ISOLATION OF PHOSPHATE SOLUBILIZING MICROORGANISMS AND OPTIMIZATION OF THEIR FERMENTATION CONDITIONS BY STATISTICAL TECHNIQUE USING AGRO-INDUSTRIAL WASTE

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Abstract. In soil phosphorus solubilizing microorganisms (PSMs) are beneficial because they have the ability to hydrolyze both organic and inorganic phosphorus to a soluble form that is easily absorbed by plants. In this study PSMs were isolated from the soil and their culture conditions were improved. Using the pour plate method and Pikovskaya Agar medium (PVK), seven PSMs were isolated from the soil collected from different areas of Lahore. *Aspergillus niger* was determined to be the isolate with the highest phosphorizer. The Response Surface Methodology (RSM) a statistical approach was applied to optimize the cultural conditions and wheat bran was used as a substrate for solid state fermentation (SSF), which produced biofertilizer. Five distinct parameters were measured and optimized using central composite design (CCD): temperature (X1), pH (X2), incubation period (X3), moisture (X4), and nitrogen source Ammonium sulphate (X5). Ammonium sulphate, which has the highest phosphorus solubility among several nitrogen sources, was chosen. Temperature (44.09°C), pH (8.31), incubation time (8.81 days), moisture content (60.90%), and nitrogen supply (2.40%) were the optimized parameters for phosphorous solubilization. It is possible to expand the global food supply without endangering the environment by employing PSMs as biofertilizers. It might be concluded that considering the *A. niger's* ability to solubilize phosphate and its ability to produce plant growth promoters, so it can be suggested as a phosphate solubilizer in an agricultural field.
Keywords: *biofertilizer, wheat bran, fermentation, RSM, SSF*

Introduction

Phosphorus is the second most vital macronutrient needed for plant growth and crop productivity (Chawngthu et al., 2020). Phosphorus is essential for the growth of roots, the strength of stems, the production of seeds and flowers, development of crops, respiration, and photosynthesis (Chen et al., 2019; Hii et al., 2020). In addition, it is also a part of numerous macromolecules, including ATP, NADPH, and nucleic acids. Plants are unable to grow properly unless they get sufficient amount of phosphorus (Djuuna et al., 2022). There are both organic and inorganic forms of phosphorus in soil (Zhang et

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al., 2018). The phosphate anions HPO_4^{2-} and $H_2PO_4^-$, which are the inorganic form of phosphorus, are absorbed by plants to fulfil the needs for phosphorus (Kirui et al., 2022). About 52.3 billion tons of soluble inorganic phosphorus is applied daily in the form of chemical fertilizers' (Soumare et al., 2020). This applied form of phosphorus quickly and readily bonds with soil cations such as calcium, iron, and aluminum to form calcium phosphate, iron phosphate, and aluminum phosphate. The phosphate anions in basic/alkaline soil react with calcium and magnesium to produce complexes that become insoluble in water, while in acidic soil they react extremely fast with iron, manganese, and aluminum (Elfiati et al., 2021). As a result, the phosphorus becomes immobilized and insoluble, and only a very small amount is used by plants for nutrition (Amri et al., 2023).

Chemical fertilizers contain an enormous amount of inorganic phosphorous in the soluble form nearly 52.3 billion tons (Soumare et al., 2020). Overuse of chemical fertilizers damages the ecosystem, resulting in eutrophication, contaminated groundwater, and decreased soil fertility (Ming et al., 2024; Islam et al., 2019; Timofeeva et al., 2022). Research studies were conducted to determine alternate methods of phosphorus availability for plants in response to these detrimental effects of phosphate chemical fertilizer. Furthermore, it is believed that using the phosphorus that is already stored in soil is more appropriate than increasing the amount of phosphorus through chemical fertilizers and dealing with the adverse environmental effects (Qarni et al., 2021).

The costly and dangerous chemical formulations have been replaced by the current trends in sustainable and agronomically feasible fertilizers, sometimes known as biofertilizer (Naveed et al., 2025; Naveed et al., 2023). The term biofertilizer refers to substances that are prepared or formulated using live microbes to improve the fertility of soil and availability of nutrients (Zameer et al., 2023; Khan et al., 2022). The soil is full of microorganisms that are particularly powerful at solubilizing phosphorus and giving it to plants in a soluble state. The phosphate solubilizing microbes are capable of solubilizing phosphorus, and their productivity and sustainability in agriculture have been observed (Ducousso-Détrez et al., 2024). These microorganisms comprise numerous kinds of bacteria and fungus, such as *Penicilium*, *Bacillus*, *Pseudomonas*, *Rhizobium*, and *Aspergillus* (Doilom et al., 2020; Hii et al., 2020).

Most of the microorganisms produce organic acids as a result of their different metabolic activities that reduce the pH of soil and help in solubilization of phosphorus (Yang et al., 2025; Kalayu, 2019). Some of microorganisms also produce enzymes like phytase and phosphatase in addition to organic acids that also have the tendency in hydrolyzing phosphorus that are present in soil (Naveed et al., 2024; Abbasi et al., 2024; Mahmood et al., 2022). Response surface approach is a statistical technique utilized in process development and optimization (Ahmed et al., 2021). Different designs, such as the Box-Behnken or central composite design (CCD), can be used to perform the RSM. These techniques have been used as reliable tools in the engineering and biological sciences for process modelling and optimization in recent years (Barin et al., 2022). The aim of the study is to isolate microorganisms that solubilize phosphate from a variety of soil samples, identify cultures with high efficiency, and adjust cultural conditions to facilitate fermentation-based biofertilizer synthesis (Pan et al., 2025; Han et al., 2024). By utilizing microbial properties to improve crop yield and soil fertility, this research tackles the need for sustainable agricultural methods.

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Materials and methods

Isolation and screening of phosphorus solubilizing microbes

Twenty soil samples, or n = 20, were collected at a depth of 0–20 cm from different Lahore localities. A 10-fold serial dilution ratio was applied to each soil sample until the 10-5 dilution factor was reached. For isolation PVK agar medium was used, and its chemical composition was (g/L): $C_6H_{12}O_6$, 10; $(NH_4)_2SO_4$, 0.5; $MgSO_4.7H_2O$, 0.1; Yeast extract, 0.5; KCl, 0.2; NaCl, 0.2; $Ca_3(PO_4)_5$, 5; and to solidify the media, 15 g of agar was added. Initial pH of medium was adjusted to 5.5. The isolates that showed clear zones around their colonies were considered phosphorus solubilizing strains.

Secondary screening of phosphorus solubilizing microbes

The solubility of phosphorus was assessed using PVK broth media. A loop containing each culture was inoculated in the media and kept in a rotary shaker for seven days at 121 rpm and 28°C ± 2°C. After that, culture was centrifuged at 2000 rpm for 20 min and supernatant was used for the determination of soluble phosphorus. (Nasr et al., 2021).

Identification

Strain of *Aspergillus niger* was identified by its morphology and microscopy. Morphologically identification was conducted by the study of shape, size and color. Portion of mycelium was helpful to examine the fungi under microscope (Bhatnagar et al., 2023).

Screening of nitrogen source

Five different organic and inorganic nitrogen sources (yeast extract, peptone, trypton, ammonium sulfate and ammonium nitrate) were used to enhance the phosphate solubilizing efficacy of *A niger*.

Optimization of the cultural conditions

Response surface methodology was used for the optimization, and the model was employed to establish the ideal conditions needed for phosphate solubilization. Temperature (X1), pH (X2), incubation time (X3), moisture (X4), and nitrogen source concentration (X5) were the five variables that were optimized. To optimize these characteristics, thirty tests using central composite design (CCD) were carried out (*Tables 1* and *2*).

The relation between the five variables for phosphorus solubilization was explained by the regression equation. The equation is

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_{11} X^2_1 + \beta_{22} X^2_2 + \beta_{33} X^2_3 + \beta_{44} X^2_4 + \beta_{55} X^2_5 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{15} X_1 X_5 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{25} X_2 X_5 + \beta_{34} X_3 X_4 + \beta_{35} X_3 X_5 + \beta_{45} X_4 X_5.$$

The response variable in this equation is denoted by Y, while the coefficient of regression is represented by β^0. The linear effect of the coefficient is represented by β1, β2, β3, and β4, the quadrant effect is represented by β11, β22, β33, β44, and β55, and the interaction effect among the five variables is represented by β12, β13, β14, β23, β24, β34, and β45.

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Here, Y is representing the Soluble Phosphorus and X_1, X_2, X_3, X_4 and X_5 representing the Temperature, pH, Incubation Period, Moisture and Nitrogen Source respectively (Table 1 and Table 2).

Table 1. *Coded values of different variables for RSM-optimization*

Designated values	-2	-1	0	1	2
Temperature X1	25°C	30°C	35°C	40°C	45°C
pH X2	4.5	5.5	6.5	7.5	8.5
Incubation Period X3	3 days	5 days	7 days	9 days	11 days
Moisture X4	50%	60%	70%	80%	90%
N. Source (NH4)2SO4 X5	0.50%	1.00%	1.50%	2.00%	2.50%

Table 2. *Experiments design by RSM*

Experiment No	Temp	pH	Incubation	Moisture	N. Source	Yield observed	Yield predicted
1	1	1	1	1	-1	30.93439	30.70106
2	1	1	1	-1	1	38.15694	39.72361
3	1	1	-1	1	-1	30.69347	31.46014
4	1	1	-1	-1	1	45.71052	42.27719
5	1	-1	-1	1	-1	31.65547	31.75547
6	1	-1	1	-1	1	32.76152	32.66152
7	1	-1	1	1	-1	32.03356	31.13356
8	1	-1	-1	-1	1	32.93411	33.83411
9	-1	1	-1	1	-1	25.65364	25.75364
10	-1	1	1	-1	1	28.54669	28.44669
11	-1	1	1	1	-1	22.18272	21.28272
12	-1	1	-1	-1	1	24.87027	25.77027
13	-1	-1	-1	1	-1	31.66672	34.10006
14	-1	-1	1	-1	1	28.44327	28.67661
15	-1	-1	1	1	-1	28.81481	28.24814
16	-1	-1	-1	-1	1	25.38586	24.61919
17	2	0	-1	0	0	35.141	35.80767
18	-2	0	0	0	0	22.312	21.64533
19	0	2	0	0	0	22.54167	23.20833
20	0	0	0	0	0	23.77833	23.11167
21	0	0	0	0	0	17.86767	15.86767
22	0	0	2	0	0	10.56933	12.56933
23	0	0	-2	2	0	21.6611	21.2611
24	0	0	0	-2	0	24.1079	24.5079
25	0	0	0	0	2	24.1079	24.5079
26	0	0	0	0	-2	22.7611	22.3611
27	0	0	0	0	0	28.864	28.864
28	0	0	0	0	0	28.864	28.864
29	0	0	0	0	0	28.864	28.864
30	0	0	0	0	0	28.864	28.864

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Statistical analysis

To construct the plots and data version 7 software of STATISTICA was used for statistical analysis. In RSM, ANOVA was used to determine the significance of parameters. To conclude the performance of regression model, the R^2 and adjusted R^2 were calculated. Optimum levels of variables were obtained from the desirability chart. SPSS software was used for comparison of means by Post Hoc test.

Results and discussion

Twenty (20) soil samples were taken from various areas of Lahore. These samples produced seven PSMs that were isolated. These isolates comprised of one yeast strain, three bacterial strains, and three fungal strains. With the exception of the bacterial strains, every isolate that was obtained after the initial screening created halo zones surrounding its colonies as shown in *Figure 1*. These strains were inoculated into broth media; it appeared that the media containing the yeast and bacteria inoculation kept their milky look and hence indicated lower solubility of phosphorus, but the media containing the fungal strains were quite transparent.

By estimating the amount of soluble Phosphorus, *Aspergillus niger* determined to produce the maximum amount of soluble phosphorus. Phosphorus solubilization by *Aspergillus niger* is reported more than many other strains in the literature. Mazrou et al. (2020) reported that *Aspergillus niger* prominently improved the growth parameters of tomato and cucumber during field trials. Elfiati et al. (2021) tested the efficiency of *Aspergillus niger* as biofertilizer for sustainable crop production and soil fertility. Jyothi et al. (2022) recommended *Aspergillus niger* as the phosphate solubilizer due to its production of plant growth promoters and phosphate solubilizing capacity in agriculture field. Therefore, the *Aspergillus niger* with maximum solubility of phosphorus was selected and proceed further. Additionally, prior research has demonstrated that fungi are more capable than bacteria in solubilizing phosphorus (Hao et al., 2021; Waday et al., 2022; Yang et al., 2022; Zhang et al., 2023).

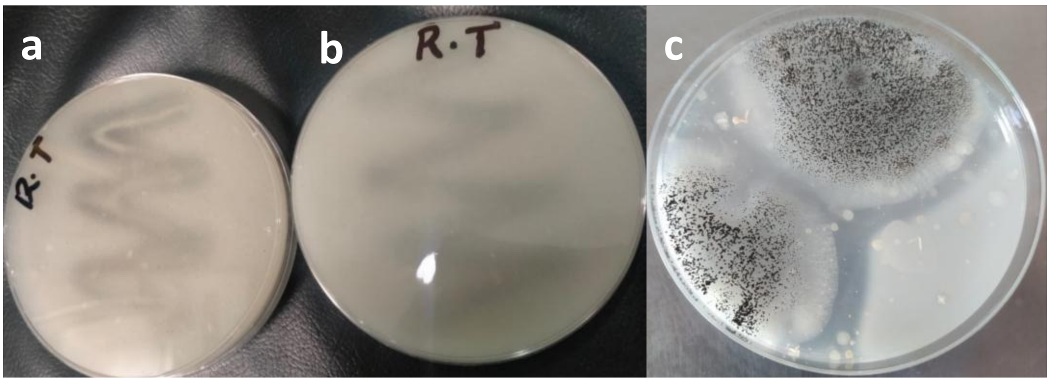

Figure 1. *Clearance zone of isolated strain on PVK media (a) bacterial strain (b) bacterial strain (c) fungal stain Aspergillus sp*

Nitrogen source

For phosphate solubilization different organic and inorganic nitrogen were screened to find out the appropriate source. Ammonium sulphate provided the highest amount of

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soluble phosphorus among these sources at 43.24 ppm, followed by ammonium nitrate at 38.93 ppm, trypton at 36.61 ppm, and peptone at 32.47 ppm and then yeast extract at (25.02 ppm). In other studies, ammonium salts were considered as the best sources among the other nitrogen sources consumed by microbes (Fatima et al., 2022; Nasr et al., 2021; Wang et al., 2023). Moreover, in the previous studies, it also revealed that *Aspergillus niger* gave maximum soluble phosphorus with ammonium sulfate rather than other nitrogen sources (Bhattacharya et al., 2015; Jain et al., 2022; Xiao et al., 2015) (*Table 3*).

Table 3. *Amount of phosphorus solubilized by nitrogen sources*

Sources of nitrogen	Soluble phosphorus in ppm ± SD
Yeast extract	25.02 ± 1.05[a]
Peptone	32.47 ± 1.08[bc]
Trypton	36.61 ± 1.32[ac]
Ammonium nitrate	38.93 ± 1.69[d]
Ammonium sulfate	43.24 ± 2.34[ab]

Each value of the above data was statistically compared with three replicates. ± indicates the standard deviation among tree replicates. The small (abc) alphabetic letters the individual columns differ significantly from each other

Optimization by using RSM

Second-order quadratic model was employed to ascertain the link between the different independent variables and the (Response) dependent variables. Phosphorus solubility was predicted by using model.

$$Y = 0.000 + 1.09960\ X_1 + 2.98092\ X_2 + 4.87754\ X_3 + 10.13161\ X_4 + 10.82293\ X_5 - 0.06783\ X_1^2 - 2.61420\ X_2^2 - 3.64532\ X_3^2 - 2.94984\ X_4^2 - 1.16335\ X_5^2 + 2.45597\ X_1 X_2 - 1.44572\ X_1 X_3 - 2.20869\ X_1 X_4 + 0.000\ X_1 X_5 - 0.36034\ X_2 X_3 - 2.73367\ X_2 X_4 + 0.000\ X_2 X_5 + 0.49605\ X_3 X_4 + 0.000\ X_3 X_5 - 7.55289\ X_4 X_5$$

In this case, Y stands for soluble phosphorus, and X1, X2, X3, X4, and X5 stand for the temperature, pH, incubation period, and moisture content and nitrogen source respectively. The model's positive signs imply that the factors have a positive impact on the response, while the negative signs indicate that the factors have a negative impact.

By calculating the statistical parameters, the model is regarded as highly significant. The greater the F value and p values less than 0.05 are deemed significant, just like in the ANOVA. The estimated value of F and p are 137.5922 and 0.000000 respectively. The coefficient of determination R^2 is used to assess the fitness of model. The reliability of model is demonstrated by the R^2 value of 0.970, which is quite close to 1. Considering that an R^2 value of more than 0.9 indicates a strong correlation between the predicted and observed data (Waday et al., 2022). Wang et al. (2023) used Box Behnken design for optimization of phosphate solubilization and suggested R^2 value of 0.9621. Only a tiny portion of data around 0.03% is not predicted by the calculated model, as indicated by the R^2 value. Conversely, the model's significance and accuracy also predicted by the adjusted value of R2 that is 0.929.

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The significance of the effects in an ANOVA is shown by the p values for the main effects, quadratic effects and interactions effects of the variables. The information is ranked according to how the factors affect productivity using the F value of each variable. The significance of the effects of the variables with higher F values is greater than that of the variables with lower F values (*Table 4*).

Table 4. *ANOVA table represents the significance of variables and model*

Effect	SS	Degree of freedom	MS	F	p
Intercept		0			
Temp		0			
Temp^2	0.0252	1	0.0252	0.0085	0.927941
pH		0			
pH^2	43.3808	1	43.3808	14.6777	0.002391
Incubation		0			
Incubation^2	285.9876	1	285.9876	96.7627	0.000000
Moisture		0			
Moisture^2	47.6726	1	47.6726	16.1298	0.001711
Nitrogen		0			
Nitrogen^2	39.3060	1	39.306	13.299	0.003346
Temp*pH	53.1733	1	53.1733	17.991	0.001144
Temp*incubation	27.3529	1	27.3529	9.2547	0.010233
pH*incubation	1.9072	1	1.9072	0.6453	0.437428
Temp*moisture		0			
pH*moisture		0			
Incubation*moisture		0			
Temp*nitrogen		0			
pH*nitrogen		0			
Incubation*nitrogen		0			
Moisture*nitrogen	406.6615	1	406.6615	137.5922	0.000000
Error	35.4667	12	2.9556		

The effects of interaction, for (Moisture – pH) and for (Incubation – Temperature), is significant and explained in “3 D Surface-Plot” in *Figures 2* and *Figure3*.

A 3D surface plot (*Figure 2*) illustrates the relative impact of moisture and pH on the solubilization of phosphate. The way that moisture and pH interact suggests that they have a detrimental influence on the solubilization of phosphorus. The graph’s declining trend for both variables indicates that there is a negative interaction effect. Their declining pattern explains that by raising one component has a detrimental impact on the other. It implies that using both of the components at once lowers their overall effectiveness. Therefore, due to the interaction effect of both variables, the solubilization of phosphorus reduces.

A 3D surface plot (*Figure 3*) illustrates the relative impact of incubation period and temperature on the phosphorus solubilization. The yield is also negatively impacted by the interaction between the incubation period and temperature, as seen by the graph, which shows that as temperature rises, solubilization of phosphorus increases. Furthermore, prolonging the incubation period will, to some extent, promote the

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solubilization of phosphorus. However, if the incubation period is prolonged past that point, the solubility of phosphorus will continue to decline.

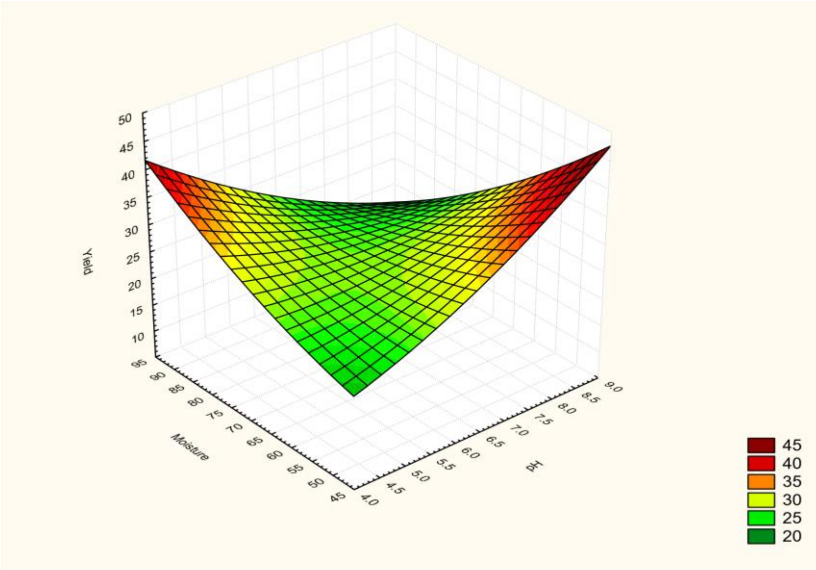

Figure 2. *3D surface represents the interaction effects of moisture and pH*

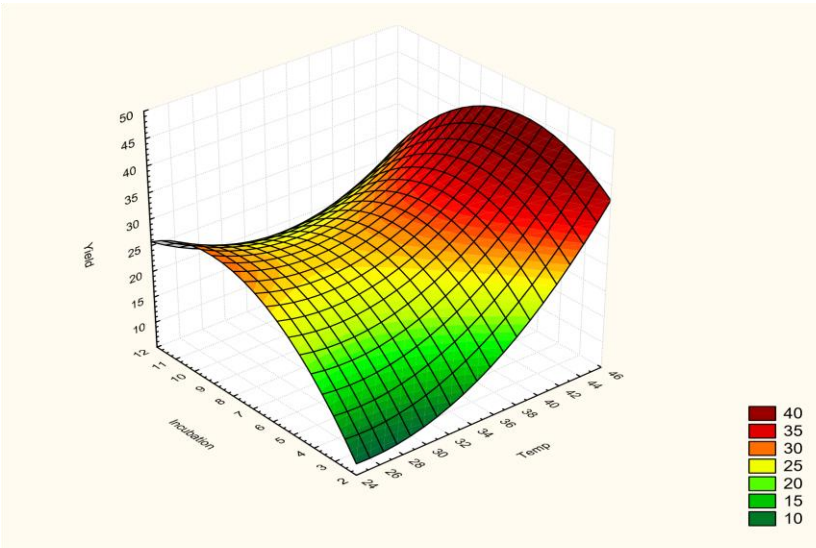

Figure 3. *3D surface plot represents the interaction effects of incubation and temperature*

The RSM-CCD model found the significant parameter settings in addition to analyzing the interaction effect of the important factors. It indicates that the following conditions are ideal for phosphorus solubilization: temperature (44.09°C), pH (8.31), duration of incubation (8.81 days), moisture content (60.90%), and nitrogen supply (2.40). Padmavathi et al. (2015) carried out a study and according to their findings, which were obtained using the CCD-RSM design, the ideal conditions for *Aspergillus niger's* phosphorus solubilization are glucose (2 g), ammonium sulphate (0.2 g), and tri-calcium phosphate (1 g) per 50 mL of media (*Figure 4*).

Figure 5 represents a comparison of the observed and predicted values. The model's predicted values are largely within the straight line and rather close to the observed ones. The graph demonstrates the accuracy and reliability of the model since it appears that the observed values closely match the predicted values. The model's ability to accurately predict the solubility of phosphorus is indicated by the fact that all of the observed and predicted data points fall within a 45° angle.

The optimal level for the observed yield is 45.710 ppm, and for the predicted yield is 42.277 ppm.

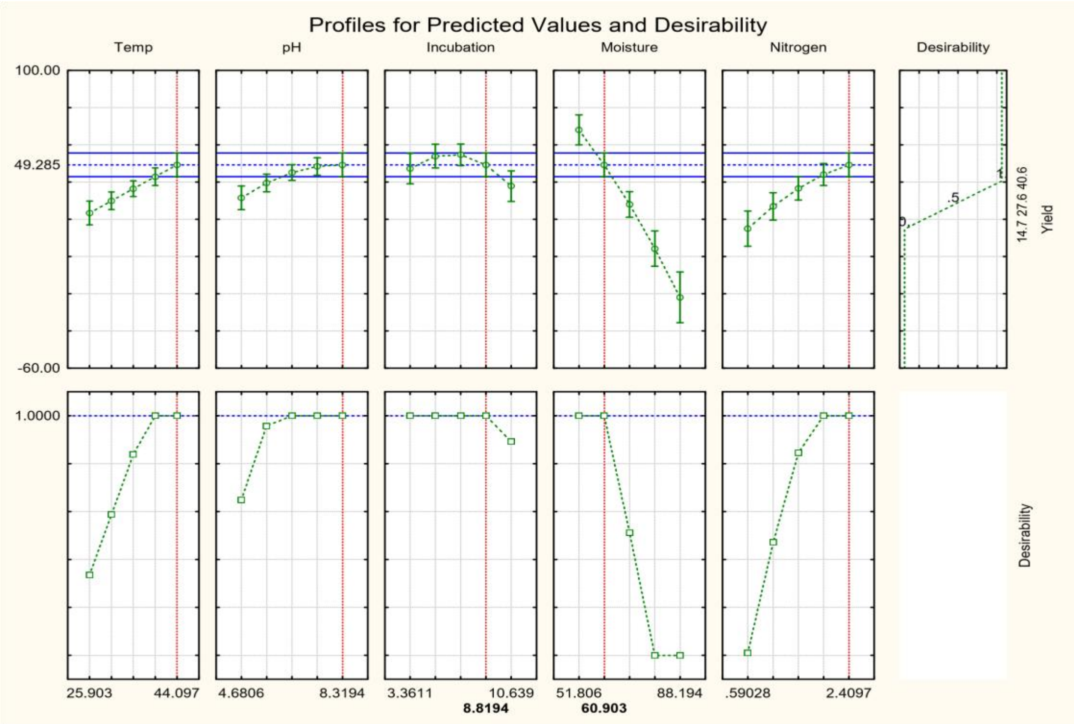

***Figure 4.** Desirability chart represents the optimum conditions of independent variables*

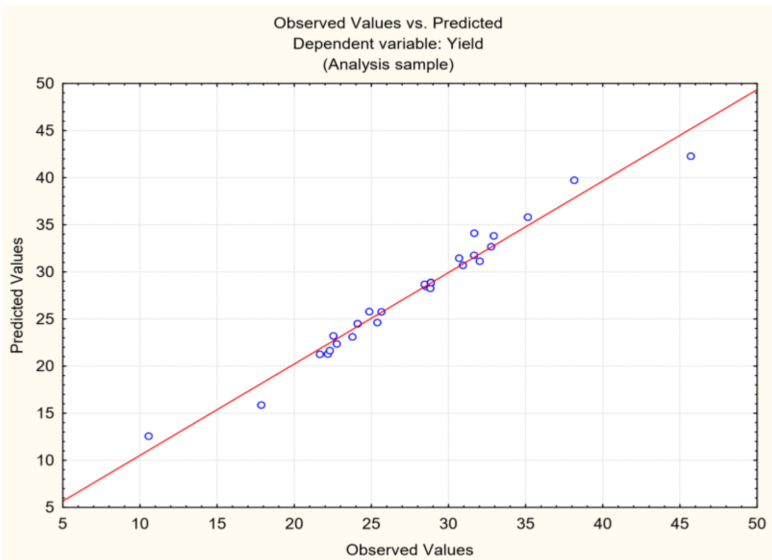

***Figure 5.** Graph between observed values vs. predicted values for phosphorus solubilization*

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Response surface method is the statistical technique that is used to achieve the optimum values of the cultural conditions to get the desired product. In biochemical processes, RSM has been used widely and has become now the successful approach to analyze many microbial products (Bhattacharya, 2021; Breig et al., 2021). A 2.4-fold increase in rock phosphate solubilization by *Aspergillus niger* was achieved after the optimization of process parameters by response surface methodology (Mendes et al., 2015). Similarly, 1.9-fold increase in phosphate solubilization achieved by *Aspergillus niger* after the optimization of the parameters by Plackett Burman and Response Surface Methodology (Padmavathi et al., 2015). Hoseini et al. (2020) reported the maximum solubilization of phosphorus from Aspergillus Sp. in microbial fertilizer using CCD-RSM design. The optimized cultural conditions were vermicompost 58%, rock phosphate 23.3% and sulfur 18.7%.

Conclusion

The current research concluded that the isolated strain of *Aspergillus niger* demonstrated effective phosphorus solubilization in PVK medium. From the study it was concluded that this strain has a lot of potential as a biofertilizer. The prospective applications of this strain as a biofertilizer are quite promising. As a result, using PSM (phosphate solubilizing microorganisms) effectively produces new chances to increase agricultural yield performance and production without endangering the soil’s health. Consequently, actions had to be done to carry out comprehensive and reliable studies to better detect and describe PSM for their final use in outdoor environments. Furthermore, ammonium sulfate was found the best nitrogen source. RSM was found to be an appropriate statistical tool for precisely and accurately optimizing a bioprocess.

Conflict of interests. All the authors declare no conflict of interests.

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