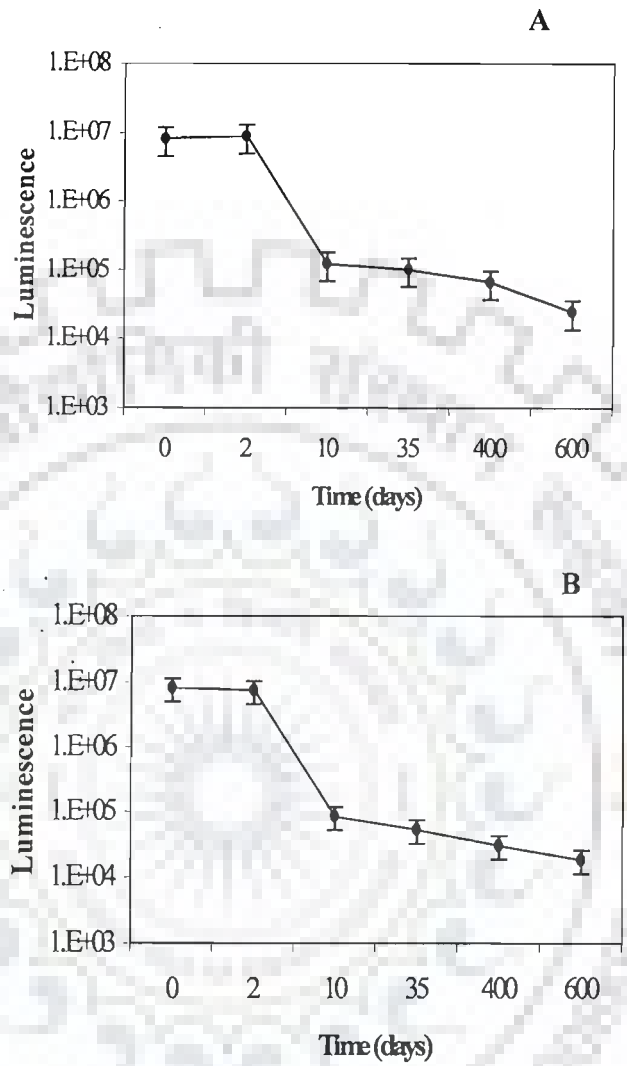


Figure 37. Survival of genetically engineered *Pseudomonas putida* bound to clay-minerals (stored as lyophilized powder at room temperature) as determined by luminescence measurement

A. Survival of lyophilized kaolin-bound engineered *P. putida*

B. Survival of lyophilized vermiculite-bound engineered *P. putida*

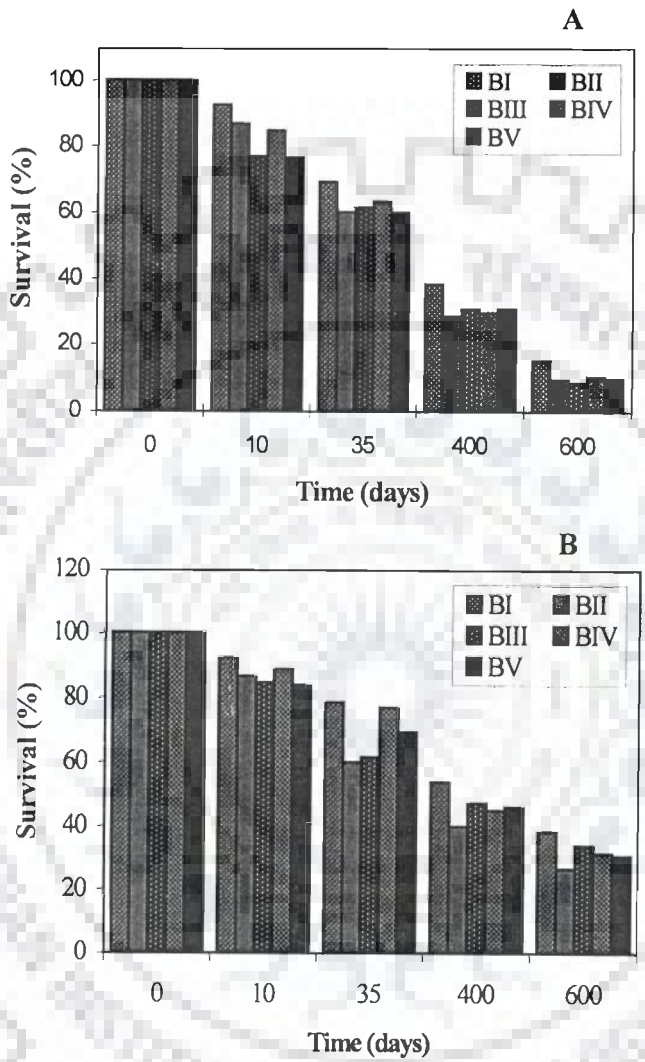


Figure 38. Long-term survival of clay-mineral bound genetically engineered *Pseudomonas putida*

A. Survival of lyophilized kaolin-bound engineered *P. putida* measured by viable cell enumeration

B. Survival of lyophilized vermiculite-bound engineered *P. putida* measured by viable cell enumeration

(Histograms BI-BV - represent results obtained with different lyophilization conditions in a typical assay)

significant proportion of engineered *Pseudomonas* cells survived during the 600-day incubation period. Viable cell counts enumerated upto 600 days demonstrated 10-15% survival in a typical experiment, for all the conditions evaluated, thereby reflecting the suitability of clay-minerals in combination with lyophilization as potential microbial carriers (Figures 37 and 38). The ability of clay-minerals, kaolin and vermiculite to serve as support for growth and survival of *Pseudomonas putida* was validated by the enhanced luminescence exhibited by immobilized cells and a 1000 to 10,000-fold increase in viable cell counts observed between 2-6 days. The viable counts subsequently stabilized at 10^8 CFU/g matrix, equivalent to the initial inoculum added, after 30 days thereby demonstrating the applicability of utilizing nutrient-supplemented kaolin and vermiculite as support for growth and survival of bacterial inoculants. In case of vermiculite and kaolin, a ratio of 1:1 (w/v) provided the best moisture content with the highest increase in cell numbers observed at this nutrient to carrier ratio. The other carrier/nutrient ratios evaluated exhibited a 100-fold increase in viable cell counts between 2-6 days. However, the highest increase in viable cell counts was obtained at a ratio of 1:1(w/v). The luminescence signal obtained also exhibited a similar trend with enhanced luminescence values seen between 2-6 days. The viable cell counts and luminescence monitored upto 30 days subsequently stabilized at the initial levels after 8 days incubation at room temperature (Figure 39).

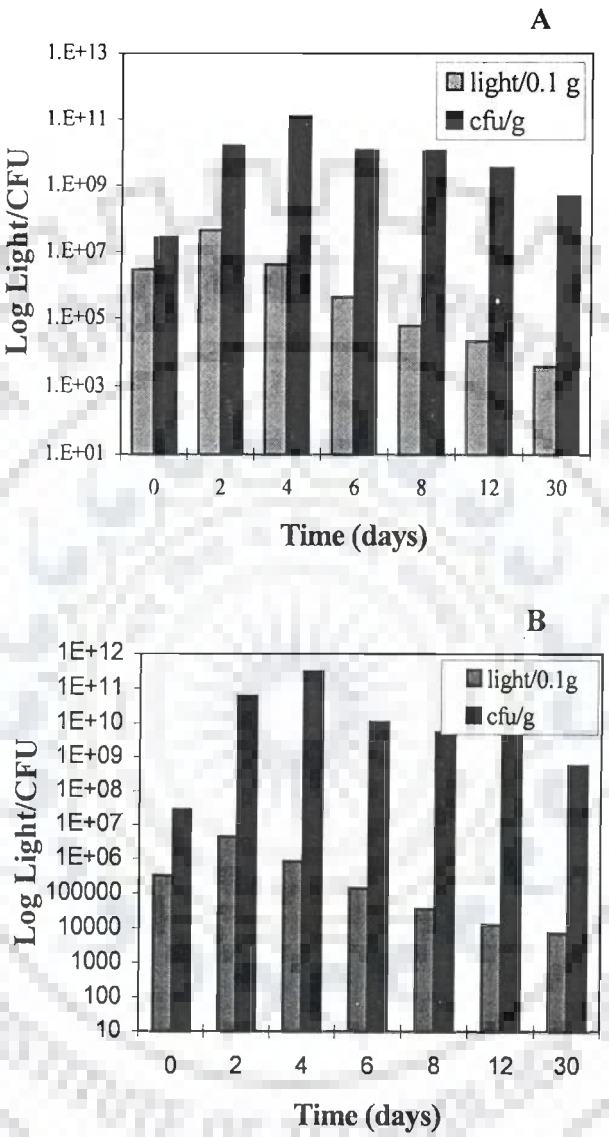


Figure 39. Proliferation and survival of genetically engineered *Pseudomonas putida* on nutrient-supplemented binding matrix

A. Kaolin amended with 1:1 (w/v) Luria-Bertani medium

B. Vermiculite amended with 1:1 (w/v) Luria-Bertani medium

6.3.5.1 Metabolic competence of immobilized Pseudomonas putida co-expressing cytochrome P-450 cam and luciferase

The stability of the engineered traits of clay-bound cells of *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase even after storage for 14 weeks was evident from the effectiveness in dehalogenating hexachloroethane to tetrachloroethylene in soil slurry confirmed by capillary GC and GC-MS (Figure 40).

6.4 Discussion

Bioluminescence is a rare phenotype among rhizosphere bacteria and has been applied to detect engineered bacteria in soil in laboratory studies and limited field application (Fravel *et al.*, 1990; Rattray *et al.*, 1990; Shaw *et al.*, 1992; Ford *et al.*, 1999). The present study evaluated the potential of the luciferase marker to serve as a sensitive detection system for engineered cells added to soil slurry. The luminescence signal has been demonstrated to be directly proportional to the biomass concentration in several lux-marked strains in cell suspensions and following inoculation into soil (Rattray *et al.*, 1990; Prosser *et al.*, 1996). Similar observations were made in this study with the light output closely related to the viable cell concentration both in liquid culture and in soil suspensions (Figure 32). Optimization of the conditions for measuring light output in soil was required as the sensitivity of detection in soil was reduced compared to liquid culture. It has been observed that the presence of soil particles reduces the sensitivity of detection by at least one order of magnitude attributed to masking and absorption of light by soil particles (Rattray *et al.*, 1990; Prosser *et al.*, 1996; Amin-Hanjani

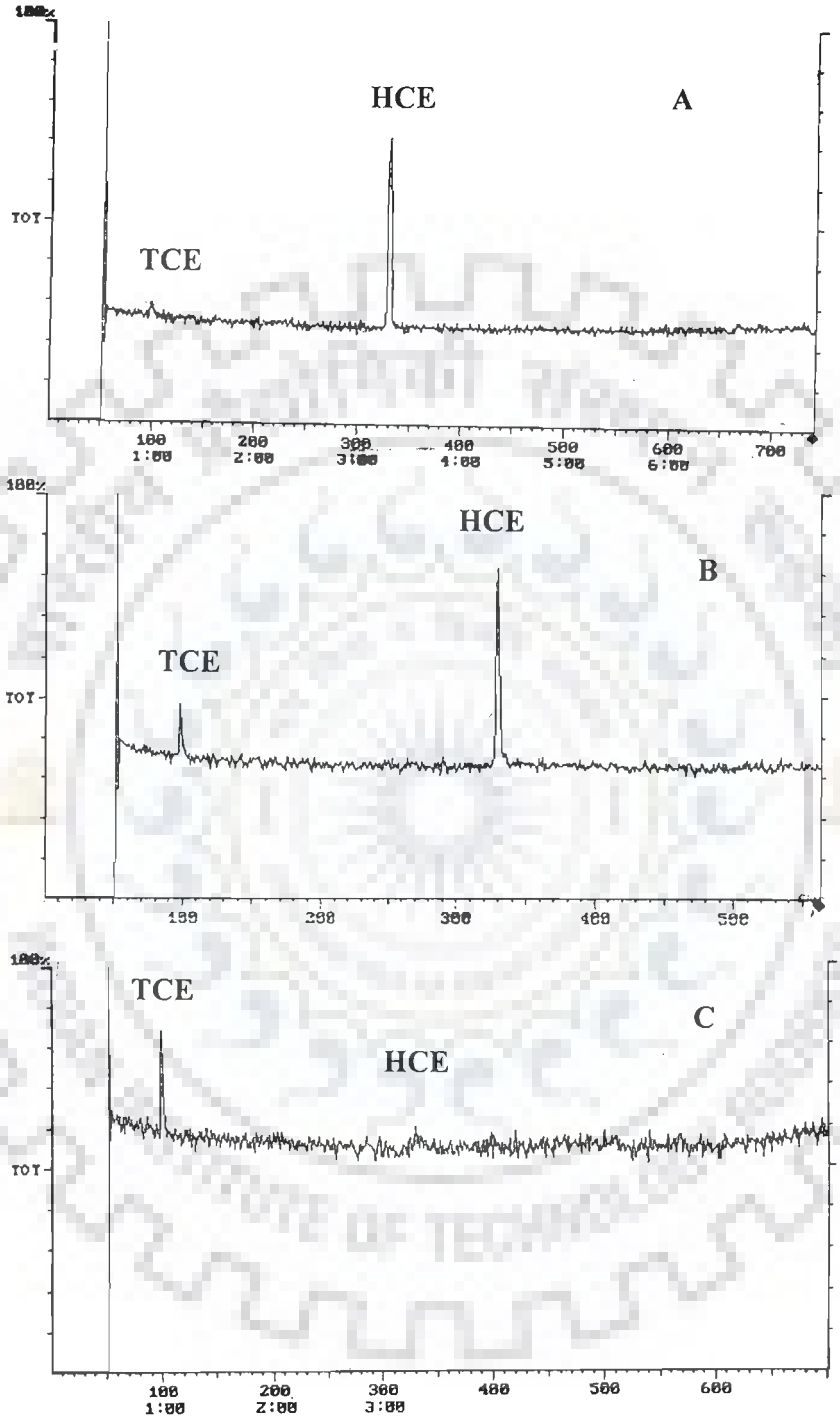


Figure 40. Dehalogenation of Hexachloroethane by clay-mineral bound *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase

[A – Non-amended control; B – Soil slurry inoculated with GEM; C – Soil slurry inoculated with GEM and supplemented with citrate and benzoate]

et al., 1993). The results obtained in the present study concur with this observation and the sensitivity of luminescence detection in soil slurry was approximately two orders of magnitude lower than liquid culture, due to quenching and masking of light by soil particles. Luminescence enumeration in a scintillation counter afforded higher detection sensitivity than luminometric assays, both in liquid culture and in soil suspensions. In cell suspensions, as low as 10^2 CFU/ml could be detected (Figure 32). Both the techniques however exhibited a linear correlation between the cell concentration and luminescence signal, reflecting their applicability for sensitive and rapid detection of lux-marked strains amongst indigenous populations.

The present study compared the stability of genetically engineered traits, encoded chromosomally or on a plasmid, under conditions of nutrient stress. The capacity of bacteria to resist starvation has been observed to influence their survival in oligotrophic conditions and studies have demonstrated that starvation susceptible bacteria do not persist in environments that are nutrient-deficient (Sinclair and Alexander, 1984; Acea *et al.,* 1988; Thompson *et al.,* 1990). Thus a bacterium's capacity to tolerate starvation conditions *in vitro* may be a useful indicator of its survival in natural environments. In this study, both the wild-type and engineered *Pseudomonas* strains exhibited significant survival with retention of the engineered functions in the recombinant strain when subjected to multiple nutrient starvation. Engineered *Pseudomonas* introduced into soil microcosms exhibited significant survival evidenced by luminescence signal even after a three-week incubation in soil. The survival of the organism was improved and substantial recoveries from

nutrient stressed situation were possible by the periodic amendment of the soil with low concentrations of carbon substrates like citrate and benzoate without providing any drug resistance based selection in a highly microbially competitive environment (Figure 33). The higher survival observed in sterile soil is in agreement with previous reports on the importance of biological factors on the survival and growth of bacteria introduced into soil (Liang *et al.,* 1982; Acea *et al.,* 1988). The survival and recovery of viable and culturable cells after slow desiccation of engineered *Pseudomonas*, even after 99 days in soil slurry, is appreciable considering the presence of dual engineered traits, both plasmid and chromosomally encoded (Figure 34). The survival of *Pseudomonads* is reported to be different from that of other gram-negative organisms with no decrease in viability in drinking water even after 95 days (Byrd *et al.,* 1991). *Pseudomonas fluorescens* 10586s/FAC510 chromosomally labelled with luciferase remained viable and culturable at 4°C and 30°C throughout the 54 day incubation period (Duncan *et al.,* 1994), but De Weger *et al.,* (1991) reported loss of plasmid pUCD 615 with a complete *lux* cassette in 80-90% cells of *Pseudomonas fluorescens* WCS374 after only six days in the rhizosphere of soy-bean. These and other reports establish that the loss of expression and viability for the same species with different plasmids or chromosomally encoded traits is probably due to intraspecies strain differences (Caldwell *et al.,* 1989; Ryder, 1994; van Overbeek *et al.,* 1990, 1995). However, none of the studies examined the stability of plasmid and chromosomally encoded traits in the same host organism.

In the present study, engineered *Pseudomonas* co-expressing cytochrome P-450 cam and luciferase was observed to survive, even after moisture loss from soil,

without any selective pressure of antibiotics in the soil slurry throughout the long incubation period (Figure 34). The observations made in the present study support the hypothesis that the survival of strains of soil *Pseudomonads* is likely to be dependent more on the physiological state and the ability to compete for nutrients with indigenous microflora rather than presence of one or more engineered traits (Kozdroj, 1996). A significant observation made in the present study is the functional stability of two engineered traits in the same cells even under nutrient stress contrary to the assumption that genetically engineered microorganisms have a selective disadvantage (Brill, 1985; Kozdroj, 1996). The engineered *Pseudomonas* was not only able to sustain a small population in soil slurry but could also be selectively enriched and reactivated *in situ* by addition of specific nutrients, leading to increase in cell numbers.

It has been demonstrated that cellular light output is more closely linked to biomass concentration in chromosomally-marked strains than in the ones which possess plasmid-borne lux genes (Amin-Hanjani *et al.,* 1993; Kragelund *et al.,* 1995; Prosser *et al.,* 1996). Moreover, as demonstrated by Amin-Hanjani *et al.,* (1993) in chromosomally-marked *Pseudomonas fluorescens*, the expression of lux genes and production of luciferase did not affect the specific growth rate, whereas in the plasmid-marked cells, the specific growth rate was reduced to about 84% of the parent strain. This suggests that ideally marker genes should be located on the chromosome. Furthermore, the chromosomally-encoded luciferase marker genes have been shown to be highly stable for up to 200 generations with no loss reported in soil on incubation for 50 days (Amin-Hanjani *et al.,* 1993). The

stabilty of chromosomally-marked luciferase genes was also validated in the present study when engineered *Pseudomonas* was subjected to desiccation stress. In addition, the luciferase marker was also highly stable in bacterial formulations of engineered *Pseudomonas* immobilized on clay-based matrices (Figures 35-37). These results therefore endorse the utility of chromosomally marked lux strains for assessment of survival and activity of microbial inocula.

Studies were conducted to elucidate the survival attributes of genetically engineered *Pseudomonas putida* immobilized on binding matrices like clay or the clay-minerals vermiculite and kaolin. Longer shelf-life with preservation of engineered functions are desirable attributes for microbial inocula (Fages, 1990; 1992; Smith, 1992). Though a variety of inoculant carriers have been successfully employed including peat, alginate, polyacrylamide (Somasegaran and Halliday, 1982; Strijdom *et al.,* 1981; Roughley, 1982; Cassidy *et al.,* 1996; Bashan, 1986; Digat, 1991; Paul *et al.,* 1993; Thomas and White, 1990), their use is limited either due to the toxic by-products generated or the expense involved in immobilizing the micro-organisms (Parker and Vincent, 1981; Graham-Weiss *et al.,* 1982). The use of inoculant carriers like peat is further restrained due to its limited availability as also the requirement for costly gamma-irradiation facilities (Somasegaran, 1985). In addition, natural and non-toxic biodegradable carriers may be preferred for environmental applications (Cassidy *et al.,* 1997). Therefore, the beneficial attributes of clay-minerals, kaolin and vermiculite viz. convenience of handling, their non-toxic nature and cost-effectiveness confers an advantage to their use as microbial carriers.

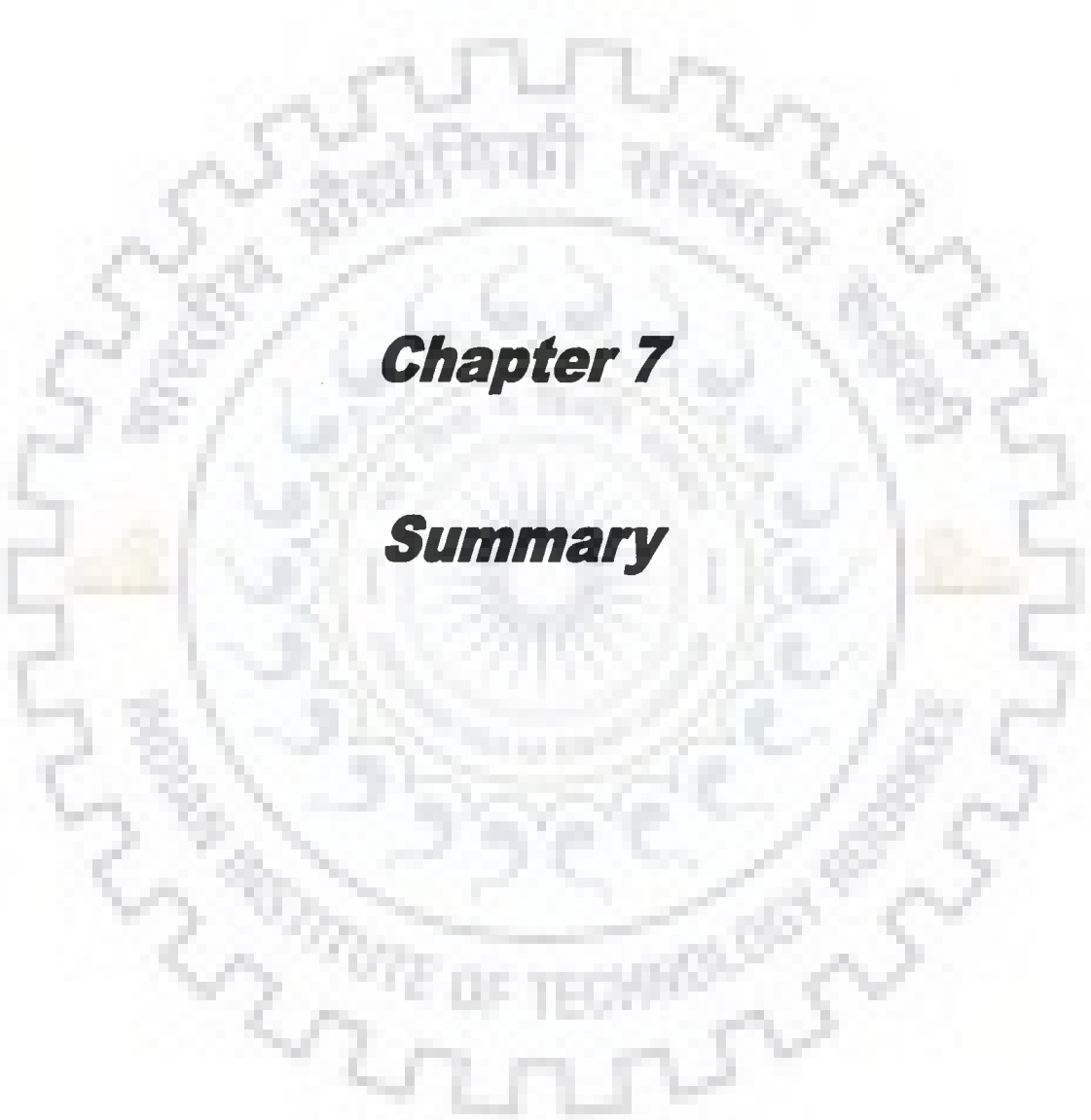
The use of lyophilization in conjunction with the immobilization of cells of engineered *Pseudomonas* on clay-minerals provides a feasible option for better inoculant production meant for long-term applications. Lyophilization with the ability to concentrate cells, is the method most commonly used for the preservation of natural microorganisms (Thompson, 1980). Lyophilization has been used to concentrate rhizobia that were combined with an oil carrier for application as inoculants (Kremer and Peterson, 1983). Plant growth-promoting bacteria have been demonstrated to survive for months as air-dried and lyophilized inoculants (Bashan, 1986; Paul *et al.,* 1993). However, more than 99.9% cells of a Tn5 mutant strain of *Pseudomonas fluorescens* could not survive the freeze-drying procedure (van Elsas *et al.,* 1988; 1989) and hence lyophilization will only be successful if the bacterial species could survive the freeze-drying procedure. Although, survival after lyophilization and long term storage for 6-10 years have been reported for many genera of natural bacteria, yeasts and fungi, the methodology has not been explored for environmentally important organisms in terms of their metabolic competence after storage.

The demonstration in the present study of the prolonged survival of engineered *Pseudomonas* immobilized on clay minerals with full retention of the engineered traits and recovery of 10-15% cells as active catalysts validates the potential of clay-matrices as effective microbial carriers (Figure 37-38, 40). Although all the lyophilized samples exhibited long-term cell viability, consistently higher viable counts were observed for the vermiculite-bound cells (Figure 38). The better survival in the vermiculite-matrix in comparison to the

clay-mineral kaolin, may be presumably due to the differences in their structure and physical properties (Meisinger, 1984). In addition, both the minerals exhibited the ability to serve as matrices for the growth of engineered *Pseudomonas* when supplemented with a nutrient source and moisture. No special storage procedure was required and the matrices proved beneficial as support for growth with increase in cell concentrations of about 2-3 orders of magnitude and survival even after 30 days (Figure 39). The present results are consistent with the high growth rates demonstrated for *Pseudomonas putida* A2 added to nutrient-amended vermiculite serving as carrier for inoculant production (Graham-Weiss *et al.,* 1982). These results thus demonstrate the utility of clay matrices for the generation of lyophilized bacterial formulations that can be stored dry at ambient temperatures with stability of both the engineered traits.

6.5 Concluding Remarks

The results of the present study validate the robust survival attributes of genetically engineered *Pseudomonas putida* possessing dual engineered traits. The luciferase-based chromosomally encoded marker system provides a highly selective means of detecting the presence and activity of the engineered organism in soil amidst indigenous microflora. Survival of the organism even after exposure to nutrient stressed situations is indicative of the ruggedness of the host and the luciferase marker. The results also validate the possibilities of reactivation and *in situ* generation of active inocula. In addition, the results demonstrate the feasibility of clay minerals as matrices for the generation of active 'microbial inocula' of natural and engineered organisms to be deployed for environmental remediation.



Chapter 7

Summary

The utilization of microbial metabolic potential for eliminating recalcitrant environmental pollutants provides a safe and economic alternative to commonly used physico-chemical techniques. Natural microorganisms, though endowed with remarkable evolutionary capabilities to adapt to a wide range of toxic compounds, do not possess the genetic competence for degradation of recalcitrant pollutants. Hence, innovative strategies are required to construct biocatalysts with desired catabolic potential to decontaminate soils and sediments. Cytochrome P-450 enzymes known to degrade a wide range of xenobiotics are an optimum choice for bioremediation. Bacterial P-450 cam and human P-450 2E1 with their inherent broad substrate range can serve as ideal catalysts for detoxification.

The present study determined the feasibility of expression of native and *N*-terminus modified human cytochrome P-450 2E1 in *E. coli* and in the environmentally robust host, *Pseudomonas putida*. *E. coli* and *Pseudomonas putida* co-expressing luciferase, sub-cloned with native human cytochrome P-450 2E1 gene, exhibited very low yield of P-450 as determined by CO-difference spectroscopy. The low yield of the native P-450 2E1 hemoprotein necessitated modification of the *N*-terminus to attain enhanced expression in the bacterial hosts. The approach involved the deletion of 21 amino acids at the *N*-terminus of P-450 2E1 gene. Long template PCR amplification using oligonucleotides designed with the biocomputing software "Lasergene" yielded the 1.461 Kb *N*-terminus modified human cytochrome P-450 2E1 gene product. The PCR product was sub-cloned in broad host range vector and transformed into the bacterial hosts, clones screened by PCR, and selected for expression studies.

The expression of *N*-terminus modified human cytochrome P-450 2E1 was determined in bacterial cell lines, viz. *E. coli* DH5α, *Pseudomonas putida* expressing luciferase and *Pseudomonas putida* MTCC 102 in 1,50,000 xg supernatant fraction of cells, confirmed by the diagnostic CO-difference spectra with a Soret peak at 452 nm. Electrophoretic mobility of the selectively ultrafiltered 1,50,000 xg supernatant and pellet fractions indicated a protein band near 56.9 kDa for the target P-450 2E1 protein by SDS-PAGE analysis. Culture conditions facilitating cytochrome P-450 expression in the bacterial hosts, *E. coli*, *Pseudomonas putida* expressing luciferase and *Pseudomonas putida* MTCC 102 were optimized. Optimum yields of spectrally detectable *N*-terminus modified cytochrome P-450 2E1 in *Pseudomonas putida* expressing luciferase ranged from 40-50 nmol/liter after 26h incubation at 26°C. Successful co-expression of *N*-terminus modified cytochrome P-450 2E1 and luciferase was demonstrated in *Pseudomonas putida* cultures grown in Terrific broth supplemented with inducers 1 mM IPTG (cytochrome P-450) and 200 μM *m*-toluate (luciferase). *Pseudomonas putida* co-expressing cytochrome P-450 2E1 and luciferase cultured in the absence of the antibiotic, chloramphenicol exhibited increased expression of both the engineered traits.

The possibility of luciferase dependent photo-reduction of *N*-terminus modified P-450 2E1 was explored by monitoring CO-difference spectra in cell fractions obtained from *Pseudomonas putida* possessing P-450 2E1 and *Pseudomonas putida* possessing luciferase lux AB. Diagnostic CO-difference spectra recorded by varying the luciferase concentration (as RLU in a

luminometer) yielded the characteristic absorbance at 452-453 nm with the higher luciferase concentrations ($80x10^5$ and $40x10^5$). Spectra recorded with the lower concentration of luciferase ($16x10^5$) did not yield the desired absorbance at 452-453 nm but indicated maxima at 445-446 nm and 423-424 nm. Diagnostic CO-difference spectra obtained on photoreduction (luciferase dependent luminescence $74x10^5$) indicated the characteristic peak at 452-453 nm and did not require the addition of the reductant, sodium dithionite. Similarly, CO-difference spectra obtained for the dark reaction (in the absence of both n-decanal and the chemical reductant, sodium dithionite) indicated the characteristic absorbance at 452-453 nm. This reaction required the addition of higher concentrations of luciferase ($37x10^6$ RLU equivalent) in comparison to those obtained in the presence of light. The observations indicate that luciferase could serve as an electron transfer partner for *N*-terminus modified P-450 2E1.

The present study explored the metabolic activity of *Pseudomonas putida* co-expressing *N*-terminus modified human cytochrome P-450 2E1 and luciferase. Resting cultures mediated the reductive metabolism of carbon tetrachloride to chloroform as confirmed by GC-MS. The demonstration of reductive dehalogenation activity effectively establishes the ability of luciferase to serve as electron transfer partner *in vivo* for catalytically active hemoprotein.

Studies evaluating the detoxification potential of *Pseudomonas putida* co-expressing bacterial cytochrome P-450 cam and luciferase in soil slurries demonstrated effective dehalogenation of hexachloroethane and γ-hexachloro-cyclohexane to tetrachloroethylene and γ-3,4,5,6-tetrachlorocyclohexene,

respectively as confirmed by GC and GC-MS. The survival and concomitant dehalogenation in soil slurries validated the *in situ* expression and stability of the catabolic potential of *Pseudomonas putida* co-expressing cytochrome P-450 cam and luciferase under oligotrophic conditions. The soil slurries amended with the engineered organism exhibited nearly 60 to 65% removal of the halogenated substrates in comparison to uninoculated controls, validating the ability of the engineered organism to metabolise relevant pollutants, and survive nutrient stress conditions often found at polluted sites. The improved removal is feasible in serial arrangement of contained *ex-situ* bioreactors.

Pseudomonas putida co-expressing cytochrome P-450 cam and luciferase was evaluated for its ability to survive under nutrient stress in soil slurry microcosms. More than 74% cells of engineered *Pseudomonas* were culturable after 7 days of multiple nutrient starvation (C,N,P). The diagnostic CO-difference spectra and luminescence emission for the dual engineered traits could be detected in a significant fraction of the surviving population. The engineered organism could be revived after repeated desiccation and starvation using Luria-broth, benzoate or citrate as nutrients. The survival and recovery of viable and culturable cells after slow desiccation even after 99 days in the soil slurry is appreciable considering the dual engineered traits, both plasmid and chromosomally encoded.

The luciferase-based chromosomally encoded marker system provides highly selective and efficient detection of the introduced organism amidst indigenous soil microflora that lack luciferase. The luminescence of cells added to

liquid culture and soil slurry closely related to the viable cell concentration indicating the potential of the luciferase marker to serve as a sensitive detection system for engineered cells.

The efficacy of clay minerals, kaolin and vermiculite to serve as effective bacterial carriers was validated by the ability of engineered *Pseudomonas* to retain initial activity upto 5 weeks on storage as a wet paste. The effective use of lyophilization in combination with the clay minerals for immobilizaton of cells of engineered *Pseudomonas* was demonstrated in samples stored as dry powder for extended periods. Survival characteristics of the lyophilized matrix bound cells monitored by the light-emitting function and enumeration of viable counts substantiate the stability of the recombinant traits with about 10-15% cells recovered as active catalysts even after prolonged storage upto 600 days. The ability of the lyophilized engineered *Pseudomonas* cells to effect the dehalogenation of hexachloroethane after storage for 14 weeks establishes the utility of the clay minerals as immobilization matrices. In addition, the ability of the clay minerals, supplemented with nutrients and moisture, to serve as support for the growth and survival of engineered *Pseudomonas* cells indicates their desirable attributes as microbial carriers.

The present study thus provides significant leads on the feasibility of expression of selected bacterial and mammalian cytochrome P-450 monooxygenase enzymes in environmentally robust hosts such as *Pseudomonas putida*. The study validates the viability of cytochrome P-450 as a remediation enzyme for the detoxification of halogenated hydrocarbons, as also the utility of

luciferase as an alternate electron transfer partner to harness the detoxification potential of multiple P-450 isozyme(s). The study underscores the feasibility of recruitment of specific bacterial and/or mammalian P-450 isozymes in designed bacterial consortium for detoxification of hazardous wastes in contained systems. The study also demonstrates the feasibility of utilizing clay matrices for the generation of active 'microbial inocula' for bioremediation applications.

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