Construction and characterization of a *lux*-marked phenanthrene degrading bacterium

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Abstract
Construction of a luminescent microbial biosensor as a sensitive and rapid biotechnological tool for monitoring survival and activity of genetically engineered microorganisms (GEMs) in the contaminated environment has been the main focus of this study. Four rifampicin resistant phenanthrene-degrading bacteria viz., Commamonas testosteroni GZ38A, C. testosteroni GZ39, Pseudomonas putida GZ44 and P. stutzeri P16 were made using gradient plate techniques. All resistant strains were transformed with the plasmid harboring the mini-Tn5-tet transposon cassette, containing the luxAB genes to confer stability. C. testosteroni GZ38A and P. stutzeri P16 were successfully lux-marked in this manner. It was found that lux-marked strains of C. testosteroni GZ38A were not able to degrade phenanthrene. Although the maximum growth rate of lux-marked strain was significantly lower ($P < 0.05$) than that of the wild type P. stutzeri P16, the P. stutzeri P16-luxAB4 was selected because it has the following criteria: the stability of expression of tet-resistance over 180 generations, phenanthrene - degradability and high level of luminescence light output in the absence and presence of phenanthrene. The addition of 1 litter (0.5 % v/v) n-decyl aldehyde produced consistently high levels of luminescence at various stages of selected strain. Results clearly indicated that lux-marked strain was appropriately constructed and it is a novel biodegradative luminescent biosensor which enables us to monitor the fate of a phenathrene bacterial degrader genetically or non-genetically made within a polluted environment.
Keywords: *Lux AB gene; Phenanthrene; Molecular Marker and Degradative bacteria.*

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INTRODUCTION

Large amounts of toxic chemicals are released into the natural environment every year through catastrophic events such as cargo breakage, oil tank explosions and industrial waste disposal. Microorganisms, because of their wide range of substrates utilisation, have been considered as useful tools for degradation of such pollutants (Bedard and Quensen III, 1995; Bennet and Faison 1997; Bossert and Compeau, 1995; Semple *et al*., 1999). In general, however, a single microbe is not able to degrade complex compounds and or application of consortia of microbes may also not result in complete degradation, as microbes within the consortia may compete with each other (Dwyer *et al*., 1988). genetic engineering methods can be used to improve the metabolic activity of introduced biodegradative microorganisms (Ensley and Deflaun, 1995; Garbisu and Alkorta, 1997; Shaw *et al*., 1992). There are several barriers for application of Genetically Engineered Microorganisms (GEMs) in the environment (Smit *et al*., 1992). Monitoring of GEMs is one of these problems which should be considered before their release. Several genes have been used for tracking GEMs in soil and water, such as *lac*Z, *xyl*E, *gas*, *gap* and *lux* (Brandt *et al*., 2002 ; Haddix *et al*., 2004 and Lindow, 1995). The bioluminescence marker system has been shown to be a good marker system for monitoring the survival and activity of bacteria in the environment (Hollis *et al*., 2000; Grant *et al*., 1991; Ritchie *et al*., 2003; Shao *et al*., 2002 and Shaw *et al*., 1992). *lux* marking of bacteria which can degrade two-ring Polycyclic Aromatic Hydrocarbons (PAHs) has been reported (King *et al*., 1990) but, to date, *lux* marking of strains capable of degrading PAHs with three or more

rings has not been reported. In this study, the phenanthrene degrading bacteria, *Pseudomonas stutzeri* P16, *Comamonas testosteroni* GZ38A, *C. testosteroni* GZ39 and *P. putida* GZ44 were chosen, as phenanthrene, a tricyclic aromatic hydrocarbon, has been used as a model for degradation of high molecular weight and carcinogenic PAH (Boldrin *et al.,* 1993; Narro *et al.,* 1992). Furthermore, *P. stutzeri* P16 was selected, because it as this strain is a natural soil isolate (Stringfellow and Aitken, 1994; Goyal and Zylstra, 1994). Mini-Tn*5* *lux*-transposon carrying *lux*AB genes was used for *lux*-marking the above strains because of resulting in high light output, and less effective on wild type genes.

MATERIALS AND METHODS

Bacterial strains used in this study: *C. testosteroni* GZ38A, *C. testosteroni* GZ39 and *P. Putida* GZ44 were provided by Dr. GJ Zylstra (Rutgers University, New Brunswick, USA). *Pseudomonas stutzeri* P16 was donated by Prof. MD Aitken (University of North Carolina, North Carolina, USA) and *Escherichia coli* SM10 (λ pir) containing the mini-Tn5 transposons on the plasmid PUT (Herrero *et al.,* 1990) was provided by Dr LA Glover (University of Aberdeen, Aberdeen, UK).

Growth conditions: All strains were initially grown in either Luria-Bertani (LB) broth (Oxoid, Ltd) (tryptone: 10 g, Yeast extract agar: 5 g, NaCl: 10 g, distilled water: 1 litter). They were also grown in Minimal Base Medium (MBM) [(NH_4cl: 0.5 g, $NaH_2PO_4.H_2O$: 0.5 g, KH_2O_4: 0.5 g, $MgSO_4$. $7H_2O$: 0.5 g, NaCl: 4 g, trace element solution: 1 ml, Distilled water: 1 litter, pH 7) (trace element solution was composed of: $CuSO_4$. $5H_2O$: 0.64 g, $FeSO_4.7H_2O$:0.11 g, $MnCl_2.4H_2O$: 0.79 g $ZnSO_4$. $7H_2O$: 0.15 g and distilled water: 100 ml)] or modified Hutner's base medium (Cohen-Bazire 1957 and Hutner, 1950) supplemented with phenanthrene as sole carbon source. All strains grown at 30°C except *E. coli* SM10 which was grown at 37°C.

LB agar plates containing rifampicin in gradients from 0 →12.5 μg ml^{-1}, 12.5 μg ml^{-1} →25 μg ml^{-1}, 25 μg ml^{-1} → 50 μg ml^{-1}, 50 μg ml^{-1} → 75 μg ml^{-1}, and 75 μg ml^{-1} → 100 μg ml^{-1} were made as previously described (Cappuccino and Sherman, 1996). Phenanthrene degrading strains were either streaked on the surface of agar plates or added as a droplet.

Samples of an transposion produce: Overnight culture of *E. coli* SM10 (λ pir) (100 μl) were mixed with each of the phenanthrene degrading strains (50 μl) in 5 ml of sterile NaCl (0.85% w/v), and filtered through a 45 mm diameter, 0.47 μm nitro cellulose filter (Whatman Ltd.). Filters were then placed on the surface of LB agar plates and incubated overnight at 30°C. The colonies grown on the filter surface were suspended in sterile phosphate buffer (0.01 M, pH 7) and spread on LB agar containing tetracycline (12.5 μg ml^{-1}) and rifampicin (25 μg ml^{-1}).

Before and after transposon mutagenesis, overnight cultures of the above strains were transferred into minimal basal medium agar plates as previously described (Kioyhara *et al.,* 1982). Phenanthrene (10% w/v) was uniformly sprayed over the surface of the agar plates by a spray gun (Richard Kohnsten Ltd.). Phenanthrene utilisation by *E. coli* and wild type, rifampicin resistant and luminescent strains of *P. stutzeri* P16 were also checked by growing them in TEP medium (Na_2HPO_4 $7H_2O$: 1.5 g, KH_2PO_4: 1g, NH_4Cl: 2g, $Na_2S_2O_3.5H_2O$: 0.02 g, tap water: 1 litter, Phenanthrene 2 g l^{-1}, PEP medium (peptone: 5 g, distilled water: 1 litter, phenanthrene: 2 gl^{-1}) and PW medium (peptone: 5 g, distilled water: 1 litter). Phenanthrene was sterilized before use by filtration (Stringfelow and Aitken, 1994).

The stability of *lux*AB genes was assessed by repeated subculturing in non-selective LB medium at 2 day intervals, over 14 days. Insert stability was calculated as the percentage of total cells (plated on LB) expressing resistance to tetracycline (plated on LB containing tetracycline).

Bioluminescence measurement: Optimal conditions for maximal light output from *lux*-marked *P. stutzeri* P16 *lux*AB4 were determined by incubating 1 ml samples of early exponential (OD_{600}= 0.03), mid exponential (OD_{600}= 0.3) and late exponential phase (OD_{600}= 0.7) at 30°C. Luminescence measurement were carried out at 25°C using an automated temperature controlled luminometer LKB125 following incubation of 1 ml batch culture sample with 1 μl n-decyl aldehyde (0.55 v/v) for 5 minutes at room temperature. Luminescence values were recorded as an average of triplicate readings and expressed in relative light units (RLU: 1 RLU is equivalent to 1 mV 10 S^{-1}) (Amin-Hanjani *et al.,* 1993). Colonies could also be detected using a nitrogen cooled, slow scanning type charge couple device (CCD) camera. The camera was encased in a light-tight box. Images were stored on a Dell system 310 computer. A 0.02 S exposure was required for bright field images, and a 2 min exposure for dark field images. Culturable counts were determined by the drop plate technique (Hoben and Somasegaran, 1982).

RESULTS

Growth and mineralisation of phenanthrene was detected as the formation of colonies surrounded by transparent (clear) zones on phenanthrene sprayed agar plates. *C. testosteroni* GZ39 and *P. ptuida* GZ44 did not form a clear zone when strains were streaked on minimal base medium (using tooth picks). However, thay grew when a drop of overnight culture was transferred to minimal base medium, and as growth increased, clearer zones developed by all four strains (Table 1). The rifampicin resistant mutants that grew on the highest concentration of rifampicin, were selected and from these strains of *C. testosteroni*, *lux*-marked cells of strain GZ39A were detected on tetracycline resistant agar plates. Three *lux*-marked GZ38A strains: *C. testosteroni* GZ38A *lux*AB5, *C. testosteroni* GZ38A *lux*AB9 and *C. testosteroni* GZ38A *lux*AB12 were checked for phenanthrene degradation, but none of these could grow on phenanthrene and produce clear zone on agar plates (Table 2). From 12 *lux*-marked colonies obtained from *P. stutzeri* P16, mutants 3, 4 and 8 gave higher luminescence than others. *P. stutzeri* P16 *lux*AB3, *P. stutzeri* P16 *lux*AB4, *P. stutzeri* P16 *lux*AB8, rifampicin resistant mutant and wild type strains of *P. stutzeri* P16 could all grow on PW medium (peptone + tap water), PEP medium (peptone + phenanthrene), and TAP medium (tap water + phenanthrene) while *E. coli* SM10 (λ pir) grew just on PEP and PW medium (Table 3). From the above three *lux*-marked strains of *P. stutzeri* P16, *P. stutzeri* P16 *lux*AB4 was selected for further characterization, because the percentage of cells expressing *tet* resistance with each successive samples was stably maintained (Fig. 1). Ability of *P. stutzeri* P16 *lux*AB4 to degrade phenanthrene was confirmed on solid medium and visualized in the dark by CCD camera (Fig. 2). The concentration of aldehyde required for optimal

Table 1. Growth of phenanthrene-degrading strains on mineral base agar plates supplementd with phenanthrene inoculated either by tooth stick streaking or adding droplet. (+) is a symbol for the density of culture. More (+) indicates denser culture and better growth.

Strain	Growth			
	streaking		droplet	
	≤ 2 days	> 2 days	≤ 2 days	> 2 days
C. testosteroni GZ38A	+	+++	++	++++
C. testosteroni GZ39	_	_	+	++
P. putida GZ44	_	_	+	++
P. stutzeri P16	+++	++++	++++	++++

Table 2. Growth of wild type, rifampicin resistant and *lux*-marked strains of *C. testosteroni* GZ38A on different medium supplemented with appropriate antibiotics. More (+) indicates denser culture and better growth.

Strain	Medium				
	MBM + phen	LB + rif	LB + rif + tet	LB + tet	LB medium
C. testosteroni GZ38A (wt)	+	_	_	+	_
C. testosteroni GZ38A (Rif^r)	+	+	_	+	_
C. testosteroni GZ38A *lux*AB5	_	++	++	++	++
C. testosteroni GZ38A *lux*AB9	_	++	++	++	++
C. testosteroni GZ38A *lux*AB12	_	++	++	++	++

MBM = minimal base medium, rif = rifampicin, tet = tetracycline, Rif ^{r} = rifampicin resistant, wt = wild type, phen = phenanthrene.

Table 3 Growth of wild type, rifampicin resistant and *lux*-marked strains of *P. stutzeri* P16 on different media. (+) is a symbol for the density of culture. More (+) indicates denser culture and better growth.

Strain	Medium				
	PEP	PW	TAP	TWB	LB
P. stutzeri P16 (wild type)	++	+	+	_	+++
P. stutzeri P16 (rifampicin^r)	++	+	+	_	+++
P. stutzeri P16 *lux*AB3	++	+	+	_	+++
P. stutzeri P16 *lux*AB4	++	+	+	_	+++
P. stutzeri P16 *lux*AB8	++	+	+	_	+++
E. coli	++	++	_	_	+++

PEP: Phenanthrene + peptone, PW: Peptone + water, TAP: Tap water + Phenanthrene, TWB: Tap water + Buffer, LB: Luria Bertani

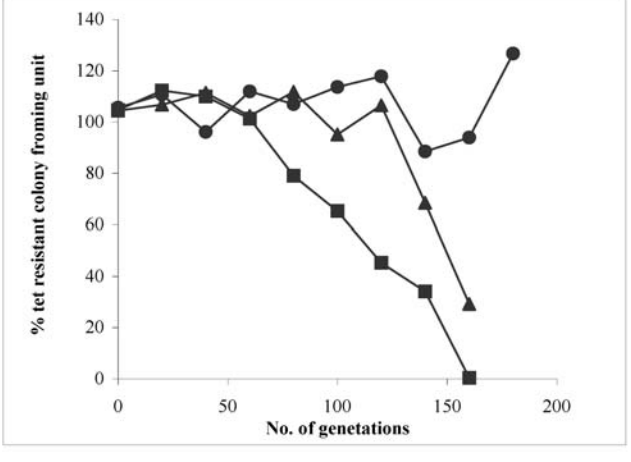

Figure 1. The stability of the *lux*AB *tet* gene construct in *P. stutzeri* P16 *lux*AB3 (▶), *lux*AB4 (♦) and *lux*AB8 (■) presented as the percentage of tetracycline resistant cells remaining after repeated subculturing in a non-selective medium.

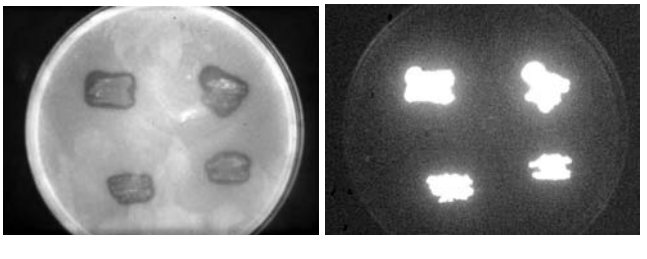

Figure 2. Bright-field image (a) and CCD image-enhanced dark field image (b) of utilization of phenanthrene by *P. stutzeri* P16 *lux*AB4

levels of light output from *P. stutzeri* P16 *lux*AB4 was determined by incubating cells for varying times in the presence of a range of concentrations of n-decyl aldehyde and the substrate. This showed that the addition of 1 µl (0.5 % v/v) n-decyl aldehyde produced consistently high levels of luminescence at various stages of growth (Fig. 3).

Growth and bioluminescence profiles for *P. stutzeri lux*AB4 in LB medium were monitored with time. Light output per unit biomass increased during growth, as illustrated in figure 4. Plots of light output against biomass concentration were linear (Fig. 5) confirming that light output was directly proportional to cell density during growth. The lower limit of detection for cells of *P. stutzeri* P16 *lux*AB4 was approximately 28.3 $\times 10^3$ cells ml^{-1}.

Figure 6 illustrates the growth curve for the wild type and *lux*-marked *P. stutzeri* P16 *lux*AB4. The mean maximum growth rate of *lux*-marked strain was 0.49 h^{-1}, significantly lower ($P < 0.05$) than the mean maximum growth rate of the wild type *P. stutzeri* P16.

DISCUSSION

Advantages of bioluminescence-based marker systems for monitoring the activity of GEMs have often been reported in the literature (Meikle *et al.,* 1992; Prosser, 1994; Prosser *et al.,* 1996 and Rattray *et al.,* 1990). Such a this marker systems, was chosen in our studies to trace phenanthrene degrading strains. Although the rate of phenanthrene degradation varied greatly in the

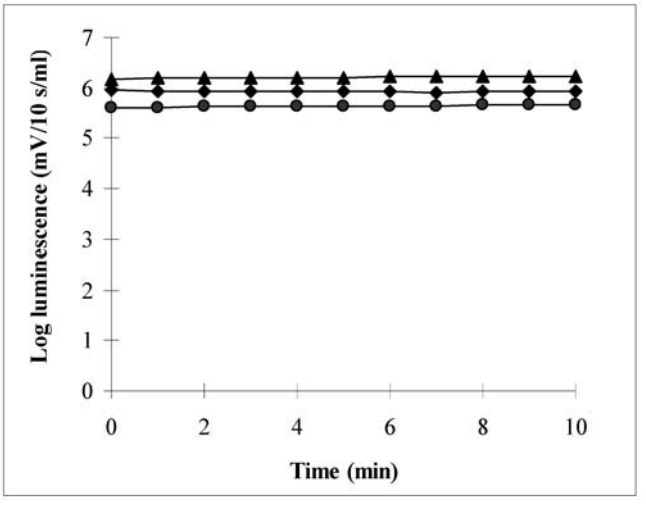

Figure 3. Bioluminescence following the addition of 1 µl of 0.5% of n-decyl aldehyde to lag phase (•), mid-exponential phase (♦) and late exponential phase (▸) cultures of *P. stutzeri* P16 *lux*AB4.

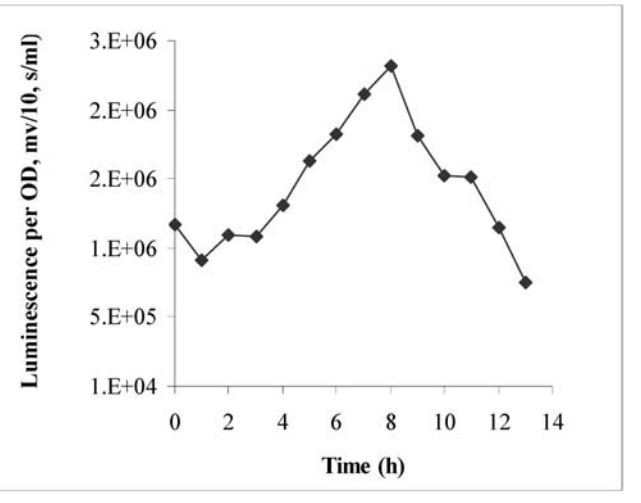

Figure 4. Changes in light output per unit biomass during growth of *lux*-marked *Pseudomonas stutzeri lux*AB4 in LB medium.

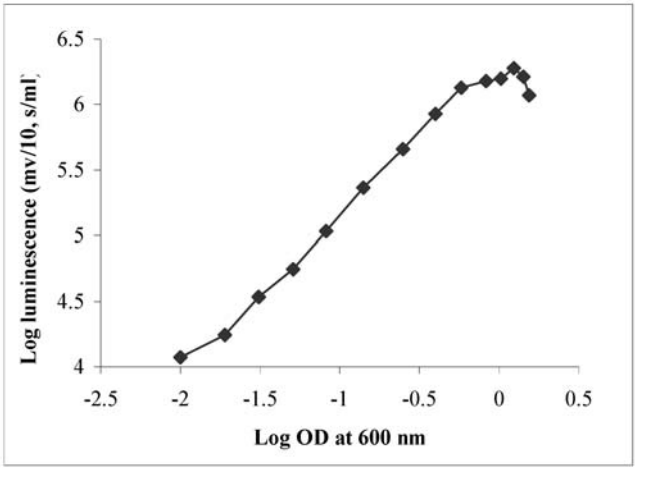

Figure 5. The relationship between light output and biomass concentration of exponentially growing cells of *P. stutzeri* P16 *lux*AB4.

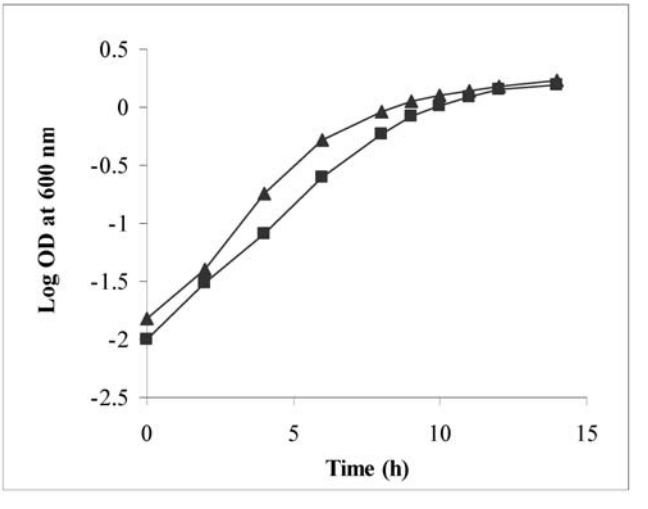

Figure 6. Growth characteristics of wild type (▸) and *lux*-marked (■) *Pseudomonas stutzeri* P16 in batch culture. Results represent mean values from triplicate cultures.

four wild type strains selected for this study. These strains were generally able to degrade phenanthrene in less than 2 days.

The gradient plate technique was found to be a good approach for obtaining rifampicin resistant mutants. Spreading of inocula on the surface of agar plates gave better results than streaking. The reason for this is unclear, but may be due to a higher number of introduced cells using the spread technique, resulting in a higher chance of obtaining an antibiotic resistant mutant.

Lux-marking of *C. testosteroni* GZ39 and *P. putida* GZ44 was unsuccessful, possibly because of instability, degradation of inserted genes within the recipient strains (*C. testosteroni* GZ39 and *P. putida* GZ44), or unsuitability of the vector that would prevent them receiving the plasmid.

Despite many advantages of mini-tn*5* transposns for DNA insertion into Gram-negative bacteria, there are some problems associated with this DNA introductory system (de Lorenzo *et al.*, 1998). For example, disruption of chromosomal sequence causing disruption of cell metabolism is possible probably due to disruption of genes in the phenanthrene degrading pathway. Therefore All 3 *lux*-marked *C. testosteroni* GZ38A (*lux*AB5, *lux*AB9, *lux*AB12) were not able to utilize phenanthrene sprayed over the minimal base medium, therefore they were discarded. However, *lux*-marked

strains of *P. stutzeri* P16 which could produce a clear zone on agar plate covered with phenanthrene, showed that insertion of the genes did not effect degradation ability of these strains. This supports pervious studies (Rattray *et al.,* 1992; Ritchie *et al.,* 2003 and Van Dyke *et al.,* 1996) on the advantage of the use of *lux*AB gene for monitoring the biodegradative GEMs in the environment.

Despite high light output from two of the *lux*-marked strains, *P. stutzeri lux*AB3 and *P. stutzeri lux*AB8, these constructs were not stable in the absence of selection pressure (tetracycline), and therefore, were also discarded. The *lux*AB *tet* cassette was stable over 180 generations in *P. stutzeri* P16 *lux*AB4. A *lux*-marked *Aeromonas salmonicida* showed similar *lux*AB gene stability over 140 generations (Ferguson *et al.,* 1995). Significant differences were observed between growth rates of *lux*-marked and wild type strains of *P. stutzeri* P16 *lux*AB4, and also between chromosomally *lux*-marked strains of *Aeromonas salmonicida* and wild type the strain. However, no significant differences in the responses of these two *A. slamonicida* strains to starvation stress were found (Ferguson *et al*., 1995). Ferguson *et al*. (1998) als provided an assessment of the use of *lux*-based marker system in investigating the survival and activity of *A. salmonicida* in aquatic environment.

In conclusion assessment of construct stability of *P. stutzeri* P16 *lux*AB4 under non-selective conditions showed that the *lux* genes were stable. Light output was proportional to cell concentration and optical density over several orders of magnitude. Although there was a significant difference between the specific growth rate of wild type and *lux*-marked strains of *P. stutzeri* P16, this may have been due to the increased metabolic burden of replicating the foreign DNA. Degradation of phenanthrene by this strain was not affected by insertion of foreign DNA. Therefore this strain can be used for further studies in monitoring biodegradative GEMs in the environment.

References

Amin-Hanjani S, Meikle A, Glover LA, Prosser JI and Killham K (1993). Plasmid and chromosomally encoded luminescence marker systems for detection of *Pseudomonas fluorescens*. *Soil Mol Ecol*. 2: 47-54.

Bedard DL, Quensen III JF (1995). Microbial reductive dechlorination of polychlorinated biphenyls. In *Microbial transformation and degradation of toxic organic chemicals,* Young LY, Cerniglia ed., Wiley Liss Inc., New York CE. pp: 127-217.

Bennett JW, Faison BD (1997). Use of fungi in biodegradation. In: *Manual of environmental Microbiology*, Hurst CJ, Knudsen GR, McInerney MJ, Stetzenbach LD, Walter MV ed., ASM Press, Washington DC. pp: 758-765.

Boldrin B, Tiehm A, Fritzche C (1993). Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobateriun* sp. *Appl Environ Microbiol*. 59: 1927-1930.

Bossert ID, Compeau GC (1995). Cleanup of petroleum hydrocarbon contamination in soil. In *Microbial transformation and degradation of toxic organic chemicals,* Young LY, Cerniglia CE ed., Wiley Liss Inc., New York, pp: 77-126.

Brandt KK, Pedersen A and Sorensen J (2002). Solid-phase contact assay that uses a lux-marked Nitrosomonas europaea reporter strain to estimate toxicity of bioavailable linear alkylbenzene sulfonate in soil. *Appl Environ Microbiol.* 68:3502-3508

Cappuccino JG, Sherman N (1996). *Microbiology a laboratory manual*. The Benjamin/Cummings Publishing Company, Redwood City, California.

Cohen-Bazire G, Sistrom WR, Stanier RY (1957). Kinetic studies of pigment synthesis by non-sulfur purple bacteria. *J cel comp physiol.* 49: 25-68.

de Lorenzo V, Herrero M, Sanchez JM, Timmis KN (1998) .Mini-transposons in microbial ecology and environmental biotechnology. *FEMS Microbiol Ecol*. 27: 211-224.

Dwyer DF, Rojo F, Timmis KN (1988). Bacteria with new pathways for the degradation of pollutants and their fate in model ecosystems. In: *Risk assessment for deliberate release.*(the possible impact of genetically engineered microorganisms on the environment), Klingmüller W ed., Springer-Verlag, Berlin pp:100-109.

Ensley BD, DeFlaun MF (1995). Hazardous chemicals and biotechnology: past successes and future promise. In: *Microbial transformation and degradation of toxic organic chemicals*, Young LY, Cerniglia CE. Wiley Liss Inc., New York, pp: 603-629.

FergusonY, Bricknell IR, Glover LA, MacGreogor DM, Prosser JI (1998). Colonisation and transmisson of *lux*-marked and wild-type *Aeromonas salmonicida* strains in Atlantic salmon (salmo salar L.). *FEMES Microbiol Ecol*. 27: 251-260.

FergusonY, Bricknell IR, Glover LA, MacGreogor DM, Prosser JI (1995). Survival and activity of *lux*-marked *Aeromonas salmonicida* in seawater. *Appl Environ Microbiol*. 61: 3494-3498.

Garbisu C, Alkorta I (1997). Bioremediation: Principles and future. *J Clean Technol, Environ Toxicol Occup Med*. 6: 351-366.

Goyal AK, Zylstra GJ (1996). Molecular cloning of novel genes for polycyclic aromatic hydrocarbon degradation from *Comoamonas testosteroni* GZ39. *Appl Environ Microbiol*. 62: 230-236.

Grant FA, Glover LA, Killham K, Prosser JI (1991). Luminescence-based viable cell enumeration of *Erwinia carotovora* in the soil. *Soil Biol Biochem*. 23:

1021-1024.

Haddix PL, Shaw NJ, LeChevallier MW (2004). Characterization of bioluminescent derivatives of assimilable organic carbon test bacteria. *Appl Environ Microbiol*. 70: 850-854.

Herrero MA, de Lorenzo V, Timmis KN (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol*. 172: 6557-6567.

Hoben HJ, Somasegaran P (1982). Comparison of the pour, spread and drop plate methods for enumeration of *Rhizobium* spp in inoculants made from presterilized peat. *Appl Environ Microbiol*. 44: 1246-1247.

Hollis RP, Killham K, Glover LA (2000). Design and application of a biosensor for monitoring toxicity of compounds to eukaryotes. *Appl Environ Microbiol*. 66: 1676-1679.

Hutner SH (1950). Anaerobic and aerobic growth of purple bacteria (Athiorhodaceae) in chemically defined media. *J Gen Microbiol*. 4: 286-293.

Kiyohara H, Nagao K, Yana K (1982). Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. *Appl Environ Microbiol*. 43: 454-457.

Lindow SE (1995). The use of reporter genes in the study of microbial ecology. *Mol Ecol*, 4: 555-566.

Meikle A, Killham K, Prosser JI, Glover LA (1992). Luminometric measurement of population activity of genetically modified *Pseudomonas fluorescens* in the soil. *FEMS Microbiol lett.* 99: 217-220.

Narro ML, Cerniglia CE, Van Baalen C, Gibson DT (1992). Metabolism of phenanthrene by the marine cyanobacterium *Agmenellum quadruplicatum* PR-6. *Appl Environ Microbiol*. 58: 1351-1359.

Prosser JI (1994). Molecular marker systems for detection of genetically engineered micro-organisms in the environments. *Microbiology*, 140: 5-17.

Prosser JI, Killham K, Glover LA, Rattery EAS (1996). Luminescence-Based systems for detection of bacteria in the environment. *Crit Rev Biotechnol*. 16: 157-183.

Ritchie JM, Campbell GR, Shepherd J, Beaton Y, Jones D, Killham K, Artz RRE (2003). A stable bioluminescent construct of *Escherichia coli* O157:H7 for hazard assessments of long-term survival in the environment. *Appl Environ Microbiol*. 69: 3359-3367.

Rattray EAS, Prosser JI, Glover LA, Killham K (1992). Matric potential in relation to survival and activity of a genetically modified microbial inoculum in soil. *Soil Biol Biochem*. 24: 421-425.

Rattray EAS, Prosser JI, Killham K, Glover LA (1990). Luminescence-based nonextractive technique for in situ detection of *Escherichia coli* in Soil. *Appl Environ Microbiol*. 56: 3368-3374.

Semple KT, Cain RB, Schmidt S (1999). Biodegradation of aromatic compounds by microalgae. *FEMS Microbiol Lett.* 70: 291-300.

Shao CY, Howe CJ, Porter AJR, Glover LA (2002). Novel cyanobacterial biosensor for detection of herbicides. *Appl Environ Microbiol*. 68: 5026-5033.

Shaw JJ, Dane F, Geiger D, Kloepper JW (1992). Use of bioluminescence for detection of genetically engineered microorganism released into the environment. *Appl Environ Microbiol*. 58, 267-273.

Smit E, van Elsas JD, van Veen JA (1992). Risks associated with the application of genetically modified microorganisms in terrestrial ecosystems. *FEMS Microbiol Rev*. 88: 263-278.

Stringfellow WT, Aitken MD (1994). Comparative physiology of phenanthrene degradation by two dissimilar pseudomonads isolated from a creosote-contaminated soil. *Can J Microbiol.* 40: 432-438.

Van Dyke MI, Lee H, Trevors JT (1996). Survival of *lux*-marked *Alcaligenes eutrophus* H850 in PCB- contaminated soil and sediment. *J Chem Technol Biotechnol*. 65: 115-122.