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Optimization of Phosphate Solubilization by Bacterial Consortium Isolated from Rhizosphere Soil in Obio Akpor, Rivers State, Nigeria

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ABSTRACT

Phosphate solubilization is an important characteristic of rhizobacteria which help them to contribute substantially to the growth of plants. Phosphate solubilizing bacteria are able to convert insoluble phosphate into forms absorbable by plants, thereby improving the yield of crops. This study investigated the optimization of phosphate solubilization by a consortium of two *Pseudomonas* spp and two *Bacillus* spp. isolated from rhizosphere soil from Obio Akpor, Rivers State using response surface methodology based on Box Behnken Design (BBD-RSM). From the data obtained, optimum phosphate solubilization occurred at alkaline pH (pH 8 to 9), inoculum concentration (2.5 to 3.5 %), TCP 1,2,3-Tricalcium phosphate (TCP) concentration (1 to 1.6 mg/L) and mesophilic temperature (33 to 45^{o}C). However, the highest phosphate solubilization (6.5 mg/L) was achieved specifically at pH 7, inoculum concentration 7.5%, TCP concentration 4 g/L and mesophilic temperature of 35^{o}C. A significant ($p = 0.0225$; $F = 3.06$; $R^2 = 0.7535$) quadratic model was obtained for the BBD design, indicating ability of the model to effectively navigate the design space. This study therefore has revealed the capacity of bacterial consortium comprising two *Bacillus* spp and two *Pseudomonas* spp isolated from the rhizosphere of maize to solubilize tricalcium phosphate to orthophosphate, a form that is absorbable by plants. This is an indication that this bacterial consortium can be deployed as suitable bioinoculant for enhanced plant growth.

Keywords: Phosphate Solubilization, Crop Yield, Response Surface, Box Behnken Design, *Bacillus*, *Pseudomonas*.

Introduction

Plants require nutrients in absorbable form to be able to function well. Some essential nutrients such as nitrogen and phosphorus may not be in a readily absorbable form and thus may require conversion to forms absorbable by the plant (Karthika *et a*l., 2018). Phosphorus is an essential nutrient for plants' growth. It is one of the seventeen essential plant's nutrients. Therefore when it is not available, plant's growth and productivity may be greatly affected in the soil (Balemi & Negisho, 2012). In certain soils, these nutrients are not usually in sufficient form and mostly exist in forms not immediately utilizable by plants. Certain bacteria called rhizobacteria associated with the rhizosphere of plants can play beneficial roles including the conversion of minerals present in the soil into soluble forms that plants can readily utilize for their growth and productivity (Ahemad & Kibret, 2014; Elshahat *et al*., 2016; Etesami & Adl, 2020). Rhizobacteria have been comprehensively documented as effective phosphate solubilizers.

Rhizobacteria achieve phosphate solubilization by secreting certain organic acids including citric, formic and gluconic acids (Emami *et al*., 2020). The ability of many bacteria to solubilize phosphate allows them to convert insoluble phosphate into forms absorbable by plants, thereby improving the yield of crops (Hariprasad & Niranjana, 2009). These rhizobacteria spread across different genera but *Bacillus* species have been heavily implicated (Khan *et al*., 2013).

Thus, phosphate solubilization is a very important mechanism for enriching soil with absorbable phosphate form especially in matrices deficient of nutrient (Kumawat *et al*., 2017). This makes phosphate-solubilizing microorganisms one of the most important soil components as they contribute to the fertility of the soil (Khan *et al*., 2013). In order to achieve maximum yield of soluble phosphate by rhizobacteria, the fermentation condition must be set at optimum values. Certain cultural and nutritional conditions capable of enhancing the production of phosphate by rhizobacteria include factors such as:

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Inoculum concentrations (Patel & Panchal, 2020), pH (Marra *et al*., 2015 and Sanchez-Gonzalez *et al*., 2022), concentration of the inorganic phosphate (Zhang *et al*., 2021), temperature (Barin *et al*., 2022) and several others.

Several studies have deployed response surface methodology (RSM) for the optimization of fermentation parameters for enhanced phosphate solubilization by microorganisms. RSM is a statistical and mathematical tool for optimizing a response that is influenced by some independent variables. It can simultaneously investigate the effect of different parameters on specific response(s) without the need to carry them out one at a time as in the case of one-variable-at-time (OVAT) method (Boodaeng *et al*., 2023).

This study was designed to optimize cultural and nutritional variables for enhanced phosphate solubilization by rhizobacterial consortium from the rhizosphere of Maize (*Zey mays*) using response surface design-Box Behnken Design (BBD).

Materials and Methods

Isolation of Rhizobacteria from Soil

Plant growth-promoting bacteria were isolated from the rhizosphere soil of *Zea mays* located at Obio-Akpor, Rivers State, Nigeria. The Map of the study area is presented in Figure 1.

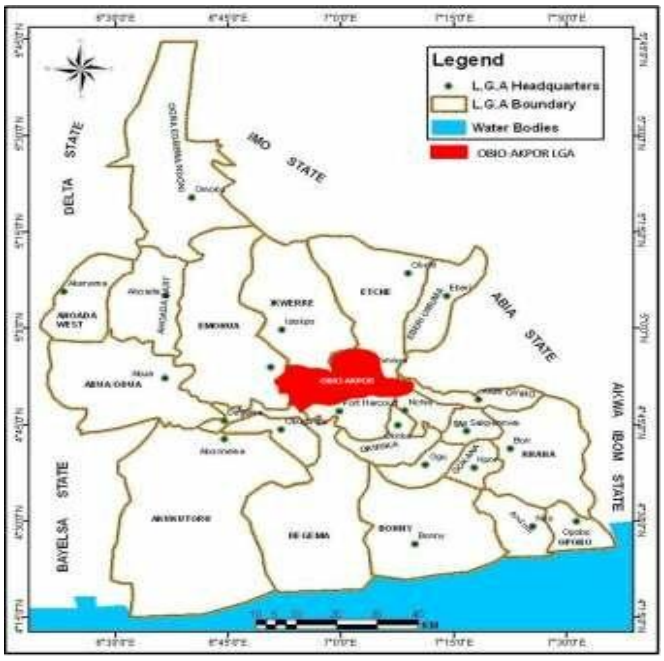

Figure 1: Map of Obio Akpor in Rivers State, Nigeria (Njoku-Tony *et al*., 2020)

Soil samples were collected following standard methods described by Pepper et al. (2015). Physicochemical analysis carried out on the rhizosphere soil included soil texture, pH and temperature. Bacterial isolation was carried out as follows: 10g of rhizosphere soil was weighed into a 250-mL flask. Ninety millilitres (90 mL) of sterilized distilled water was added to the soil sample and mixed properly using a votex mixer. The flask was incubated at 120 rpm for 10 min on a rotary shaker. Afterwards, one millilitre of the homogenized mixture was diluted in a ten-fold serial dilution and 0.1 mL of dilutions 10^{-4} and 10^{-6} were spread-plated on sterilized Nutrient Agar (Himedia, India). The inoculated medium was incubated at 30°C for 3 days. Discrete colonies were picked from the plates and streaked on freshly prepared nutrient agar plates. Colonies of pure bacterial isolates were observed for morphological characteristics and stored in an agar slant for further analysis.

Screening of bacterial isolates for phosphate solubilization

A total of thirty (30) rhizobacteria bacteria were isolated from the rhizosphere soil of *Zea mays* located at Obio-Akpor. These isolates were screened for their ability to solubilize phosphate using the method described by Gupta *et al*. (2022). This method is both qualitative and quantitative. For the qualitative method, Pikovskaya's agar plates were prepared. Wells, measuring 10 mm diameter, were made with sterile cork borer on the agar plates. The bacteria were first grown in nutrient broth for 72 h at 28 ± 2°C and 10 ml cell-free supernatant from each of the bacterial strain was dispensed into each well. The plates were incubated at 28 ± 2°C for 1 day and the formation of yellow zone around the wells confirmed phosphate solubilization. Phosphate solubilization was determined by measuring the zone of clearance (mm) around the colonies as proposed by Nguyen *et al*. (1992).

Quantitative estimation of soluble phosphate (orthophosphate) was carried out according to the method described by Gupta *et al*., (2022). Spectro vanadomolybdate phosphoric yellow colour method was adopted based on the calibration of orthophosphate using KH_2PO_4. Five milliliter (5 mL) of ammonium molybdate reagent was added to 10 mL culture supernantant, and the mixture was shaken vigorously for proper mixing. Thereafter, 1 mL of chlorostannous acid (prepared by mixing 0.25g of $SnCl_2{\cdot}2H_2O$ in 1 mL of concentrated HCl, and then diluting to 10 mL with distilled water).

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The optical density (OD) was measure at 600 nm. The concentration of soluble phosphate was determined from KH_2PO_4 calibration curve.

Identification of the phosphate-solubilizing bacteria

The bacterial isolates were subjected to several biochemical tests as described by Alsina & Blanch (1994). In addition, the morphological and microscopic characteristics of the isolate were recorded.

Experimental design for RSM optimization of Phosphate production by the bacterial consortium

Four (4) independent variables; temperature (25 to 45 ^oC), pH (5 to 9), inoculum concentration (2.5 to 7.5 %) and tryptophan concentration (10 to 30 µL/mL) at three (3) levels, screened through twenty-nine (29) different experimental runs (Tables 1 and 2), with the insignificant ones eliminated to obtain a smaller and more fitting collection of factors were performed. Minimum and maximum values of independent variables investigated in cultivation medium and their centre points are given in Table 1. The Box Behnken Design (BBD) comprised eight (24) factorial points and five (5) centre points. The centre point was repeated to obtain a reliable estimate of the experimental error. This ensured adequate estimation of the variation of the response, thereby providing the required number of degrees of freedom for sufficiently testing the model. On establishing the critical factors, the BBD was used to generate a quadratic model that comprised factorial trials used in estimating quadratic effects and central points to determine the variability of the pure process with Phosphate (mg/L) as the response. Design Expert version 13 was used in designing, analysing and interpreting experimental data obtained through BBD. The system's behaviour is explained by quadratic equations given in Eq. 1 and 2 (Behera et al., 2018).

Table 1: Factors and their levels in the optimization of phosphate production by bacterial consortium using Box Behnken Design - response surface methodology (BBD-RSM).

Code	Variables	-1	0	+1
A	pH			
B	Tricalcium phosphate, TCP (g/L)	1	2.5	4
C	Inoculum concentration (%)	2.5	5	7.5
D	Temperature (°C)	25	35	45

$$Y = \beta o + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j + \varepsilon \quad \textbf{Eq. 1}$$

Where:
Y = the response variable,
X_i (where i=1,2,3,4 = 1, 2, 3, 4i=1,2,3,4) = the coded levels of the four factors,
β_0 = intercept (constant) term,
β_i = linear coefficients (main effects of each factor),
B_{ii}= quadratic coefficients (squared terms for each factor),
β_{ij} = interaction coefficients (interactions between pairs of factors
Ɛ = the error term.

With the expanded quadratic model given as:

$$Y= \beta_0+\beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4 + \epsilon \quad \textbf{Eq. 2}$$

Where:
Y = Phosphate in mg/L,

β0 = Intercept,
$\beta_1, \beta_2, \beta_3, \beta_4$ = linear coefficients (main effects of each factor)
$\beta_{11}, \beta_{22}, \beta_{33}, \beta_{44}$ = quadratic coefficients (squared terms for each factor)
X_1, X_2, X_3 and X_4 = Factors 1, 2, 3 and 4 in this case pH, TCP concentrations, inoculum concentration and temperature, respectively,
$\beta_{12}, \beta_{13}, \beta_{14}, \beta_{23}, \beta_{24}, \beta_{34}$ = interaction coefficients (interactions between pairs of factors)
Ɛ = the error term.

Results

The result of the physiochemical characteristics of the rhizosphere soil is presented in Table 2. It revealed that the soil contained 0.473 mg/Kg phosphate, 8.15% organic matter, 1.08% nitrogen and 170.52 µS/cm electrical conductivity.

Table 2: Physicochemical characteristics of *Zea mays* rhizosphere soil

Parameter	Value
Phosphate (mg/kg)	0.473
E.C (µS/cm)	170.52
Organic Matter (%)	8.15
Nitrogen (%)	1.08

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Individual screening characteristics of the bacteria isolated from the rhizosphere soil of *Zea Mays* revealed efficient phosphate solubilizing capacity of the bacteria and the result is presented in Table 3. Isolates AO2, NFV2 and AO4, DCC6 had zones of clearance with diameters of 13mm, 9mm, 8mm and 5mm, respectively. These four top isolates with the highest zones of clearance were selected to constitute the bacterial consortium. The bacterial consortium prior to optimization had a maximum phosphate concentration of 3.22 mg/L.

Table 3: Clearance zones of the different isolates screened for phosphate solubilization

Sample Code	Halo zone (mm)
AO1	-
AO2 (S1)	13
AO3	-
AO4 (S2)	8
DCC1	-
DCC2	-
DCC3	-
DCC4	-
DCC5	-
DCC6 (S3)	5
NFVI	
NFV2 (S4)	9
NFV3	-
NFV4 (S5)	-
NFV5	-
NFV6	3
DFR1	-
DFR2	-
DFR3	-
NASG1	-
NASG2	-
NASG3	-
NASG4	-
NASG5	-
DR1	-
DR2	2
DR3	-
DR4	-
DR5	-

The bacterial consortium was identified as members of *Pseudomonas* and *Bacillus* genera based on morphological, microscopic and biochemical. Morphological, microscopic and biochemical characteristics of the bacteria isolates are presented in Table 4.

Table 5 presents results of the optimization of soluble phosphate production using RSM- BBD.

The composition of various experiments of the BBD for independent variables: pH, TCP concentration, inoculum concentration and temperature, and response phosphate (mg/L) are presented in Table 5. The Table shows the actual values for phosphate produced at different experimental runs using the bacterial consortium.

The fitting of model and ANOVA for the production of phosphate by the bacterial consortium:-

Summary of ANOVA for response surface quadratic models for phosphate production (mg/L) by the bacterial consortium is given in Table 6. Model F-value of 3.06 was obtained, with P-value of 0.0225 (<0.0500) implying that the model is significant. This means that there is only a 2.25%% chance that an F-value this large could occur due to noise**.** Model terms AB, BC, BD, B^2, D^2 were significant.

The Lack of Fit F-value of 0.64 means that the Lack of Fit is not significant relative to the pure error. There is a 73.98% chance that a Lack of Fit F-value this large could occur due to noise. Coefficient of determination (R^2) obtained from the model was 0.7535.

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Table 4: Cultural, Morphological, Biochemical Characteristics and Probable Identity of the Phosphate Solubilizing Bacteria that Constituted the Consortium

Isolate Code	Cultural Morphology		Microscopy		Biochemical							Sugar Fermentation					Probable Organism
	Color	Shape and Appearance of Colony	Gram Reaction	Cell Shape	Catalase	Motility	Oxidase	Citrate	Gas Production	Indole	Starch	Glucose	Sucrose	Galactose	Lactose	Maltose	
AO2	Cream	Flat, entire, opaque & smooth	- ve	rod	+	+	+	-	+	-	-	A	-		A	A	*Pseudomonas* sp.
AO4	Cream	Flat, entire, opaque & smooth	- ve	rod	+	-	+	-	+	-	-	A	-		A	A	*Pseudomonas* sp.
DCC6	Orange	Entire, flat, opaque & smooth	+ ve	rod	+	+	+	-	+	-	+	A	-		-	A	*Bacillus* sp.
NFV2	Cream	Flat, entire, opaque & smooth	- ve	rod	+	-	+	-	+	-	-	A	-		A	A	*Pseudomonas* sp.

Key: + = positive; - = negative; A = acid

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Table 5: RSM-BBD employed for independent variables and various experiments' composition

Run	A: pH	B: D. TCP (g/L)	C: Inoculum (%)	D: Temp. (°C)	Phosphate Conc (mg/L)
1	7	4	7.5	35	6.5
2	7	1	5	45	6.1
3	5	4	5	35	5.8
4	7	1	2.5	35	4.8
5	9	2.5	7.5	35	4.6
6	7	2.5	5	35	4.6
7	9	1	5	35	4.5
8	7	2.5	5	35	4.5
9	7	4	5	25	4
10	7	1	7.5	35	3.8
11	5	2.5	7.5	35	3.8
12	7	4	5	45	3.7
13	9	2.5	2.5	35	3.6
14	7	2.5	5	35	3.5
15	9	2.5	5	45	3.2
16	9	2.5	5	25	3.2
17	5	2.5	2.5	35	3.1
18	7	2.5	7.5	45	3
19	7	4	2.5	35	3
20	9	4	5	35	2.9
21	7	2.5	5	35	2.9
22	7	2.5	2.5	45	2.4
23	7	2.5	2.5	25	2.3
24	7	2.5	5	35	2
25	7	2.5	7.5	25	1.8
26	7	1	5	25	1.7
27	5	1	5	35	1.7
28	5	2.5	5	25	1.6
29	5	2.5	5	45	1.5

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Table 6: Equation of the parameters for phosphate production by the bacterial consortium as the function of temperature, initial pH, inoculum concentration and tryptophan concentration in coded factors

Source	F-value	p-value
Model type	Quadratic	
Model	3.06	0.0225
A-Temperature	1.85	0.1950
B-pH	1.48	0.2444
C-Inoculum concentration	1.57	0.2301
D-Tryptophan	3.68	0.0758
AB	9.04	0.0094
AC	0.0250	0.8765
AD	0.0028	0.9587
BC	5.64	0.0324
BD	6.15	0.0265
CD	0.3367	0.5709
A^2	0.4665	0.5058
B^2	5.48	0.0346
C^2	0.1536	0.7011
D^2	5.58	0.0332
Residual		
Lack of Fit	0.6437	0.7398

Key: A = Temperature; B = pH; C = Inoculum concentration; D = Tryptophan concentration

Final equation in terms of coded factors

Equation in terms of coded factors for the production of phosphate by the bacterial consortium is given in Equation 3. The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Phosphate (mg/L) = +3.48 + 0.3738A + 0.3338B + 0.3446C+0.5561D-1.42AB +0.0750AC+ 0.0263AD + 1.13BC-1.23BD+0.2887CD-0.2542A^2+0.8708B^2 + 0.1458C^2-0.9693D^2 **Eq. 3**

Where: A, B, C. and D represent the values for the temperature, pH, Inoculum concentration, and Tryptophan concentration, respectively.

The combined effects of pH-temperature, pH-inoculum concentration and pH-tryptophan concentration on phosphate production by the bacterial consortium are presented in Figure 2. The pH ranges that had the optimum effect on phosphate production for the bacterial consortium is 8 to 9.

Combined effects of TCP concentration-pH, TCP concentration-inoculum concentration and TCP concentration-temperature phosphate production is presented in Figure 2. The TCP concentration range that had the optimum effect on phosphate production was 1 to 1.6 g/L.

The combined effects of inoculum concentration-pH and inoculum concentration-tryptophan concentration and inoculum concentration-temperature on phosphate production is presented in Figures 2. The inoculum concentration range that had the optimum effect on phosphate production was 2.5 to 5.5 %.

The combined effects of temperature-pH, temperature-TCP concentration and temperature-inoculum concentration on phosphate production is presented in Figure 2. The temperature range that had the optimum effect on phosphate production was 33 to 45 °C.

The interaction effects of the many variables on phosphate production by the bacterial consortium studied by plotting 3D curves against any two given independent variables, while keeping others at central level. Figure 3 reveals the plots of predicted values against actual values.

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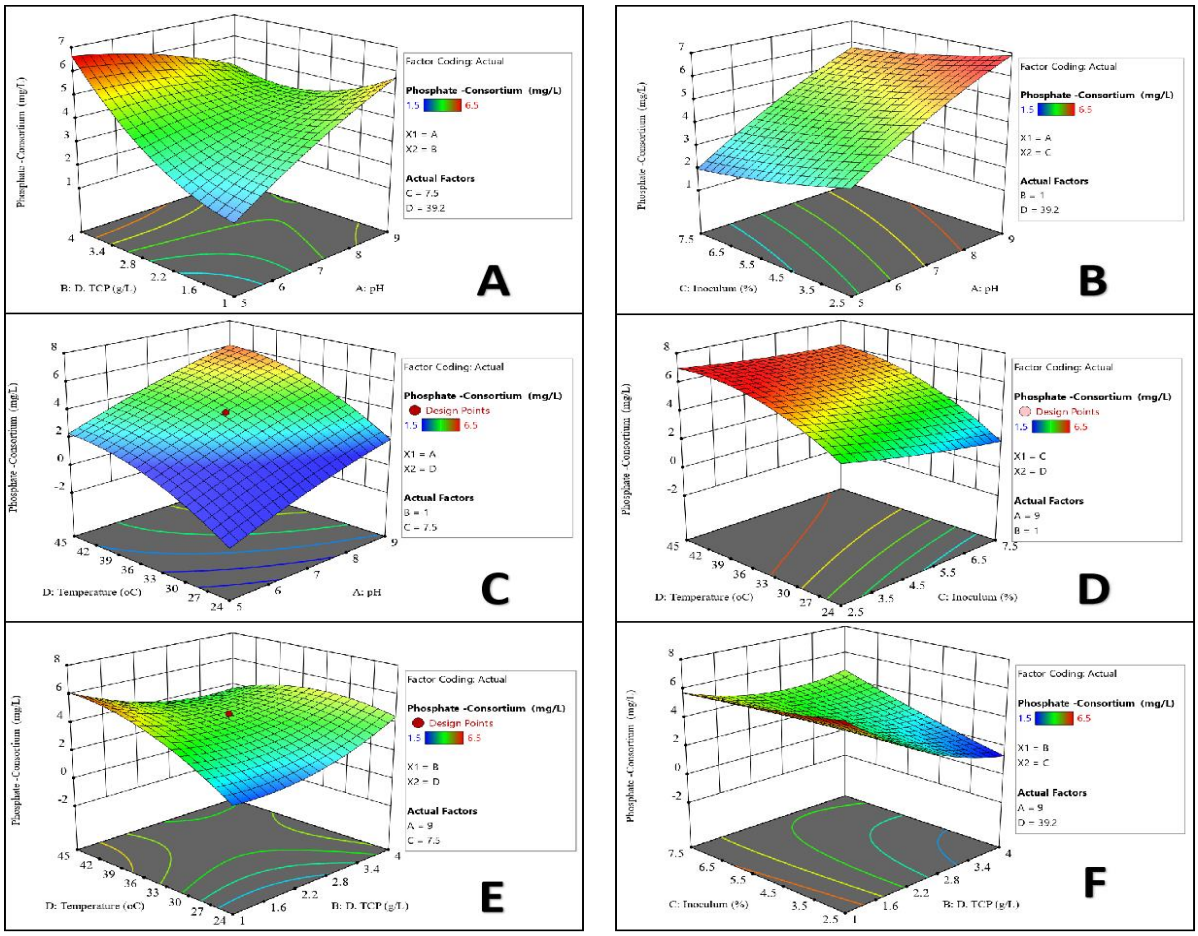

Figure 2: Response surface 3D for phosphate production from the bacterial consortium in batch fermentation as a function of pH and TCP concentration (g/L) (A), pH and inoculum concentration (%) (B), pH and temperature (ºC) (C), TCP concentration (g/L) and inoculum concentration (%) (D) TCP concentration (g/L) and temperature (ºC) (E), and (ºC) and inoculum concentration (%) temperature (ºC) (F).

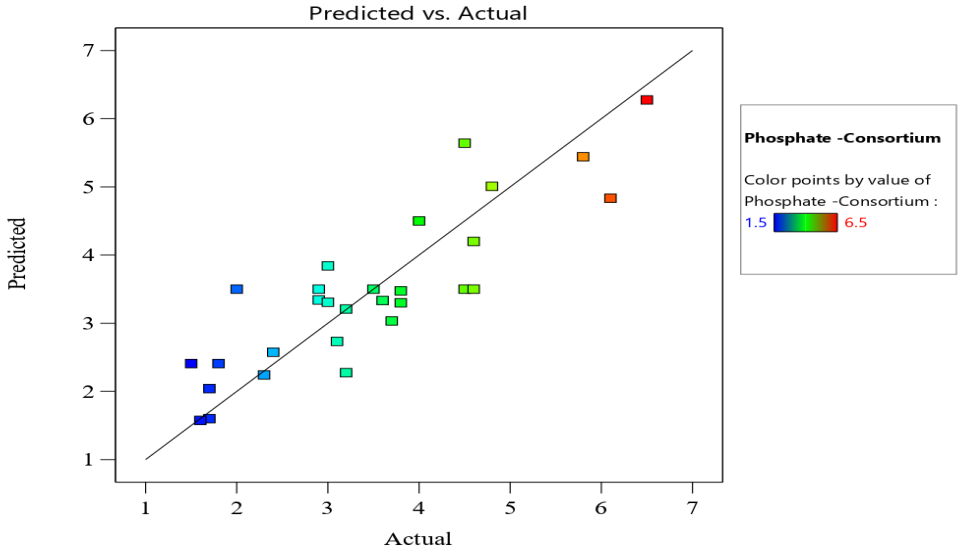

Figure 3: Actual and predicted values from phosphate production based on quadratic modelling of the interactions between independent factors

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Discussion

In this study the capacity of bacterial consortium isolated from rhizosphere of soil to solubilize phosphate was investigated and the parameters that affect this activity optimized using response surface methodology - Box Behnken Design. Rhizosphere bacteria play vital role in the soil, one of which include conversion of inorganic phosphorus to soluble phosphate utilizable by plants (Pan & Cai, 2023), a characteristic this present study corroborates.

Reports of rhizobacterial solubilization of phosphate abound but few had investigate this activity by bacterial consortium. This is important in mimicking the natural mechanism. The bacterial consortium were identified as belonging to the genera *Bacillus* and *Pseudomonas*. Many studies have reported the capacity of *Bacillus* spp and *Pseudomonas* spp to solubilize phosphate. Saeid *et al*. (2018) reported efficient solubilization of phosphate by *Bacillus* sp enriched with fish, with up to 483 ± 5 mg/L of released phosphate. In another study, Bakki *et al*. (2024) reported that phosphate solubilizing *Pseudomonas* and *Bacillus* combined with rock phosphates to promote tomato growth by reducing bacterial canker disease.

Therefore the phosphate solubility bacteria reported in this study are well known for this specific activity. However, not many studies have investigated their synergistic effect as carried out in this present study.

A few reports however that different bacterial species could benefit from a synergistic effect in their production of soluble phosphate exist. A study by Blanco-Vargas *et al*. (2020) reported that phosphate-solubilizing *Pseudomonas* sp., and *Serratia* sp. co-culture enhanced the growth of *Allium cepa* L. Also, Ríos-Ruiz *et al*. (2024) reported enhanced phosphate solubilization by co-inoculation of phospahte-solubilizing bacteria (*Micrococcus* sp. Sfcm-14-01, *Agrobacterium* sp. Sfl-043-09, and *Enterobacter* sp. Sfcm-014-02 and Sfcm-054-06) along with rhizobia (*Ensifer terangae* R1-012-02 and *Bradyrhizobium glycinis* Rcm-025-01).

Bacterial co-culture tend to do better in solubilizing phosphate than single bacterium as the combined activity of the bacteria usually result in higher release of phosphate.

Certain conditions must be optimal for efficient phosphate solubilization. The optimal conditions observed for phosphate-solubilizing bacterial consortium in this study were alkaline pH (pH 8 to 9), inoculum concentration (2.5 to 3.5 %), TCP concentration (1 to 1.6 g/L) and mesophilic temperature (33 to 45^{o}C). These are in agreement with the reports of Agboola *et al*. (2023) on efficient production of soluble phosphate at optimal pH of 9 and temperature of 35^{o}C. Different bacteria have their optimum growth conditions, which subsequently impacts on their biosynthetic characteristics. Temperature and pH are key factors that affect biosyntheic capacity of microorganisms. This is because, the biosynthetic enzymes are temperature and pH sensitive and therefore function best at optimum conditions (Thakur *et al*. (2019) and Tyc *et al*. (2017). However, other researchers such as Sanchez-Gonzalez *et al*. (2022) and Marra *et al*. (2015) have reported efficient phosphate production at pH 5 to 5.5. Similarly, the findings of this study on the effect of temperature, differs from that reported by Sarikhani *et al*. (2019). Their report showed that higher temperature up to 55^{o}C supported phosphate solubilization by the rhizobacterial strain.

Inoculum concentration and TCP concentration were the other factors examined for their effect on phosphate solubilization. The respective optimum inoculum concentration and TCP concentration were 7.5% and 4 g/L. Bacterial seed inoculum size can affect phosphate solubilization (Zhang *et al*. 2021). This is because the size of bacterial cells can significantly affect the yield of biomass in the fermentation medium, which subsequently affects the the rate at which the organisms converts the inorganicc phosphorus to soluble phosphate. High inoculum concentration may result in excess competition for the substrate thereby reducing yield whereas too low inoculum size may not be sufficient to result in significant yield. The seeding bacteria concentration must therefore be optimum for efficient phosphate solubilization. The findings of this study are consistent with Patel and Panchal (2020), who reported that a higher inoculum concentration generally leads to increased phosphate production due to the higher biomass available for solubilization activities. Significant interactions observed between pH, TCP concentration, inoculum density, and temperature show how complex microbial processes are and their dependence on specific conditions.

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Similar findings were noted by Barin *et al*. (2022), where RSM-based models also highlighted interaction effects as critical factors influencing microbial efficiency.

The model's R^2 value of 0.7535 obtained in this study and significant model terms such as AB (temperaure and pH), and BD (inculum and tryptophan concentration) are indicators of the role of these factors in enhancing phosphate solubilization. Therefore, when taken into consideration their interactive effect can result in efficient phosphate solubilization.

Conclusion

This study revealed that the bacterial consortium comprising two *Bacillus* spp and two *Pseudomonas* spp isolated from the rhizosphere of maize had the capacity to solubilize tricalcium phosphate to orthophosphate, a form that is absorbable by plants. The bacterial consortium converted 4 g/L of tricalcium phospahte to a maximum of 6.5 mg/L of orthophosphate under the following conditions: pH 7, temperature of 35ºC and 7.5% inoculum concentration. This finding is therefore an indication that these bacteria consortium can be deployed as suitable bioinoculant for enhanced plant growth.

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