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Green-fertilizer, *Rhodospirillum rubrum*, for agricultural development on fly-ash without any toxic metal ion release

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ABSTRACT

Fly-ash has now been taken environmentalists’ attention as a potential occupational hazard. ‘Fly-ash stress’ on environment is increasing day by day and polluting biosphere severely. An experimental attempt has been taken to reuse fly-ash in agricultural fields with the help of green-biofertilizer (photosynthetic bacteria). 48 bacterial isolates have been isolated from cow (*Bos indicus*) dung through selective purple non-sulphur bacteria (PNSB) culture media. Based on different biochemical tests results, 7 isolates have been separated to check some inorganic phosphates solubilizing ability. With the help of those phosphate solubilizing PNSB-strains, *Oryza sativa* L. seed germination as well as sprout growth promoting ability have been tested on fly-ash. All 7 isolates have good mineral solubilizing and plant growth promoting activities on bacterized fly-ash but out of them WF11 (*Rhodospirillum rubrum* Strain ATCC 11170; GenBank accession number X87278) has best biofertilizing ability on fly-ash with highest Vigor-Index (990) value. This bacterial isolate helps in calcium (Ca$^{2+}$), magnesium (Mg$^{2+}$) and zinc (Zn$^{2+}$) cations mobilization from fly-ash by secreting different organic acids (here, phosphoric acid, tartaric acid and lactic acid have been identified by High Performance Liquid Chromatography). But no toxic metal ion release from fly-ash has been found after atomic absorption spectrophotometric assay.

Keywords: Coal fly-ash, Purple non-sulphur bacteria, *Oryza sativa* L., High Performance Liquid Chromatography, Atomic Absorption Spectrophotometer

INTRODUCTION

Phosphorus (P) is present in soil as both organic and inorganic forms (Requejo and Eichler-LÖbermann, 2014). It helps in plant growth and health development (White and Brown, 2010). Plants need and absorb inorganic phosphorus from soil. But the level of soluble inorganic phosphorus is very low in the soil because of their insoluble forms (Sharma et al., 2013).
Microorganisms have the ability to solubilize these insoluble inorganic phosphates by secreting different organic acids, extracellular polysaccharides and thus they can maintain the soil fertility (Rodriguez and Fraga, 1999). Among different phosphate solubilizing bacteria, fluorescent *Pseudomonas* have been considered as an important group of bacteria due to their biofertilizing and bio-control properties (Naik et al., 2008).

The use of inorganic phosphate solubilizing microbes in agricultural fields rather than phosphatic chemical fertilizers can not only minimize the agricultural cost but also help in insoluble phosphorus mobilization from anthropogenic sources (Sharma et al., 2013). Application of phosphate solubilizing bacteria (PSB) as biofertilizer is now emerging as an important technique to improve soil fertility (Qian et al., 2010). Biofertilizers are also getting preferences as they are eco-friendly, non-hazardous and non-toxic (Bhardwaj et al., 2014). These microbes are known to improve plant health by secretion of different phyto-hormones (Ahemad and Kibert, 2014), growth promoting substances and extracellular polysaccharides (Gupta et al., 2015) and by increasing the availability of various macro- and micro-nutrients (Rana et al., 2012).

Beside other plant growth promoting bacteria, photosynthetic bacteria have more probability in providing polysaccharides to plants. Purple bacteria use anoxygenic photosynthesis (Hoogewerf et al., 2003). In the absence of light, most purple bacteria are non-heterotrophy but some species carry out fermentations both anaerobically and aerobically (facultative in nature) (Schaafl et al., 2015). These microbes are known to improve plant health by secretion of growth promoting substances and by increasing the availability of micronutrients with the action of low molecular weight organic acids which are produced in the periplasmic space of some Gram-negative bacteria through a direct glucose oxidation pathway (Sashidhar and Podile, 2010). These groups of bacteria are also been reported as phosphate solubilizing bacteria (Lee et al., 2008; Wu et al., 2013).

Fly-ash is a finely dispersed mineral residue resulting from the combustion of coal in different thermal power plants. It is now the largest amount of industrial waste and occupational hazards in the World (Senapati, 2011). Beside various technical advantages, the use of coal fly-ash in cement industry is not only a glorious achievement of civil engineers but also give ecological benefits like an efficient reduction of greenhouse gases (O’Brien et al., 2009). Indian coal fly-ashes are rich in micro- and macro-nutrients of plants (Shaheen et al., 2014). In this experimental study, an attempt has been taken to use coal fly-ash as a source of plant nutrients (different inorganic phosphates and mineral cations). Photosynthetic purple non-sulphur bacteria have been mixed with fly-ash for insoluble minerals (present in fly-ash) solubilization. This experimental study has been carried out to get photosynthetic facultative biofertilizer strain(s) from cow dung for future agricultural improvement on fly-ash pond or using fly-ash with soil.

**MATERIALS AND METHODS**

**Isolation and characterization of bacterial isolates**

Cow dung samples, as a source of different photosynthetic bacteria (Rana et al., 2014a), were collected in sterile auto-cleavable biohazard bags (HIMEDIA, India) from Rangamati, VIH–Campus, Paschim Medinipur (Latitude- 22° 25' 00'' to 22°57'00'' north, Longitude- 87°11’ east, Altitude- 23 meters from mean sea level), West Bengal, India. 1 gm of cow dung samples were dumped separately in 100mL selective purple non-sulphur bacteria broth (PB) [Composition (in gm/L): CH$_3$COONa- 0.10; Yeast extract- 4.0; K$_2$HPO$_4$- 1.0; MgSO$_4$.7H$_2$O- 0.20; Na$_2$S$_2$O$_3$.5H$_2$O- 0.10; CaCl$_2$. 0.02] (Hoogewerf et al., 2003) and incubated at 28±2°C. After ten days, 1mL of those diluted inoculums (500 µL) were pour-plated with PB-agar (1.5%) medium and incubated at 28±2°C for 24 hr. Distinct single colonies were picked up, sub-cultured for purify on same medium. The isolates were identified by following the tests given in Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994). After purification and selection each isolates were stored in 40% glycerol at -20°C.

Thermal power plant ash was collected from Kolaghat Thermal Power Plant, Purba Medinipur and West Bengal, India.

**Screening of different phosphate solubilizing bacteria**

For inorganic phosphate bio-solubilization assay,
bacterial isolates were grown on different phosphate salt-agar medium at room temperature (28±2°C) for 7 days. Aluminum phosphate (AlPO₄), tri-calcium phosphate (Ca₃(PO₄)₂), ferric phosphate (FePO₄), magnesium phosphate (Mg₃(PO₄)₂) and zinc phosphate (Zn₃(PO₄)₂) were taken for this experimental study. Bacterial isolates producing clear-halos around the colonies were considered to have salt solubilizing activities (Rana et al., 2014b).

Seed bacterization for growth promotion

A field experiment had been carried out during Rabi seasons 2013-14 and 2014-15 at the Sabang area, Paschim Medinipur, West Bengal, India. A separate field was prepared by mixing the soil with fly-ash with the help of a tractor. Seeds of Oryza sativa L. were surface-sterilized with 70% ethanol, 1% sodium hypochlorite solution and sterilized double-distilled water repeatedly and sequentially up to 5 min and dried under a sterile air stream. Then seeds were bacterized according to ISTA protocol. Some seeds were not bacterized and they were taken as control for this study. After the seasons, plant growth-promoting activity of selected microbes was assessed based on the seedling Vigor Index (VI) and Percent Disease Incidence (PDI) by the standard method (Ramamoorthy et al., 2002). The investigation had been carried out throughout the seasons to see the plants crop production ability and toxicity assay of product crop.

Coal fly-ash solubilization assay and toxic metal release analysis

After 7 days all the bacterized fly-ash samples (seven samples) had taken to a particle size analyzer (Malvern, UK, E Ver. 5.2, Sl. No. MALY1017204) and their particles size had been analyzed with respect to a normal fly-ash sample (non-bacterized). Some of the bacterized samples had also been collected from the field and dissolved in water (HPLC grade; Merck India) and mixed properly. The mixtures had left at room temperature for 15 min to settle down the ash-particles at the bottom. Water supernatants had been collected with the help of a bacteria filter (pore size- 0.22 µm; HIMEDIA) and pH of each water samples had been recorded. Identification of organic acid(s) in each sample had been carried out by HPLC (Perkin Elmer, USA, Series-200) following Rana et al. (2015).

The released cations had been identified and their concentrations had been measured by following Sarode et al. (2010) using an atomic absorption spectrophotometer (AAS) (Perkin Elmer, USA, Model No. AA700, S/N 700S4030201). Same protocol had been followed during the analysis of product crop dust.

Identification of bacterial isolates

Genomic DNA had been extracted and sequenced according to the procedure described in Rana et al. (2014b). Sequence analysis had been carried out using BLAST (BLASTn) search tool (http://www.ncbi.nlm.nih.gov) available on the NCBI homepage. A Maximum Likelihood (ML) tree with 16s rRNA sequences of identified bacterial strains had constructed using MEGA 6.0 software to study phylogenetic relationship among them (Rana et al., 2015).

RESULTS

Isolation and preliminarily biological characterization of all isolates

48 bacterial isolates have been collected randomly. Purified isolates are Gram-negative, small-spiral or coccus in shape and showed purple colored circular colonies on PB agar plates (Figure 1 below). After different biochemical tests 7 bacterial isolates have been selected for inorganic phosphate ion mobilization and Oryza sativa L. seed germination as well as their growth promotion on fly-ash. All 7 isolates can utilize citrate and have catalase activity. Among all seven isolates only WF711-isolate has tryptophanase and amylase activity. From above studies we can summarize that cow dung contain beneficial phototrophic purple non-sulphur bacteria and those are facultative in nature (bacterial isolates have been screened anaerobically with in glass BOD-bottles but isolated and purified aerobically). They have phytohormone secretion ability.

Evaluation of salt solubilizing activities

All 7 selected isolates can grow on every inorganic phosphate salts containing agar-plates but no one can produce halo-zones on aluminum phosphate (AlPO₄) and ferric phosphate (FePO₄). While those bacterial isolates can produce clear
Figure 1. (a) Screening of PNSB strains from cow dung and (b) single colonies isolation of different facultative PNSB-isolates

Table 1. Different inorganic phosphate solubilizing efficiency (SE) values and Seed germination and germinated plant growth result of seven selected isolates.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>SE of Ca$_3$(PO$_4$)$_2$</th>
<th>SE of Mg$_3$(PO$_4$)$_2$</th>
<th>SE of Zn$_3$(PO$_4$)$_2$</th>
<th>Mean VI values of sprouts on treated fly-ash</th>
<th>Per cent of Disease Incidence (PDI) of total sprouts treated by bacteria</th>
<th>Germination Index (GI) of total sprouts treated by bacteria</th>
<th>Germination energy (%) of total seeds treated by bacteria</th>
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halo-zones on Ca$_3$(PO$_4$)$_2$, Mg$_3$(PO$_4$)$_2$ and Zn$_3$(PO$_4$)$_2$ with different solubilizing efficiency (SE) (Table 1).

Response to seed germination and plant growth

Table 1, reflects a good variation of different inorganic phosphate solubilization efficiency (SE) among different PNSB strains. Vigor-Index value of the sprouts has increased with respect to the control.
Figure 2. (a) Plant growth promotion on fly-ash by different selected PNSB-strains and (b) Vigor-index calculation of the sprout on fly-ash treated with WF

Figure 3. HPLC mediated identification of different organic acids from the water supernatant of WF

(non-treated seeds) with the help of those selected microbes. The non-treated seeds have been germinated on the fly-ash mixed soil. But the sprouts have been died after one week. Among seven isolates WF
, can influence Oryza sativa L. growth smarter than other in all cases. Every time Oryza sativa L. seeds have been germinated (germination energy 50%) and sprouts have highest average growth. Mean Vigour-index (VI) values and percent disease incidence (PDI) values of different sprouts after a week has been shown in Table 1.
Toxicity and particle size analysis

Metal ions mobilization phenomena from fly-ash happen due to the secretion of different acidic secondary metabolites by different PNSB-strains. High Performance Liquid Chromatography (HPLC) analysis helps to identify the presence of phosphoric acid (1.99 min), tartaric acid (2.36 min) and lactic acid (3.39 min) (Figure 3 above) from the water supernatant of WF\textsuperscript{7,11}. But two acids cannot be identified. WF\textsuperscript{7,11} can decrease the particle size of fly-ash (mean volume weight has been decreased from 11.576 to 7.674 µm, mean surface weight has been decreased from 1.797 to 1.794 µm and 90% particles diameter have been decreased from 17.640 to 16.047 µm) by the secretion of these acids within normal pH range (6.5-7.5).

During atomic absorption spectrophotometric (AAS) assays, we have found the presence Ca\textsuperscript{2+}, Mg\textsuperscript{2+} and Zn\textsuperscript{2+} ions in different water samples but surprisingly not a little concentration of other toxic metal ions (like Mn, V, Ni, Cd, As, Hg, B, Cu, Co, Cd, Se, Zn, Mo, Pb). This result reveals this bio-technique by different PNSB-isolates is safe for mankind. The best PNSB isolate, WF\textsuperscript{7,11} can extract Ca\textsuperscript{2+} (20 mg/L), Mg\textsuperscript{2+} (10 mg/L) and Zn\textsuperscript{2+} (4 mg/L) during the fly-ash mineralization to promote plant growth.

Phylogenetic affiliation of identified bacterial isolate

Among seven selected PNSB bacterial isolates, WF\textsubscript{7,11} has been found to have best fly-ash utilizing and plant growth promoting activity. 16S rRNA gene sequencing reveals that the bacterial isolate is 100% similar with a reported purple non-sulphur bacterial strain *Rhodospirillum rubrum* Strain ATCC 11170 (GenBank accession number X87278) (Munk et al., 2011). A Maximum Likelihood Phylogenetic tree (Figure 4) is constructed with nearest twenty neighbors using
MEGA 6.0 software (Rana et al., 2015). This phosphate solubilizing and phytohormone secreting (IAA) bacterial strain (*Rhodospirillum rubrum* Strain ATCC 11170) may help in conversion of unfertile fly-ash to fertile-ash or use of coal fly-ash as plant growth nutrients reservoir in agricultural field instead of chemical fertilizer.

**DISCUSSION**

Indian thermal power plant ash is a huge source of silica, aluminum, iron and calcium ions (Ibrahim, 2015), which play as micro-nutrients in plants nutrition. Microbes not only play vital role in physical weathering of parental rocks to generate fertile land or soil (Rana et al., 2015; Schuiz et al., 2013) but also help in plant nutrition by microbes-plant symbiotic interaction (Goh et al., 2013; Bonfante and Genre, 2010; Kawaguchi and Minamisawa, 2010). Thus, if we apply microbes on fly-ash, microbes may not only make fly-ash useful for plants growth but also may suppress environment pollution.

But there is a problem during microbial minerals mobilization. Microbes may extract heavy metals present in fly-ash, which will harm living organisms directly (Singh et al., 2011; Sijakova-Ivanova et al., 2011). In this experimental study, we want to isolate a bacterial isolate from cow dung which has plant growth promoting ability through its phytohormone secretion and plant growth nutrient extraction ability from fly ash rather than heavy metals. We have selected photosynthetic bacterial community due to their carbohydrate generation and delivery probability to plants additionally.

We have found 48 purple non-sulphur bacterial isolates from cow dung and separated 7 isolates for this experimental investigation based on their different biochemical characters. 16S rRNA gene sequencing and their phylogenetic affiliation reveal that, the bacterial isolates belong to the purple non-sulphur bacterial community and out of them WF7 is 100% similar with *Rhodospirillum rubrum* Strain ATCC 11170 (Figure 4). This bacterial isolate can solubilize different mineral-phosphates and helps in plant growth promotion (Table 1) on both fly ash and soil through the secretion of different organic acids like phosphoric acid, tartaric acid and lactic acid (Figure 3). Similarly, this bacterial isolate has been extracted Ca++, Mg++ and Zn++ from fly ash without any toxic heavy metal ion. The product crops are also free from any toxic metal ion. Figure 2 shows how fly-ash and green-biofertilizer mixture helps in *Oryza sativa* seeds germination, sprouts growth promotion and crop production without any chemical fertilizer in two successive seasons.

**CONCLUSIONS**

Use of autotrophic biofertilizer can help in replacement of chemical fertilizers in future by providing the plants useful nutrients for their growth and development. They may also help in cleaning the environment by utilizing fly-ash and greenhouse gases during photosynthesis. Here photosynthetic bacteria play vital role in extracting useful nutrients from fly ash without any toxicity. Thus, this is a win-win option near mankind for fly ash pollution control and sustainable agricultural development on fly ash using green-biofertilizer. However, the same results may vary for other seeds rather than *Oryza sativa* L. or same bacterial strain isolates from other cattle manure.

**REFERENCES**


