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Optimization of Biofertilizer Formulation for Phosphorus Solubilizing by *Pseudomonas fluorescens* Ur21 via Response Surface Methodology

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Abstract: This study aimed to analyze and quantify the effect of different ratios of vermicompost, phosphate rock, and sulfur on P solubilization and release by *Pseudomonas fluorescens* Ur21, and to identify optimal levels of those variables for an efficient biofertilizer. Twenty experiments were defined by surface response methodology based on a central composite design (CCD), and the effects of various quantities of vermicompost, phosphate rock, and sulfur (encoded by −1, 0, or +1) on P solubilization was explored. The results show that the CCD model had high efficiency for predicting P solubilization (R^2 = 0.9035). The strongest effects of the included variables on the observed P solubilization were linear effects of sulfur and organic matter (vermicompost), a quadratic effect of phosphate rock, and an interactive effect of organic matter × phosphate rock. Statistical analysis of the coefficients in the CCD model revealed that vermicompost, vermicompost × phosphate rock, and phosphate rock × phosphate rock treatments increased P solubilization. The optimal predicted composition for maximal P solubilization by *P. fluorescens* Ur21 (at 1684.39 mg·kg^{-1}, with more than 90% of the added phosphate dissolved) was 58.8% vermicompost, 35.3% phosphate rock, and 5.8% sulfur. ANOVA analysis confirmed the model's accuracy and validity in terms of *F* value (10.41), *p* value (<0.001), and non-significant lack of fit.

Keywords: biofertilizer; central composite design; modeling; phosphate solubilizing bacteria

1. Introduction

The soils of arid and semi-arid regions are mainly calcareous and have high pH, with low organic matter content, and hence a low content of both macro- and micro-nutrients for plants [1,2]. Together with nitrogen and potassium, phosphorus (P) is one of the most important nutrients for plants, and is also a major component of agricultural fertilizer [3]. P plays key roles in myriads of physiological processes in plants, including cell division, photosynthesis, respiration, energy transfer, and the synthesis of nucleic acids, proteins, and carbohydrates [4]. In soils, it is present in both organic forms (nucleic acids, phospholipids, phosphoproteins, inositol phosphates, and phosphosugars) and inorganic forms (calcium, magnesium, iron, and aluminum phosphates) [5]. Plants absorb phosphorus from soil in the form of phosphate, but P readily forms low-solubility complexes with soil materials [6]. Thus, even when the P concentration in soil is high, it generally has low mobility and availability to plants. The concentration of soluble P in soil is usually, at most, about 1 mg·kg^{-1} [7], and its lack of availability frequently limits plant growth.

In addition, substantial proportions of P in chemical fertilizers are converted to insoluble forms and fixed in the soil. Thus, agricultural soils commonly contain high quantities of

insoluble P, and it is essential to release it from its insoluble forms to increase its availability to crops [8]. Water-soluble chemical fertilizers, such as superphosphate, $Ca(H_2PO_4)_2 \cdot H_2O$, are commonly applied in conventional farming. However, their use should be minimized due to the fixation of their P contents in the form of iron/aluminum phosphate in acidic soils [9] and calcium phosphate in neutral to alkaline soils [10]. This may have adverse environmental impacts on soil health, as well as terrestrial and freshwater resources [11], including the eutrophication of surface waters [12], microbial diversity [13], and microbial respiration [14]. Phosphate rock (Ca_5 $(PO_4)_3$(F, Cl, OH)) is also extensively used in organic farming to increase soil P contents, but it has low solubility [15].

Clearly, there are urgent needs to increase the efficiency of soil applications of P for meeting crops' requirements, reducing the use of phosphate fertilizers by developing alternative, sustainable methods. An increasingly widely applied strategy is to exploit phosphate-solubilizing microorganisms in the soil as biofertilizers. For example, several plant growth-promoting rhizobacteria have been effectively used to increase crop yields, soil fertility, and the sustainability of agriculture and forestry [16,17]. According to a common definition, a biofertilizer is a "formulated product containing one or more microorganisms that enhance the nutrient status (the growth and yield) of the plants by either replacing soil nutrients and/or by making nutrients more available to plants and/or by increasing plant access to nutrients" [18].

Some microorganisms can promote plant growth through other mechanisms, such as the production of phytohormones, environmental stress relief, or prevention of plant diseases [16]. However, application of efficient phosphate-solubilizing microorganisms is a key strategy to meet plants' P requirements, and this is the focus of this study. These microorganisms convert mineral and unavailable P into soluble forms via processes such as acidification, chelation, and exchange reactions, thereby increasing plant P uptake and reducing the need for phosphate-containing chemical fertilizers [19]. Several species of soil bacteria and fungi can convert low-solubility P compounds into plant-absorbable forms [20,21]. The mechanisms involved are not completely understood, but controlled experiments indicate that they reduce the pH of their surroundings and solubilize these compounds by the partial oxidation of sugars and the production of organic acids, e.g., acetic, lactic, oxalic, tartaric, succinic, citric, and gluconic acids [22–25]. Various bacteria generally solubilize phosphate more effectively than fungi and are abundant in most soils [26]. These include members of the genera *Pseudomonas*, *Bacillus*, *Achromobacter*, *Burkholderia*, *Rhizobium*, *Pantoea*, *Agrobacterium*, and *Flavobacterium* [27]. Taxa shown to have high potential utility for solubilizing insoluble mineral phosphates include the following species of *Pseudomonas*: *P. putida*, *P. fluorescens* and *P. aeruginosa* [28]. However, diverse factors, such as types and abundance of carbon and nitrogen sources, temperature, pH, aeration, and incubation period affect these microorganisms' P solubilization efficiency [29]. Therefore, it is important to consider the effects of these factors when investigating the microbial solubilization of P.

An important application of phosphate-solubilizing microorganisms is in the production of microbial fertilizers containing specific microbes, minerals, organic matter, chemical fertilizers, and fillers, which are applied similarly to chemical fertilizers. For example, granulated phosphate microbial fertilizer, which comes in a solid granulated or powdered form and contains P-solubilizing microbes, is commonly used in agriculture to meet crops' P demands [30]. This reportedly exhibits efficacy similar to triple superphosphate in maize cultivation at farms in several Iranian provinces [31]. The phosphate microbial fertilizer used in the cited study comprised 60% phosphate rock, 20% sulfur, 16% organic matter, 4% zinc sulfate, and at least 10^5 phosphate-solubilizing *Bacillus coagulans* bacteria per gram. Another (greenhouse) study showed that the application of phosphate microbial fertilizers with six bacterial strains in a base substrate of phosphate rock, sulfur, and bagasse (in a 14:15:30 *w/w/w* ratio) significantly improved ($p < 0.05$) maize plants' root and shoot fresh and dry weights, chlorophyll index, and both root and shoot P uptake [32]. Further-

more, some microbial fertilizer treatments resulted in higher yields than chemical triple superphosphate treatment.

Due to the key role of biofertilizers' filler components in solubilizing low-solubility phosphates, there have been extensive efforts to identify the most effective substrates [33]. Optimization of both the substrate and ratio of microbial fertilizer components is important to maximize P solubilization. In conventional optimization approaches, one factor is changed at a time, while the others are kept constant. However, this is time-consuming, costly, and may not identify optimal combinations of factors due to interactive effects [26]. Thus, statistical methods, such as the Plackett–Burman design and response surface methodology (RSM) can be highly useful for identifying metabolically optimal conditions [34]. These techniques involve the use of statistical and mathematical methods to design experiments and develop predictive mathematical models for assessing responses of a dependent variable to selected factors (independent, predictor variables) [35]. RSM is widely used for modeling processes in which a response of interest is affected by multiple independent variables, and the objective is to optimize the response [36]. Given the significance of the composition and ratios of biofertilizer constituents, the aim of the study presented here was to model the effects of different levels of organic sources and sulfur in a P biofertilizer formulation on phosphate rock solubilization using a promising bacterial strain, *P. fluorescens* Ur21, using RSM [37].

2. Materials and Methods

2.1. Inoculant Preparation

The *Pseudomonas fluorescens* strain Ur21 was isolated from a sample of soils obtained from corn and wheat farm*s* in Urmia County, Iran, and then purified and identified by the Department of Soil Science, Urmia University, Urmia, Iran [37]. The strain was cultured in a nutrient broth (NB) medium and shaken at 120 rpm at 28 °C for 24 h. Next, an inoculant was prepared at a population of 10^8 cfu mL^{-1}.

2.2. Sample Preparation and Phosphate Solubilization Modeling

Vermicompost (organic matter), phosphate rock, and sulfur were powdered and passed through a 140-mesh screen. A central composite design (CCD) was used to model and predict the effects of different levels of organic matter (vermicompost), sulfur, and phosphate rock in the biofertilizer (independent variables) on the bacterial strain's P solubilization capability (assayed using the molybdate/vanadate method described below) as the dependent variable. The ranges of the variables were organic matter (5–50 g), sulfur (0–30 g), and phosphate rock (20–80 g) and were applied in the empirical experiments at five levels, determined by the CCD model described below (Table 1).

Table 1. The ranges of experimental values of the variables included in modeling.

Factors	Coded	Levels				
	x_i	+1.68	+1	0	−1	−1.68
Organic matter (g)	x_1	50	40.9	27.5	14.1	5
Sulfur (g)	x_2	30	23.9	15	6.1	0
Phosphate rock (g)	x_3	80	67.8	50	32.2	20

In total, 20 experimental variations were designed using Minitab 14 software, by combining different levels of encoded values of the variables (Table 1) based on the defined ranges and number of independent variables (Table 2). The water retention capacity of the compositions was determined, and the amount of water required to drench the samples was calculated. The samples were sterilized in an autoclave at 121°C and 1.5 atm pressure [38]. Finally, the amount of water determined for each composition, along with 1 mL of *P. fluorescens* Ur21 inoculant (with a population of 108 cfu/mL), was poured into a polyethylene package and sealed. The packages were kept at 28 ± 2 °C for 2 months;

then the P content of the solution was estimated using the molybdate/vanadate method by measuring its absorbance at 470 nm using a spectrophotometer [39].

Table 2. The matrix of encoded values of variables in modeling the central composite design method.

Experiment No.	Encoded Values of Variables			Measured Soluble P Mg/kg
	Organic Matter	**Phosphate Rock**	**Sulfur**	
1	0.00	0.00	1.68	102.9
2	1.68	0.00	0.00	794.5
3	1.00	−1.00	−1.00	1259.6
4	1.00	−1.00	1.00	839.2
5	−1.00	−1.00	−1.00	103.6
6	−1.00	1.00	1.00	350.3
7	0.00	0.00	0.00	295.7
8	0.00	0.00	0.00	344.6
9	0.00	−1.68	0.00	1046.6
10	0.00	0.00	−1.68	903.2
11	1.00	1.00	1.00	610.5
12	0.00	0.00	0.00	571.5
13	0.00	0.00	0.00	99.7
14	−1.00	1.00	−1.00	840.2
15	1.00	1.00	−1.00	888.2
16	1.00	−1.00	1.00	73.5
17	−1.68	0.00	0.00	100.8
18	0.00	0.00	0.00	187.4
19	0.00	1.68	0.00	1645.3
20	0.00	0.00	0.00	227.9

The real values of the variables were encoded by Equation (1):

$$X_i = \frac{x_i - x_0}{\Delta x_i} \tag{1}$$

Here, X_i is the encoded value of variable i, x_i is the real value of the variable (x_1–x_3), $x0$ is the mean range of the variable, and Δx is the step change of each parameter.

The real values of the encoded values (1.68, 1.00, 0, −1.00, and −1.68) were 50, 40.5, 27.5, 14.1, and 5 g for organic matter, 30, 23.9, 15, 6.1, and 0 g for sulfur, and 80, 67.8, 50, 32.2, and 20 g for phosphate rock, respectively.

Following RSM [36], a quadratic polynomial function (Equation (2)), with linear, quadratic, and interaction terms based on encoded values of the variables was used to predict the dependent variable:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_0 X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j + \varepsilon \quad i \neq j \tag{2}$$

Here, Y is the response variable (amount of solubilized P), X_i and X_j are the encoded independent variables, k is the number of independent variables, ε is the model residuals (difference between observed values and values estimated by the model), and β_0, β_i, β_{ii}, and β_{ij} are the effects of the y-intercept, linear functions, quadratic function, and interaction of the variables, respectively. To facilitate interpretation of the modeling results and rank the effects of the parameters included in the CCD model (Equation (2)), the percentage effect of each parameter was estimated by Pareto analysis using Equation (3):

$$P_i = \left[\frac{\beta_i^2}{\sum \beta_i^2} \right] \times 100 \quad i \neq 0 \tag{3}$$

Here, P_i is the percentage effect of each variable included in the CCD model, and β_i are the coefficients of the polynomial Equation (2).

3. Results

It should be noted that preliminary experiments showed that phosphorus release was very low (P_{Olsen} = 4.58 mg·kg^{-1}, 7.5% phosphorus as P_2O_5 according to XRF results) under the experimental conditions without the addition of microorganisms. Table 2 presents the composition of the media used in the experiments defined by the CCD with the three variables vermicompost, phosphate rock, and sulfur, as well as amounts of soluble P empirically determined at the end of each experiment. The highest amount of solubilized P (1645.3 mg/kg) was obtained with medium levels of organic matter (27.5 g) and sulfur (15 g), with the maximum level of phosphate rock (80 g). The lowest amount of P (73.5 mg/kg) was obtained with organic matter and phosphate rock levels of −1 and a sulfur level of 1 (Experiment 2, Table 2).

The coefficients for the linear, quadratic, and interaction parameters of the variables are presented in Table 3, based on the CCD and inputs in Table 2 for the independent variables (i.e., organic matter, phosphate rock, and sulfur levels) and the response variable (amount of dissolved P at the end of the experiment). Organic matter (vermicompost) and sulfur had significant linear effects ($p < 0.01$) on the dissolved P concentration, and phosphate rock had a significant quadratic effect ($p < 0.01$). Further, the organic matter × phosphate interaction significantly affected P solubilization ($p = 0.014$).

Table 3. The results of the analysis of variance (ANOVA) of the polynomial model for dissolved phosphate.

Source		Df	Sum of Squares	Mean Square	F-Value	p-Value
Model		9	3,460,020	384,447	10.41	0.001 *
Linear		3	1,473,790	491,263	13.30	0.001 *
	OM	1	844,719	844,719	22.87	0.001 *
	P-Rock	1	147,697	147,697	4.00	0.073 ns
	S	1	481,373	481,373	13.03	0.005 *
Square		3	1,644,226	548,075	14.84	0.001 *
	OM × OM	1	5665	5665	0.15	0.704 ns
	P-Rock × P-Rock	1	1,640,757	1,640,757	44.42	0.000 *
	S × S	1	22,376	22,376	0.61	0.454 ns
2-Way interaction		3	342,004	114,001	3.09	0.077 ns
	OM × P-Rock	1	325,466	325,466	8.81	0.014 *
	OM × S	1	3962	3962	0.11	0.75 ns
	P-Rock × S	1	12,577	12,577	0.34	0.572 ns
Error		10	369,403	36,940		
Lack-of-fit		5	236,594	47,319	1.78	0.271 ns
Pure error		5	132,809	26,562		
Total		19	3,829,424			

P-Rock, phosphate rock; OM, organic matter; S, sulfur; *, statistically significant; ns, not statistically significant. R^2 = 90.35%, Adj-R^2 = 81.67%.

Based on the significant results in Table 3, and associated coefficients of the CCD polynomial function, the following function for predicting the amount of dissolved P at the end of the experiment was formulated (Equation (4)):

$$\text{Dissolved P (mg kg}^{-1}) = 293.4 + 248.7X_1 + 104X_2 - 187.7X_3 + 19.8X_1^2$$
$$+ 337.4X_2^2 + 39.4X_3^2 - 201.7X_1X_2 - 22.3X_1X_3 - 39.6X_2X_3 \tag{4}$$
$$R^2 = 0.9035$$

Here, X_1, X_2, and X_3 are the encoded values of organic matter, sulfur, and phosphate rock levels, respectively (Equation (4)). The equation clearly shows the positive effect of vermicompost on P solubilization. To assess the accuracy of the model derived by CCD

(Equation (4)), the quantities of P released in the CCD experiments were plotted against the quantities predicted by the CCD model (Figure 1). The results show that the model appropriately estimated the amount of P released, accounting for 90.35% of the variation in P solubilization.
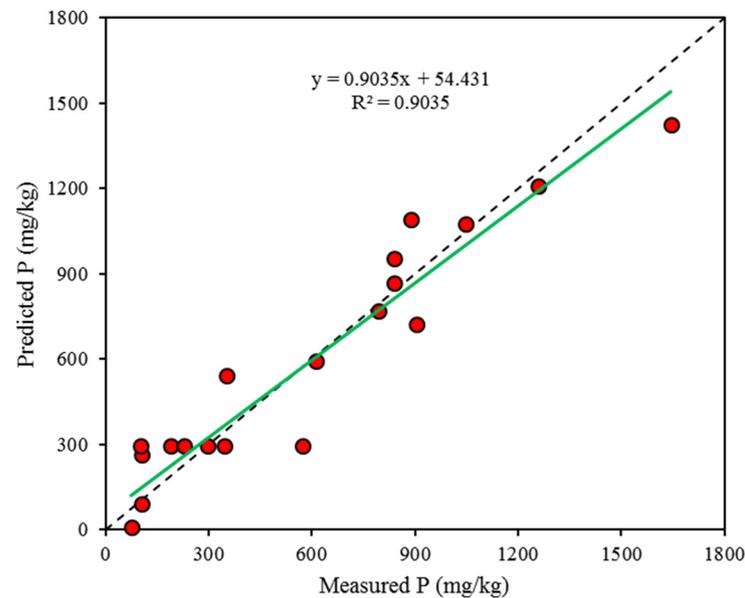

Figure 1. The comparison of the observed dissolved phosphate concentrations and the concentrations predicted by the central composite design model.

Figure 2 shows that the residuals of the CCD model were distributed normally. Based on the results of the residual values, it can be concluded that the model did not systematically overestimate or underestimate the results, and the direction of its error was random rather than a function of the dissolved P content.
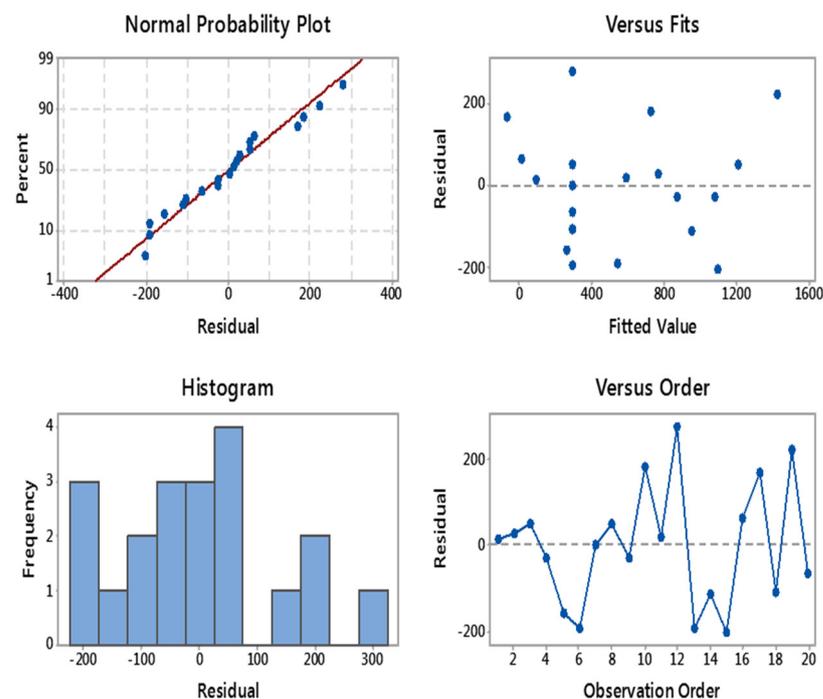

Figure 2. The distribution of the residuals of the central composite design model.

To visualize the ranked effects of the CCD model parameters (Equation (2)), their percentage effects are shown in the Pareto graph in Figure 3. The linear effects of organic matter (vermicompost) and phosphate rock, the quadratic effect of phosphate rock, and the organic matter × phosphate rock interaction were the strongest tested effects on P solubilization, with percentage effects of 42.72, 23.21, 15.27, 13.23, and 4.06%, respectively. The total percentage effect of these five factors was 98.45%.
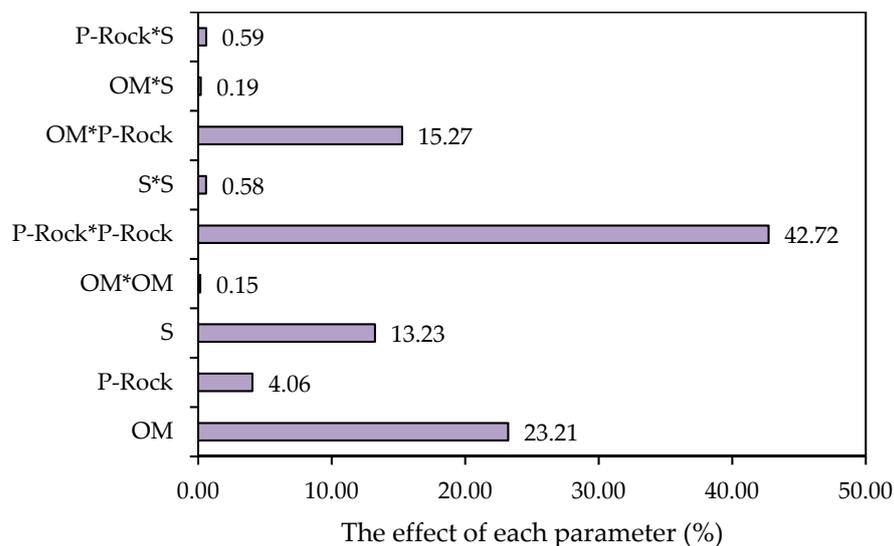

Figure 3. The results of the Pareto comparison of the effects of the input parameters of the central composite design model on P solubilization. P-Rock, phosphate rock; OM, organic matter; S, sulfur.

To show the combined effect of the variables, including organic matter (vermicompost), sulfur, and phosphate rock, three-dimensional (3D) and contour plots (2D) of the variations in P solubilization were constructed for these variables on a pairwise basis, based on the CCD model (Figure 4).

Figure 4a shows the combined effect of vermicompost and phosphate rock on the amount of solubilized P. An increase in vermicompost significantly increased P solubilization, especially at low phosphate rock levels. Similarly, increasing levels of phosphate rock significantly increased P solubilization, especially at low levels of organic matter. The highest P solubilization was obtained with the maximum level of phosphate rock.

The results presented in Figure 4b show that increasing the sulfur level in the presence of organic matter slightly increased the amount of dissolved P. Increasing the sulfur level also increased P solubilization at low levels of phosphate rock, but had no significant effect at high levels of phosphate rock (Figure 4c).

The optimal formulation of the biofertilizer to maximize the amount of dissolved P was determined based on the significance of the factors underpinning P solubilization in the CCD model. Table 4 shows the predicted fertilizer composition for maximal P solubilization (1648.39 mg·kg^{-1}) by *P. fluorescens* Ur21: 58.8% vermicompost, 35.3% phosphate rock, and 5.8% sulfur.
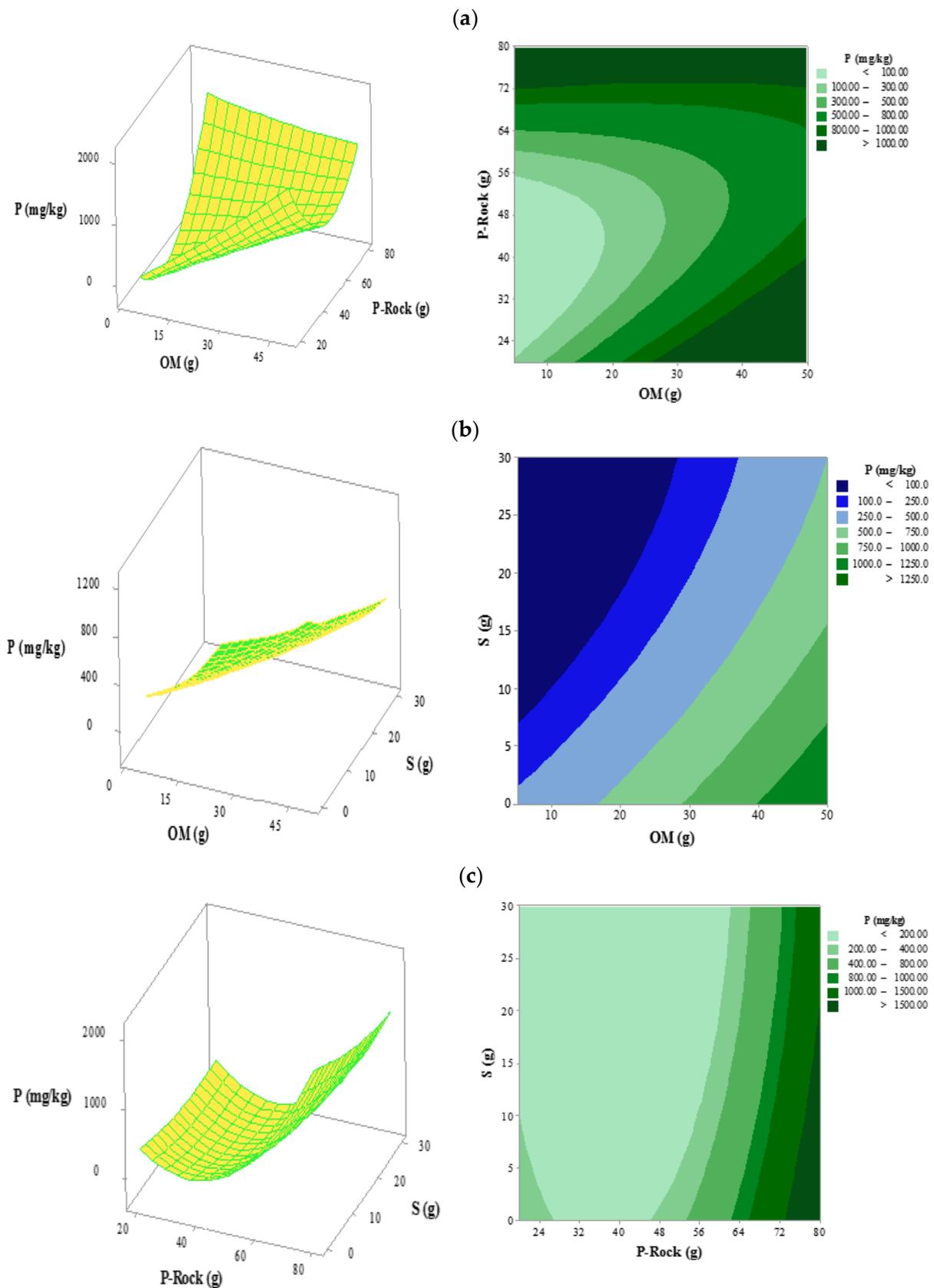
Figure 4. Three-dimensional (3D) and contour (2D) plots showing the interactive effects of (**a**) organic matter and phosphate rock levels, (**b**) organic matter and sulfur levels, and (**c**) phosphate rock and sulfur levels, on P solubilization. P-Rock, phosphate rock; OM, organic matter; S, sulfur.

Table 4. Optimized values of the model input parameters to obtain the maximum predicted dissolved P concentration.

Variable	Unit	Optimum Value	Predicted Dissolved P (mg·kg^{-1})
OM	g	50	1684.39
P-Rock	g	30	
S	g	5	

P-Rock, phosphate rock; OM, organic matter; S, sulfur.

4. Discussion

As previously mentioned, earlier research has shown that various factors can influence the efficiency of P solubilization, including the types and abundance of carbon and nitrogen sources, temperature, pH, and aeration [29]. Organic materials are crucial sources of carbon and other nutrients for the growth and metabolic activities of microorganisms. These activities include the synthesis of organic acids, which (together with phosphatases synthesized by phosphate-solubilizing microorganisms) can release P from low-solubility phosphate sources and increase P solubility [40–42]. Acid strength amounts of dissolved Ca, and both types and positions of chelating ligands, can also affect rates of P release [43]. Thus, the careful optimization of solubilization treatments is required. In the conditions applied in this study, increasing the sulfur level in the presence of vermicompost and phosphate rock slightly increased the amount of dissolved P at the end of experiment (Figure 4b,c). As sulfur promotes acid production, its application with phosphate rock, organic matter, and appropriate microorganisms should theoretically reduce the pH, thereby increasing P solubility and promoting the gradual release of P from the phosphate rock [44]. Thus, increasing the sulfur level alone was expected to increase the P release from phosphate rock. This did not happen, possibly due to the relatively short incubation period (two months), the slow rate of chemical oxidation of sulfur, and the absence of sulfur-oxidizing microorganisms [45]. However, in the unsterilized soil of arable lands, sulfur oxidizes more rapidly due to the presence of chemoorganotrophs and chemolithotrophs. Therefore, adding sulfur together with phosphate rock will be more effective for releasing P in these soils than using phosphate rock alone. Accordingly, in a previous study, the application of both sulfur and phosphate rock provided significantly higher soybean growth and yields than applications of phosphate rock, sulfur, phosphate rock + sulfur, superphosphate, magnesium sulfate, or a control treatment with no additives [46]. Sulfur and phosphate rock also resulted in higher shoot N and P contents than application of phosphate rock or sulfur alone.

As shown in Table 4, the optimal organic matter:phosphate rock:sulfur ratio (by weight) in the phosphate microbial fertilizer for P release was found to be 10:6:1. Similarly, the use of microbial fertilizer with an organic matter:phosphate rock:sulfur ratio of 3:2:1 resulted in higher maize yields than chemical fertilization by triple superphosphate in a greenhouse trial [32]. Another previous study found that increases in levels of organic matter increased P release from phosphate rock [47], and 4 parts of organic matter to 1 part of phosphate rock was an effective composition for P release. However, it should be noted that variations in experimental conditions, the quality of the phosphate rock (including its fragmentation), and soil type can influence the optimal ratio of these variables [48]. We found that a phosphate rock:sulfur ratio of 6:1 was optimal for P solubilization by our microbial fertilizer. This is consistent with previous indications that phosphate rock + sulfur fertilizers with ratios of 1:1 to 7:1 can be as effective as superphosphate, if the phosphate rock fragmentation is sufficiently high [49]. The application of sulfur and phosphate rock is reportedly more effective than phosphate rock alone for improving soil fertility and P availability, even in acidic soils, and sulfur levels should be increased with high levels of organic matter to improve efficiency [15]. A study of the effects of varying phosphate rock to sulfur ratios from 1:1 to 20:1 concluded that low (1:1 and 2:1) phosphate rock to sulfur ratios resulted in crop yields similar to those obtained with chemical P fertilizers [50]. In

addition, a 10:1 ratio of phosphate rock to sulfur has been found to be more effective than a lower ratio (38:1) in conjunction with molasses (as a carbon source) [48].

As always, this study has several limitations that would ideally be addressed in future investigations. It would be interesting to compare the P-solubilizing capacities of a consortia of bacteria with those of single bacterial strains and fungi. As SRM can assess the effects of multiple factors over wide ranges, it would be highly suitable for such experiments. Moreover, greenhouse and field tests with various plants in diverse geographical areas are required to assess the generality of the efficiency of *Pseudomonas* bacteria for solubilizing P from low-solubility compounds and increasing its availability for plants. Economic comparison of its costs and benefits with those of chemical fertilizers is also required. It would also be interesting to study the efficiency of the optimized fertilizer presented here in greenhouse and field trials with different plants, as well as the amounts and fractions of insoluble soil phosphorus compounds before and after such trials.

5. Conclusions

We used a central composite design (CCD) to generate data for a model to predict the effects of different levels of organic matter (vermicompost), sulfur, and phosphate rock in a biofertilizer on P solubilization. The results allowed the prediction of the optimal fertilizer composition (vermicompost + phosphate rock + sulfur) on the solubilization capacity of *Pseudomonas fluorescens* at a laboratory scale. Increasing the ratio of vermicompost to phosphate rock increased P solubilization, but increasing the amount of phosphate rock had less influence on P solubilization, despite its effect on increasing dissolved P, versus vermicompost. Increasing sulfur in the presence of organic matter did not increase the amount of dissolved P significantly. More than 90% of the added P was dissolved with the optimized formulation. ANOVA verified the model's accuracy and validity with respect to the F value (10.41), p value (<0.001), and non-significant lack of fit. To maximize the P solubilization (at 1684.39 mg·kg^{-1} under our conditions) from phosphate rock by *P. fluorescens* Ur21, the microbial fertilizer package should contain 58.8% vermicompost, 35.3% phosphate rock, and 5.8% sulfur (a ratio of 10:6:1).

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