Modeling and Optimization of Phosphate Solubilizing Bacteria Isolated from Rhizospheric Soils of the Coffee Plant using Artificial Neural Network (ANN) and Response Surface Methodology (RSM).

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Modeling and Optimization of Phosphate Solubilizing Bacteria Isolated from Rhizospheric Soils of the Coffee Plant using Artificial Neural Network (ANN) and Response Surface Methodology (RSM).

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Abstract

Phosphorus is often found inaccessible to plants, as it forms precipitates with cations and can be converted to accessible forms by using Phosphate solubilizing bacteria (PSB). In the present study, isolation and characterization of phosphate solubilizing bacteria from rhizospheric soil of coffee plants were performed. The influence of four independent variables (incubation temperature, incubation time, pH, and inoculum size) was investigated and optimized using an artificial neural network and response surface methodology on the solubility of phosphate and indole acetic acid production. The bacterium that can dissolve phosphate were isolated in Pikovskaya's agar containing insoluble tricalcium phosphate. Total, six Phosphate Solubilizing Bacteria were isolated and three of them (PSB1, PSB3, and PSB4) were found to be effectively solubilizing phosphate. Based on phosphate solubilizing index results Pseudomonas bacteria (PSB1) was selected for modeling. The results showed that both models performed reasonably well, but properly trained artificial neural networks have the more powerful modeling capability compared to the response surface method. The optimum conditions were found to be incubation temperature of 37.5 ^{o}C, incubation time of 9 days, pH of 7.2, and inoculum size of 1.89 OD. Under these conditions, the model predicted solubility of phosphate of 260.69 µg/ml and production of IAA of 80.00µg/ml with a desirability value of 0.947. Generally, the isolated Pseudomonas bacteria is a promising Phosphate solubilizing capability that enhances plant growth and this research is a base for recommending the use of this bacterial strain for biofertilizer, as an alternative to synthetic fertilizer.

Keywords Artificial neural network, Optimization, Phosphate solubilization, Response surface methodology, Rhizobacteria,

Introduction

Phosphorus (P) is a vital macronutrient for all life forms to carry out numerous metabolic activities since it is a fundamental component of the most important energy source adenosine 5'-triphosphate (ATP), and nucleic acid [1]. In plants, phosphorus aids photosynthesis, proper plant development, and stress relief [2] Phosphorus in soils can exist in both organic and inorganic forms; the inorganic forms of phosphorus are estimated to account for 35 – 70 % of total P in soil [3]. Although, both organic and inorganic forms are present in sufficient concentration and solubility in soils is very low [4]. In soil, phosphorus always forms complexes with other compounds in the form of phosphates. The fixation of inorganic phosphorus into insoluble complexes renders these compounds inaccessible for absorption by plants, and thus, leads to severe insufficiency in both acidic and alkaline soils. To address the problem of P deficiency in different crops, phosphatic fertilizers are added in various amounts in the soil. However, a large proportion of the soluble forms of P fertilizers are precipitated in insoluble form soon after application and become unavailable to plants [5]. According to Elhaissoufi et al [6] the excessive and repeated application of soluble P fertilizers can cause economic constraints as well as pose a serious threat to groundwater. To solve such problem, biofertilizers is a sustainable and eco-friendly to environmental protection and give nutrient to plant in a safe way when they needed.

The effect of process parameters on the phosphate solubilizing rhizobacteria isolated from rhizospheric soil of different plants have been studied by several researchers [7,8] however, to the best of our knowledge there was no report on the solubility of phosphate and the production of indole acetic acids (IAA) rhizosphere bacteria isolated from rhizospheric soil of coffee plant by coupling artificial neural network (ANN) and response surface methodology (RSM). RSM is widely used in the optimization of chemical and physical processes that integrates mathematical modeling and experimental design. ANN has been widely used due to its suitability for modeling and simulation of various processes in real engineering applications [9]. ANN does not require a mathematical description of the phenomena in the process, so the simulation of the complicated systems could be performed more efficiently [10]. Compared with the RSM focusing on the statistical importance of the linear process parameters and their interactions via analysis of variance (ANOVA), ANN is more reliable in capturing the nonlinear relationship between the dependent variable and independent process variables. Although ANN is an efficient tool to predict and optimize any complex process parameters, it cannot guarantee the globally optimal solution [11].

In the present study, the combination of ANN and genetic algorithm can generate the relationship between the optimal input operating variables and the output process under study. Subsequently, the result predicted by the ANN and RSM techniques were statistically compared, using various parameters, such as coefficient of determination (R^2), root means square error (RMSE), mean absolute error (MAE), standard error of prediction (SEP), mean of squared errors (MSE) and absolute average relative deviation (AARD) based on the validation data set for their predictive and generalization capabilities.

The phosphate-solubilizing ability and production of IAA of isolated bacteria were designed by response surface statistical experimental method using central composite design (CCD) was selected to establish the regression model. Therefore, this study was conducted to investigate the effect of four parameters (incubation temperature, incubation time, pH, and inoculum size,) on the solubility of phosphate and production of IAA and to optimize these processes variables by employing RSM and ANN. The interaction effect of the independent variables with the dependent variables using the response surface plots was illustrated. Meanwhile, the back-propagation ANN model was also developed and the optimal number of hidden neurons was determined by the trial and error method. The predictive abilities and modeling competencies of the two models are compared and confirmed. Furthermore, the study characterizes the microorganisms at the morphological and phenotypic levels.

Materials and methods

Soil samples collection and isolation of rhizobacteria

The study sites were located in Jimma Zone, Oromia regional state, in the southwestern part of Ethiopia. Jimma town is the capital and administrative center of the zone, located 346 km away from the capital city of Ethiopia. The town's geographical coordinates are approximately 7°41' N latitude and 36° 50'E longitude with annual maximum and minimum temperature of 30 °C and 14 °C respectively [12]. The soil used for bacterial isolation was excavated from the 15-20 cm depth of roots of coffee and collected in sterilized plastic bags and stored at 4 °C in a refrigerator until the time of analysis.

Isolation of rhizosphere bacteria was done based on [13] method. In this method, 1 g of soil sample was transferred in 9 ml of sterilized distilled water and mixed by vortex shaker for 20 seconds. The suspension was then diluted up to 10^{-9} dilutions. From each dilution, 0.1 ml of the diluted sample was poured onto a sterilized surface plate of nutrient agar (NA) medium supplemented with 100 µg/ml of cycloheximide to defeat fungal growth. Finally, the plate was incubated at 30ºC for 5days [14]. Individual bacterial isolates on nutrient agar were further sub-cultured on the PVK agar medium to obtain a pure culture by streak plate method. Highly grown bacteria culture on PVK agar media was maintained at 4 °C in the refrigerator for further analysis and studies. All of the experiments were done aseptically to disinfect unnecessary microbes.

Characterization of the isolate

Morphological characterization of the isolates

Morphological characteristics such as shape, color, edge, motility, and endospores formation**,** and gram stain of the isolate were examined according to the [15] method.

Phenotypic identification of bacteria Isolates

Indole test, Urease test, Catalase test, Voges-Proskauer (VP) test, Methyl Red (MR) test, and Citrate test of the isolated bacteria were conducted according to Bergey's manual of systematic bacteriology [16].

Determination of phosphate solubilization index (PSI)

Qualitative estimation of phosphate solubilization was conducted using plate assays on PVK agar. Phosphate solubilization is indicated by the development of a clear zone around a growing colony and this is determined by calculation of PSI through the following formula [17].

$$PSI = \frac{\text{Total diameter (colony + halo zone)}}{\text{colony diameter}} \quad (1)$$

P-Solubilization efficiency of isolate in broth media

The isolated bacteria that showed higher PSI was selected and quantitative phosphate solubilization efficiency of the isolate was determined in PVK broth media with some modification [17]. The isolate (500 µl) was inoculated into 100 ml PVK broth media in 250 ml conical flask containing 5 g/l insoluble phosphates in the form of tricalcium phosphate (TCP) and incubated on a shaker incubator at 120 rpm for incubation temperature (25 - 45 ^{o}C), incubation time (3 -15 days), pH (5- 9) and inoculum size (1-3 OD). The process variables ranges were selected based on [18,19] Five ml of samples were withdrawn from each treatment and analyzed for phosphorus solubilization.

Quantification of soluble phosphorus

The phosphorus in the supernatant was determined according to [20]. The withdrawn samples were centrifuged at 15,000 rpm for 15 min to determine the optical density (OD) of the supernatant using a spectrophotometer with 700 nm wavelength and the quantity of P-solubilized by strain was extrapolated from the standard curve.

Assay for Indole-3-acetic acid (IAA) production

Quantitative production of IAA was determined using the colorimetric method as described by Lebrazi et al.[21]. The bacterial isolates were cultured in Erlenmeyer flasks containing 50 ml of YMB supplemented with 0.1 % DL-tryptophan, adjusted to different incubation temperatures (25-45 ^{o}C), incubation time (3-15 days), pH values (5-9), and inoculum size (1-3 OD) on a shaker incubator at 180 rpm. The production of IAA was checked after culture centrifugation using Salkowski reagent [22]. The resulting solution was examined in a spectrophotometer at 530 nm and the concentration of IAA produced was estimated using a standard IAA curve and expressed in terms of µg/mL.

Experimental design and statistical analysis

The relation between different parameters was evaluated by the response surface statistical experimental method using the design expert software version of 11.1.2.06. This study was conducted using RSM, CCD by considering incubation temperature (A) and incubation time (B), pH (C) and, inoculum size (D) on solubilization of phosphate and IAA production. Generally, CCD involves sixteen factorial points, eight axial points, and six points at the center were carried out with a total of 30 experiments as shown in equation (2). All four factors have to be adjusted at five

coded levels (-α, -1, 0, +1, + α). The relationship between the coded and the actual value of the variables is shown in Table 1. The variables were coded according to the equation:

$$N = 2^n + 2n + n_c = 2^4 + 2*4 + 6 = 30 \quad (2)$$

where N is the total number of experiments required, n is the number of variables, and n_c is the number of replicates.

Table 1: Independent variables and levels used in the CCD for the phosphate solubilization and IAA production of the isolate

Factors	Unit	Coded symbol	Coded Levels				
			-α	-1	0	+1	+α
Incubation Temperature	°C	A	25	30	35	40	45
Incubation time	days	B	3	6	9	12	15
pH	-	C	5	6	7	8	9
Inoculum size	OD	D	1	1.5	2	2.5	3

The second-order polynomial multiple quadratic regression equation was used to determine the relationship between independent variables and responses.

$$Y = b_O + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} X_i X_j \quad (3)$$

where Y is the predicted response (i.e., phosphate solubilization or IAA production), n is the number of independent variables, b_0 is the constant coefficient, b_i is the linear coefficient, b_{ij} is the second-order interaction coefficient, b_{ii} is the quadratic coefficient, and x_i and x_j are the coded values of the independent variables. The coefficient of determination R^2, adjusted R^2, and predicted coefficient R^2, lack of fit from ANOVA were used in the determination of the quality of the developed model.

ANN modeling

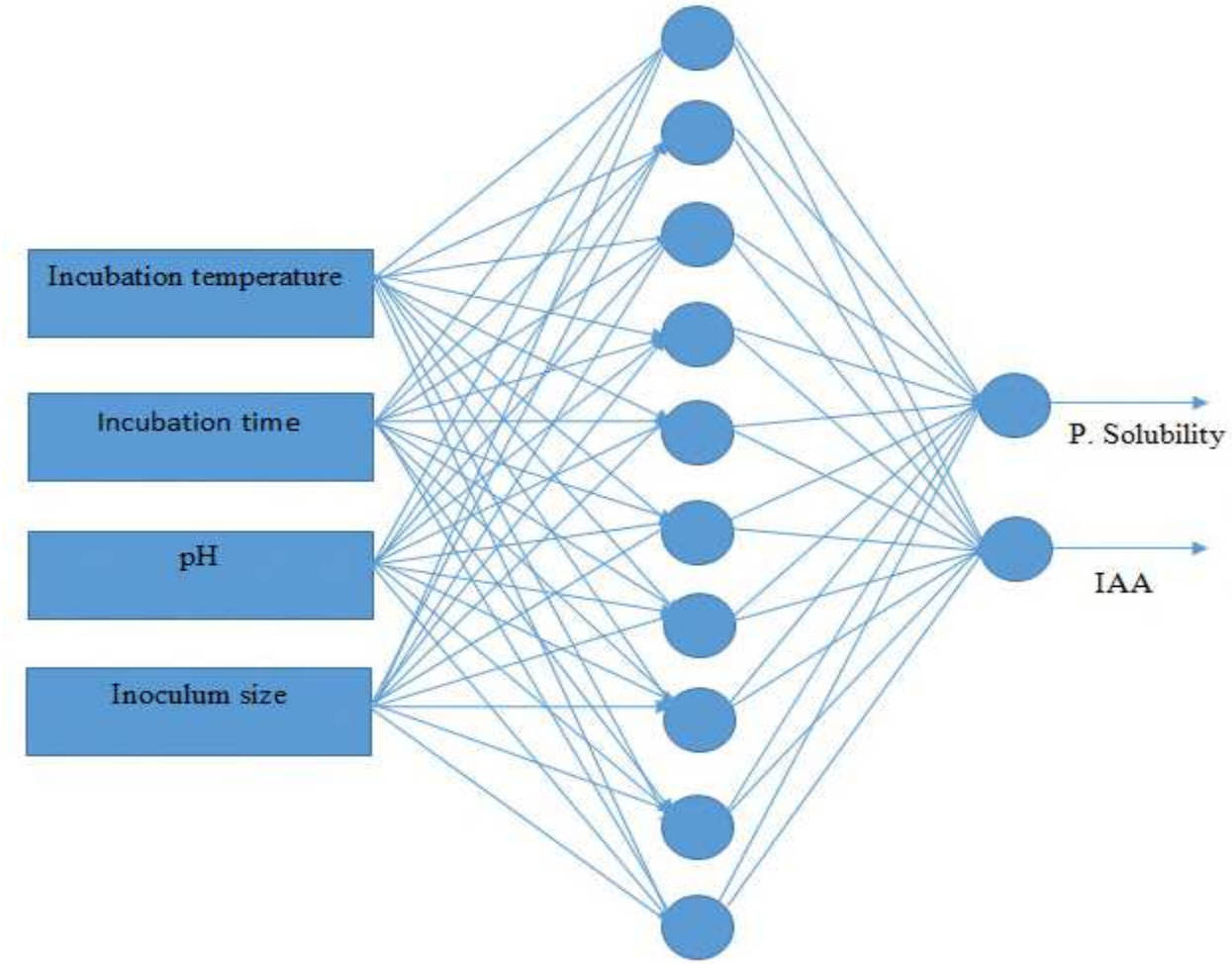
The feed-forward architecture of ANN also known as multilayered perceptron (MLP) was selected to develop a predictive model with a layer of four neurons (incubation temperature, incubation time, pH, and inoculum size) as input, two layers of neurons (solubility of phosphate and IAA production) as output, and 10 hidden layers as indicated in Figure 1. 70% of the data points were selected for training to develop the neural network, 15% of the data set used for validation and 15% data sets for testing. Figure 1 depicts a simplified representation of the ANN structure. The following expression was used to calculate the number of neurons in the hidden layer [9].

156 $$2(n+m)^{0.5} \text{ to } 2n+1 \quad (4)$$

157 where n is the number of neurons in the input layer and m is the number of neurons in the output layer. Each network
158 is trained separately, and therefore, the best network is selected based on the accuracy of the predictions within the
159 testing phase. The feed-forward ANN in this study was trained using the Levenberg-Marquardt algorithm represented
160 in Equation (5). To achieve fast convergence to the minimum mean square error (MSE), the inputs and outputs are
161 scaled within the uniform range of 0 (new X_{min}) to 1 (new X_{max}) by the following equation (5) to ensure uniform
162 attention during the training process.

163 $$X_{normal} = \frac{X_i - X_{\min}}{X_{\max} - X_{\min}} (newX_{\max} + newX_{\min}) + newX_{\min} \quad (5)$$

164 where X_{norm} is the normalized data, X_i is the input/output data (data of independent and dependent variables), X_{max}
165 and X_{min} are the maximum and minimum values of the particular variable, respectively. ANN Modeling using
166 Toolbox of MAT LAB version 8.1(R2013a) was applied to evaluate the goodness of fitting model using RSM data
167 output.



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Input layer Hidden layer Output layer

Figure 1: Feed-forward with the backward propagation neural network used in the current study

Comparative analysis of RSM and ANN models

For this study, the performance of ANN and RSM have evaluated the goodness of the fitting and prediction accuracy of the constructed models by using the following error functions; RMSE, SEP, R^2, MAE, MSE, and AARD. The models were acceptable as R^2 was closer to the value of 1 and the other error function values were as small as possible [23]. The equations representing each error function are presented by Eqn. 6 to 11. To study the comparison of experimental with predicted values obtained by the RSM and ANN models, the values are tabulated and compared with the corresponding experimental values.

Table 2: Error functions and its equations with references

Error function	Equation		Reference
Mean-squared error	$MSE=\frac{1}{n}\sum_{i=1}^{n}(Yi,p-Y_{i,e})^2$	(6)	[24]
Root mean square error	$RMSE=\sqrt{\frac{\sum_{i=1}^{n}(Y_{i,e}-Y_{i,p})^2}{n}}$	(7)	[25]
Standard error of prediction	$SEP=\frac{RMSE}{Y_e}\times 100$	(8)	[26]
Coefficient of determination	$R^2=1-\frac{\sum_{i=1}^{n}(Y_{i,p}-Y_{i,e})^2}{\sum_{i=1}^{n}(Y_{i,e}-Y_e)^2}$	(9)	[27]
Mean absolute error	$MAE=\frac{1}{n}\sum_{i=1}^{n}\left\|Y_{i,e}-Y_{i,p}\right\|$	(10)	[28]
Absolute average relative deviation	$AARD=\frac{1}{n}\sum_{i=1}^{n}(\left\|\frac{Y_{i,p}-Y_{i,e}}{Y_{i,e}}\right\|)x100$	(11)	[29]

where, $Y_{i,e}$ is the experimental data, $Y_{i,p}$ is the predicted data obtained from either RSM or ANN, Y_e is the mean value of experimental data and n is the number of the experimental data.

RESULTS AND DISCUSSION

Isolation and quantification of phosphate solubilization Index (PSI)

In the present study, the rhizosphere bacteria was isolated from rhizospheric soil of coffee plants on NA medium supplemented with 100 µg/ml of cycloheximide. Individual bacterial isolates on nutrient agar were further sub-cultured on the PVK agar medium to evaluate in vitro for P solubilizing bacteria. A total of 6 phosphate solubilizing bacterial colonies were isolated on PVK agar medium, containing tricalcium phosphate (TCP). Out of 6 bacterial isolates, 3 isolates (PSB_1, PSB_3, and PSB_4) were found to be potent phosphate solubilizers showing a clear halo zone around its colony.

Qualitative estimation of P-solubilization potential was anticipated by observing the clear zones around the bacterial colonies on PVK plates after incubation at 30 ºC for 5 days. The halo diameter around the bacterial colony and colony diameter used to calculate PSI were measured. The solubilization index based on colony diameter and halo zone for each PSB isolate is presented in Table 3. The results showed that Maximum PSI was observed by PSB_1 (5.092) followed by PSB_3 (4.65) and PSB_4 (4.45). The bacterial isolates produced the largest halos of approximately 5.0 mm to 15.1 mm within 5 days of incubation. This result was in agreement as reported by Anzuay et al. [30] and Pandey et al.[31].

Table 3: Qualitative estimation of phosphate solubilization efficiency of isolates

Bacterial Isolates	Colony diameter, mm	Halo zone diameter, mm	PSI
PSB_1	3.69	15.1	5.092
PSB_2	2.94	4.70	2.60
PSB_3	1.42	5.20	4.65
PSB_4	1.74	6.0	4.45
PSB_5	9.3	5.3	1.57
PSB_6	2.49	5.0	3.01

Morphological and biochemical analysis of bacterial isolate

To identify the strain of the isolated PSB, morphological and biochemical tests were conducted. Morphological characterization of three PSB showing a larger clear halo zone around its colony was performed, and characteristically, all the isolates were Gram-negative, round raised colonies and of rod-shaped, motile, and most of with shiny surface (Table 4). The isolates were further characterized by a series of biochemical reactions such as Citrate utilization, Catalase Test, Indole test, Urease test, Voges-Proskauer (VP) test, and Methyl Red (MR) test to determine phenotypic properties of the isolate, and the results were summarized in Table 4.

Table 4: Morphological and biochemical characterization of PSB_1, PSB_3, and PSB_4 isolates

Tests		Bacterial Isolate		
		PSB_1	PSB_3	PSB_4
	Color	Reddish	White	Cream

Morphological	Shape	Rod-shape	Rod-shape	Rod-shape
	Gram staining	-	-	-
	Motility	Motile	Motile	Motile
	Endospores formation	+	+	-
Biochemical	Indole test	-	-	+
	Catalase Test	+	+	-
	Urease test	-	-	-
	Voges-Proskauer (VP) test	-	+	+
	Methyl Red (MR) test	-	-	-
	Citrate utilization	-	-	+

All 3 bacteria have shown negativity for the urease and MR test. Bacteria isolate PSB_1 and PSB_3 were negative for the Citrate and indole test. However, bacteria isolate PSB_4 was positive for VP, Citrate, and indole test. The morphological and biochemical test results of PSB_1 are consistent with the many phenotypic characteristics of the genus Pseudomonas [32], whereas PSB_3 and PSB_4 are comparable with Bacillus sp. [33], and Enterobacter sp. [34] respectively. Thus, it was concluded that the isolated PSB were identified as Pseudomonas sp., Bacillus sp., and Enterobacter sp. These bacterial sp. were also identified as phosphate solubilizers by several authors [17,35,36]. Based on their PSI, PSB1 was selected for further analysis.

Modeling and prediction using RSM

The experimental results obtained from the solubility of phosphate and IAA production based on CCD are presented in Table 4. A total number of 30 experiments were performed with different combinations of process variables to investigate and optimize the influence of independent variables (incubation temperature, incubation time, pH, and inoculum size) on the responses (solubility of phosphate and IAA production).

Table 5: Composite design matrix and experimental yields

Run	Coded variable				Decoded Variable				Dependent Variable	
	A	B	C	D	Temp (°C)	Time (day)	pH	Inoculum (OD)	SizeSoluble P(µg/ml)	IAA(µg/ml)
1	0	0	0	0	35	9	7	2	239.68	78.23
2	1	-1	-1	1	40	6	6	2.5	181.62	46.05
3	-1	-1	-1	-1	30	6	6	1.5	130.85	53.16
4	-1	1	-1	-1	30	12	6	1.5	150.58	45.16
5	1	1	1	1	40	12	8	2.5	175.44	63.18

6	-α	0	0	0	25	9	7	2	201.75	50.12
7	0	α	0	0	35	15	7	2	135.43	57.03
8	1	-1	1	-1	40	6	8	1.5	195.41	62.68
9	1	1	-1	-1	40	12	6	1.5	218.31	52.36
10	-1	1	1	1	30	12	8	2.5	225.53	52.07
11	0	0	0	0	35	9	7	2	266.34	78.62
12	0	-α	0	0	35	3	7	2	128.85	52.31
13	-1	-1	1	-1	30	6	8	1.5	190.83	45.28
14	0	0	0	α	35	9	7	3	133.38	55.56
15	0	0	0	0	35	9	7	2	260.14	81.02
16	0	0	α	0	35	9	9	2	235.59	56.01
17	1	-1	1	1	40	6	8	2.5	156.56	45.34
18	α	0	0	0	45	9	7	2	270.12	61.72
19	0	0	0	-α	35	9	7	1	165.34	58.12
20	-1	-1	-1	1	30	6	6	2.5	125.67	49.26
21	-1	1	-1	1	30	12	6	2.5	142.01	48.95
22	0	0	0	0	35	9	7	2	255.51	81.54
23	-1	1	1	-1	30	12	8	1.5	230.57	46.52
24	-1	-1	1	1	30	6	8	2.5	185.74	42.58
25	1	1	-1	1	40	12	6	2.5	202.06	50.12
26	0	0	0	0	35	9	7	2	245.65	78.62
27	1	1	1	-1	40	12	8	1.5	211.91	64.64
28	1	-1	-1	-1	40	6	6	1.5	221.16	54.52

29	0	0	0	0	35	9	7	2	249.71	77.54
30	0	0	-α	0	35	9	5	2	147.34	51.63

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220 Table 6: Analysis of variance for response surface quadratic model of soluble phosphate and IAA

Source	Sum of Squares	Df	Mean Square	F-value	p-value
(A) Soluble phosphate					
Model	60323.45	14	4308.82	32.12	< 0.0001
A-Temperature	4198.41	1	4198.41	31.29	< 0.0001
B-Inoculum time	1376.07	1	1376.07	10.26	0.0059
C-pH	5897.88	1	5897.88	43.96	< 0.0001
D- Inoculum size	1996.73	1	1996.73	14.88	0.0015
AB	245.16	1	245.16	1.83	0.1965
AC	8435.96	1	8435.96	62.88	< 0.0001
AD	718.64	1	718.64	5.36	0.0352
BC	234.47	1	234.47	1.75	0.2060
BD	31.16	1	31.16	0.2323	0.6368
CD	15.82	1	15.82	0.1179	0.7361
A^2	262.58	1	262.58	1.96	0.1821
B^2	23135.59	1	23135.59	172.45	< 0.0001
C^2	5539.71	1	5539.71	41.29	< 0.0001
D^2	16785.17	1	16785.17	125.11	< 0.0001
Residual	2012.40	15	134.16		
Lack of Fit	1535.05	10	153.51	1.61	0.3130
Pure Error	477.35	5	95.47		
Cor Total	62335.85	29			
(B) IAA					
Model	4173.62	14	298.12	35.31	< 0.0001
A-Temperature	260.77	1	260.77	30.88	< 0.0001
B-Inoculum time	46.96	1	46.96	5.56	0.0324

C-pH	41.27	1	41.27	4.89	0.0430
D- Inoculum size	42.37	1	42.37	5.02	0.0406
AB	23.26	1	23.26	2.75	0.1177
AC	114.86	1	114.86	13.60	0.0022
AD	65.00	1	65.00	7.70	0.0142
BC	85.24	1	85.24	10.10	0.0062
BD	90.49	1	90.49	10.72	0.0051
CD	1.64	1	1.64	0.1948	0.6652
A^2	1156.37	1	1156.37	136.96	< 0.0001
B^2	1270.36	1	1270.36	150.46	< 0.0001
C^2	1350.93	1	1350.93	160.00	< 0.0001
D^2	1075.90	1	1075.90	127.43	< 0.0001
Residual	126.65	15	8.44		
Lack of Fit	113.51	10	11.35	4.32	0.0599
Pure Error	13.13	5	2.63		
Cor Total	4300.27	29			

Multiple linear regression results and analysis of the adequacy of the fitted model

The ability of phosphate solubilizing bacteria (Pseudomonas sp.) to dissolve phosphate and produce IAA was investigated in this study, and ANOVA was used to determine the interaction between the process factors and the responses. Table 6 summarizes the model regression coefficients for each ANOVA response found. The results of the ANOVA were statistically significant, implying that at least one of the model parameters could explain the experimental variance in phosphate solubility and IAA production. For soluble phosphate and IAA production, the ANOVA result revealed a perfect fit of the quadratic regression model (F-value of 32.12 and $p < 0.0001$) and F-value 35.31 and $p < 0.0001$), respectively. The p-values for "Lack of Fit" for soluble phosphate and IAA were 1.61 ($p > 0.313$) and 4.32 ($p > 0.06$), respectively, showing that lack of fit was not significant when compared to the pure error. Therefore, the results obtained verified that the models generated were accurate enough to predict the soluble phosphate and IAA production within the range of the variables studied (Equation 12 and 13). The results demonstrate that A, B, C, D, AC, AD, B^2, C^2, D^2 had a significant effect on soluble phosphate, whereas AB, BC, BD,

CD, and A^2 had no significant effect. In the case of IAA, A, B, C, D, AC, AD, BC, BD, A^2, B^2, C^2, D^2 were found to have a significant effect on the IAA, while AB and CD, were not significant (Table 6).

According to the findings, the regression model was highly significant, with R^2 values of 0.9677 and 0.9705 for phosphate solubility and IAA production, respectively, showing that the model suited the experimental data well and that the experimental error was small. Moreover, the value of the adjusted R^2 for soluble phosphate and IAA were 0.9376, and 0.9431 respectively, confirming the significance of the model, indicating that the experimental and predicted values for the dependent variables were in good agreement. Adjusted R^2 and predicted R^2 should be within 20% to be in good agreement as suggested by Le Man et al. [37]. This requirement is satisfied in this study with a predicted R^2 value of 0.8471 and 0.8436 for soluble phosphate and IAA production, respectively. The coefficient of variation (CV %) and standard deviation of the two responses in this study was fairly low and acceptable, showing high accuracy and high reliability of the experiments conducted (Table 7).

Table 7: Regression coefficients of the predicted second-order model for the response variables

S. No	Response parameter	Soluble phosphate	IAA production
1	Std. Dev.	11.58	2.91
2	Mean	195.97	58.00
3	C.V%	5.91	5.01
4	R^2	0.9677	0.9705
5	Adjusted R^2	0.9376	0.9431
6	Predicted R^2	0.8471	0.8436
7	Adeq Precision	18.010	17.4029
8	Model suggested	Quadratic	Quadratic

Development of regression model equation

By applying multiple regression analysis on the experimental data, second-order polynomial equations were developed for the responses (solubility of phosphate (Y) and IAA production (X)) which can express the relationship between process variables (incubation temperature (A), incubation time (B), pH (C), and inoculum size (D)) and the responses. The final equations obtained in terms of coded factors after excluding the insignificant terms were given in Equations (12) and (13), respectively. Note the equation (12) and (13) only apply under the conditions tested: 25°C < incubation temperature < 45°C, 3 day < incubation time < 15 day, 5 < pH< 9 and 1< pH < 3.

Phosphate solubilization (Y) = + 252.84 + 13.23A + 7.57B + 15.68C – 9.12D – 22.96 AC
-6.70AD- 29.04B^2 - 14.21C^2 - 24.74D^2 (12)

IAA production (X) = + 79.26 + 3.30A + 1.40B + 1.31C - 1.33D + 2.68AC - 2.02AD + 2.31BC
+2.38BD - 6.49A^2 - 6.81B^2-7.02C^2 - 6.26D^2 (13)

The positive signs in the models signify the synergetic effects of factor, while the negative sign indicates the antagonistic effect. According to the linear coefficients of the second-order equation, incubation temperature, incubation time, pH showed a positive effect, while, and inoculum size had a negative influence.

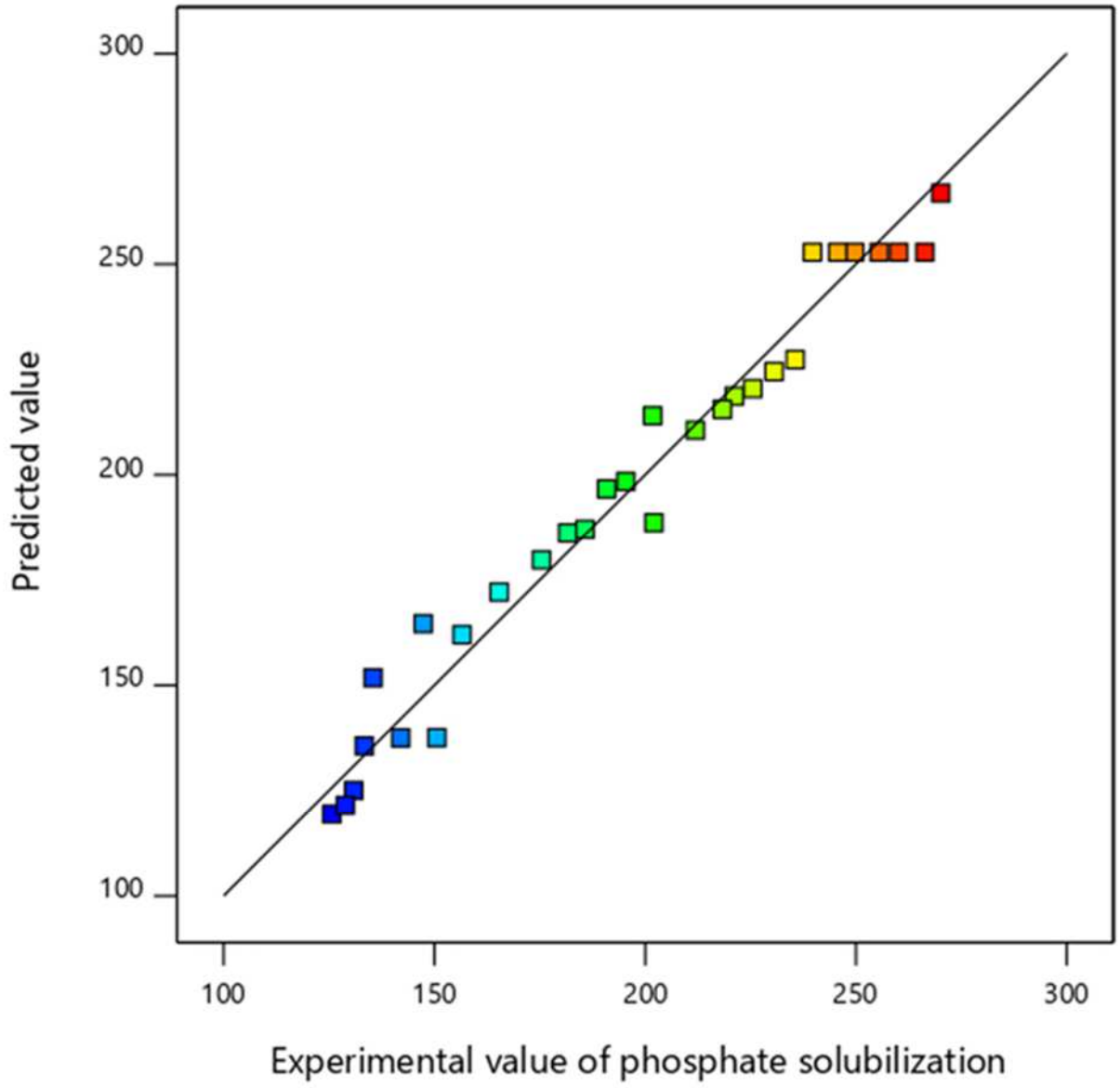

Figure 2: Correlation between the experimental and predicted value of soluble phosphate

Figures 2 and 3 demonstrate that the predicted values were quite close to the experimental values and lie reasonably close to the straight line and indicated the adequate agreement with the actual data. All predicted and experimental responses fell in 45^{o} lines, showing that the established model is suitable for predicting phosphate solubility and IAA production. From the graph, it is clear that the values derived experimentally match closely with the constructed model. Similar research has been done on optimizing a low-cost medium for the rhizobacterial strain

Pseudomonas putida Rs-198 culture isolated from salinized soils [38] and rhizobacterial strains isolated from Acacia cyanophylla root nodules [21].

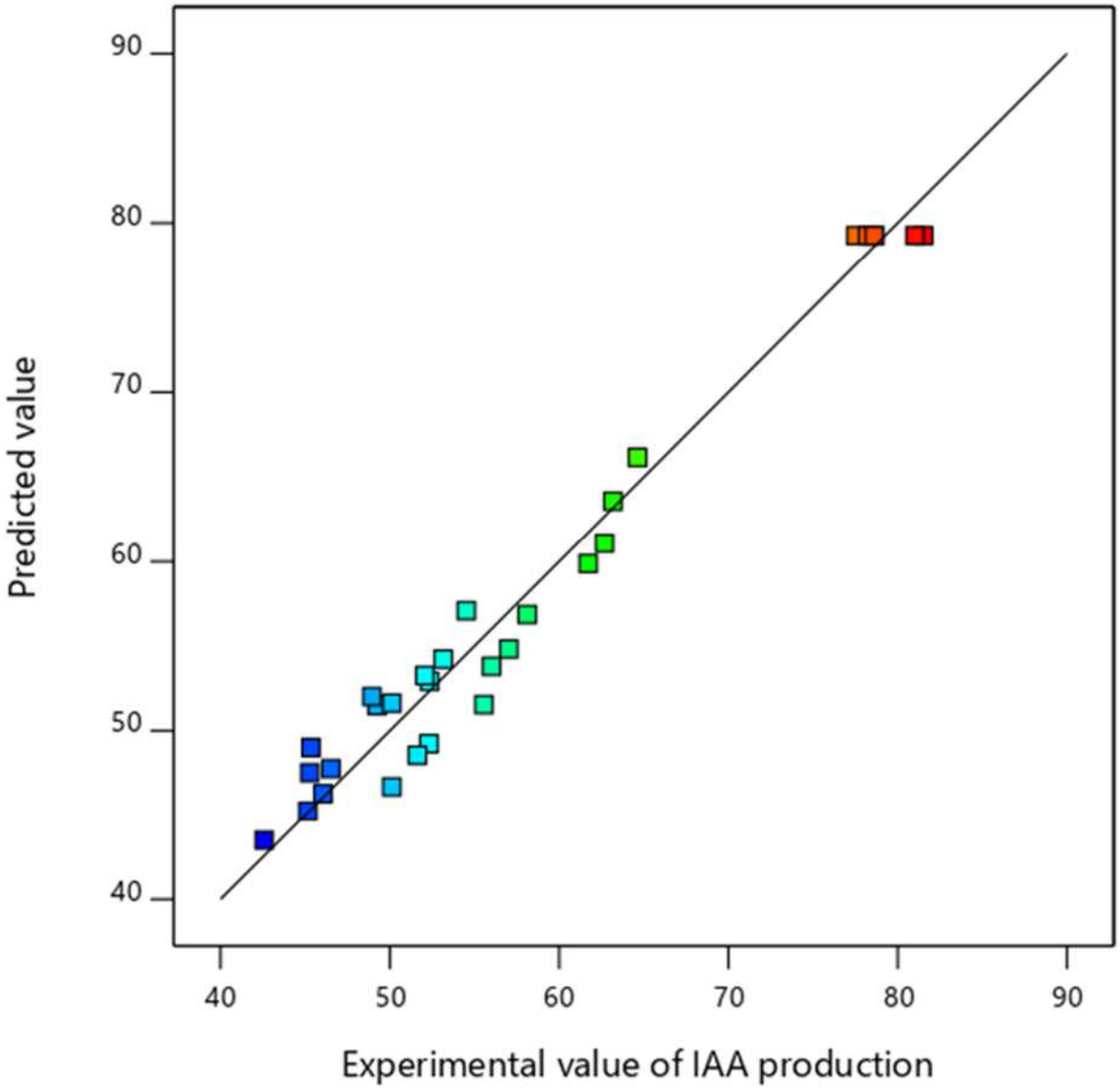

Figure 3: Correlation between the experimental and predicted value of IAA production

Response surface analysis of extraction process

To study the interaction relationship between response and process parameters, three-dimensional (3D) surface plots were generated based on the fitted polynomial equation. The 3D response surfaces plots were drawn to account for the most significant interaction effects of the independent variables on the solubility phosphate and IAA production. The most significant parameters for each response are demonstrated in Figures 4 and 5 through 3D response surface

plots. In these figures, two factors are held constant at the optimum level, while the other two factors are varied within their experimental ranges.

Effect of process variables on the phosphate solubilization

Table 5 shows the phosphate solubilization values obtained from Pseudomonas bacteria isolated from the rhizospheric soil of coffee plants, according to the CCD matrix, with corresponding process variables. Solubility of phosphate obtained in this experiment was found to be in the range of 125.67 µg/ml - 270.12 µg/ml (Table 5), which is comparable to the phosphate solubilizing bacteria (PSB) isolated from the rhizosphere of Allium hookeri Thwaites (124.8–266.4 µg/mL) [39] and higher than phosphate solubilizing bacteria (PSB) isolated from rhizobacterial salinized soils (44.00 – 63.90 mg/L) [38]. In the present work, the higher solubility of phosphate suggests that bacterial strain increases the soluble phosphate content of the soil, thereby improving phosphate absorption. However, incubation temperature, incubation time, pH, and inoculum size are vital to process variables that could have a remarkable effect on the solubility of phosphate.

Phosphate solubilization was directly proportional to incubation temperature, incubation time, and pH, and indicated that increasing any of those parameters would result increase the phosphate solubilization until the optimum value was achieved (Eq.12). The solubility of phosphate always increased if incubation temperature, incubation time, and pH increased (with constant inoculum size), because each of these factors increases the solubility of phosphate, giving suitable conditions of medium composition required by most of the bacterial growth in biofertilizer formulation. However, further increase in incubation temperature, incubation time, and pH resulted in a decrease of phosphate solubilization activity since too high those variables would lead to a reduction in the effectiveness of the bacterial population to affect phosphate release from the tricalcium phosphate (TCP) substrate.

The result shows that the phosphate solubilizing activities increase with an increase in incubation time this suggesting that there was the microbial mobilization of phosphate during this period of incubation time.

The incubation temperature, incubation time, pH, and inoculum size show a significant ($p < 0.0001$) effect on the solubility of phosphate. The solubility of phosphate increases as the incubation time increased until optimum values are achieved. However, as the incubation time increased beyond the optimum value the solubility of phosphate decreased. This reduction is may be due to the availability of a soluble form of phosphate, which has an inhibitory effect on further phosphate solubilization and the formation of an organophosphate compound induced by organic metabolites released, which in turn, reduce the amount of available phosphate [6].

The pH has the most significant effect on the solubility of phosphate whose F value is 43.96, followed by incubation temperature, inoculum size, and incubation time (Table 6). The pH was directly proportional to the solubility of phosphate and indicated that increasing the value of pH would result in an increasing percentage of solubility of phosphate (Eq.12). The solubility of phosphate increases with increasing pH value; this is might be due to phosphate solubilizing microorganisms produce a variety of organic acids from simple carbohydrates under which they solubilize insoluble inorganic phosphates. However, the solubility of phosphate attained maximum value and decline with further changes in the pH due to bacterial activity. A similar effect was noted in the phosphate solubilizing activity of bacterial sp from solid-state culture [17]. The solubility of phosphate decreases with

increasing inoculum size, this is might be due to the amount of phosphate was immobilized by the bacteria. The effect of inoculum size in this study is similar to the previous work of [40].

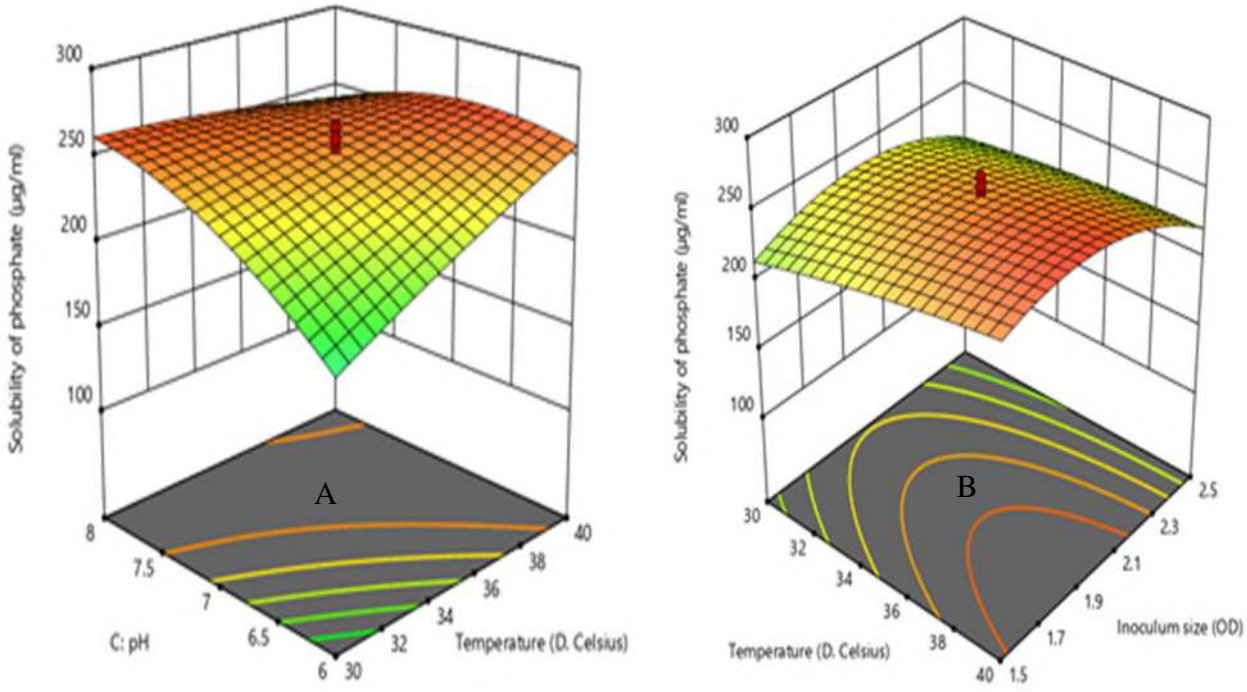

Figure 4: 3D response surface plot of solubility of phosphate: (A) effects of incubation temperature and pH at constant incubation time and inoculum size and (B) effects of incubation temperature and inoculum size at constant pH and incubation time

Based on the results of the experiment conducted, it was observed that there is a negative interaction effect of incubation temperature with pH and incubation temperature with inoculum size have a significant ($p < 0.05$) effect on the solubility of phosphate (Table 6). Figure 4 A shows a 3D response surface plot of the solubility of phosphate as a function of incubation temperature and pH at a fixed incubation time and inoculum size. The interaction between incubation temperature and pH has the most significant effect on the solubility of phosphate (Table 6). The solubility of phosphate was observed to rapidly decrease with an increased pH compared to that of incubation temperature. Increasing the combined effect between incubation temperature and pH generally decreased the solubility of phosphate; the highest solubility was achieved when both variables were at the minimum point. This shows that the solubility of phosphate reduces with an increased interaction effect of incubation temperature with pH.

The interactive effect of incubation temperature and inoculum size on the solubility of phosphate at a constant pH and incubation time is shown in Figure 4 B. The solubility of phosphate was observed to rapidly increase with an increase in incubation temperature compared to that of inoculum size. There is a negative significant interaction between the incubation temperature and inoculum size. This shows that the solubility of phosphate reduces with an increase in the combined effects of incubation temperature and inoculum size.

The effect of process variables on the indole acetic acid (IAA) production

In the present study, the produced IAA within the range of 42.58 – 81.54 µg/ml (Table 5), which is comparable to the phosphate solubilizing bacteria (PSB) isolated from the rhizosphere of medicinal plant Stevia rebaudiana (34-91.7 µg/ml) [41] and higher than phosphate solubilizing bacteria (PSB) isolated from rhizobacterial salinized soils (44.00 – 63.90 mg/L) [38]. Production of IAA was slightly lower than IAA produced by the cultures growing the isolates in YEM broth amended with 0.2 % of L-tryptophan (52.9–186.65 µg/mL) [39]. IAA production is an indicator of plant growth-promoting rhizobacteria. The results were consistent with previous work of 31.05-88.26 µg/ml IAA [18].

In this study, IAA is found to be significantly affected by all linear process variables (Equation 13). Based on the ANOVA, the IAA production was significantly affected by linear, interactions and quadratic between process variables. Incubation temperature, incubation time, pH, and inoculum size showed significant ($p < 0.05$) effects on the IAA production (Eq. 13). The IAA production was positively influenced by incubation temperature, incubation time, and pH. The results obtained from the ANOVA showed that incubation temperature has the most significant effect on the IAA production, followed by incubation time, inoculum size, and pH. Regarding the incubation time, linear effects were verified to be statistically significant for IAA production, as indicated by the p-value in Table 6.

The interactions among the four parameters investigated for the IAA production were examined using 3D surface plots (Figures 5 C and D). The interaction between incubation temperature and pH exhibited a strong significant ($p < 0.05$) effect on the IAA production. It can be seen from Figures 5 (C and D) that the response surface of the interaction of incubation temperature with pH and incubation time with inoculum size for IAA production has a steep slope, indicating significant interaction between the two factors. Significant interaction indicates that the factors work independently, whilst the presence of interaction indicates that the difference in IAA production at different levels of a factor is not the same at all levels of another factor. From F value and P-value in Table 4, it can be concluded that the order of influence of interaction term on the IAA production is: AC > BD > BC > and AD.

Figure 5 (C) shows a 3D response surface plot of the IAA production as a function of incubation temperature and pH at fixed incubation time and inoculum size. RSM plot showed that IAA production increased with temperature and pH up to an optimum point where further increase in temperature or pH resulted in a decrease of IAA production activity. IAA production was generally increased as the interaction between incubation temperature and pH increased (Eq.13). Figure 5D shows a 3D response surface plot of the IAA production as a function of incubation time and inoculum size at a fixed pH and incubation temperature. For the interaction between incubation time and pH, a linear effect was detected for the IAA production, which confirms that the increase in time and pH improves the solubility of phosphate. The quadratic of incubation temperature, incubation time, pH, and inoculum size has a significant effect on the IAA production. The quadratic of pH has the most significant effect on the IAA production whose F value is 160.00 (Table 6). Similar findings were reported by another researcher [18].

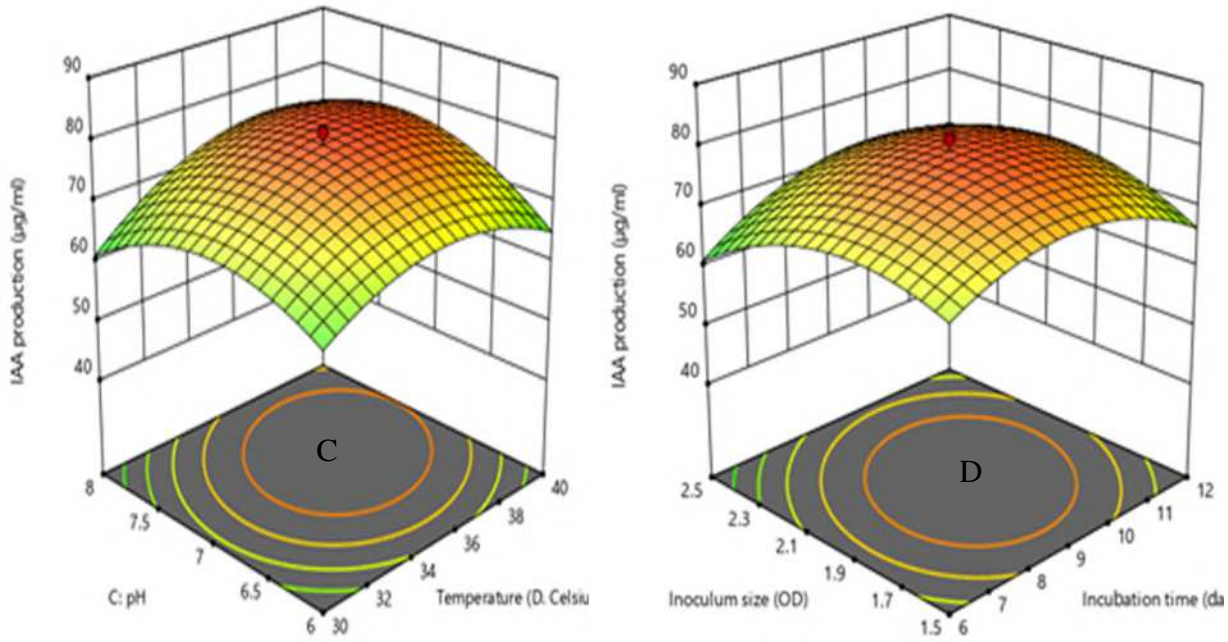

Figure 5: 3D response surface plot of solubility of phosphate: (C) effects of incubation temperature and pH at constant incubation time and inoculum size and (D) effects of incubation time and inoculum size at constant pH and incubation temperature

Artificial neural network-based modeling

The network used in this study consists of four input neurons (incubation temperature, pH, incubation time, and inoculum size) in the first layer, two output neurons (solubility of phosphate and IAA production) in the third layer, and 10 hidden neurons are shown in Figure b (a). Figure 6 (b) shows the scatter diagrams that compared the actual versus the computed neural network data for training, testing and validation networks. The correlation coefficients (R) values for training (0.99913), test (0.9802), validation (0.98834), and whole data sets (0.99268) indicating that the ANN model shows better regression and fitting compared to the RSM model. Nearly the whole point has been scattered around the 45° line an indication of excellent compatibility between the experimental results and ANN predicted output data values. As can be seen from Figure 6 the ANN model indicating that prediction for training, validation, and testing has sufficient reliability and can be used for predicting the solubility of phosphate (µg/ml) and IAA production (µg/ml).

392 Table 8: Validation data set for experimentally determined ANN and RSM predicted values of solubility of phosphate
393 (µg/ml) and IAA production (µg/ml)

Exp. No	Solubility of phosphate (µg/ml)			IAA production (µg/ml)		
	Experimental	Predicted value		Experimental	Predicted value	
		RSM	ANN		RSM	ANN
1	239.68	252.84	238.46	78.23	79.26	78.22
2	181.62	186.21	180.28	46.05	46.29	46.05
3	130.85	125.05	129.45	53.16	54.24	53.12
4	150.58	137.58	148.78	45.16	45.25	45.17
5	175.44	179.76	175.36	63.18	63.59	63.18
6	201.75	214.01	205.61	50.12	46.70	49.89
7	135.43	151.81	134.91	57.03	54.84	56.89
8	195.41	198.42	195.02	62.68	61.10	62.18
9	218.31	215.53	218.24	52.36	52.93	52.36
10	225.53	220.46	225.56	52.07	53.26	53.07
11	266.34	252.84	264.65	78.62	79.26	79.63
12	128.85	121.52	127.63	52.31	49.24	51.45
13	190.83	196.66	190.05	45.28	47.53	45.56
14	133.38	135.64	133.78	55.56	51.55	54.65
15	260.14	252.84	259.78	81.02	79.26	80.98
16	235.59	227.34	237.12	56.01	53.81	56.25
17	156.56	161.99	156.75	45.34	49.01	46.12
18	270.12	266.91	270.08	61.72	59.88	60.98
19	165.34	172.13	164.43	58.12	56.87	58.06
20	125.67	119.41	123.96	49.26	51.50	50.54
21	142.01	137.52	141.26	48.95	52.02	49.03
22	255.51	252.84	255.46	81.54	79.26	80.56
23	230.57	224.50	229.59	46.52	47.77	47.17
24	185.74	187.04	185.64	42.58	43.50	43.00
25	202.06	188.66	200.12	50.12	51.64	50.65
26	245.65	252.84	246.96	78.62	79.26	78.16
27	211.91	210.60	211.91	64.64	66.17	66.37
28	221.16	218.66	221.16	54.52	57.09	54.29
29	249.71	252.84	248.85	77.54	79.26	77.36
30	147.34	164.64	146.56	51.63	48.57	50.16

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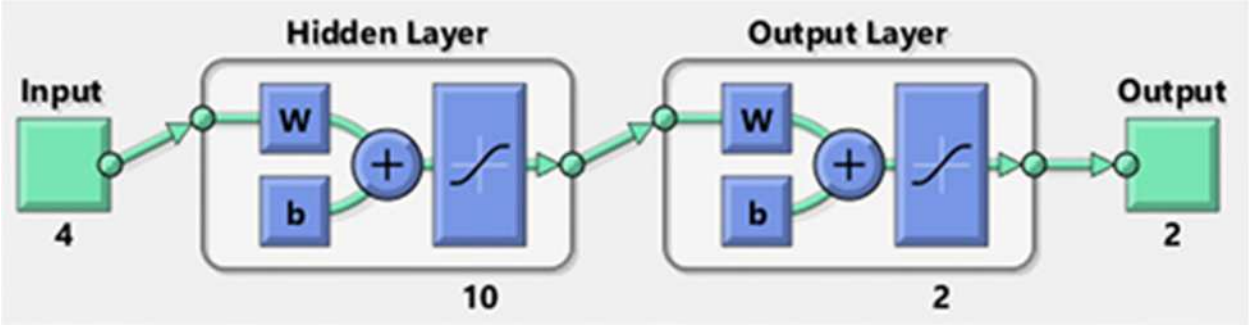
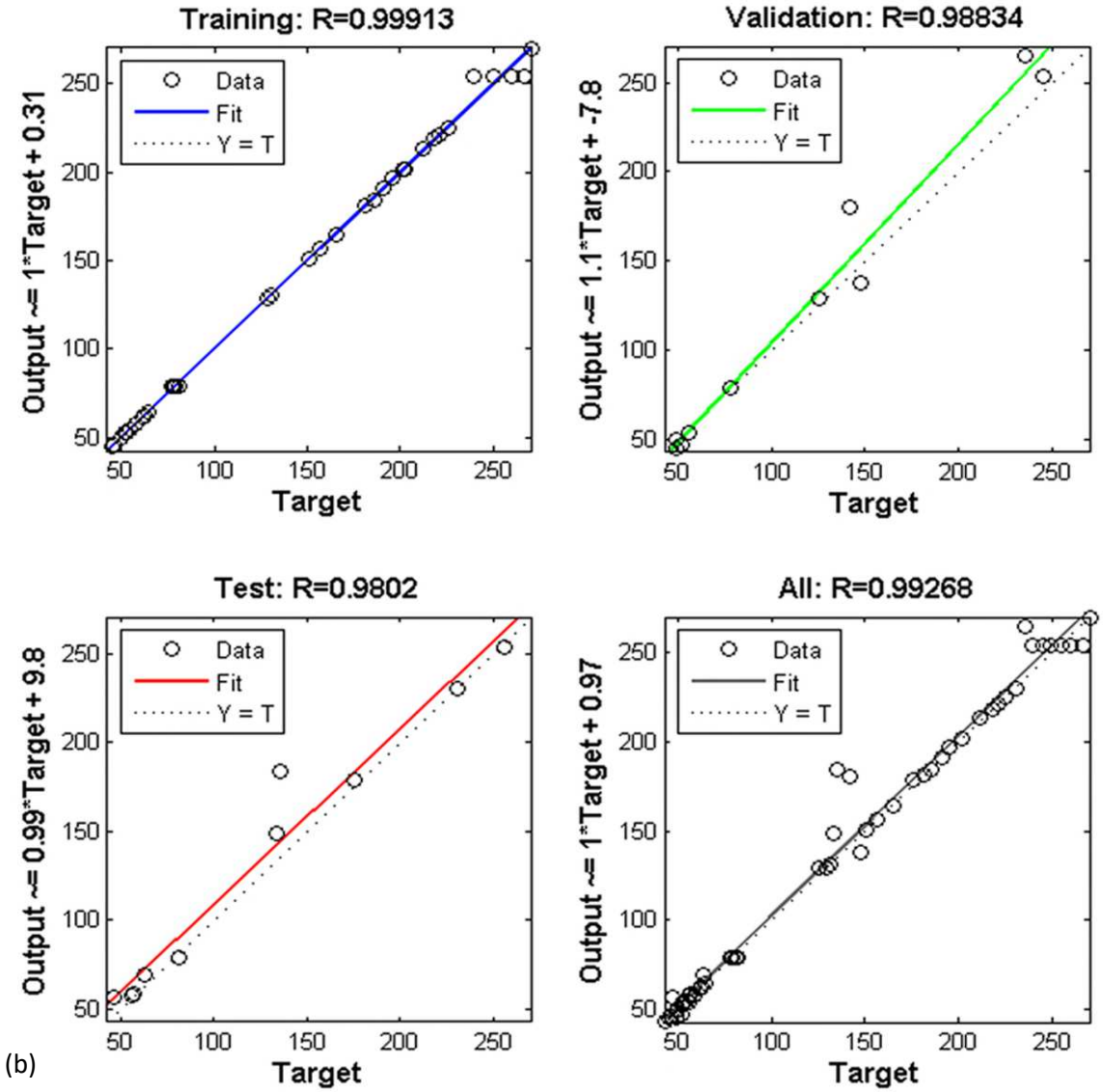


(a)



(b)

Figure 6: Network architecture (a), correlation plots of predicted versus experimental values (b) for the developed ANN model

Performance assessment of the predictive capability of the developed models

The effectiveness of the developed RSM and ANN models to predict the solubility of phosphate and IAA production were measured statistically in terms of the RMSE, R^2, MSE, SEP, MAE, and AARD. The value of R^2 should be close to 1 for a good correlation between experimental and predicted values, indicating a good fit of the model. Although the results showed that both models performed well, the ANN model has stronger modeling potential than the RSM model for solubility of phosphate and IAA production. As can be seen, the predicted ANN values are considerably closer to the experimentally measured data, which shows that the ANN model is superior to the RSM model in terms of predictability (Table 9). The performance of the neural network for predicting the solubility of phosphate and IAA production by Pseudomonas bacteria isolated from rhizospheric soil of coffee plants is very impressive and provides a deeper understanding of the nonlinear system. The results of the statistical analysis showing the comparison between ANN and RSM models are depicted in Table 9.

Table 9: Comparison of predictive abilities of RSM and ANN models

	Solubility of phosphate (µg/ml)		IAA production (µg/ml)	
Parameters	RSM	ANN	RSM	ANN
RMSE	8.190	1.205	2.054	1.686
R^2	0.968	0.999	0.971	0.979
AARD (%)	0.961	0.487	3.234	2.532
MAE	6.863	0.877	1.776	1.396
SEP	4.179	0.615	3.541	2.907
MSE	67.077	1.452	4.219	2.842

Optimization and Validation of the model by using response surface modeling

The main objectives of this study were to determine the optimal operating parameters for the maximum phosphate solubilization and production of IAA by phosphate solubilization bacteria isolated from rhizospheric soils of the coffee plant. The numerical optimization of phosphate solubilization and production of IAA were performed by using statistical experimental design techniques. Solubility of phosphate and production of IAA was set at maximum values while the value of process variables was set in the range under study. To obtain the maximum phosphate solubilization and production of IAA, the predicted combination of process variables were as follows: incubation temperature of 37.5 ºC, incubation time of 9 days, pH of 7.2, and inoculum size of 1.89 OD. Under these conditions, the model predicted solubility of phosphate of 260.69 µg/ml and production of IAA of 80.00 µg/ml with a desirability value of 0.947. To validate the optimum conditions predicted by the model using desirability ramp, triplicate tests were performed under the predicted process parameters, yielding means phosphate solubilization of 260.45 µg/ml and production of IAA of 79.65 µg/ml were obtained. The validity of the estimation models built through the statistical experimental design was verified by the small differences (< 4 %) between the experimental and the predicted responses. The results are closely related to the data obtained from optimization analysis using desirability

functions, indicating CCD incorporates with desirability function could be effectively used to optimize the parameters that affect the solubility of phosphate and production of IAA.

Discussion

Many studies deal with phosphate solubilizing bacteria isolated from rhizospheric soil [30,41-43]. In the present study, the coffee rhizosphere was selected for the isolation of phosphate solubilizing bacteria. Analysis of phosphate solubilizing ability in PVK media containing tricalcium phosphate indicated that all bacteria had phosphate solubilizing ability. Out of six bacteria isolate, three of them observed maximum PSI, PSB1 (5.092) followed by PSB_3 (4.65) and PSB_4 (4.45). The formation of halo in Pikovskaya's agar media is attributed to the production of different organic acids and acid phosphatase production by the PSB isolates [5,17]. This result was in agreement as reported by Anzuay et al.[30,31]. Among all the tested strains, isolate pseudomonads (PSB1) exhibited significant phosphate solubilization as indicated by PSI. A previous study showed that pseudomonas ps. Known to as phosphate solubilizer [44,45].

In addition, Morphological and biochemical tests performed for the PSB isolates led to their probable identification up to genus level. All the isolates were Gram-negative, round raised colonies and of rod-shaped, motile, and most of with shiny surface (Table 4). Results for some of the biochemical tests performed are listed in Table 4. As shown in Table 4, All 3 bacteria have shown negativity for the urease and MR test. Bacteria isolate PSB_1 and PSB_3 were negative for the Citrate and indole test. However, bacteria isolate PSB_4 was positive for VP, Citrate, and indole test. Phosphate solubilization potential has been attributed to the strains ability to produce soluble phosphate and IAA [30,46,5,17,44,47]. The present study showed that *pseudomonas* has the potential to produce IAA and soluble phosphate.

Solubility of phosphate obtained in this experiment was found to be in the range of 125.67 µg/ml - 270.12 µg/ml (Table 5), which is comparable to the phosphate solubilizing bacteria (PSB) isolated from the rhizosphere of Allium hookeri Thwaites (124.8–266.4 µg/mL) [39] and higher than phosphate solubilizing bacteria (PSB) isolated from rhizobacterial salinized soils (44.00– 63.90 mg/L) [38]. In the present work, the higher solubility of phosphate suggests that bacterial strain increases the soluble phosphate content of the soil, thereby improving phosphate absorption. However, incubation temperature, incubation time, pH, and inoculum size are vital to process variables that could have a remarkable effect on the solubility of phosphate. The pH has the most significant effect on the solubility of phosphate whose F value is 43.96, followed by incubation temperature, inoculum size, and incubation time (Table 6). As pH increase from 6–12, it showed an increase in soluble phosphate and attained maximum production (270.12 µg/ml) at a pH value of 7and started decreasing when moved through the pH range 8–12. The least production was 15.33 µg/ml observed at pH 12 [48] showed the maximum phosphorus solubilization of 197 µg/ml at pH 6 in *Bacillus subtilis* KA (1) 5r. The effect of temperature was studied in the range of 25- 45 °C whereby the maximum yield of P-solubilization (184 µg/ ml) was observed at 35 °C (table 6). Present results are in line with [50] (197 µg/m), where the optimum temperature for P-solubilization was at 35 °C using *Bacillus subtilis* KA (1) 5r isolated from the medicinal herb. In the presence of TCP, maximum amounts of P maintained the highest values until

the optimum incubation period of 9 days. The positive correlation of incubation period with P-solubilization was reported by Muleta et al [5]. The solubility of phosphate decreases with increasing inoculum size, this is might be due to the amount of phosphate was immobilized by the bacteria. The effect of inoculums size in this study is similar to previous work [40].

Production of IAA was slightly lower than IAA produced by the cultures growing the isolates in YEM broth amended with 0.2 % of L-tryptophan (52.9–186.65 µg/mL) [39]. IAA production is an indicator of plant growth-promoting rhizobacteria. The results were consistent with previous work of 31.05-88.26 µg/ml IAA [18]. In this study, IAA is found to be significantly affected by all linear process variables (Equation 13). Based on the ANOVA, the IAA production was significantly affected by linear, interactions and quadratic between process variables. Incubation temperature, incubation time, pH, and inoculum size showed significant ($p < 0.05$) effects on the IAA production (Eq. 13). The effect of pH variation on IAA production showed that *pseudomonas* sp. produced more IAA at pH 7, but this production decreased with the further increasing pH. Similarly, previous studies found that the maximum amount of IAA was produced at neutral to slightly alkaline pH [49-51]. Other scholars showed that the maximum yield of IAA was obtained at pH 9 [21,52]. Maximum IAA production of 81.49 µg/mL at a pH of 7 was reported by Baliyan et al.[18]. The variation of IAA production may due to the source of isolation of the microorganisms at particular pH. In the present study, the Maximum yield of IAA (81.02 µg/mL) at 35°C was obtained [53] showed the maximum IAA production at a temperature of 37° in Kl. *Pneumonia* whereas [14] reported the maximum production of 25 µg/mL IAA using various organisms at 30°C. The result indicated that variation of IAA production with temperature may depend on the bacterial species as well. The IAA production was significantly increased when the incubation period increased from 3 to 12 days. Studies on the production of IAA revealed exponentially increasing IAA production with an increase in the incubation period [53,54]. According to Bharucha et al. [49] reported that maximum production of IAA was observed by *Pseudomonas* putida at 96hrs whereas [18] at 8 days.

Conclusions

The results of the present study provide substantial evidence that phosphate solubilizing rhizobacteria isolated from rhizospheric soil of coffee plants facilitate solubilization of free phosphate in soil, and also enhance the growth of the plant. Three of the best P dissolving strains were characterized using different morphological and biochemical tests and the isolate strains were identified as Pseudomonas, Bacillus, and Enterobacter species. Pseudomonas species demonstrated high P dissolving capacities (PSI=5.092) followed by Bacillus (PSI=4.65) and Enterobacter (PSI=4.45). The performance of both the models was compared based on prediction accuracy of the solubility of phosphate and production of IAA. The study revealed that all four variables linearly affect the solubility of phosphate and the production of IAA. Based on the values of errors functions for validation data sets, the ANN model was demonstrated to be more efficient than the RSM model both in data fitting and prediction capabilities. The finding of the study indicated that incubation temperature of 37.5 °C, incubation time of 9 days, pH of 7.2, and inoculum size of 1.89 OD is the best combination of process parameters that result in the highest phosphate solubilization of

260.45 µmg/ml and production of IAA of 79.65 µg/ml. The significant increase in the P content of PSB-treated plants emphasizes the potential of an economically and eco-friendly means of achieving higher levels of phosphorus. Therefore, these P-solubilizers can be used as a plant biofertilizer and may be used to develop an economic cultivation strategy from the rhizospheric soil of coffee plants.

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Competing of Interest: The authors have no conflict of interests to declare that are relevant to the content of this research article.

Ethics approval: Not applicable.

Consent to participate: Not applicable

Consent for publication: Not applicable

DECLARATION: I hereby declare that this manuscript entitled "Modeling and optimization of phosphate solubilizing bacteria isolated from rhizospheric soils of the coffee plant using artificial neural network (ANN) and response surface methodology (RSM)" is the original work not previously published in similar form and not currently under consideration by another Journal. Ana also each Author has seen and approved the content of the submitted manuscript.

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