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Original Research Paper

Bacterial Population Dynamics in Fly Ash Amended Soil With and Without Amelioration by Earthworms

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ABSTRACT

The experiment was conducted to study the bacterial population in fly ash amended soil with and without earthworm inoculation in laboratory by dilution plate method. It was found that in presence of *Drawida willsi* at 5% fly ash (FA) amendment showed maximum population of $43.2 \pm 1.15 \times 10^9$ cfu/ g soil from initial population. At 10% and 15% FA amendment gradual decline to $14.3 \pm 0.4 \times 10^9$ and $7.63 \pm 0.6 \times 10^9$ cfu/g soil from $21.7 \pm 0.36 \times 10^9$ and $15.33 \pm 1.1 \times 10^9$ cfu/g soil over 90 days of experiment at an interval of 15 days was observed. In absence of earthworms gradual decline in population was seen in the three concentrations of fly ash amendment to $22.7 \pm 0.5 \times 10^9$, $11.6 \pm 0.7 \times 10^9$ and $3.43 \pm 0.7 \times 10^9$ cfu/g soil respectively. ANOVA test showed that with earthworms the effect of concentration of FA on the bacterial population was significant ($F = 69.9$, $df = 6, 2$, $p \leq 0.05$) and in the absence of earthworm both concentration and time interval played a significant role ($F = 155.1$, $df = 6, 2$, $p \leq 0.1$; $F = 10.5$, $df = 6, 2$, $p \leq 0.001$). Lower dose of 5% fly ash in soil proved to be optimum for the bacterial activity in the presence of earthworms. Morphological details of different bacterial colonies were assessed and found to be of punctiform, irregular, circular and filamentous shapes with punctiform dominating the culture.

INTRODUCTION

Every year Indian thermal power plants produce more than 100 million ton of fly ash, which is expected to reach 175 million ton in near future (Vimlesh & Giri 2011). Disposal of this huge quantity of ash is a great problem due to its limited utilization in manufacturing of bricks, cements and other civil construction activities. This would further bring to changes in land use pattern and contribute to land water and atmospheric degradation, if proper management options for fly ash handling are not undertaken (Pathak et al. 1996). Use of fly ash in agriculture provides a feasible alternative for its safe disposal to improve the soil environment and enhance crop productivity (Sharma & Kalra 2006).

Various soil constituents have a great capacity to retain environmental contaminants, especially those with polar molecules or positively charged divalent and trivalent ions. Consequently, the soil is a net sink for pollutants. 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. Microorganisms by virtue of the coenzymatic activities are considered as primary decomposers among the decomposer organisms in soil due to their key role in mineralization and demineralization process facilitating cycling of minerals in biosphere (Anderson 1982). Bacterial population can influence carbon or mineral cycles and have the ability to colonize harsh environments.

Microorganisms play a unique role in the soil ecosystem, because of their contributions to soil fertility (Al Gaidi 2010). Collins & Stotzky (2001) stated that microorganisms interact with metals in various ways. Many metals are essential to microorganisms, because they are electron acceptors or cofactors in enzymes, whereas other metals are toxic. The fly ash (FA) contains a high concentration of toxic heavy metals such as Cu, Zn, Cd, Pb, Ni, Cr, etc. (Tiwari et al. 2008) along with low nitrogen and phosphorus content and pH ranged from 4.5 to 12.0 depending on the S-content of parental coal. Increase microbial activity may help to reduce the detrimental effect of fly ash. The microbes are the important elements of the soil environment as they participate in the degradation of the organic matter and make the nutrients available to other soil organisms. The ill effect of fly ash can be further reduced by the presence of earthworms which act as biological fertilizers by bringing about physical and chemical changes in the soil. Earthworms are the natural factories, which serve as bio-catalytic agents to enhance the soil fertility through physical, chemical and biological processes (Garg et al. 2006).

As the ameliorating effect of earthworm in particular reference to bacterial population dynamics is virtually not studied in detail, the present work was conducted to study the bacterial population in the amended coal fly ash in presence and absence of earthworms providing the effective dose of fly ash to be utilized in agricultural prospects.

MATERIALS AND METHODS

The laboratory experiment was conducted using coal fly ash collected from the ash ponds of Patratu Thermal Power Plant and soil from the agro-ecosystem of Ranchi University campus.

Bacterial population was estimated from CFA amended soil with and without earthworm inoculation at an interval of 15 days for 90 days experiment by dilution plate count method (Waksman 1922). Each dilution was plated in Petri plates containing Czapak Dox Agar (Thom & Raper 1945) media for the bacterial culture. The medium was prepared using peptone -10 g/L, NaCl - 5g/L, Beef extract - 10g/L, Agar - 15g/L and the pH was maintained at 7. For each amendment three replicates of Petri plates were prepared. After 24 hr incubation of the Petri plates at an ambient temperature of 38 ± 2°C for 48 h, the bacterial colonies were counted. Further, the bacterial colonies were isolated for Gram staining.

RESULTS

Morphological analysis of the cultured bacterial colonies from the amended soil sample with and without earthworm inoculation provided a detail of qualitative assessment. The bacterial colonies developed on nutrient agar Petri plates were inferred according to their shape, margin, elevation and the colour. They were found to be basically of four types viz., punctiform, circular, irregular and filamentous with entire, undulate margins as exhibited in the Table 1 and 2 respectively in accordance to the absence and presence of earthworms. In 5% fly ash amendment without earthworms, 45% of the developed colonies were punctiform with entire margin, 20% irregular, 25% circular and 1% filamentous. The elevation of the punctiform was either 30% raised or 70% flat with 100% umbonate for irregular group, and entire circular ones showed 65% raised, 20% flat or 15% convex margins. The punctiform colonies exhibited 76% white, 14% green and 10% yellow colonies, irregular showed white (35%) cream (45%) and green (20%). Circular ones were white colonies with filamentous being 100% yellow. In 10% fly ash amendment the colonies were punctiform entire (65%), circular (20%) and irregular undulate (15%). Circular colonies showed 70% raised 5% convex and 25% flat elevation. The irregular ones had 80% umbonate or 20% flat margins. In 15% amendment the four colonies morphology was 66% punctiform, 22% circular, 12% irregular with the rare filamentous group missing as might not be resistant to high concentration of fly ash. 32% slight reddish circular colonies were observed along with white (94%), cream (10%), white circular (68%) and white irregular (90%).

In presence of earthworms, the same four types of bacterial colonies were observed with varied percentage. In 5% fly ash amendment about 68% of the colonies were punctiform, 32% irregular, 8% circular with 2% filamentous. The elevation of entire punctiform was 80% flat and 20% raised with umbonate for irregular group and circular ones showed about 45% raised, 40% flat and 15% convex. The colonies were mostly whitish in colour with 15% being greenish and 15% yellowish. The filamentous colony was the rarest one. In 10% amendment about 58% entire punctiform, 32% circular, 17% irregular undulate ones and only 1 % filamentous colonies were observed. The colonies were mostly white with few creamish ones. In 15% fly ash amendment punctiform (56%), circular (24%) and irregular (18%) cultured colonies were inferred.

On Gram staining it was found that punctiform-entire bacterial colony was coccus bacterium, which responded negatively to Gram's stain thus being G-ve cocci. The circular colonies were G+ve cocci, irregular-undulate G+ve bacilli and filamentous were also G+ve bacilli.

The bacterial colonies were enumerated and represented as the number of colony forming units (cfu) per g of the soil sample in both presence and absence of earthworms (*Drawida willsi*) over a period of 90 days in an interval of 15 days (Table 3)

In 5% fly ash amended soil maximum population was observed in the presence of earthworms (Fig. 1). The value showed continuous increase from $31 \pm 0.52 \times 10^9$ to $43.2 \pm 1.15 \times 10^9$ cfu/g soil till 75^{th} day of experiment and then a decline to $42.6 \pm 2.83 \times 10^9$ cfu/g soil on the 90^{th} day when the soil fly ash mixture was inoculated with earthworm *Drawida willsi*. In 10 and 15% FA amendment, with earthworm's presence the population varied from $21.7 \pm 0.36 \times 10^9$ to $14.3 \pm 0.4 \times 10^9$ cfu/g soil and $15.33 \pm 1.1 \times 10^9$ to $7.63 \pm 0.6 \times 10^9$ cfu/g soil showing gradual decline with increase in the incubation period.

In absence of the earthworms a decreasing trend was observed from 1^{st} day of incubation till 90th day in all the three levels of fly ash amended soil as shown in Fig. 2. The bacterial population varied from $31 \pm 0.52 \times 10^9$ to $22.7 \pm 0.56 \times 10^9$ cfu/g soil, $21.7 \pm 0.36 \times 10^9$ to $11.6 \pm 0.75 \times 10^9$ cfu/g soil and $15.33 \pm 1.1 \times 10^9$ to $3.43 \pm 0.75 \times 10^9$ cfu/g soil respectively in 5, 10 and 15% FA amendment. A continuous increase was observed in 5% fly ash amended soil in presence of earthworms showing the concentration to be high enough to be suitable for the microbial growth.

Two way ANOVA revealed that in presence of earthworms *Drawida willsi*, the variation in bacterial population was significant between the fly ash amendments ($F = 69.9$, $df = 6,2$, $p \leq 0.05$) and nonsignificant between the days ($F = 0.026$). In absence of earthworms both the fly ash concen-

Table 1: Morphological characterization of the bacterial colonies observed in Petri plates cultured from the fly ash amended soil in presence of earthworms.

Fly ash %	Shape	Margin	Elevation	Colour	Gram-stain
5% FA	68% punctiform	68% entire	80% flat 20% raised	70% white 15% greenish 15% yellowish	-ve cocci
	32% irregular	46% undulate	100% umbonate	55% white 45% creamish	+ve bacilli
	8% circular	55% entire	45% raised 40% flat 15% convex	90% white	+ve cocci
	2% filamentous		100% flat	100% yellow	+ve bacilli
10% FA	58% punctiform	60% entire	80% flat 20% raised	83% white 17% yellowish	-ve cocci
	32% circular	40% entire	70% raised 15% convex 15% flat	60% white 35% creamish	+ve cocci
	17% irregular	20% undulate	80% umbonate 20% flat	100% white	+ve bacilli
	1% filamentous		100% flat	100% white	+ve bacilli
15% FA	56% punctiform	56% entire	100% flat	100% white	-ve cocci
	24% circular	37% entire	85% flat 15% raised	83% white 17% reddish	+ve cocci
	18% irregular	25% undulate	85% umbonate 15% flat	90% white 10% yellow	+ve bacilli

Table 2: Morphological characterization of the bacterial colonies observed in Petri plates cultured from the fly ash amended soil in absence of earthworms.

Fly ash%	Shape	Margin	Elevation	Colour	Gram- stain
5% FA	45% punctiform	65% entire	70% flat 30% raised	76% white 14% greenish 10% yellowish	-ve cocci
	20% irregular	30% undulate	100% umbonate	35% white 45% creamish 20% greenish	+ve bacilli
	25% circular	20% entire	65% raised 20% flat 15% convex	90% white	+ve cocci
	1% filamentous		100% flat	100% yellowish	+ve bacilli
10% FA	65% punctiform	60% entire	80% flat 20% raised	80% white 25% yellowish	-ve cocci
	20% circular	20% entire	70% raised 5% convex 25% flat	60% white 10% slight reddish 25% creamish	+ve cocci
	15% irregular	27% undulate	80% umbonate 20% flat	100% white	+ve bacilli
15% FA	66% punctiform	56% entire	80% flat 20% raised	88% white 10% cream	-ve cocci
	22% circular	40% entire	75% flat 25% raised	68% white 32% reddish	+ve cocci
	12% irregular	25% undulate	25% flat 75% umbonate	85% white 15% yellow	+ve bacilli

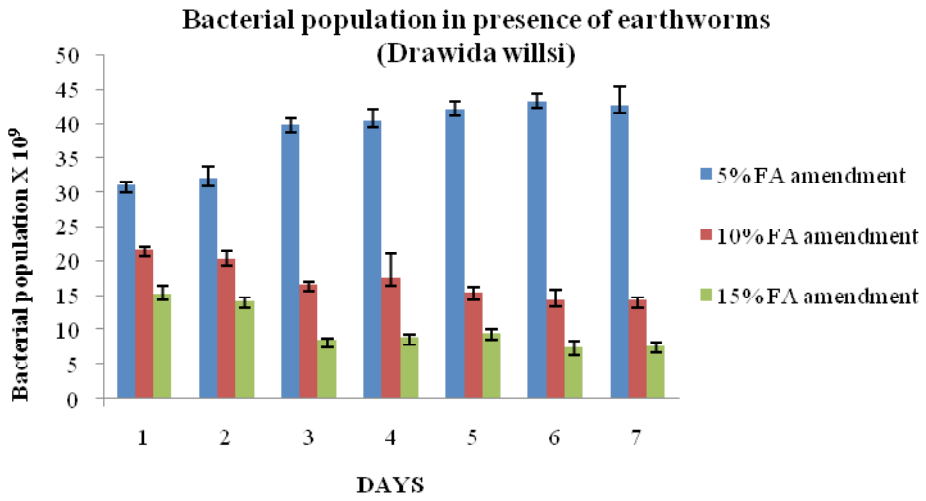

Fig. 1: Bacterial population in graded level of fly ash amendments in presence of earthworms.

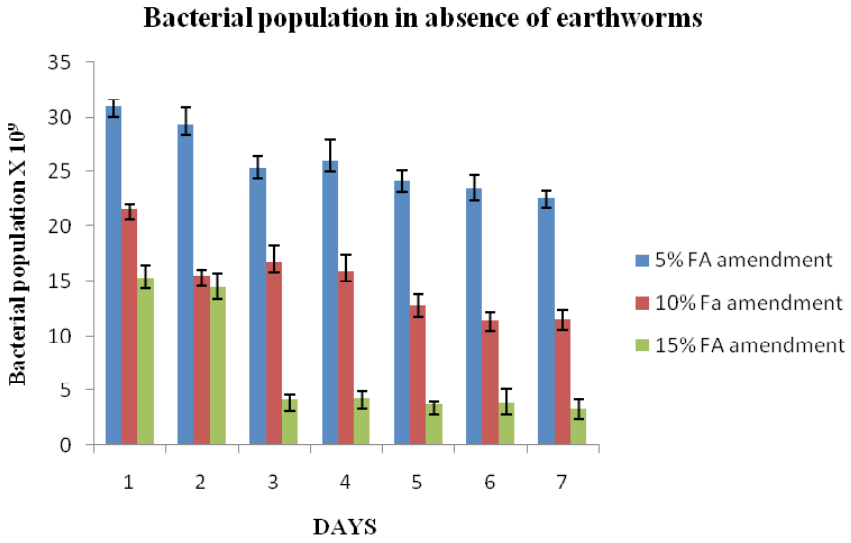

Fig. 2: Bacterial population in graded level of fly ash amendments without earthworms.

tration and the time interval played a significant ($F = 155.1$, $df = 6, 2$, $p \leq 0.1$; $F = 10.5$, $df = 6, 2$, $p \leq 0.001$) role on the bacterial population in fly ash amended soil (Tables 4 and 5).

DISCUSSION

Enumeration of bacterial population revealed that at 5% concentration the bacterial count was more in comparison to 10 and 15% fly ash amendment. Similar observation was made by Lal et al. (1996) where higher microbial activity was found up to 8% fly ash level. It is reported by some researchers that at higher concentration of CFA some heavy metals become more active and hinder the microbial activity (Dorans & Martens 1972). Rippon & Wood (1976) observed enhancement in microbial activity at low level of fly ash application due to availability of more nutrients. When fly ash is added at levels more than 10%, a decline in microbial activity was observed. This could have been due to a decrease in substrate availability associated with accumulation of persistent lignite-derived organic carbon compounds. Lal et al. (1996) and Rajkumar (2000) observed similar increase in the bacterial population due to combined application of fly ash and NPK, and fly ash increased the microbial count significantly. Kulkarni et al. (2002) found enhancement in microbial population and enzyme activity at 5-6% fly ash amendment. Surridge et al. (2009) have reported that fly ash addition has a liming effect on the soil leading to increased mobility of

Table 3: Bacterial population in fly ash amended soil in presence and absence of earthworms (Number of bacterial colonies × 10^9 cfu/g soil).

Days	5% FA (E)	5% FA (WE)	10% FA (E)	10% FA (WE)	15% FA (E)	15% FA (WE)
1st	31±0.52	31.0±0.5	21.7±0.36	21.7±0.36	15.33±1.1	15.33±1.10
15th	31.9±1.85	29.4±1.49	20.33±1.2	15.53±0.41	14.2±0.55	14.46±1.25
30th	39.8±1.05	25.36±1.05	16.56±0.404	16.76±1.5	8.46±0.32	4.16±0.45
45th	40.4±1.62	26.03±2.0	17.4±3.58	15.96±1.47	8.86±0.35	4.33±0.58
60th	42.1±1.06	24.2±0.9	15.4±0.79	12.8± 1.04	9.56±0.50	3.83±0.20
75th	43.2±1.15	23.5±1.24	14.36±1.4	11.46±0.75	7.36±1.02	3.90±1.20
90th	42.6±2.83	22.7±0.56	14.3±0.4	11.6±0.75	7.63±0.61	3.43±0.757

E - with earthworms; WE - without earthworms

Table 4: Two-way analysis of variance of bacterial population in presence of *Drawida willsi* in CFA amended soil.

Source of Variation	Sum of Square	Degree of freedom	Mean Square	Variation ratio F	Significance
Between Days	3.522524	6	0.587087	0.026528	NS
Between Concentration	3094.828	2	1547.414	69.92076	≤ 0.1
Error	265.5716	12	22.13097		

Table 5 : Two-way analysis of variance of bacterial population in absence of *Drawida willsi* in CFA amended soil.

Source of Variation	Sum of Square	Degree of freedom	Mean Square	Variation ratio F	Significance
Between Days	258.9463	6	43.15772	10.56102	≤ 0.001
Between Concentration	1268.288	2	634.1439	155.1798	≤ 0.1
Error	49.03813	12	4.086511		

calcium and hydroxide ions, ultimately causing an increase in bacterial species richness. However, fly ash also has a high content of toxic heavy metals (Page et al. 1979), which can hinder normal microbial metabolic processes when added in the soil at higher concentrations.

With inoculation of *Drawida willsi* the bacterial population was enhanced in the three varied concentrations of fly ash but with increase in the incubation period gradual decline was observed in 10% and 15% CFA amendment. At 5% FA amendment with earthworm the population showed gradual increment till the 75th day of experiment. Some factors such as pH, salinity, toxicity of trace elements and poor physical conditions can limit colonization of microorganisms as well as plants in the FA (Carlson & Adriano 1993). Pati & Sahu (2004) found little or no inhibition of microbial activity, soil respiration and enzyme activities up to 2.5% FA amendment in presence of *Drawida willsi*. With further addition of FA, all the above activities were significantly decreased. On the other hand, significant stimulation of soil respiration and microbial activities were observed up to 5% FA amendment when the soils contained earthworms. This may be due to increased microbial activity induced by substrates that are produced by the earthworms. Co-application of FA and earthworms at lower doses can thus be considered to stimulate soil biological activity and thereby improve nutrient cycling in acidic soil. The enhancement in the population with earthworms might be due to the ameliorating effect of the earthworms (Jabeen et al. 2010). Earthworm activity may raise N_2O emissions from agro-ecosystems. Rather than emitting N_2O themselves, earthworms are thought to enhance soil microbial activity (nitrification, denitrification and nitrifier denitrification) by changing physico-chemical properties, excreting mucus and increasing available carbon (Lubbers et al. 2010). On qualitative assessment the punctiform colonies were found to be dominating in all the bacterial culture plates and were gram negative. This has also been documented by Schutter & Fuhrmann (2001) that Gram negative bacteria are tolerant to fly ash. It was further observed that the filamentous colonies grew only in presence of earthworms and in its absence only at 5% concentration fly ash.

Thus, 5% fly ash amendment proves to be the optimum dose for being utilized in agriculture with inoculation of earthworms as maximum bacterial activity was observed at low concentration of FA with the presence of earthworms.

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