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Optimized production of indole acidic acid with phosphate solubilizing activity using isolated *Bacillus pumillus*

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ABSTRACT

The aim of the present study was isolating indole acetic acid producing bacteria with phosphate solubilising activity. For the identification and characterizing of selected bacteria, 16S rRNA sequencing was carried out and simultaneously phylogenetic tree was constructed. Optimized production of indole acetic acid was analysed by various methods like OFAT, plankett- burman and response surface methodology. The most significance factor affecting IAA production was found to be Lactose, Tryptophan and Peptone.

INTRODUCTION

Different bacteria are involved in various biotic activities of the soil ecosystem to enrich the nutrient turn over and sustainable for crop production (Ahemad, 2009). Predominantly, rhizobacteria plays major role in recycling the soil nutrients and subsequently, they are crucial for soil fertility (Glick, 2012). Generally, plant growth promoting rhizobacter promote plant growth by either make possible to gain minerals (nitrogen, phosphorus and essential minerals) or improving plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth (Glick, 2012). Among the various mechanisms involved for improvement of plant growth, synthesis of phytohormone auxin (indole acetic acid) by microorganism contributed towards important role in improving biofertility (Spaepen and Vanderleyden, 2011). Beside these, making availability of phosphate from inorganic form to organic form reduces deficiency of phosphate (Mckenzie and Roberts, 1990).

MATERIALS AND METHODS

Isolation of bacteria: The root (*Catharanthus roseus)* samples were collected from different regions in Chennai. Freshly collected roots were carefully washed with tap water for adhering soil (G.E. Dawwamth et. al., 2013). The roots were surface sterilized using 70% ethanol for 30 s and 2% sodium hypochlorite for 5 min and then washed twice with sterilized distilled water. The sterilized roots were carefully cut into small sections with 0.8% saline solution. These were serially diluted from 10^{-1} to 10^{-5}. From these serial dilutions, 10^{-1} to 10^{-5} dilutions were inoculated into Yeast Extract Mannitol Agar Medium (YEMA) (Vincet, 1970) and Pikovskaya's Agar medium (PVK) (Pikovskaya, 1948) and the plates were incubated at 37 °C for 3-10 days.

Screening of bacterial isolates for their indole acetic acid production: Bacterial isolates were grown in 50 mL yeast extract mannitol broth supplemented with 0.1 g/L of L-Tryptophan and incubated in dark at 30 °C for 7 Days (Vincet 1970, Sarwer and Kremer 1995). The ability of indole acetic acid production were determined by 2 mL of culture supernatant was mixed with 2 drops orthophosphoric acid and 4 ml of Salkowsky's reagent was added. The standard procedure were followed further. (Gordon and Weber 1951; Sarwart, 1992).

Determination of phosphate solubilizing activity: Spot inoculation of the isolates were done in the center of the Pikovaskay's medium amended with bromophenyl blue. These plates were then incubated at 37 ºC for 48 to 72 h. Phosphate solubilizing acitivity were determined in the form of a clear yellow colour halo formed around the colony representing the production of organic acids as a possible mechanism of the phosphate solubilization. Quantitative phosphate solubilization was carried out in liquid Pikovaskay's medium in 250 mL flasks for 14 d. The concentration of the soluble phosphate in the supernatant was estimated by Stannous Chloride (SnCl2. 2H2O) method (Gaur 1990).

16S rRNA gene sequencing: The bacterial isolates were identified by 16S rRNA sequencing analysis using with 16SF and 16SR primers. Total genomic DNA were extracted from cells cultured on nutrient agar, and 16S rRNA sequence were performed using ABI 3730xl Genetic Analyzer.

Optimization of media:

OFAT design: The factors considered, analyzed and optimized for the production of indole acetic acid were various carbon sources (glucose, lactose, sucrose), various organic nitrogen sources (yeast extract, peptone, beef extract), pH (5 to 9) and temperature (25, 30, 35, 40, 45 ºC). OFAT was used to determine the possible optimum levels of these factors.

Plackett-Burman design: The purpose of the optimization was to identify the significance of the ingredients of the media for the production of indole acidic acid. For screening purpose, various components have been evaluated using Plackett-Burman statistical design. (Plackett and Burma, 1946). A set of 12 experiments were constructed

using MINITAB 15 software for 5 components: Lactose, Tryptophan, Peptone, pH, Temperature. Each components was tested at two different concentration levels. The experiments were carried out in 250 mL conical flask containing 100 mL media at 120 rpm and 37 °C. The response was measured as indole acetic acid activity.

The central composite design: The central composite design (CCD) under the response surface methodology (RSM) (Box and Wilson, 1951) was employed in order to illustrate the nature of the response surface in the experimental region and elucidate the optimal conditions of the most significant independent variables. The independent variables and its levels were chosen based on that could be obtained from the results of OFAT experiments and PB design.Three major variables namely Lactose, Tryptophan, Peptone were included in this model. Each parameter was studied at three different levels (-1, 0, +1). A matrix of 20 experiments with 3 factors was generated using the software package MINITAB 14. The Indole acetic acid (µg/ml) produced was taken as the dependent variable or response. This resulted in an empirical model that related the response measured in the independent parameters. The model was constructed based on the variables at the 95 % confidence level. Each parameter was studied at three different levels (-1, 0, +1). All the parameter were taken at central coded value considered as 0. The minimum and maximum ranges of parameter were investigated and full experimental plan with respect to their values. According to the CCD for three the variables, 20 experimental runs were executed and their observations were fitted to the following second order polynomial model:

$$\mathbf{Y= \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC}$$

where, Y is the dependent variable (Indole acetic acid yield); A, B and C are the independent variable (Lactose, Tryptophan and peptone); β_0 is the regression coefficient at center point; β_1 , β_2 , and β_3, are the linear coefficients; β_{11}, β_{22} and β_{33} are the quadratic coefficients and β_{12}, β_{13}, and β_{23} are the second order interaction coefficient. The developed regression model was evaluated by analyzing the values of regression coefficients, analysis of variance (ANOVA), p- and F-values. The quality of fit of the polynomial model equation was expressed by the coefficient of determination, R^2.

RESULT AND DISCUSSION

Identification of Bacteria: A total of twenty isolates of were isolated from rhizospheric soil and tentatively identified on the basis of biochemical test and as described in Bergy's Manual of Determinative Bacteriology. Out of the 20 isolates, 60% of isolates produced IAA in broth medium (YEMA) containing L-Tryptophan. Among the producers, it was noted that the bacterial strain p13 and p15 was the stronger producer of phyto hormone IAA than the remaining 18 bacterial strains of the test isolates as weak producer of IAA. And further both IAA productivity and phosphate solubilising activity was compared between bacterial strains p13 and p15. Among all the isolates selected, isolate p15 appeared to be better in terms of both IAA yield and phosphate solubilising activity. So, we identified these isolates in greater detail. On the basis of 16S rRNA sequencing the selected isolate was identified to be *Bacillus pumilus.*

Phylogenetic tree was constructed and shown in Fig. 1. The evolutionary relationships between identified isolate and their relatives in the Gene bank. The evolutionary distances were computed using Maximum composite likelihood method (Tamura *et al.,* 2004).

Indole acetic acid standard: The standard graph was prepared by varying the indole acetic acid concentration. The graph was drawn as indole acetic acid concentration versus OD absorbance at 540 nm and shown in Fig.2. The absorbance values were plotted and a linear line was drawn. (R^2= 0.996).

Optimization of media:

Effect of pH on IAA Production: It was concluded that pH at which there is maximum production of IAA. The Fig. 3 shows that there was sudden increase in IAA production with as increasing in pH level and reaches maximum and further increase in pH leads to decrease in IAA yield. Thus, pH 7 was selected , fixed and followed for further analysis.

Effect of Temperature on IAA Production: In this study, the temperature was varied from low to appreciably optimum values and the effect of the microorganism upon the production of IAA was analysed. Fig.4 showed that temperature at 35 ° C was maximum for the production of IAA production.

Combined effect of carbon sources and nitrogen sources on IAA production: In the below Fig.5 we concluded that among the various combination of carbon and nitrogen sources, the combined effect of lactose and peptone showed the maximum yield of indole acetic acid.

Statistical optimization:

Variable screened by PB Design: The data obtained from screening experiments by PB design showed a variation of IAA production. Estimated coefficient and the main effect of each variable were tabulated (Table 4). Main effect of Lactose, Tryptophane, Peptone showed positive effect and other variables employed for the screening showed the negative effect on the IAA production. If the effect is positive, it is significant in higher concentration and if negative then it is significant at low concentration. Tryptophan was identified as the most significant medium component with the confidence levels of 99.9 % followed by lactose (94.1 %) and Peptone (90.9 %) on IAA production.

Response surface estimation: The observed experimental values given in the table 5 were subjected to multiple linear regression analysis. The significance of regression coefficient were determined by performing students t test. The statistical analysis results including the regression co efficient and p values for linear quadratic and interaction effects were given in the table 6 with 95% significance levels. Low p value indicates more significance of the corresponding coefficient and its effect on the IAA (µg/mL). Significance of each coefficient and the interaction between the variables were evaluated by the p value.

It was observed that the co efficient for the linear effect of Lactose ($p< 0.005$) were found more significant than the other linear and combined effect of medium variables. The second order polynomial model for the response, IAA activity (µg/mL) was constructed by using the regression coefficient (in coded units) from Table 6 is given by,

$$\mathbf{Y = 459.73 + 2.000\ A - 0.3000\ B + 0.900\ C - 9.591\ A^2 - 3.091\ B^2 - 4.091\ C^2 - 1.500AB + 0.750BC - 0.250AC}$$

The statistical significance of the model equation was evaluated by the F test of ANOVA table and the residuals analysis was performed to validate the model at 95% confidence levels. The ANOVA statistical for the response, IAA concentration (µg/mL) was shown in table Table 7. The ANOVA table indicates that linear and quadratic effect between the variable in the second order polynomial model were highly significant and adequate to represent the relationship between IAA activity (µg/mL) and Lactose, Tryptophan, Peptone.

To determine the optimum conditions for the extraction of total phenolic content, the three- dimensional plots were constructed (Fig 6). The influence of lactose and tryptophan on IAA yield at a fixed peptone (g/L) was shown in Fig. 6A. IAA yield increased with lactose concentration and reaches maximum at middle value of the lactose concentration. The effect of tryptophan and peptone on IAA yield was shown Fig. 6B. The third variable was kept constant at their middle level. It was observed that IAA yield was high at middle value of tryptophan and at middle level of peptone. The effect of lactose and peptone on IAA yield was shown Fig. 6C. The third variable was kept constant at their middle level. It was observed that IAA yield was high at middle value of both lactose and at the middle level of peptone. Based on the model the maximum yield of IAA was reported to be 463 µg/mL.

Table.1.Variable levels for PB design experiment

Variables	Symbol	Lower levels (-1)	Higher levels (+1)
Lactose (g/L)	X_1	4	6
Tryptophan (g/L)	X_2	4	6
Peptone (g/L)	X_3	0.25	0.75
Ph	X_4	6	8
Temperature (^{0}C)	X_5	30	40

Table.2.Experimental range and variable levels of Central composite design experiment.

Variables	Symbol	Lower levels (-1)	Higher levels (+1)
Lactose (g/L)	A	4	6
Tryptophan (g/L)	B	4	6
Peptone (g/L)	C	0.25	0.75

Table.3.Levels of variables, experimental design matrix for PB experiments with observed and predicted response

Experimental design					Responses	
X_1	X_2	X_3	X_4	X_5	IAA (µg/ml)	
					Observed	Predicted
4	4	0.75	8	40	440	440.6667
6	4	0.75	6	30	442	441.3333
5	5	0.5	7	35	460	459
4	4	0.25	8	40	445	443
6	4	0.25	6	40	441	444.3333
6	6	0.75	6	40	450	447
5	5	0.5	7	35	458	459
5	5	0.5	7	35	459	459
4	6	0.25	6	30	445	446
4	6	0.75	6	40	443	444.3333

4	4	0.25	6	30	443	441
4	6	0.75	8	30	444	445
6	6	0.25	8	30	450	450
6	4	0.75	8	30	442	442.6667
6	6	0.25	8	40	451	450.6667

Table.4.Estimated coefficient, calculated t value, p value and confidence level as per Experimental Design for IAA (µg/mL)

Components	Symbol	Main effect	Estimated co-efficient	Standard Error	t value	P value	Confidence level
Lactose (g/L)	X_1	2.667	1.333	0.6067	2.20	0.059	94.1
Tryptophan (g/L)	X_2	5.000	2.500	0.6067	4.12	0.003	99.97
Peptone (g/L)	X_3	-2.333	-1.167	0.6067	-1.92	0.091	90.9
Ph	X_4	1.333	0.667	0.6067	1.10	0.304	69.6
Temperature (°C)	X_5	0.667	0.333	0.6067	0.55	0.598	40.2

Table.5.Central composite design matrix with observed and predicted response

Run order	X_1	X_2	X_3	IAA (µg/mL)	
				Observed	Predicted
1	5	5	0.5	462	459.7364
2	5	5	0.25	454	454.7455
3	4	4	0.25	443	439.3636
4	5	5	0.5	462	459.7364
5	4	5	0.5	442	448.1455
6	5	5	0.5	463	459.7364
7	4	6	0.25	441	442.2636
8	5	5	0.75	453	456.5455
9	4	4	0.75	440	440.1636
10	6	6	0.25	443	441.7636
11	5	4	0.5	454	456.9455
12	5	5	0.5	458	459.7364
13	6	5	0.5	454	452.1455
14	6	4	0.75	451	448.6636
15	5	5	0.5	460	459.7364
16	6	6	0.75	442	444.5636
17	5	5	0.5	462	459.7364
18	5	6	0.5	455	456.3455
19	6	4	0.25	442	444.8636
20	4	6	0.75	446	442.0636

Table.6.Estimated regression coefficient for IAA (µg/mL)

Terms	Constant	Co-Efficeint	p-Value	Significance
Constant	β_0	459.736	0.000	Significant
X_1	β_1	2.000	0.029	Significant
X_2	β_2	-0.300	0.809	
X_3	β_3	0.900	0.044	Significant
X_1^2	β_{11}	-9.591	0.002	Significant
X_2^2	β_{22}	-3.091	0.209	
X_3^2	β_{33}	-4.091	0.016	Significant
X_1X_2	β_{12}	-1.500	0.293	
X_1X_3	β_{13}	0.750	0.591	
X_2X_3	β_{23}	-0.250	0.857	

Table.7.Analysis of Variance (ANOVA) values for polynomial model for IAA (µg/mL) production

Sources	Degrees of freedom	Sum of squares	Mean square	F value	*p* value
Regression	9	1152.52	128.058	8.77	0.001
Linear	3	49.00	16.333	1.12	0.387
Square	3	1080.52	360.174	24.66	0.000
Interaction	3	23.00	7.667	0.53	0.675
Residuals	10	146.03	14.603		
Errors					
Lack-of-Fit	5	129.19	25.839	7.067	0.022
Pure Error	5	16.83	3.367		
Total	19	1298.55			

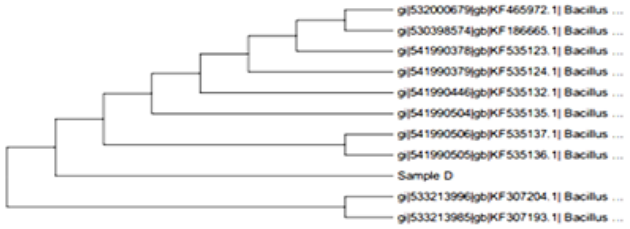

Fig.1.Phylogenetic tree constructed for Sample D, which denotes *Bacillus pumillus*

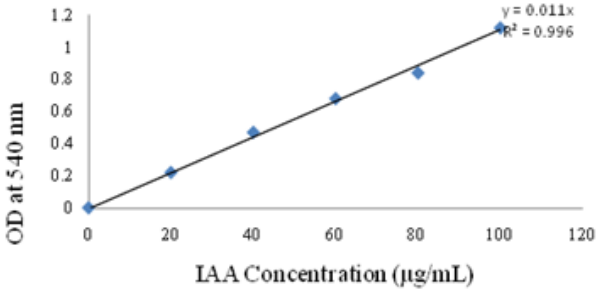

Fig.2.Standard graph for Indole acetic acid

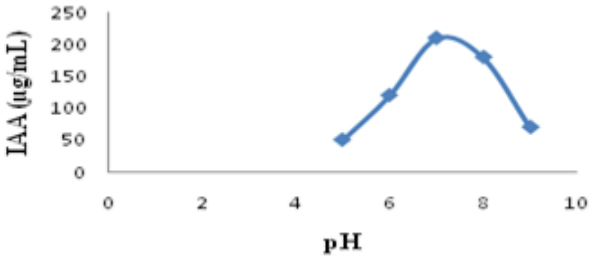

Fig.3.Effect of pH on the production of IAA

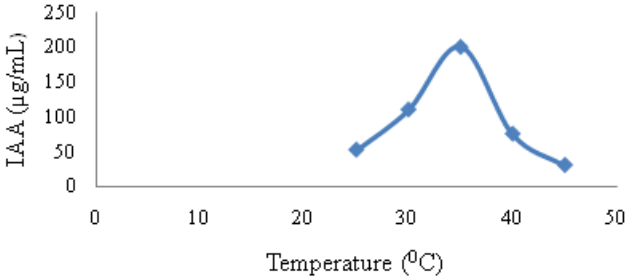

Fig.4.Effect of Temperature on the production of IAA

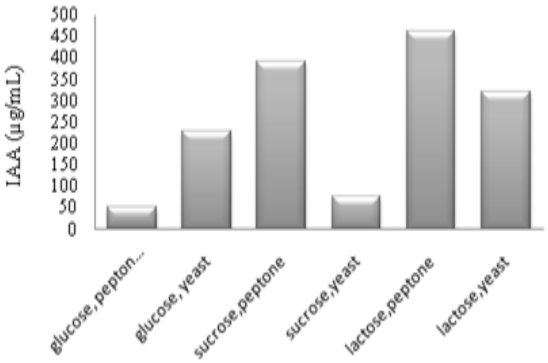

Fig.5.Combined effect of carbon, nitrogen sources on IAA production

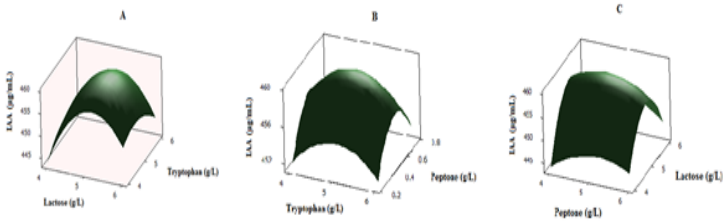

Fig.6.Response surface plot of Indole acetic acid (IAA) (µg/mL)

CONCLUSION

In this work, *Bacillus pumilus* strain was isolated from root samples and various parameters for their growth and the consequent production of IAA were analysed. The organism that produced the maximum IAA at a specified pH and Temperature was found to be 7 and 35°C, respectively. The production media parameters like Carbon source, Nitrogen source are optimized by single factorial method. The optimum yield of IAA was reported in the presence of tryptophan. Three medium components with two variables were studied in the Plackett-Burman experimental design. The most significance factor affecting IAA production was found to be Lactose, Tryptophan and Peptone. The medium optimization was carried out using Response Surface Methodology. A full –factorial central composite design was employed for experimental design and analysis of the results.

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