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Isolation and Characterization of Lead Resistant Indole Acetic Acid Producing Rhizobacteria from Fly Ash Contaminated Site Near Thermal Power Plant

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ABSTRACT

Coal Fly Ash is a major by-product of a coal-fired thermal power plant along with bottom ash, exhaust gas and effluent. Safe storage and disposal of Fly Ash is a global challenge of modern era due to the presence of different heavy metals i.e. Pb, Al, Ni, Cd, Zn, As, Co etc. Most of Fly Ash is used for landfill purposes and only a small portion is used in other industries like pawer blocks, cement and agriculture. Fly Ash has been reported the as soil amendment agent for agricultural land. However, Fly Ash has also been reported to have a negative effect on soil microbial community due to the presence of heavy metals. Hence, Study was carried out to isolate and characterize heavy metal resistant IAA producing Rhizobacteria from the rhizosphere of *Arachis hypogaea L* plant which was collected from the farms adjacent to the Ukai thermal power plant. Highest IAA producing isolated strain of Bacillus cereus was used for optimization. The three factors explored in the current study were pH (Factor A), Temperature (Factor B) and Tryptophan concentration (Factor C). The effect of these three factors variations on the response of IAA produced (Y) was investigated using a 2^3 Full Factorial Design (FFD). A 2^3 Full Factorial Design model was built and Regression was done using R software.

Keywords : Coal Fly Ash, Full Factorial Design, Heavy Metals, IAA, Rhizobacteria, PGPR, R software

I. INTRODUCTION

Power is considered to be the growth engine of any country, especially for developing countries like India. India is 3rd largest coal producer in the world (Ghosh *et al.,* 2015). India is dependent majorly upon coal-fired thermal power plants (TPPs) for power generation. In India, total 67% power is generated by TPPs. Out of this 59.1% power is generated by coal-fired TPP. Coal ash is a byproduct of coal combustion in TPP which can be further classified into three types: (1) Slag (2) Bottom ash (3) Fly Ash (FA). FA is the residue collected by Electro Static Precipitators- ESP (Santos *et al.,* 2014).

FA is a heterogeneous mixture of crystalline and amorphous particles which contains majorly Al, Fe, Zn, Cu, Ni, Pb, Cr, Cd, Mn, Na, Ti, Mo, Se, Si, Mg, Ca, B, P, S, K etc. (Lokeshappa and Dikshit, 2011, Ghoshal *et al.,* 1995, Nayak *et al.,* 2015). According to CEA report, only about 57.93 % FA was utilized during the first half of the year 2016-17 which leaves a large portion of FA to be either stored or disposed of. The percentage utilization of FA for various purposes is shown in Fig. 1. It indicates that only 0.99 % of FA is used for agriculture (CEA Report on FA generation and utilization 2016-17).

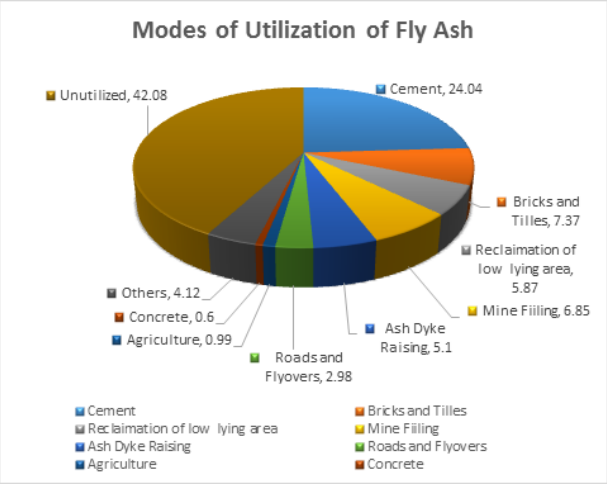

Figure 1. Utilization of Fly Ash (Central Electricity Authority India Report 2016-17)

Some researchers have reported the use of fly ash as soil amendment agent for agricultural land (Kumar *et al.,* 2005, Jala and Goyal, 2006, Basu *et al.,* 2009, Patil *et al.,* 2013). However, Fly Ash has also contained various heavy metals like Pb, Ni, Al, Cr etc. which shows detrimental effects to plants (Chaudhari *et al.,* 2016) and microorganisms (Nayak *et al.,* 2015).

The Rhizobacteria which stimulates plant growth are known as Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Schroth, 1978). PGPR can exert their plant growth promoting activities by various mechanisms i.e. (1) Nitrogen Fixation (2) Phosphate Solubilisation (3) Indole Acetic Acid (IAA) production (4) Siderophore Production (5) HCN production (6) Biological Control etc. (Kloepper *et al.,* 1989). Bacterial species including *Azospirillium, Azotobacter, Alkaligenes, Arthrobacter, Burkholderia, Bacillus, Enterobacter, Pseudomonas, Serratia* have been reported to possess PGPR activities. (Bharucha *et al.,* 2013). IAA is one of the most physiologically active auxin which increases the root length, number of root hairs and number of lateral roots. (Mohite 2013).

The primary aim of this study was to isolate and characterize a heavy metal resistant IAA producing PGPR which can exert its plant growth activity in FA amended soil.

II. MATERIALS & METHODS

Sample Collection

Healthy plant of *Arachis hypogaea L.* was selected and up-rooted carefully from the field from the near vicinity of the Ukai thermal power station. The sample was collected in zip lock bags and transported to the laboratory immediately after collection. The sample was stored in a refrigerator at 4 ℃ and processed for isolation on the same day.

Isolation of Rhizobacteria

The up-rooted plant was shaken slightly in order to remove extra soil and approximately 1 gm of rhizospheric soil was collected in 100 ml of sterile distilled water. The flask was then kept at 150 RPM on a rotary shaker at 27 ° C for 30 minutes. After 30 minutes flask was removed from shaker and allowed to stand at 27 ° for 1 hour. After 1 hour supernatant was collected and serial dilutions were made. 100µl of inoculum was spreaded on sterile nutrient agar plate. N-Agar plates were incubated at room temperature for 4-5 days. After incubation period is over, different colonies were selected on the basis of difference in their colony characteristics (Majeed *et al.,* 2015). Further, pure cultures were obtained and maintained on nutrient agar slant by sub-culturing.

Heavy Metal Resistance

Isolates were streaked on Nutrient agar plates containing 5 ppm of Pb as lead nitrate and Nutrient agar plates containing 1% Fly Ash to check their heavy metal resistance.

Screening for IAA production

An active culture of all the isolates was inoculated in 50 ml of sterile nutrient broth containing L-Tryptophan (50µg/ml) and incubate on an environmental shaker for five days. After Incubation

period is over, 15ml of the sample was withdrawn from each flask and subjected to centrifugation at 8000 RPM for 20 minutes. 2 ml of the supernatant was collected in a separate tube and 4 ml of Salkowski's reagent (2% 0.5 $FeCl_3$ in 35% $HCLO_4$ solution) was added to it. After proper mixing tubes were allowed to stand at room temperature for 30 minutes in dark and absorbance of pink colour developed was measured at 530 nm using UV-Visible Spectrophotometer. Quantification was done using 20 µg/ml Indole Acetic Acid (aqueous) as a standard solution (Gordon and Weber, 1951). The highest IAA producing isolate was selected as best IAA producer.

Characterization

The best IAA producing organism was characterized by various biochemical test i.e. Indole, MR, VP, Sugar fermentation, Gelatinase, Urease, Citrate dehydrogenase etc. Further, 16s sequencing was performed and obtained sequence was aligned by BLAST tool from NCBI.

Optimization study for IAA production was further carried out using the best IAA producing isolate. The three factors explored in the current study were pH (Factor A), Temperature (Factor B) and Tryptophan concentration (Factor C). The effect of these three factors variations on the response of IAA produced (Y) was investigated using a 23 Full Factorial Design (FFD). Factors A, B and C were varied at two levels. Low-level values and high-level values assigned to each factor were (-1) and (+1) respectively. Factors and level used in FDD are presented in Table 1. Factor A varied between pH 6 to pH 7, Factor B varied between 27°C to 37°C and Factor C varied between 25 µg/ml to 50 µg/ml.

Table 1. Factors and Levels Used

Factor	A	B	C
Low Level (-1)	6	27°C	25µg/ml
High Level (+1)	7	37°C	50µg/ml

Total 8 experiments were run in 23 Full Factorial Design. Briefly, Inoculation of the selected isolate was done in nutrient broth containing different pH and temperature conditions and incubated on a shaker for five days. After incubation period quantification of IAA was done using Salkowski's reagent as described earlier. The concentration of IAA produced was taken as a response (Y) and a model was built using R software (R version 3.3.1, https://cran.r-project.org). Design Matrix and the Result of IAA produced are shown in Table 2.

Table 2. Design Matrix and the Result of IAA produced

Standard Order	Actual Order	pH (Factor A)	Temperature (°C) (Factor B)	Tryptophan Conc. (mg/ml) (Factor C)	IAA Produced (µg/ml)
1	7	6	27	25	51
2	1	7	27	25	12
3	8	6	37	25	97
4	3	7	37	25	80
5	2	6	27	50	174
6	4	7	27	50	167
7	6	6	37	50	225
8	5	7	37	50	128

III. RESULTS AND DISCUSSION

Isolation and Screening

Total 15 strains were isolated based on differences in their colony characteristics out of which seven strains shown good growth on 5ppm lead-containing N-Agar plate. These seven strains were coded as AI_1, AI_2, AI_3, AI_4, AI_5, AI_6 and AI_7. The amount of IAA produced by all strains is presented in Table 3. Strain IA_2 shown maximum production (127 µg/ml) followed by Strains IA_4, IA_5 and IA_3. Strain IA_7 and IA_1 show very less IAA production. Strain IA_2 was selected as best IAA producing strain and used for optimization experiments.

Table 3 . IAA Production by Isolated strains

Sr. No.	Strain	IAA Production (µg/ml)
1	IA_1	65
2	IA_2	127
3	IA_3	109
4	IA_4	119
5	IA_5	110
6	IA_6	41
7	IA_7	98

Characterization

Biochemical characterization of strain IA_2 indicated that IA_2 is Grams positive bacilli. Further IA_2 was found to give positive results for Indole, MR, Gelatinase activity, Catalase activity, Glucose, Maltose and Lactose fermentation. IA2 strain was further identified by 16s sequencing. Obtained sequence was aligned using BLAST tool from NCBI. IA_2 strain was found to be related to Bacillus cereus. Chagas *et al.,* (2014) and Jetiyanon *et al.,* (2008) have also reported IAA production by *Bacillus cereus* and *B. thuringiensis* isolated from soil.

Optimization of IAA Production

Three factors 2^3 FFD optimization was run. A linear regression model was built using R software. Pareto plot (Fig.2) were made to understand the magnitude of effects.

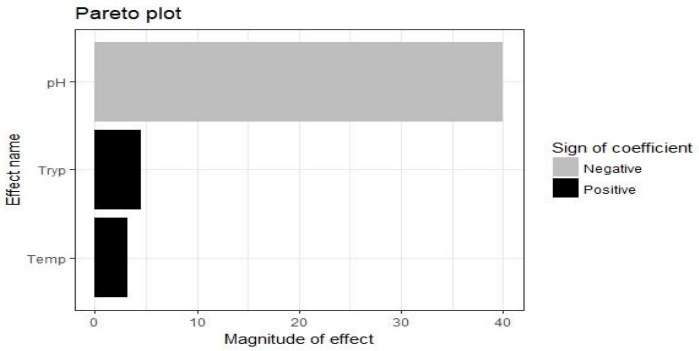

Figure 2. Pareto plot for the model

As shown in Figure 2. ParetoPlot indicates that Factor A (pH) has a negative effect with the highest magnitude. Factor B (Temperature) and Factor C (Tryptophan concentration) show positive effects. Which means that maximum IAA production can be achieved at lower pH and Higher temperature. Tryptophan concentration (Factor C) shows highest positive effect indicating that IAA production increase with an increase in Tryptophan concentrations.

Regression analysis of the model was done using R software. The statistical data obtained for the model is shown in Table 4.

Table 4. Statistical Analysis

Sr.No.	Parameter	Value
1	Residual Standard Error	30.56
2	Degree of Freedom	4
3	R^2	0.8923
4	Adjusted R^2	0.8116
5	F Value	11.05 on 3 and 4 DF
6	P value	0.02094

Statistical analysis shows that model is having statistically significant P value and R^2 as well as

Adjusted R^2. The formula based on this model is as follows.

$Y = 105.7 - 40X_A + 3.15X_B + 4.54X_C$

The formula shows that Intercept value for the model is 105.7.

IV. CONCLUSION

The present study shows that *Bacillus cereus* can produce IAA efficiently at low pH, high temperature and high tryptophan concentration. Furthermore, R software can be used to build the model for optimization. Pot studies and field studies should be carried out further to establish application of heavy metal resistant PGPR for FA amended soil.

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