# Genetic monitoring of microbes in soils amended with fly ash

**Submitted as a major project in partial fulfillment of the requirements for the award of the degree of**

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**By**

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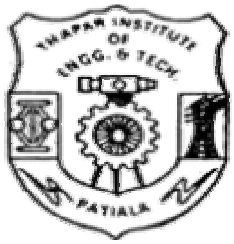


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# Certificate

This is to certify that the thesis entitled "Genetic monitoring of microbes in soils amended with flyash" submitted by Rajiv Kumar in partial fulfillment of the requirements for the award of Degree of Masters of Science in Biotechnology to Thapar Institute of Engineering and Technology (Deemed University), Patiala, is a record of student's own work carried out by him under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.


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## Summary

Coal fly ash may prove as a valuable soil amendment agent especially to coarse-texture soils mainly due to the presence of silt and clay like particles and plant nutrients. Its effect on soil chemical and physical properties has been studied, but little is known regarding its effect on soil microbial community.

Present study was focused to monitor the behavior of inoculated transformant S2: pMMB277 in pure soil and soil amended with flyash @ 0, 5, 10, 20 and 30% (w/w basis) under sterile and non-sterile conditions over a period of four weeks and also to study the effect of fly ash in general on soil microbial activity. Phosphate solubilizing bacteria (S2) isolated form fly ash affected soil was transformed with a wide host range, low copy number plasmid containing *cat* and *LacZ* gene responsible for chloramphenicol resistance and functional β-galactosidase which were used as molecular markers. Dehydrogenase activity was used as an indicator of total microbial activity in soil. Application of fly ash @ 10% in conjunction with bacterium S2: pMMB277 had optimum effect on its establishment and colonization and provides favorable environment for the growth of native as well as inoculated bacterium.

Addition of fly ash causes an increase in the electrical conductivity of soils and heavy metals Pb, Fe, Zn, Co, Ni, Cu, Cd and Cr content increased as proportion of fly ash was increased in soil from 5 to 30%. Also there was an increase in available phosphorus, available sulphur and organic carbon content of soil with concomitant increase in the fly ash percentage. Application of unweathered fly ash @ 10 % (w/w) basis has positive impact in soil on soil biological properties where as at higher concentration it had negative impact.

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# Introduction

Soils are central to the sustainability of our ecosystem. The applications of wastes to soil as a recycling option can only be sustained if there are demonstrable 'ecological benefits' which is usually justified in terms of elevated organic carbon and its effect on soil conditions and stimulation of microbial activity and nutrient supply and this is sustainable only if threshold levels of pollutants does not exceed. The analysis of microbial communities is potentially a sensitive way of detecting changes in soil functioning and could therefore be employed to evaluate the effectiveness of soil protection policies.

India generates more than 100 m t of fly ash per annum. Fly ash is a solid waste produced as a bye product of coal burning in thermal power plants. Besides its several other uses, its use in agriculture and forestry has become another important thrust area. Generally fly ash is dumped on open land, as land fill material or in ash ponds covering nearly 70,000 acres of land, which otherwise could be put for raising forestry plantations etc. Its effect on agriculturally important crop plants and forest tree species has been studied. Bacterial population can influence carbon or mineral cycles and have the ability to colonize harsh environments. However, little efforts have been made in studying the microbial ecology of such soils. In such soils or sites affected with fly ash introduction of beneficial soil microorganisms and their establishment, colonization and survival along with their role in improving soil fertility and interaction with plant roots will reveal more information on developing strategies for faster remediation of such sites.

To monitor microbes in nature, various methods have been developed which are primarily divided into two categories namely, nucleic acid based detection methods and methods based on phenotypic detection of a marker gene. The most straightforward way is tracking specific nucleic acid sequences or genes of interest using PCR or direct

hybridization of DNA or RNA probe with nucleic acid extracts from environmental samples. An alternative to tracking marker genes at the DNA level is also to track the phenotype of the marker gene. A number of marker gene have recently been described. Antibiotic resistant genes such as the *nptII* gene encoding resistance to kanamycin, was the first to be employed as marker. Genes encoding metabolic enzymes have also been used as non-selective markers. These include *xylE* (encoding catechol 2,3 oxygenase), *lacZY* (encoding β-galactosidase and lactose permease) and *gusA* (encoding β-glucuronidase (GUS). The *xylE* gene product can be detected by the formation of a yellow colored catabolite (2-hydroxymuconic semialdehyde) from catechol .The enzymes encoded by *lacZ* and *gusA* cleave the uncolored substrates X-gal or X-glc, respectively, to produce blue colored products. The difference with this method, compared to that above, is that it does not rely on incorporation of an inhibitory compound to the agar medium. Increasingly, it is becoming apparent that the best solution for tracking a microbe is to use more than one marker simultaneously.

Wide-host-range vectors have become very useful tools in studies and manipulation of non-enteric bacteria. Among the replicons used for construction of these vectors RSF1010 is the best-known system in terms of replication and complete nucleotide sequence. It has several desirable properties such as small size, wide host–range among gram negative bacteria, and good efficiency of mobilization by the *incpα* conjugation system. Several cloning vectors have been constructed based on this replicon, but among them, the auto regulated expression vector based on pMMB66 has been especially useful. Four of these new vectors code for $Cm^r$ that is more reliable marker in *Pseudomonas* than $Ap^r$, other incorporate a *lacZY* fragment directly after the Ptac or Ptaclac promoter.

The present study examines the feasibility of using a wide-host-range, control expression vector pMMB277 that retain all the properties of pMMB66 series ($Cm^r$) with

the added capability for direct screening of plasmid by *LacZ* expression and to insert this reporter gene in beneficial soil bacteria such as phosphate solubilisers and study their multiplication and colonization in fly ash amended soils after their introduction in order to assess the optimum dose of fly ash required for their survival and to determine its possible negative impact on soil microbes.

# Review of Literature

## 2.1 Microbes and microbial processes

Microbial processes represent the largest reservoir of novel useful gene products (Tiedja, 1995). In nature they play an important role in biogeochemical recycling of minerals and essential plant nutrients and are promising tools for bioremediation of toxic chemicals. Many microorganisms have been isolated that have remarkable capability of enzymatic transformation of traditionally recalcitrant harmful compounds. Only a subset of the micro-organisms in an ecological system is crucial for these chemical transformation (Mark A.Schneegurt, 1998). Therefore it is important to understand the dynamics of this active community and to develop ways of detecting its presence and measuring its activity both in terrestrial and aquatic eco-systems. Several microorganisms have been isolated from different habitats that are capable of useful enzymatic transformations, which play an important role in biogeochemical cycling (Schneegurt and Kulpa, 1998). Biotechnology involves primarily the techniques to use complex microbial processes and optimizing a product for its commercial success. Another promising way is the construction of GMO's and releasing these for various environmental and agricultural applications.

## 2.2 Methods of monitoring microorganisms

Traditional method of bacterial enumeration is often insufficient for monitoring the specific microbes critical for important biochemical reaction in complex, mixed microbial communities. Molecular method have been developed that can detect and quantify phylogenetic group on the bases of rDNA sequences and relevant structural genes. Many of these techniques rely on PCR for amplification of DNA sequences that might be in low abundance in a mixed microbial community. Reverse transcriptase can be coupled to PCR

for measuring gene expression on the basis of RNA abundance. Microbial diversity and community structure can be addressed by further examination of PCR products by various separations techniques and restriction analysis. Microscopic examination of the architecture of the intact microbial community such as bio-film and flocks can by fluorescent labeled with population of specific rRNA probes.

Methods for monitoring of microorganisms

- Phenotype detection of marker gene
- Nucleic acid based detection methods

### 2.3 Phenotype detection of marker gene

An alternate to tracking marker gene at DNA level is to track the phenotype of the marker gene. Antibiotic resistant gene, such as the *npt*II gene encoding resistance to kanamycin was the first gene to be employed as marker (Jansson, 1995). These phenotype marker genes are generally falling out of contributing to the undesirable spread of antibiotic resistance in nature giving misleading results in selective plating. Gene encoding metabolic enzymes has also been used as non-selective markers. These include *xylE* (encoding catechol 2, 3 oxygenase), *lacZY* (encoding B- galactosidase and lactose permease gusA (encoding β-glucuronidase). Some advantage and disadvantages of these phenotype markers have recently been discussed like, a useful application of *xyl*E is the specific detection of intact and viable cells, because catechol 2,3 oxygenase is inactivated by oxygen and rapidly destroyed outside the cell (Saunders *et al*., 1995). The GUS marker has been proposed particularly useful for plant-associated microbes, such as rhizoid in nodules where essentially no endogenous GUS background exist (Wilson *et al*, 1994). Disadvantages of these marker genes are potential background enzyme activity in the indigenous microbial population and requirements for growth and cultivation in the

detection methods. To increase the specificity of detection combination of methods have become more relevant and the best solution for tracking a microbe in the environment is to use either several markers simultaneously or multiple detection methods (Table 1, 2). Antibiotic selection in combination with bioluminescence, *xyl*E or *Gus*A has been found to be very effective and useful for selection of low number of tagged cells (Flemming *et al*., 1994).

### 2.3.1 Plasmid Transfer

Genetically modified microorganisms may be constructed by engineering of the chromosome or by introduction of plasmid containing gene encoding desired traits. Plasmids are extra-chromosomal genetic elements and some can be transferred to other microorganisms. The potential for plasmid transfer from the GMM to known recipients member of the indigenous microbial community can be monitored directly by tagging the mobile DNA molecules with appropriate marker genes like, different GFP variants with different excitation and emission spectra may be used to follow donor, recipient and transconjugants in the environmental samples. Simultaneously this can be achieved by tagging the conjugative plasmid with a cassette comprising one constitutive fluorescent marker and the recipient being chromosomally tagged with another fluorescent marker gene. In this case the donor cells will appear as emitting light in one color, recipient cells in another, and the transconjugants as a combination of both colors. These methods while developed specifically for plasmid conjugation studies may be modified with little effort to be useful in study of transduction or transformation. For example the R-galactosidase promoter *Plac*, can be fused to the *gfp* gene on the plasmid of interest. The GMM should also contain the lac repressor protein *lacI* integrated into its chromosome. In this case the *plac* promoter will be repressed in the indigenous microorganisms lacking the *lacZ* gene, the lac promoter will be active and detected by microscopic method (Jansson, 1995).

A series of wide host range, low copy number plasmid that allows direct screening of recombinants was constructed based on wide host range plasmid pMMB66EH. Some of these new vector code for ∝-peptide of β-glycosidase and allow the direct screening of recombinant colonies by inactivation of ∝-complementation. The *bla* gene was replaced in some plasmid by *cat* gene of Tn-9 coding for chloramphenicol resistance, extending its use into β-lactam resistant strain. It has wide host range among gram-negative bacteria, and good efficiency of mobilization by the incp ∝-conjugation system (Morales, 1991).

## 2.4 **Nucleic acid based detection methods**

These are divided into two general categories based on **a)** Tracking specific DNA sequences by direct hybridization, **b)** Tracking specific marker DNA sequence by PCR.

### 2.4.1 Tracking specific DNA sequences by direct hybridization

The most straightforward way of detecting specific nucleic acid sequence or genes of interest is through direct hybridization of a DNA or RNA probe to microbial nucleic acid extract. Whole-cell DNA or RNA is extracted from the microbes of an experimental sample such as soil or water and fixed to a positively charged membrane (nitro cellulose, nylon). Bacterial colonies can also be replica plated from agar plates to membranes and their nucleic acid exposed to *in situ* for subsequent hybridization. The nucleic acid on the membrane is probed with a small piece (often an oligonucleotide) of DNA or RNA containing a sequence complimentary to the target sequence. The probe is labeled before hybridization with $^{32}P$, a chemiluminescent compound or digoxigenin (for later antibody-peroxides detection). DNA probes can be used to detect genes in the bacterial genome or on plasmid (southern blots) or to detect mRNA or rRNA (Northern blots). The signal from hybridization is quantitative (Schneegurt *et al*., 1998); Positive bands or spots on a

membrane can be directly compared through densitometry. The structural gene for naphthalene degradation (the *nah* operon) have been detected and quantified by using three different types of nucleic acid hybridization techniques. One of the earliest studies to use direct hybridization techniques for the monitoring of microbes with bioremediation potential, followed the TOL (for toluene degradation) and NAH (for naphthalene degradation) plasmid in soil microcosm (Sayler *et al*., 1995).

**Table 1. Marker genes and methods used to detect GMMs in environmental samples.**

| Marker gene | Microorganism | Detection method | Reference |
|---|---|---|---|
| *lac* | *Pseudomonas* sp | Selective plating and PCR | Kluepfel, 1989. |
| *lac* | P*seudomonas aureofaciens* | Selective plating & Biology metabolic tests | England. *et.al.,* 1993 |
| ***npt***II | E*scherichia coli* | Selective plating, MPN-PCR and colony hybridisation | Recorbet G *et. al*, 1993 |
| *Tn5-lux* | *Pseudomonas sp* | Luminometer and CCD-enhanced detection | Beauchamp CJ, 1993 |
| *lux* | *Bacillus subtilis* | Non-selective plating, Luminometer and CCD-enchanced detection | Cook N,1993 |
| *lux-lac* | P*seudomonas aeruginosa* | Non-selective plating, selective plating, CCD-enhanced detection, PCR and southern blottings | Masson L,1993 |
| *lux-lac* | P*seudomonas aeruginosa* | *aeruginosa* Non-selective plating, selective plating, CCD - enhanced detection , PCR and southern blotting. | Flemming *et al*, 1994 |
| *lux* | P*seudomonas fluorescens* | Luminometery, selective plating, CCD-enhanced detection | Amin-Hanjan *et al*, 1993. |
| *lux* | P.*aeruginosa* | Bioluminescent-MPN(microplate assay), luminometry and CCD-enhanced detection | Flemming *et al*, 1994) |
| *Tn7-lux* | *P.fluorescens* | Autoradiogarphy and photon counting in sintilation counter | Brennerove *et al*., 1994 |
| *luc* | *Rhizobium meliloti* | Non-selective plating, luminometry, auto photography and visualization of luminescent colonies by eye. | Cobolia *et al*., 1993 |
| *luc* | *E.coli* | Selective plating, luminometry and PCR | Moller *et al*., 1993 |
| RP4:*pat* | *P.fluorescens* | Selective plating, PCR, Fluoreantibodies, southern blotting and Slot–blot hybridization | Smalla *et al*., 1993 |
| *xyl*E,$Km^r$ | *P.putida* | Non-Selective plating and selective plating | Winstanley *et al*., 1993 |
| $Km^r$-*xyl* and *lac* | *P.aureofaciens* | Selective plating | De Leij *et al*., 1993 |
| heterologus 5S rRNA | *E.coli* | Hybridization | Hedenstierna *et al*., 1993 |
| nptII-cyrIVB$^+$ | *P.fluorescens* | Selective plating and colony hybridization | Van Elsas *et al*., 1994 |
| Ω or *nptII* | *R.meliloti* | Selective plating | Bosworth et al., 1994 |
| *Tn5-gusA* | P.*putida* | Selective plating | Wilson *et al*., 1994 |

Colony hybridization with entire plasmid as probes allows the counting of cells containing this catabolic plasmid. Similar technique was used recently to monitor genes (*xylE* and *ndoB*) involved in creosote degradation in soil microcosm (Hosein *et al*., 1997). Standard southern blot hybridization has been used to follow the bacterial population of naphthalene degraders in seeded microcosms induced with salicylate (Ogunseitan *et al*., 1991). In this study, probes specific for the *nah* operon provided a measure of the naphthalene-degradation potential of the microbial population. *nahA* transcript have also been quantified with a different technique known as ribonuclease protection assays (Fleming *et al*., 1991). Here radiolabelled anti-sense RNA was hybridized in solution to extracted RNA. The result of molecular probing have been used in conjugation with traditional most-probable number (MPN) techniques in several studies focusing on microbes capable of dechlorinating the aromatic compounds. A combination of MPN and colony hybridization was used to follow the microbial community of a flow-through lake microcosm seeded with a chlorobenzoate-degrading *Alcaligenes* strain (Fulthorpe *et al*., 1989). This study found a correlation between the size and activity of a specific catabolic population during exposure to various concentration of 3-chlorobenzoate. Similarly colony hybridization for the detection of 2,4-dichlorophenoxyacetic acid (2,4-d)-degrading genes was positively correlated with MPN analysis of population from natural waters and raw sewage (Amy *et al*., 1990).

### 2.4.2 Tracking specific marker DNA sequence by PCR

PCR amplification has become one of the most promising method for sensitive detection of specific DNA sequence in nature. It has been particularly useful for detection of microbes that are in a non-culturable state or whose cell count (by plating) have declined below the detection threshold (Jansson, 1995). In general, PCR amplification is at

least 2-3 times more sensitive than DNA hybridization methods, and its sensitivity depends on target DNA copy number, the nature of the primer (nested versus normal) and the purity of the DNA sample.

The method for quantification of DNA by PCR is rapidly growing in number but essentially these are divided into three categories 1) Extrapolation from a standard curve during the linear range of amplification by quantitative PCR, 2) Competitive PCR (cPCR or Q-cPCR), 3) Most probable number PCR (MPN-PCR) that uses published MPN tables. Quantitative PCR (Q-PCR) has been applied for the quantification of DNA in the environmental samples (Romanowski *et al*., 1993). To track a microbe in nature on the basis of these unique DNA sequence it is first necessary to isolate DNA from the environment samples to which they have been added. Procedure for DNA isolation from soil and sediment were developed in the 1980s and can involves either direct cell lyses followed by DNA purification steps or bacterial extraction followed by cell lyses and DNA purification procedure have been compiled both for methods employing direct extraction (Saano *et al*., 1995; Trevors *et al.,* 1995). In particular a novel procedure for efficient recovery of soil bacteria on density gradients is promising (Bakken *et al*., 1995). Marker DNA may be detected in total DNA isolated and purified from environmental samples by a variety of methods. DNA hybridization techniques, using labeled marker DNA as a specific probe has been used in the past for identification of specific GMM in environmental samples (Atlas *et al*., 1992).

## 2.5 Microbial activity in soil

Soil enzyme activity is highly sensitive to both natural and anthropogenic disturbances. A quick response to the induced changes in the activity of dehydrogenase is considered as an indicator of the oxidative metabolism in soil and thus of the

microbiological activity. Dehydrogenase activity being exclusively intracellular, it is linked to viable cells however the relationship between an individual biochemical property and the total microbial activity is not always obvious, especially in complex systems such as soils, where microorganisms and processes involved in the degradation of the organic compounds are highly diverse. Nevertheless, dehydrogenase activity (DHA) has been used as an indicator of the microbiological activity in various types of soils (Consuelo *et al.,* 2002). In general the technique involves the incubation of soil with 2, 3, 5-triphenyl tetrazolium chloride (TTC) either in the presence or absence of added electron donating substrates. During the incubation, the microbial dehydrogenase activity results in the reduction of water-soluble, colorless TTC to the water insoluble, red colored 1,3,5-triphenyl-tetrazolium formazan (TTF). The latter is then extracted from the soil and read calorimetrically for quantification of microorganisms. Most workers have used incubation period of 24h or longer at $37^0$C to study dehydrogenase activity (Casida, Jr. 1977).

## 2.6 Fly ash

Fly ash typically consists of a variety of trace and heavy metal elements, some essential and others toxic to both plants and animals. They are present in the form of silicates, oxides, sulphates, phosphates etc. (Page *et al*., 1979). Major matrix elements in fly ash are silicon, aluminium and Iron together with significant percentage of calcium, magnesium, boron, zinc, copper, phosphorous etc. (Rees and Sidrak, 1956). Trace elements include Lanthanum, Nickel, Chromium, Terbium, Antimony, and Strontium etc.

Fly ash has a vast potential for use in agriculture, forestry and wasteland reclamation due to its excellent soil ameliorating properties (Adriano *et al*., 1979, 1980; Capp, 1978; Fail and Wochok, 1977; Aitken and Bell, 1986; Ciravolo and Adriano,

1979). Fly ash on addition in soil is reported to decrease bulk density, improve soil porosity, increase water-holding capacity, decrease surface encrustation change soil pH and increase the electrical conductivity of soil (Chang *et al*., 1977; Page *et al*., 1979; Elseewi *et al*., 1980). On account of its heterogeneous chemical nature fly ash can be used as a microfertilizer in agriculture and forestry. An increase in the yield of cereal crops like rice wheat etc. and vegetables like tomato, cabbage, pea etc. have been reported on soils amended with fly ash @ 25% (RRL Bhopal). Industrially important aromatic grasses and medicinal plants have been grown in fly ash amended soils. Application of fly ash in low doses in the agricultural fields is suitable for better crop management (Fail and Wochok, 1977). Minimum microbiological activities in ash deposits have been reported which is attributed due to their sterility (Hodgson and Townsend, 1973).

To study establishment of inoculum, it is necessary to have a sensitive and reliable means of specifically detecting and quantifying the inoculated strain in the field. Reporter gene systems provide an opportunity for microbiologists to step beyond the confines of previous methodologies in their attempts to understand the eco-physiology of microbes in their natural habitats. Traditional approaches for studying microbial ecology in the field have used spontaneous or induced antibiotic resistance markers or various antigen detection techniques including fluorescently labeled antibodies, the ELISA technique or immuno-diffusion. In recent years, new techniques based on molecular markers or polymerase chain reaction (PCR) has been used to specifically monitor the bacteria in the field. Use of marker genes such as *gus*A, *lac*Z, *cel*B, *xyl*A, *lux* AB has become an important tool in studies on microbial ecology. Molecular tagging of inoculant strains (such as *Rhizobium, Bradyrhizobium, Azotobacter, Azospirillum, Bacillus polymyxa and Pseudomonas striata*) is essential for ecological monitoring and

to assess their performance in the field. The *lac*Z has been used to study nodule infection by *Rhizobium* (Boivin *et al*. 1990) and root colonization by *Azospirrilum* (Katupitiya *et al*. 1995). We have isolated nitrogen fixing and phosphate-solublizing bacteria form fly ash affected soils. In this proposal we intend to tag these bacteria and study their performance *in situ*.

# Materials and Methods

## 3.1 Instruments used

Instruments used during the course of this work were Atomic absorption spectrophotometer (*GBC 932AA, Australia*); Shaker incubator (Shivaki, Delhi); Cooling centrifuge (Sigma 1-15,Germany); Microfuge (Sigma 1-15,Germany) and Electrophoresis assembly (Tarson, Mumbai).

## 3.2 Chemicals and Reagents

Chemicals and microbiological media were procured from Ranbaxy Laboratories and SD fine-chem. Ltd., Fluka Biochemika, SRL and Hi Media, Mumbai. Nutrient agar and LB medium (Trypton 10g, Yeast extract 5g, NaCl 10g, in one litre of distilled water, pH 7.0) were used for culturing bacteria. Whenever a solid media was required, 15-g/l agar agar was added to the liquid medium. Chemicals used were 2,3,5-triphenyl tetrazolium chloride, 1,3,5-triphenyl tetrazolium formazan, chloramphenicol, Xgal, IPTG etc. purchased from SD fine-chem. Ltd and Hi Media, Mumbai.

## 3.3 Bacterial cultures and culture conditions

*Escherichia coli* MTCC 2842 containing plasmid pMMB277 was obtained from IMTECH (Institute of Microbial Technology, Chandigarh India and was maintained on solid LB Media containing chloramphenicol @ (10µg/ml). Three bacterial isolates S2, S3, S5, from flyash-amended soil having phosphate-solublizing ability were transformed with plasmid pMMB277 isolated from *E.coli* 2842. Transformants were maintained on nutrient agar plates supplemented with chloramphenicol @10ug/ml and X-gal, IPTG. For the preparation of X-gal, IPTG plates, 30µl of X-gal stock, 8µl of IPTG stock was mixed in 40µl of sterile water in an eppendorf tube and spread on the solid agar surface, dried and

then used for plating bacterial suspension. Stock solution of X-gal was prepared by dissolving 20 mg of X-gal in 1ml of dimethyl formamide and stock solution of IPTG was prepared by dissolving 2 mg of IPTG in 10 ml double distilled sterile water. Stock solutions were filter sterilized by 0.22 μdispensable filters and stored at 4°C for further use.

### 3.4 Plasmid isolation and purification

Alkali lysis (Brinboim and Doly, 1979) was performed for the isolation of plasmid pMMB277 from *E.coli* MTCC 2842 (Morales *et al.,* 1991). Exponentially grown bacterial cultures ($OD_{600nm}$=1.0) in Luria broth containing chloramphenicol @ 10μg/ml were harvested by centrifugation at 6,000 rpm for 6-8 min. The bacterial pellet was air-dried and resuspended in 200μl of solution I (50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA, pH 8.0), which was immediately followed by addition of freshly prepared 50 μl lysozyme (10 mg/ml). The mixture was kept on ice bath for five minutes and 400 μl of freshly prepared solution II (0.2N NaOH, 1%w/v SDS) was added and mixed gently. After this 300μl of solution III (60 ml 5M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml water, store at 4°C) was added and kept on ice bath for 20-60 min. and was centrifuged at 10,000 rpm for 10 min. and the clear supernatant was collected in a fresh eppendorf tube. Equal volume of isopropanol was added to the supernatant and kept at -4°C for 1 hr and centrifuged at 10,000 rpm for 20 min. The pellet was air-dried dissolved in 30 μl of 1X TE buffer (10 mM Tris-Cl, 1mM EDTA) and stored at -20°C.

Plasmid was also isolated and screened following procedure of Kado and Liu, 1981. Exponentially grown 1.5 ml of the bacterial culture (OD 600nm= 1) was harvested and the pellet was washed with 1 ml TE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 7.9 adjusted with glacial acetic acid) and re-suspended in 100 μl of TE buffer and 200 μl of

lysis buffer (3% SDS, 50 mM Tris, pH 12.5 adjusted with 2N NaOH). The cell suspension was incubated at 65°C for 30 min to get a clear solution. Double volume of phenol, chloroform and iso-amyl alcohol solution (24:24:1) was and mixed by gently inverting the tubes for a few minutes. The mixture was centrifuged at 10,000 rpm for 15 min at 4°C and upper aqueous layer was transferred carefully to a fresh eppendorf tube. 35µl of that aqueous layer along with 10 µl of loading dye was loaded in 0.8 % agarose gel and run at 40V at low temperature upto two third of the gel length and seen under UV light after staining with ethidium bromide (2 µg/ml).

**Purification of plasmid**

Purification of plasmid was done using low melting agarose gel. Slices of gel, containing DNA bands were melted at 65°C and mixed with equal volume of phenol, followed by centrifugation at 8000 rpm for 8 min. Upper aqueous layer (200µl) was collected in fresh eppendorf tube and residue was re-extracted with 300 µl STE buffer (by centrifugation at 8000 rpm for 8 min). Equal volume of iso-propanol was added to the supernatant followed by incubation at -4°C centrifuged at 10000 rpm for 20 min. The pellet was dissolved in 30 µl of 1X TE buffer (10 mM Tris-Cl, 1mM EDTA) and stored at -20°C for further use.

### 3.5 Transformation of bacteria

Transformation of bacterial isolate S2 by plasmid pMMB277 was performed as per the method given by Mendal and Higa, 1970. Overnight grown bacterial cultures were reinoculated in 25 ml of fresh nutrient broth and incubated at 37°C for two and half hr and immediately kept on ice to stop further growth. The cells were harvested by centrifugation at 7,000 rpm for 8 min at -4°C and re-suspended in 100 mM $CaCl_2$ and kept for 10-20 min in ice. The step was repeated and cells were resuspended in ice chilled $CaCl_2$ and kept at

-4°C for two and half hr to make cells competent. 100µl of competent cells were transferred to sterile eppendorf tube and 10 µl of plasmid DNA was added and mixed and kept on ice for 30 min. It was followed by heat shock in water bath at 42°C for 2 min. and 1.0 ml of fresh nutrient broth was added and incubated at 37°C for 1 hr. The cells were harvested by centrifugation 6000 rpm for 6 min and 100 µl of concentrated cell suspension were plated on nutrient agar plates containing chloramphenicol (10 µg/ml) and incubated at 37°C for 24-48 hr. Chloramphenicol resistant colonies were screened for their β-glactosidase expression on X-gal, IPTG plates.

### 3.6 Soil and fly ash sampling

Soil samples were collected from agricultural field of Thapar institute of engineering and Technology, Patiala. Samples were collected from 10 cm layer of soil in plastic bags. The sample were divided into two parts, one was kept at -4°C and another was oven dried at 100°C and then sieved using 0.2 mm mesh sieve and stored at room temperature for further analysis. Fly ash was collected from electrostatic precipitator of Ballarpur Industries, Yamunanagar.

### 3.7 Characterization of soil and fly ash

Fly ash from ESP was mixed with soil at different concentrations 5%, 10%, 20%, 30%, on w/w basis. Soil flyash and different soil + flyash mixtures were analysed for various physiochemical properties. The characteristic like pH (Ghosh *et al*., 1983), electrical conductivity, organic carbon (Walkley & Black, 1934), available sulphur, available nitrogen, available phosphorus and heavy metals such as Cr, Pb, Fe, Zn, K, Co, Mn, Cd Ni, and Cu were analysed. 1 g soil sample were taken in 150 ml beaker and 15ml $HNO_3$ followed by 5 ml $HClO_4$ was added slowly and contents were heated on hot plate at

150-180°C for 2-3 hr till all the acid is dried and the soil takes whitish brown colour. Dry digested sample was dissolved in 100 ml conc. HCl and water in the ratio of 1:1 and filtered through quantitative filter paper (grade equivalent to whatmen 42). The extract was analyzed for metal content by atomic absorption spectrophotometer (AAS) as per GBC, Australia guidelines.

### 3.8 Determination of soil dehydrogenase activity

Method for the determination of dehydrogenase activity was modified based on the protocol of Casida, 1977. All the glassware, water and substrate solution were sterilized for dehydrogenase determination. 10 gm of soil were mixed with $CaCO_3$ in the ratio of 100:1. 3 g of each of these samples was dispensed in screw cap glass vials and 0.5 ml of sterile water was added and incubated for 1 hr at 28°C. After that 0.25 ml of single strength substrate solution (0.1% yeast extract) was added followed by 0.25 ml sterile water and allowed to diffuse through the soil samples. The vials were then incubated for 8 hr at 28°C. After the incubation, 0.5 ml of 3% aqueous TTC (2,3,5-tri phenyl tetra-zolium chloride) and 0.25ml double strength (0.2%) yeast extract was mixed with the help of sterile glass rod and incubated again for 6 hr at 37°C and the soil was extracted immediately with 25 ml methanol and filtered through qualitative filter paper (grade equivalent to whatmen 1). The methanol extract containing red colored formazan was read at 480 nm against the extract from the non-TTC soil blank. The values obtained were compared against a formazan standard curve prepared in methanol and these were reported as µg of formazan per gram of soil.

*Standard curve*

In 100 ml of volumetric flask 5, 10, 15 and 20 ml of working solution of 100 µg/ml 1,3,5-Triphenyl tetrazolium formazan (diluted from stock solution of 1mg/ml TPF) was added

and volume was adjusted with methanol. Absorbance was measured at 480 nm using UV-VIS spectrophotometer and the standard curve of concentration vs absorbance was plotted.

### 3.9 Determination of available sulphur

Available sulphur was determined as per the procedure of Jackson, 1958. According to this 50 ml of 0.15 % $CaCl_2$ solution was added to 10 g soil samples taken in 150 ml conical flask and agitated at 130 rpm for 30 minutes on an electric shaker and filtered through quantitative filter paper (grade equivalent to whatmen 42). 1.0 g of 30-60 mesh $BaCl_2$ crystals were added in a 25 ml volumetric flask containing 20 ml of filtrate and swirled for one minute to dissolve crystals followed by addition of 1 ml of 0.25% of gum acacia solution and volume was adjusted up to the mark with distilled water and absorbance was taken at 420 nm using spectrophotometer and compared with standard plot.

*Standard curve*

Different volumes 0.25, 0.5, 1.0, 2.5 and 5.0 ml of 100 ppm standard sulphur solution. 0.5434 g of the reagent grade potassium sulphate ($K_2SO_4$) in distilled water and diluted to one litre) were taken separately in 25 ml volumetric flasks. 10 ml of 0.15% $CaCl_2$ solution and 1.0 g of 30-60 mesh $BaCl_2$ crystals were added in each flask and swirled for one minute to dissolve the crystals. 1.0 ml of 0.25% solution of gum acacia was added and the volume was adjusted with distilled water and kept for 10 minutes to develop turbidity and absorbance was measured at 420 nm using spectrophotometer. Standard curve was plotted between concentration *vs* absorbance.

### 3.10 Determination of available phosphorus

Available phosphorous was determined as per the procedure of Olsen *et al*., 1954 for alkaline soils. 25 ml of 0.5M $NaHCO_3$ was added to 2.5 g soils taken in 100 ml conical flask and agitated at 130 rpm for 30 min on an electric shaker. The mixture was filtered through quantitative filter paper (grade equivalent to whatman 42). 2 ml of aliquot was transferred to 100 ml beaker followed by addition of 0.2 ml of 2.5M $H_2SO_4$ and 3 ml of distilled water. To this mixture 3.1 ml of distilled water and 1.6 ml of Reagent B [Dissolved 1.056g of ascorbic acid in 200 ml of Reagent A (Dissolved 12g of ammonium molybdate in 250 ml of distilled water and 0.2906 g of antimony potassium tartarate in 100 ml of distilled water added these two solution in 1000 ml of 2.5M $H_2S0_4$**,** mix thoroughly and make up to 2000 ml with distilled water)]. Absorbance of the samples were read on spectrophotometer at 882 nm and compared with standard plot.

*Standard curve*

0.2, 5, 10, 15 and 20 ml of standard phosphorus solution $KH_2PO_4$ were taken in 6 different 50 ml volumetric flasks followed by addition of 10 ml of 0.5 M $NaHCO_3$ and 1.0ml 2.5 M $H_2SO_4$ to each flask. 8 ml of reagent B was added to each and volume was adjusted to 50 ml with distilled water. The phosphorous concentration of these solution will be 0.04, 0.1, 0.2 0.30 and 0.40 mg $L^{-1}$ respectively. Plotted a standard curve showing relationship between concentrations of phosphorous and absorbance.

**Calculation**

P in soil (mg/kg)= P in extract (mg/L) x 20 (the standard soil to solution ratio)

### 3.11 Determination of organic carbon in soil (Walkley and Black, 1934) rapid titration method)

1.0 g of 0.2 mm sieved soil was taken in dry 500 ml conical flask and 10 ml of $K_2Cr_2O_7$ was added and swirled for a while followed by addition of 20ml of conc. $H_2SO_4$.

Flasks were allowed to stand for 30 min and then 200 ml of distilled water was added. After incorporation of 10 ml of phosphoric acid and 0.5 g of NaF and 1.0 ml of diphenylamine indicator, the content were titrated with ferrous ammonium sulphate solution till the color flashes form blue to green. A blank without soil was used for comparison. Organic carbon was calculated as follows:

Organic C (%)=10(B-T)/B X 0.003 X 100/wt. of soil

B= Vol. in ml of ferrous ammonium sulphate solution required for blank titration.

T= Vol. in ml of Ferrous ammonium sulphate solution required for soil sample.

### 3.12 Microbial enumeration

Representative 1 gm of soil samples were drawn from incubated soils and mixed in 10 ml sterile water serially diluted in sterile water and 100µl of appropriate dilution was plated on nutrient agar plates, nutrient agar plates containing chloramphenicol (10µl/ml) and nutrient agar plates containing chloramphenicol (10µl/ml), IPTG and X-gal. Plating was done in duplicates and incubated at 37°C for 24 hr and colony forming units were counted.

### 3.13 Monitoring of phosphate solubilizing transformants (S2: pMMB277) in soil and different soil and fly ash mixtures along with native bacterial population

The experiment was set up to study the microbial behavior in sterile and nonsterile systems. 480 gm each of 0%, 5%, 10%, 20%, 30% fly ash (w/w) amended soil was taken in four sets. Two sets were sterilized twice and the other two sets were kept non-sterile. In both cases again one set was augmented with transformed bacterial culture (S2: pMMB277) while second one un-inoculated and kept as control. Bacterium S2: pMMB277 was grown in 200 ml nutrient broth at 37°C, 150 rpm for 24 hr. Cells were

harvested, washed twice with sterile water and re-suspended in 100 ml sterile water. 10 ml was transferred in a sterile sample bottle and final volume was made up to 50 ml using sterile water and was inoculated to 480 gm of soil, mixed and all non-sterile and sterile soils were incubated at 30°C for four weeks and soil samples were drawn after 7 days interval for analysis of pH, electrical conductivity, organic carbon, dehydrogenase activity and microbial enumeration. Soil moisture of 40-50% was maintained periodically by adding equal quantity of sterile water in all soils.

# Results and Discussion

The aim of present study was to find an optimum concentration of fly ash that can establish a sustainable environment for the growth of beneficial microorganisms introduced in the soil and thus results in enhancement of soil fertility. Since, little is known regarding the effect of fly ash on soil microbial communities (Schutter and Fuhrmann, 2001). Therefore to develop a sustainable system for soil improvement, the inoculated microorganism must be traced with regard to its multiplication and colonization and to observe the impact of fly ash on soil biological properties in general. For this a phosphate solubilizing bacterial strain S2, was transformed with plasmid pMMB277 containing chloramphenicol $^{r}$ and *LacZ* marker genes and was inoculated in soil amended with fly ash and its population growth was monitored periodically. The following text presents the results on physico-chemical characterization of soil and soil amended with flyash and monitoring of inoculated microbes in soil and soil amended with fly ash under sterile and non-sterile conditions.

## 4.1 Physico-chemical characterization of soil and soil amended with flyash

Alkaline soils with loam texture pH 7.7 were mixed with ESP fly ash (Yamunanagar) at different concentrations 0, 5, 10, 20 and 30% on w/w basis and were analyzed for their physico-chemical properties. These included pH, EC, organic carbon, available sulphur and available phosphorus and heavy metals Ni, Cu, Cr, Pb, Zn, Fe, Co, Cd (Table 2). The pH of soil was alkaline so its addition to soil did not resulted in drastic change and the soil pH remained almost unaltered. The electrical conductivity however increased from 0.53 mS/cm to 1.99 mS/cm after flyash amendment, which represented almost four-fold increase in soluble salt content. Addition of unweathered fly ash results in increase electrical conductivity of soil there by increasing the availability of soluble salts (Cervelli et al., 1986; Wong and Wong, 1986; Pitchel, 1990; Pitchel and Hayes, 1990), which might

have detrimental effects on microbial respiration, enzyme activity and soil N cycling etc. A significant increase in heavy metals Pb, Fe, Zn, Co, Ni, Cu, Cd and Cr was observed as proportion of fly ash was increased in soil from 5 to 30% (Fig 1-2), accept Mn which remained unchanged in range of 200 to 202 ppm (Table 2).

### 4.2 Isolation, purification of plasmid and its introduction into bacterial isolate (S2)

A low copy number, wide host range controlled expression vector plasmid pMMB277 was isolated from *E.coli* MTCC using alkali lyses method. Fig. 1 shows 0.7% agarose gel electrophoretogram (Plate 1) of plasmid having size 9.234 Kb which migrated parallel to 23.13 kb Lambda DNA cut by Hind III. The plasmid was purified from low melting agarose used for transforming S2 bacterium.

Three bacterial isolates from fly ash affected soils S2, S3 and S5 having phosphate-solubilizing ability were tested for chloramphenicol sensitivity and *LacZ* expression by streaking them on nutrient agar plates containing chloramphenicol (10µg/ml) and X-gal, IPTG. All three were chloramphenicol sensitive and $LacZ^{ve}$. Microscopic studies after Gram staining reveals that isolate S2 and S3 were Gram +ve where as S5 were Gram -ve (Plate 2-4). Bacterial strains S2 and S5 were attempted for transformation with plasmid pMMB277. Only S2 was transformed with plasmid pMMB277. The transformants were screened on chloramphenicol plates followed by X-gal, IPTG containing plates for expression of plasmid born chloramphenicol resistance and *Lac Z* marker (Plate 3).

## 4.2 Monitoring of inoculated microbes in soil and soil amended with fly ash.

Transformants S2: pMMB277 was grown on mass scale and used for further studies. Behavior of inoculated microorganism S2: pMMB277 in pure soil and soil amended with flyash @ 0, 5, 10, 20 and 30% (w/w basis) using sterile and non-sterile soils in particular and effect of fly ash in general on soil biological properties was studied over a period of four weeks at 30$^{o}$C and 40-50% soil moisture. Soil samples were withdrawn periodically and analyzed for pH, EC, organic carbon, dehydrogenase activity and microbial count on nutrient agar plates and nutrient agar containing chloramphenicol, IPTG and X-gal. pH of the soil samples remained slightly alkaline during the whole study whereas EC was found to slightly increase during the course of incubation indicating the increase in soluble salt content of the soil (Table 4). Organic carbon also showed a steady increase over a period of time and was higher in non-sterile soil conditions. There was no correlation of organic carbon in with soil dehydrogenase activity or microbial count and it increased with respect to increasing concentration of fly ash in soil.

Soil dehydrogenase activity was determined for control soil and soil + fly ash treatment under sterile and non-sterile condition with and without inoculum over weekly intervals (Table 2, Fig. 3-6). It was observed that in sterile inoculated system, fly ash amendment @ 10% leads to increase in the dehydrogenase activity after 24 days of incubation (Fig. 6) which indirectly represents microbial activity of soil and it is clear that 10 % fly ash has a positive effect on soil microbial activity. This dehydrogenase technique for measuring the metabolic activity of microorganisms in soil was modified to use a 6-h, 37 C incubation with yeast extract as the electron donating substrate in limiting concentration which does not support the growth of microorganisms but only increase their activity (Casida, 1977). The technique was used to follow changes in the overall metabolic activities of microorganisms in soil undergoing incubation with different

concentration of fly ash along with control to consider the metabolic state of the organisms in soil before dehydrogenase determination. Dehydrogenase activity decreased at all concentrations of fly ash as compared to control in the beginning and only at 10 % fly ash it tends to show increase after two weeks of incubation. These detrimental effects were partly due to excessive levels of soluble salts and trace elements present in unweathered fly ash (Schutter and Fuhrmann, 2001) however, high concentration of salts and other elements may decrease as the fly ash becomes weathered during natural leaching processes, thus reducing detrimental effects overtime (Adriano et al., 1980; Sims et al., 1995) Since soils and fly ash used in these experiments have a pH between 7-8 therefore detrimental effects due to pH is ruled out. Various comparisons of respective dehydrogenase activities have been made for variations in microbial number, oxygen consumption, $CO_2$ evolution, soil C:N ratios, soil humus content, activity of other enzymes in soil, plant growth and yields, soil particle size fractions, soil sampling depth season and so forth. These studies have not resulted in a uniformity of opinion as to which component of the soil ecosystem is contributing to the dehydrogenase response (Casida, 1977).

Aerobic heterotrophic bacterial population was studied at different time interval especially of inoculated bacterium with a marker gene to elucidate in greater detail the actual shifts that may occur among bacterial community in response to fly ash. The soil samples were periodically removed and analyzed for total microbial count and S2 transformants. It was observed that in sterile soil system, microbial count increased in 10% fly ash amended soil ($0.32x10^7$) as compared to control soil ($0.21x10^7$) after 24 days of incubation. At higher concentration of fly ash the count was much less (Fig 8). Here there was a direct correlation between microbial count and soil dehydrogenase activity. This trend was almost observed in all treatment i.e. sterile and non-sterile soils with and without bacterial inoculation. At higher concentration of fly ash there was a negative

impact of fly ash in soil biological properties. Fly ash did not exert any detrimental effect on the population of inoculated bacteria. Also at 10% fly ash the water holding capacity of the soil is increased with optimum availability of trace elements required for growth of inoculated microorganisms while a further increase in fly ash content may result in exceeding the trace element concentration more than the toxic limit for the inoculated microorganisms to survive. The application of fly ash @ 10% in conjunction with bacterial S2 has the optimum effect on its establishment and colonization also at this there is an increase in available Phosphorus, available sulphur content of soil and concordant to increase in organic carbon. It provides congenial atmosphere for bacterial growth in soil and soil biological properties including both native and inoculated bacteria.

# Conclusions

1. The pH of fly ash was alkaline so its addition to soil did not resulted in drastic change and the soil pH remained almost unaltered.
2. The electrical conductivity however increased from 0.53 mS/cm to 1.99 mS/cm after flyash amendment, which represented almost four-fold increase in soluble salt content.
3. The fly ash amendment in soil significantly increased the heavy metals Pb, Fe, Zn, Co, Ni, Cu, Cd and Cr content as proportion of fly ash was increased in soil from 5 to 30%, accept Mn, which remained unchanged. Also there was an increase in available phosphorus, available sulphur and organic carbon content of soil with concomitant increase in the fly ash percentage.
4. All the three phosphate solubilising bacterial strains S2, S3 and S5 isolated from fly ash affected soils were chloramphenicol sensitive and $LacZ^-$ and S2 and S3 were Gram +ve whereas S5 was Gram -ve. Only S2 was transformed with plasmid pMMB277.
5. Organic carbon showed a steady increase with respect to increasing concentration of fly ash and over a period of time and was higher in non-sterile soil conditions. There was no correlation of organic carbon v/s soil dehydrogenase activity or microbial count.
6. Fly ash amendment @ 10% caused increase in the dehydrogenase activity after 24 days of incubation which indirectly represents microbial activity of soil and it is clear that 10 % fly ash has a positive effect on soil microbial activity.
7. It was observed that in sterile soil system, microbial count increased in 10% fly ash amended soil ($0.32x10^7$) as compared to control soil ($0.21x10^7$), which decreased

at higher concentration of fly ash. Here there was a direct correlation between microbial count and soil dehydrogenase activity.

8. Application of fly ash @ 10% in conjunction with bacterium S2: pMMB277 has the optimum effect on its establishment and colonization and provides favorable environment for the growth of native and inoculated bacteria.

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**Table 2.** Physico-chemical characterisation of soil, fly ash and soil amended with fly ash at different concentrations.

| Parameter analysed | Soil | Soil+ 5% FA | Soil+ 10% FA | Soil+ 20% FA | Soil+ 30% FA | FA |
|---|---|---|---|---|---|---|
| pH | 7.69 | 7.73 | 7.73 | 7.67 | 7.55 | 7.67 |
| E.C (mS/cm) | 0.53 | 0.85 | 1.27 | 2.04 | 1.99 | 2.74 |
| Ava. Phosphorus (ppm) | 1.68 | 2.36 | 13.76 | 27.50 | 41.88 | 53.96 |
| Ava. Sulphur (ppm) | 32.93 | 81.87 | 208.66 | 363.12 | 440.97 | 687.8 |
| Organic carbon (%) | 0.348 | 0.356 | 0.492 | 0.645 | 0.679 | 1.606 |
| K (ppm) | 50.2 | 47.3 | 45.9 | 43.8 | 41.9 | 38.7 |
| Pb (ppm) | 85.26 | 91.26 | 95.03 | 107.4 | 116.53 | 152.2 |
| Fe (ppm) | 1821 | 1653 | 1811 | 1631 | 1519 | 1545 |
| Zn (ppm) | 51.8 | 51.53 | 58.93 | 70.23 | 81.53 | 144.7 |
| Co (ppm) | 8.266 | 8.266 | 9.30 | 10.26 | 11.90 | 17.10 |
| Ni (ppm) | 19.8 | 15.8 | 19.5 | 21.7 | 19.2 | 31.4 |
| Cu (ppm) | 14.56 | 12.7 | 17.7 | 21.66 | 30.16 | 67.73 |
| Mn (ppm) | 200 | 176 | 184 | 186.2 | 176 | 202 |
| Cr (ppm) | 14.1 | 20.0 | 21.3 | 23.04 | 23.4 | 33.3 |
| Cd (ppm) | < 0.02 | < 0.02 | < 0.02 | < 0.02 | < 0.02 | < 0.02 |

**Table 3.** Dehydrogenase activity of pure soil, soil amended with fly ash at different concentrations under sterile and non-sterile conditions at different intervals of incubation.

| Soil + %FA | Sterile/ Unsterile | Inoculated (I)/ un-inoculated (Uni) | Soil dehydrogenase activity (µg/g of soil/6hr) | | | | |
|---|---|---|---|---|---|---|---|
| Days> | | | 0 | 8 | 16 | 24 | 32 |
| 0% | Un-sterile | Uni | 48.83 | 34.44 | 22.06 | 14.02 | 22.06 |
| | | In | - | 47.02 | 17.11 | 30.10 | 19.38 |
| | Sterile | Uni | - | 21.99 | 17.11 | 13.16 | 19.38 |
| | | In | - | 31.12 | 9.90 | 26.18 | 10.09 |
| 5% | Un-sterile | Uni | 30.72 | 22.67 | 14.02 | 11.34 | 16.66 |
| | | In | - | 14.02 | 9.90 | 15.46 | 6.38 |
| | Sterile | Uni | - | 11.13 | 9.90 | 9.90 | 8.03 |
| | | In | - | 15.87 | 6.58 | 12.99 | 20.61 |
| 10% | Un-sterile | Uni | 24.22 | 13.60 | 9.90 | 11.13 | 5.56 |
| | | In | - | 14.01 | 20.61 | 9.07 | 5.77 |
| | Sterile | Uni | - | 7.03 | 6.33 | 10.09 | 5.35 |
| | | In | - | 10.10 | 6.79 | 21.44 | 7.62 |
| 20% | Un-sterile | Uni | 24.07 | 10.30 | 6.59 | 7.83 | 5.14 |
| | | In | - | 10.10 | 4.73 | 9.48 | 5.14 |
| | Sterile | Uni | - | 5.36 | 5.08 | 8.86 | 5.77 |
| | | In | - | 7.21 | 6.79 | 6.60 | 4.12 |
| 30% | Un-sterile | Uni | 4.94 | 7.00 | 6.59 | 7.83 | 5.14 |
| | | In | - | 9.69 | 4.73 | 9.48 | 5.14 |
| | Sterile | Uni | - | 5.36 | 5.08 | 8.86 | 5.77 |
| | | In | - | 8.25 | 6.40 | 6.59 | 4.12 |

**Table 4.** pH, EC and organic carbon content of soil, soil + fly ash mixtures at different intervals of incubation

| Soil + % FA | Non Sterile/ Sterile | Inoculated/ uninoculated | Chemical parameters | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | pH | | | | | EC (µS) | | | | | Organic Carbon (%) | | | |
| Days > | | | 0 | 8 | 16 | 24 | 32 | 0 | 8 | 16 | 24 | 32 | 0 | 8 | 16 | 24 |
| 0% | Non Sterile | Uni | 7.69 | 8.00 | 7.95 | 7.83 | 7.63 | 531 | 206 | 225 | 232 | 223 | 0.35 | 0.47 | 0.47 | 0.510 |
| | | In | - | 7.98 | 7.58 | 7.62 | 7.67 | - | 117 | 205 | 245 | 280 | - | 0.27 | 0.32 | 0.260 |
| | Sterile | Uni | - | 7.52 | 7.81 | 7.70 | 7.76 | - | 208 | 215 | 267 | 253 | - | 0.27 | 0.29 | 0.210 |
| | | In | - | 8.42 | 8.10 | 7.91 | 7.62 | - | 181 | 193 | 216 | 232 | - | 0.33 | 0.31 | 0.210 |
| 5% | Non Sterile | Uni | 7.73 | 7.63 | 7.52 | 7.43 | 7.40 | 859 | 284 | 290 | 279 | 260 | 0.36 | 0.47 | 0.41 | 0.320 |
| | | In | - | 7.72 | 7.70 | 7.65 | 7.65 | - | 271 | 284 | 286 | 290 | - | 0.23 | 0.31 | 0.220 |
| | Sterile | Uni | - | 7.82 | 7.51 | 7.76 | 7.67 | - | 234 | 242 | 268 | 286 | - | 0.33 | 0.28 | 0.330 |
| | | In | - | 8.24 | 7.80 | 7.73 | 7.98 | - | 274 | 286 | 311 | 265 | - | 0.25 | 0.28 | 0.310 |
| 10% | Non Sterile | Uni | 7.74 | 7.75 | 7.70 | 7.47 | 7.38 | 1274 | 452 | 504 | 614 | 510 | 0.49 | 0.67 | 0.51 | 0.547 |
| | | In | - | 7.92 | 7.65 | 7.98 | 7.70 | - | 538 | 570 | 642 | 557 | - | 0.32 | - | - |
| | Sterile | Uni | - | 7.47 | 7.55 | 7.31 | 7.45 | - | 467 | 490 | 625 | 567 | - | 0.35 | 0.42 | 0.314 |
| | | In | - | 8.08 | 7.82 | 8.15 | 8.22 | - | 540 | 552 | 632 | 620 | - | - | - | - |
| 20% | Non Sterile | Uni | 7.67 | 7.75 | 8.00 | 7.92 | 7.38 | 2041 | 615 | 635 | 617 | 644 | 0.65 | 0.62 | 0.61 | 0.670 |
| | | In | - | 7.66 | 7.46 | 7.30 | 7.70 | - | 910 | 963 | 1017 | 983 | - | 0.02 | 0.28 | 0.302 |
| | Sterile | Uni | - | 7.75 | 7.61 | 7.63 | 7.66 | - | 675 | 712 | 815 | 680 | - | 0.55 | 0.55 | 0.420 |
| | | In | - | 8.00 | 7.85 | 7.90 | 7.86 | - | 900 | 862 | 822 | 818 | - | 0.44 | 0.42 | 0.410 |
| 30% | Non Sterile | Uni | 7.56 | 7.70 | 7.64 | 7.54 | 7.69 | 2786 | 677 | 710 | 820 | 678 | 0.68 | 0.85 | 0.69 | 0.723 |
| | | In | - | 7.86 | 7.89 | 7.82 | 7.77 | - | 753 | 735 | 811 | 840 | - | 0.66 | 0.72 | 0.640 |
| | Sterile | Uni | - | 7.88 | 7.73 | 7.64 | 7.84 | - | 700 | 740 | 768 | 770 | - | 0.44 | 0.31 | 0.463 |
| | | In | - | - | 7.90 | 7.52 | 7.60 | - | 690 | 753 | 740 | 755 | - | 0.42 | 0.45 | 0.432 |

**Table 5.** Total bacterial count at different intervals of incubation

| | Sterile/ unsterile | Uninoculated/ Inoculated | Microbial count (cfu/g of soil) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Nutrient Agar | | | | Nutrient Agar + chloramphenicol + IPTG | | | | | | | |
| | | | | | | | Total | Blue | Total | Blue | Total | Blue | Total | Blue |
| Days > | | | 0 x$10^7$ | 8 x$10^7$ | 16 x$10^7$ | 24 x$10^7$ | 0 x$10^7$ | | 8 x$10^7$ | | 16 x$10^7$ | | 24 x$10^7$ | |
| 0% | Non Sterile | Uni | 240 | 45 | 0.84 | 0.7 | 4 | 1.7 | 2.5 | 1.3 | 1.52 | 1.21 | 0.768 | 0.384 |
| | | In | 13740 | - | 0.50 | 4.1 | 13504 | 13500 | - | - | 0.04 | 0.032 | 0.915 | 0.81 |
| | Sterile | Uni | 0.020.020 0. | 0.3 | 4.20 | 1.2 | 0.01 | 0.07 | 0.03 | 0.014 | - | - | 0.054 | 0.02 |
| | | In | 13500 | 3000 | 0.02 | 1.0 | 13500 | 13500 | 30.7 | 4.00 | 0.02 | 0.015 | 0.219 | 0.217 |
| 5% | Non Sterile | Uni | 160 | 300 | 2.30 | 4.1 | 2 | 0.7 | 0.2 | 0.03 | 0.02 | 0.018 | 0.024 | 0.124 |
| | | In | 13660 | - | 3.20 | 0.8 | 13500 | 13500 | - | - | 0.042 | 0.035 | 1.10 | 1.01 |
| | Sterile | Uni | 0.4 | 0.01 | 0.52 | 0.8 | 0.01 | 0.006 | 0.035 | 0.013 | - | - | 0.2 | 0.02 |
| | | In | 13500 | 1820 | 0.67 | 1.9 | 13500 | 13500 | 18.6 | 3.00 | 0.02 | 0.01 | 0.272 | 0.24 |
| 10% | Non Sterile | Uni | 110 | 8 | 6.00 | 3.2 | 1.0 | 0.23 | 0.33 | 0.03 | 0.015 | 0.01 | 0.26 | 0.153 |
| | | In | 13610 | - | 6.00 | 12.8 | 13500 | 13500 | - | - | 0.062 | 0.045 | 1.25 | 1.22 |
| | Sterile | Uni | 6.1 | 0.18 | 0.01 | 0.4 | 0.2 | 0.006 | 0.025 | 0.006 | - | - | 0.038 | 0.028 |
| | | In | 13506 | 27.9 | 0.62 | 3.0 | 13500 | 13500 | 27.9 | 4.00 | 0.032 | 0.022 | 0.373 | 0.32 |
| 20% | Non Sterile | Uni | 90 | 1.5 | - | 0.2 | 1.5 | 0.20 | 0.10 | 0.01 | 0.012 | 0.010 | 0.210 | 0.124 |
| | | In | 13590 | - | - | 0.28 | 13500 | 13500 | - | - | 0.12 | 0.04 | 0.12 | 0.04 |
| | Sterile | Uni | 15.2 | 0.001 | 0.002 | 0.4 | 0.1 | | 0.02 | 0.006 | - | - | 0.028 | 0.025 |
| | | In | 13515 | 79 | 0.32 | 0.9 | 13500 | 13500 | 10.7 | 3.00 | 0.012 | 0.01 | 0.025 | 0.020 |
| 30% | Non Sterile | Uni | 10 | 7.2 | | 0.2 | 0.4 | 0.010 | 0.2 | 0.01 | 0.015 | 0.01 | 0.204 | 0.124 |
| | | In | 13510 | - | - | 0.2 | 13500 | 13500 | - | - | 0.12 | 0.04 | 0.12 | 0.04 |
| | Sterile | Uni | 2.18 | 0.224 | | 0.03 | 0.1 | 0.009 | 0.002 | - | - | - | 0.028 | 0.024 |
| | | In | 13520 | 229 | 0.04 | 0.08 | 13500 | 13500 | 8.1 | 4.00 | 0.012 | 0.01 | 0.025 | 0.020 |

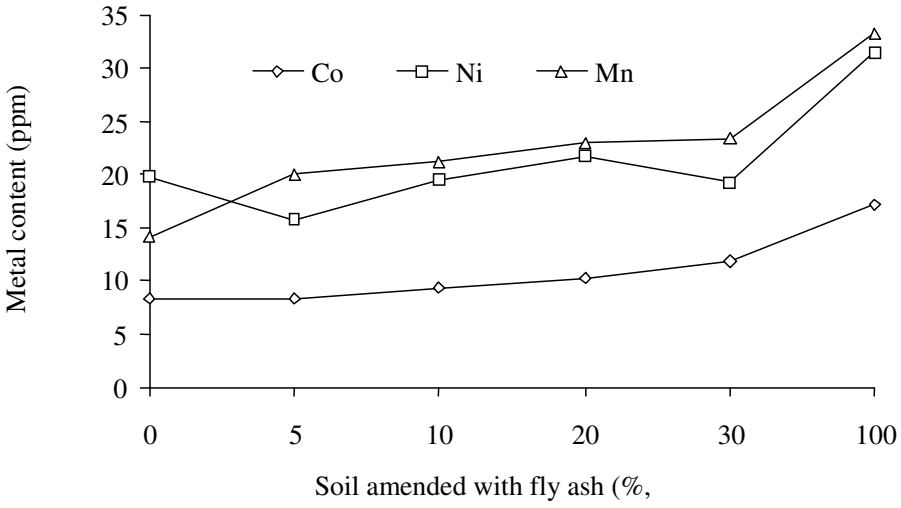



Fig.1 Co, Ni and Mn in soil, fly ash and soil + flyash

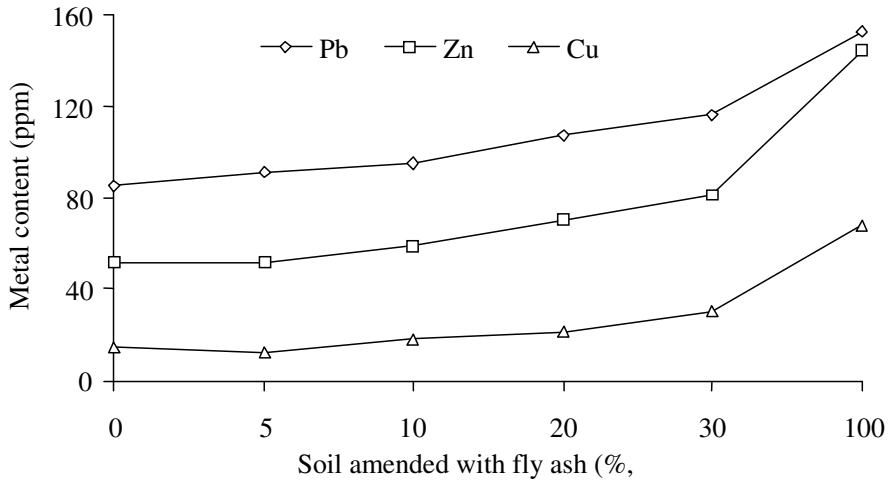



Fig. 2 Pb, Zn and Cu in soil, fly ash and soil + flyash

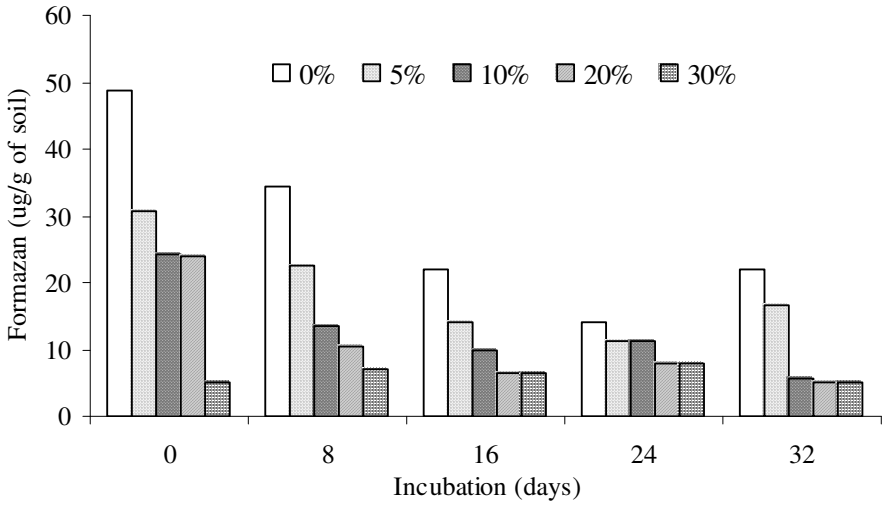



Fig. 3 Effect of flyash on dehydrogenase activity in nonsterile soil without inoculum

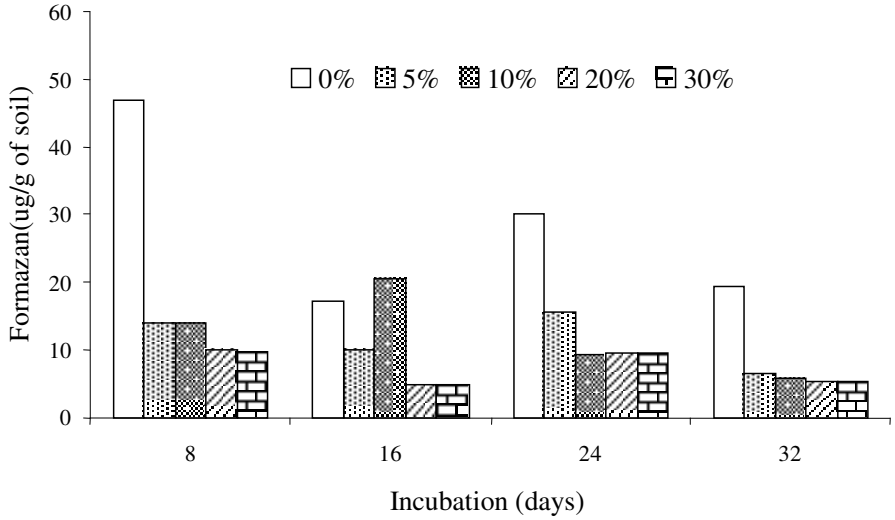



Fig. 4 Effect of flyash on dehydrogenase activity in nonsterile soil with inoculum (S2:pMMB277)

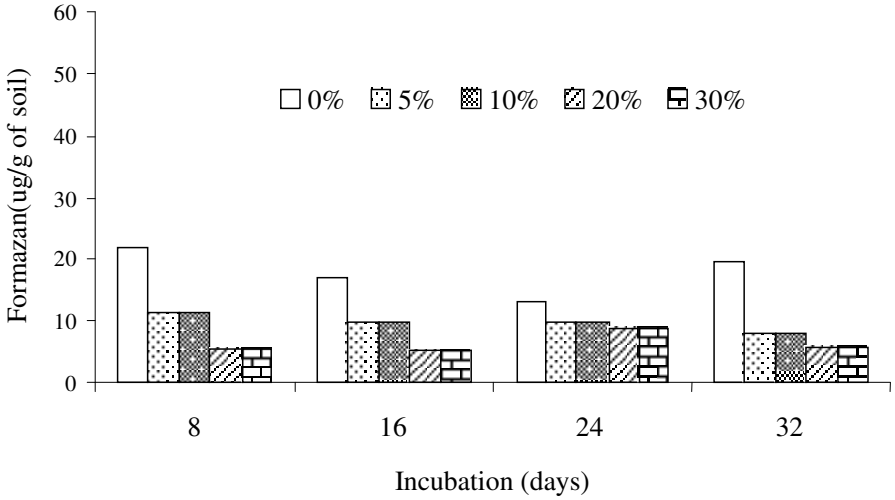



Fig. 5 Effect of flyash on dehydrogenase activity in sterile soil without inoculum

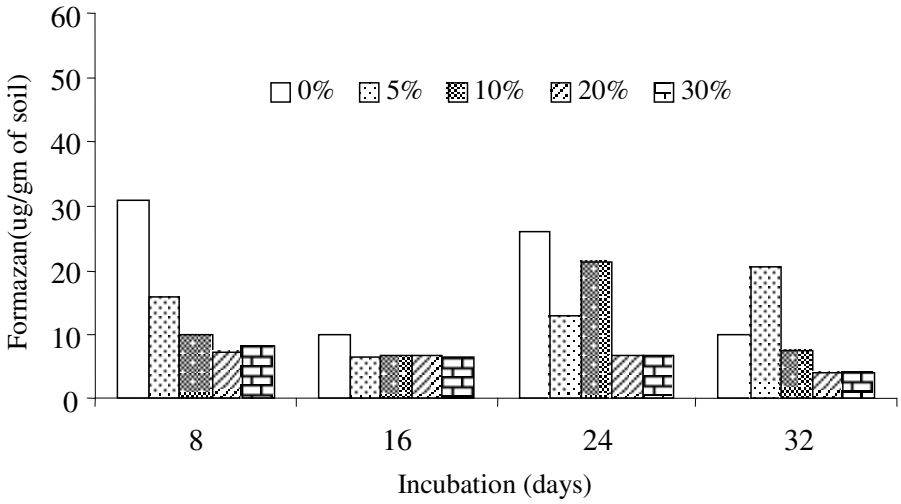



Fig. 6 Effect of flyash on dehydrogenase activity in sterile soil with inoculum (S2:pMMB277)

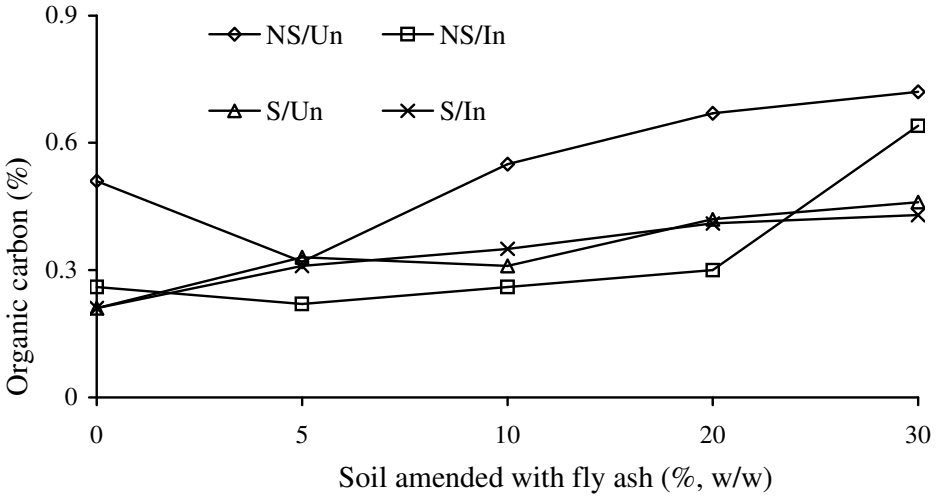



Fig.7 Organic carbon in soil and soil + fly ash mixtures after 24 days of incubation

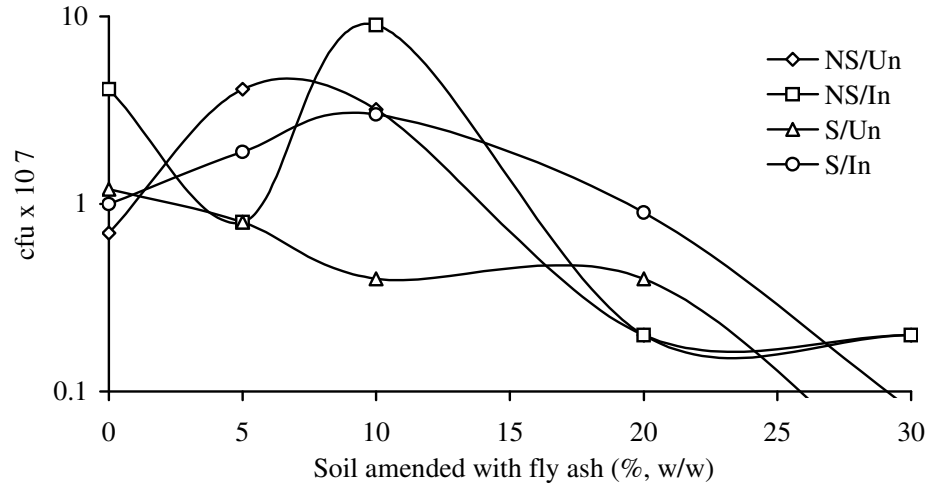



Fig. 8 Effect of fly ash on total bacterial population in non-sterile (NS) and sterile (S) soils with and without inoculation (I) after 24 days of incubation

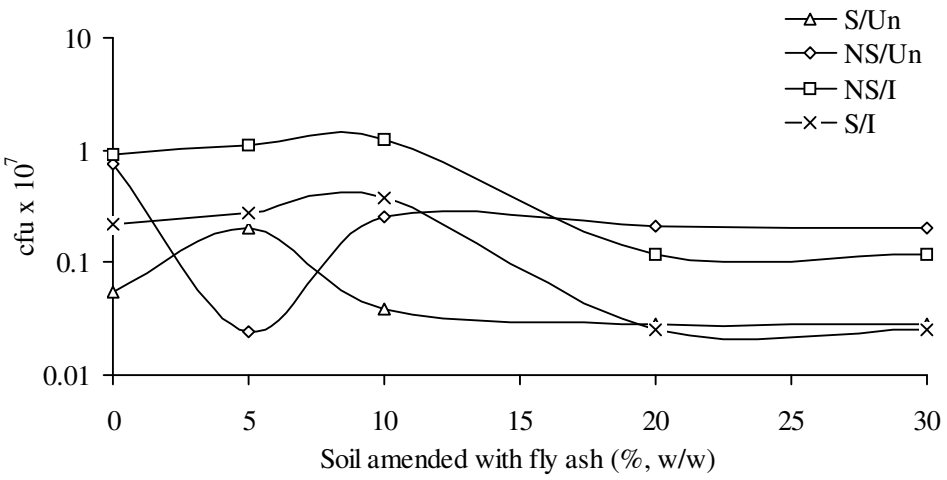



Fig. 9 Effect of fly ash on total bacterial count (chloramphenicol resistant) in non-sterile (NS) and sterile (S) soils with and without inoculation (I) after 24 days of incubation

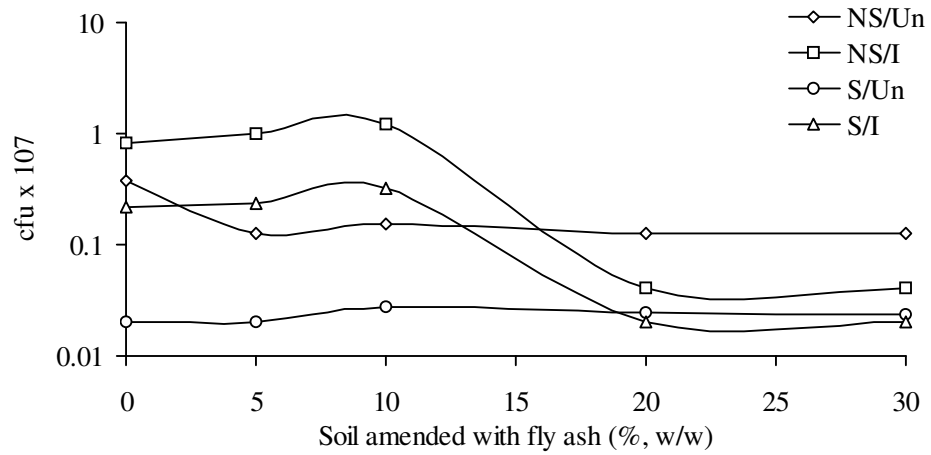



Fig. 10 Effect of fly ash on total bacterial count (chloramphenicol resistant + LacZ positive) in non-sterile (NS) and sterile (S) soils with and without inoculation (I) after 24 days of incubation

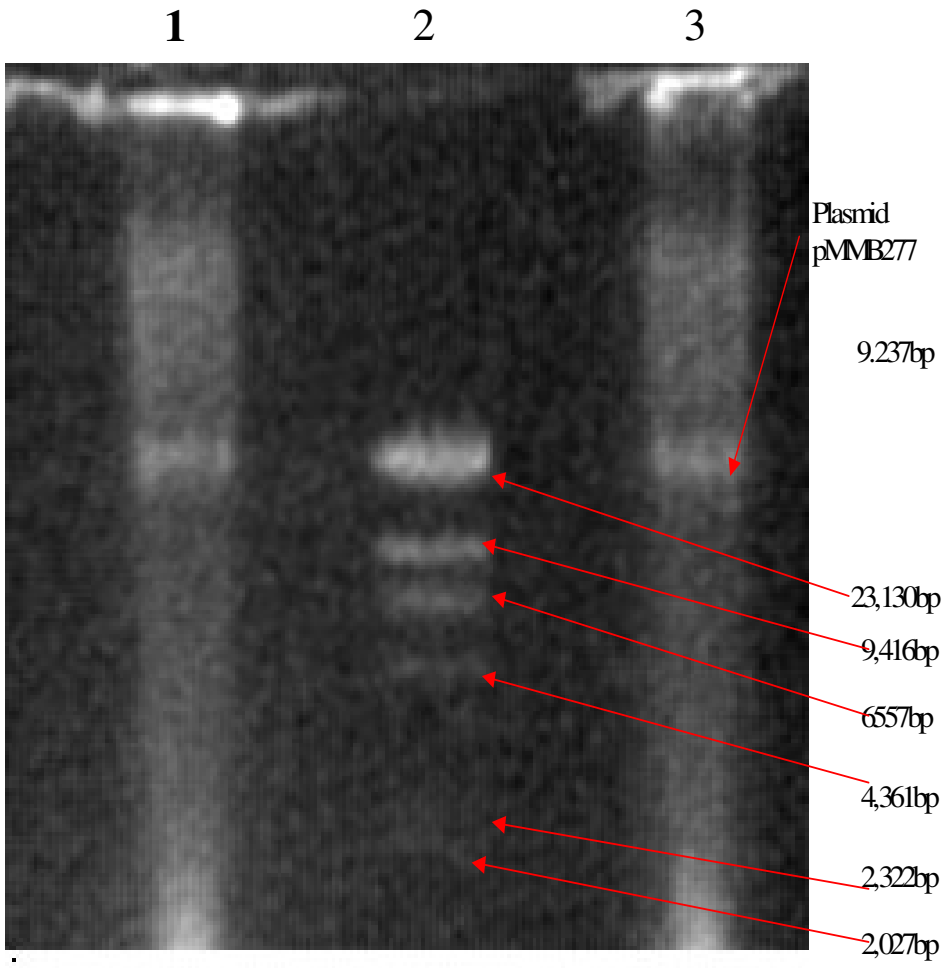
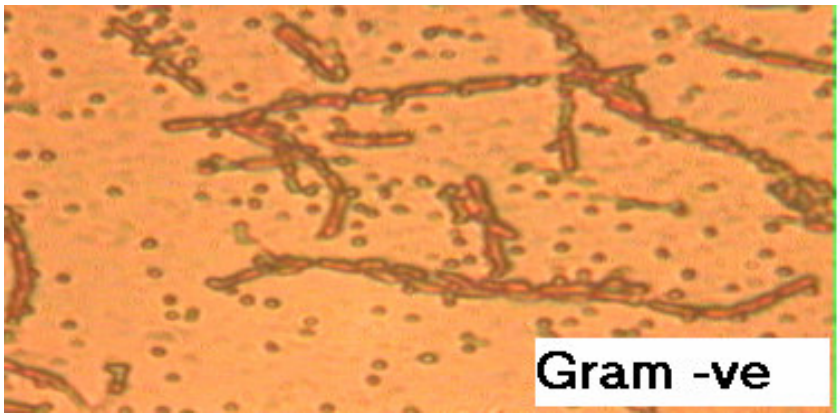
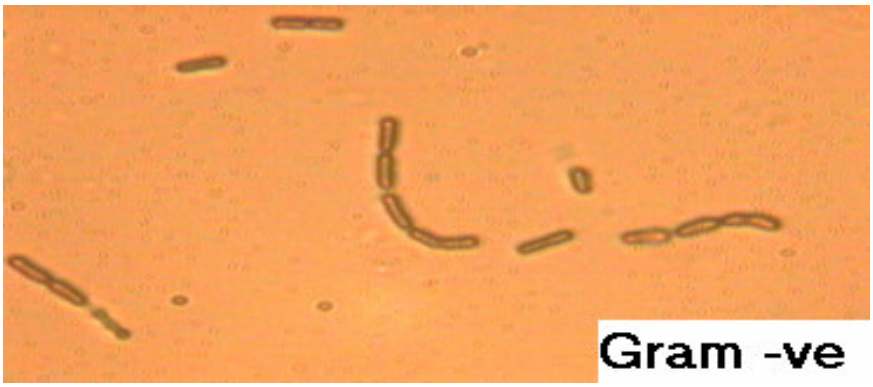


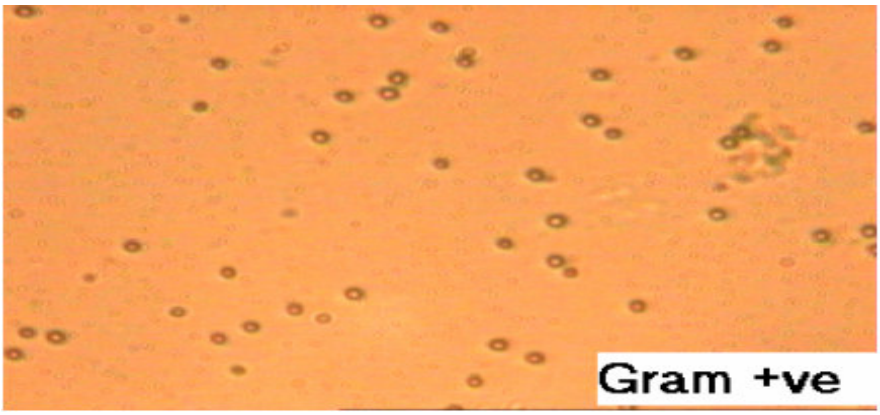

**Plate 1 0.7 % Agarose gel electrophoretogram**
**Lane 1 & 3: Plasmid pMMB277 isolated by alkaline lysis method**
**Lane 2: Lambda marker cut by Hind III.**




**(A)**




**(B)**




**(C)**

**Plate 2. Microscopic study of bacterial strains (A) S2 Gram-ve, (B) S3 Gram-ve, (C) Gram+ve.**

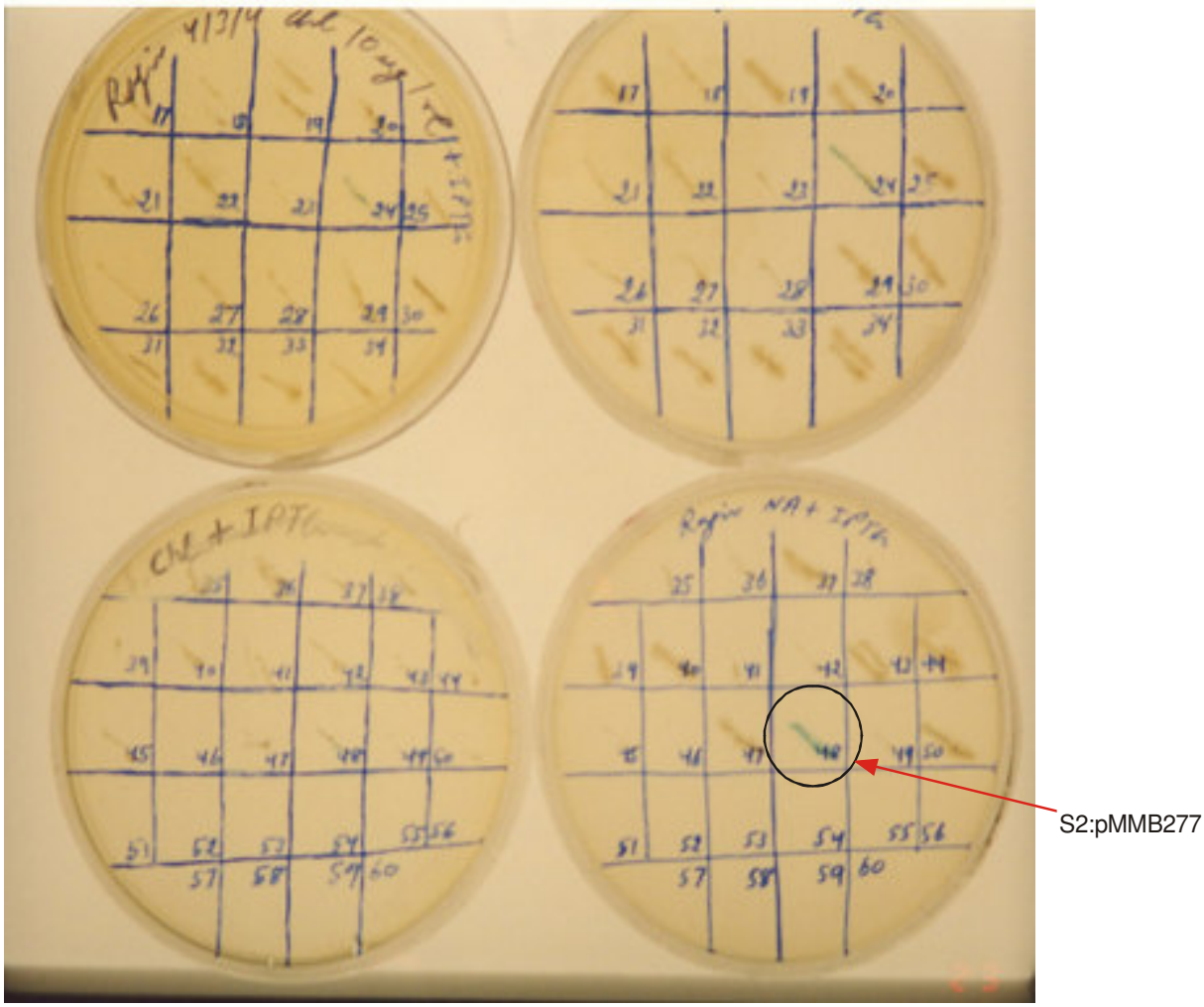



Plate 3 Transformant Bacteria S2 : pMMB277 (Blue colony) on Nutrient agar plates containing chloramphenicol with IPTG, X – Gal.


**(A)** **(B)** **(C)**

Plate 4. Enumeration of bacteria in 10% fly ash amended soil under non-sterile conditions (A) on nutrient agar, (B) on nutrient agar + chloramphenicol and **(C) Nutrient agar + chloramphenicol +X-gal, IPTG.**


**(A)** **(B)**

**Plate 5. Enumeration of *lacZ*$^{+ve}$ and *LacZ*$^{-ve}$ bacteria in 10% fly ash amended soil under non-sterile conditions (A) without S2:pMMB277 inoculation and (B) with inoculation.**