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RESEARCH ARTICLE

Optimization of phosphate solubilization by *Aspergillus niger* using plackett-burman and response surface methodology

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Abstract

Phosphorus is one of the major components required for the metabolic activities and for the growth of any organism. Many soil organisms are known to solubilize inorganic phosphates. *Aspergillus niger* isolated from the soil showed extensive solubilization of Tri-calcium phosphate.It was observed that the solubilization was due to drop in pH.Acidification was due to production of organic acids by the fungi. The effect of different medium components on the solubilization of phosphate was determined using the Packett-Burman design. It was observed that glucose and ammonium sulphate had significant effect on phosphate solubilization. Considering the Plackett-Burman results, the response surface methodology was used for optimization of these medium components along with tri-calcium phosphate on P-solublization. The analysis from RSM revealed that the optimum values for the tested variables were glucose - 2g/50ml, ammonium sulphate –0.2g/50ml and tri-calcium phosphate - 1g/50ml. Phosphate solubilization of 3.64 mg/ml was observed as comparison to original level of 1.88mg/ml, which was a 1.93-fold increase was obtained. From the HPLC analysis it was observed that oxalic acid and lactic acid were the major acids responsible for enhancing the P solubilization.

Keywords: Phosphate solubilizing fungi, Plackett-Burman, response surface methodology, organic acids, *Aspergillus niger*

1. Introduction

Phosphorus (P) is one of the major plant nutrients influencing the plant growth. Most of the agricultural soils contain high amount of phosphorus due to the application of chemical fertilizers.Most of this phosphorus is present in inorganic forms which are not assimilated by the plants. Phosphate solubilizing organisms are involved in converting the inorganic/ insoluble forms of phosphorus into soluble forms which are readily taken up by the plants.A variety of bacteria and fungi have been isolated and characterized for their capability to solubilize inorganic unavailable forms of phosphorous to available forms of phosphorous. These phosphate-solubilizing microorganisms (PSMs) have known to be used as fertilizer to increase the phosphorous uptake and plant growth (Wang *et al*. 2007).

Phosphate solubilizing rhizobacteria and arbuscular mycorrhiza play a synergestic role on growth and yield of agricultural crops (Minaxi *et al***.** 2013; Schoebitz *et al*. 2013**).**

The PSMs convert the insoluble forms of phosphorous to soluble forms by the process of acidification, chelation and exchange reactions (Sudakar Reddy *et al*. 2002).The main mechanism for the solubilization of inorganic phosphate is the production of organic acid. The production of organic acid leads to acidification of the microbial cells and their surroundings. Many fungal strains can solubilize rock phosphate, aluminium phosphate and tricalcium phosphate, such as *Aspergillus* sp*., A. niger, A. tubingensis, A. fumigatus, A. terreus, A. awamor, Penicillium* sp*., P. italicum, P. radicum, P. rugulosum, Penicillium albidum, Penicillium thomii, Penicillium restrictum, Penicillium frequentans, Gliocladium roseum, Fusarium* sp*., F. oxysporum, Curvularia lunata, Humicola* sp*., Sclerotium rolfsii, Pythium* sp*., Aerothecium* sp*., Phoma* sp*., Cladosporium* sp*, Rhizoctonia* sp*., Rhizoctonia solani, Cunninghamella* sp*., Rhodotorula* sp*., Candida* sp*., Schwanniomyces occidentalis, Oideodendron* sp*., Pseudonymnoas cussp* (Morales *et al*. 2011; Chalit *et al*. 2009). One of the important organic acids responsible for phosphate solubilization is gluconic acid. Other acids responsible for acidification of the media and in turn phosphate solubilization are citric acid, oxalic acid, succinic acid, lactic acid etc.

The fungal growth, spore production and accumulation of metabolic products are strongly influenced by the medium components such as carbon sources, nitrogen sources, various inorganic salts and trace elements. Medium optimization was therefore an important criteria for solubilization of phosphate. The factorial combination of medium optimization involving one variable at a time by keeping others at fixed level fails as it is laborious, time consuming and also does not guarantee the optimal conditions. Statistical approach such as Plackett- Burman design for the modifications of nutrients and culture conditions are useful tools for the screening of nutrients as they depict significant impact on growth rate; which in turn aids in understanding the interactions among the process parameters at different levels (Swetha *et al*. 2014; Rajendran *et al.* 2007). The use of statistical experimental design in medium optimization has gained considerable attention in recent years and also number of publications describing the application of these methods for the production of various enzymes and biomolecules has appeared in the literature (Seraman *et al.* 2010).

In the present study, *Aspergillus niger* which was isolated from the rhizospheric soil of tomato plants showed immense solubilization of tri-calcium phosphate. Medium optimization was performed to increase the phosphate solubilizing ability of this fungus. Statistical tool Plackett-Burman design, a rapid screening multifactor method was used to find the most significant independent factors for phosphate solubilization and followed by Response surface methodology for multiple regression analysis by means of quantitative data obtained from suitably designed experiments to simultaneously solve multivariable equations.

2. Materials and Methods

2.1. Microorganism, culture maintenance and inoculum preparation

The phosphate solubilizing fungi *Aspergillus niger* was isolated from the rhizospheric soils of tomato and was maintained in Potato Dextrose Agar (PDA). Slants with PDA were used for inoculum preparation. The fungal culture was allowed to grow and sporulate. The inoculum used for the experiment was in the form

of spore suspension. The spores of *Aspergillus niger* were prepared using sterile 10ml water into which a loop full of the spores were inoculated and vortexed for even distribution of the spores. The numbers of spores used for inoculation were $5x10^8$ spores/ ml.

2.2. Measurement of cell growth and pH

The culture (fungal biomass) was filtered through pre-weighed Whatman No. 1 filter paper, dried in a hot air oven at 60 ^oC for 48-72 hours. The dried filter paper along with the biomass was weighed and the weights were recorded. The growth of *Aspergillus niger* was expressed in terms of dry cell weight in grams. The pH of the culture filtrate was detected using a digital pH meter. (Whitelaw *et al*. 1999)

2.3. Phosphate estimation

The soluble Phosphorus concentration was estimated as described by Murphy and Riley (1958), where 1ml of culture filtrate and 3ml of the acid molybdate reagent was added and read at 830nm using UV-Vis spectrophotometer (Shimadzu UV-1800).

2.4. Media composition

The components of the medium used for phosphate solubilization by *Aspergillus niger* are given in the Table 1 below. The medium used was the Pikovskaya's medium consisting of yeast extract (0.5g/lt), dextrose (10g/lt), tri-calcium phosphate (5g/lt), ammonium sulphate (0.5g/lt), potassium chloride (0.2g/lt), magnesium sulphate (0.1g/lt), manganese sulphate and ferrous sulphate. The different carbon sources used were glucose, sucrose, xylose and maltose. The different nitrogen sources used were yeast extract, sodium nitrate, urea and ammonium sulfate. A 12-run Plackett-Burman design (Table 1) with a first-order polynomial equation was applied to evaluate eight factors (including four dummy variables). Each variable was examined at two levels: -1 for the low level and +1 for the high level (Swetha *et al* 2014).

2.5. Design of the Plackett-Burman experiment

The Plackett-Burman design based on the first order model was used to screen and evaluate the important media components that influence the phosphate solubilization (available phosphate), pH of the culture media and biomass. All the experiments were carried out according to designed matrix (Table 1) using the equation $Y= \beta_o +\sum\beta_i X_i$ (i= l----------k) where Y is the estimated target function-available phosphate/pH of the culture media/biomass, β_o is a model intercept/constant, β_i is the regression co-efficient. X is the independent variable and k is the number of variables (Fan *et al*, 2011; Seraman *et al.* 2010). Total of eight variables were screened where four carbon sources viz. glucose, sucrose, xylose and maltose were investigated at a high (+1)(1g/50ml) and low (-1)(0.5g/50ml) levels which represent the two different nutrient concentration. Similarly four nitrogen sources viz. yeast extract, urea, ammonium sulphate and sodium nitrate were investigated at high level (+1) (0.5g/50ml) and low level (-1) (0.025g/50ml) which represented two different nutrient concentrations. The student's t- test was performed to determine the significance of each variable employed. The regression co-efficients were determined by least square method. However this design did not consider the interactions between the variables. The variables screened by Plackett- Burman design can be optimized by using statistical and mathematical optimization tools such as response surface methodology (RSM).

2.6. Design of response surface methodology

A central composite design (CCD) of RSM was employed to optimize the two most significant factors ammonium sulphate and glucose for enhancing P solubilization by

Aspergillus niger, screened by Plackett-Burman design (Isaie and Padmavathi 2015). The third factor taken was tri-calcium phosphate which was selected by borrowing method (Fan *et al*. 2011). The three independent factors were investigated at five different coded levels (-2, -1, 0, +1, +2). The experimental design used for study is shown in Table 2 below.

2.7. Statistical analysis

MATLAB software was used for the graphical and regression analysis of the experimented data and for examining the response surface and contour plots. Statistical parameters were estimated using ANOVA.

2.8. Detection of organic acids by HPLC

The culture filtrate was detected for presence of organic acids using high performance liquid chromatography (HPLC), a modified method of Alam *et al*. 2002. Samples were injected with 20 µL injection loop into the column. The column used here was C18 column, mobile phase acetonitrile: water: 7: 3 at the flow rate of 1 mL min-1. The column was set at 27° C temperature. The samples were detected using UV detector at 210nm. The standard organic acids namely oxalic acid, succinic acid, citric acid, lactic acid, maleic acid, malic acid, acetic acid, tartaric acid and formic acid were run before the samples.

Table 1. The Plackett-Burman design variables with soluble phosphate as response.

Runs	Glucose	Sucrose	Xylose	Yeast extract	Sodium nitrate	Ammnium sulphate	Urea	Maltose	Phosphate solublization mg/ml
1	+	-	+	-	-	-	+	+	2.6
2	+	+	-	+	-	-	-	+	1.84
3	-	+	+	-	+	-	-	-	3.46
4	+	-	+	+	-	+	-	-	3.42
5	+	+	-	+	+	-	+	-	3.14
6	+	+	+	-	+	+	-	+	2.8
7	-	+	+	+	-	+	+	-	3.88
8	-	-	+	+	+	-	+	+	4.4
9	-	-	-	+	+	+	-	+	4.5
10	+	-	-	-	+	+	+	-	3.86
11	-	+	-	-	-	+	+	+	3.9
12	-	-	-	-	-	-	-	-	3.52

Table 2. Experimental design and results of the central composite design.

Runs	X1	Glucose	X2	$(NH_4)_2SO_4$	X3	$Ca_3(PO_4)_2$	Phosphate estimated mg/ml
1	-1	0.5g	-1	0.05g	-1	0.25g	0.3875
2	+1	1.5g	-1	0.05g	-1	0.25g	0.8026
3	-1	0.5g	+1	0.15g	-1	0.25g	0.1315
4	+1	1.5g	+1	0.15g	-1	0.25g	0.9999
5	-1	0.5g	-1	0.05g	+1	0.75g	0.3157
6	+1	1.5g	-1	0.05g	+1	0.75g	1.6314
7	-1	0.5g	+1	0.15g	+1	0.75g	0.2499
8	+1	1.5g	+1	0.15g	+1	0.75g	2.000
9	-2	0g	0	0.1g	0	0.5g	0.2894
10	+2	2g	0	0.1g	0	0.5g	1.9077
11	0	1g	-2	0g	0	0.5g	1.5788
12	0	1g	+2	0.2g	0	0.5g	1.6183
13	0	1g	0	0.1g	-2	0g	0.0789
14	0	1g	0	0.1g	+2	1g	1.3683
15	0	1g	0	0.1g	0	0.5g	1.3683
16	0	1g	0	0.1g	0	0.5g	1.2893

3. Result and Discussion

3.1. Plackett-Burman Data

The eight components of the media were studied using Placket-Burman experimental design. The available Phosphate values were studied using the software MATLAB. The biomass found to vary from 0.76 g /50ml to 7.79 g/50ml, pH of the culture filtrate varied from 2.9 to 3.88 and available phosphate varied from 1.84mg/ml to 4.5mg/ml.

Glucose, sucrose, ammonium sulfate and urea had a negative effect on the biomass whereas Xylose, yeast extract, sodium nitrite and maltose had a positive effect on production of biomass. The pH Pareto plot shows that glucose, sucrose, yeast extract and maltose had a positive effect on the pH whereas the xylose, sodium nitrite, ammonium sulfate and urea had negative effect on the pH.

From the Plackett-Burman data in Table 3, it was observed that Glucose (p value=0.0045) and ammonium sulphate (p value=0.0218) were relatively significant. These two components along with tri-calcium phosphate were used for RSM.

Table 3. Regression coefficient results from Plackett-Burman data.

Sl. No.	Parameter	Co-efficients	Standard error	t-value	p-value	Remarks
1	Constant	3.7033	0.5505	6.7268	0.0067	NA
2	Glucose	-2.0000	0.2577	-7.7598	**0.0045**	**Significant**
3	Sucrose	-1.0933	0.2577	-4.2420	0.0240	Significant
4	Xylose	-0.0667	0.2577	-0.2587	0.8126	NA
5	Yeast extract	6.9333	5.1548	1.3450	0.2713	NA
6	Sodium nitrite	20.0000	5.1548	3.8799	0.0303	NA
7	Ammonium sulphate	22.6667	5.1548	4.3972	**0.0218**	**Significant**
8	Urea	14.9333	5.1548	2.8970	0.0627	NA
9	Maltose	-0.4133	0.2577	-1.6037	0.2071	NA

3.2. Optimization by response surface methodology

A central composite design was employed to study the interactions between the significant factors. It was also used to determine the optimal levels of the factors. The experimental design for the factors and experimental results are represented in Table 4.The equation explaining the relationship of the three variables for phosphate solubilization is given below

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1X_2 + b_5X_1X_3 + b_6X_2X_3 + b_7X_1^2 + b_8X_2^2 + b_9X_3^2 \ldots\ldots\ldots\ldots Eq\ (1)$$

equation 1

Substituting the coefficients in equation 1, we get

Y= 0.5472 + 0.0737X_1-11.2371X_2+1.3906X_3+4.4385 X_1X_2+1.7823 X_1X_3+3.615 X_2X_3-0.2302 X_1^2+26.975 X_2^2-2.4208 X_3^2.

Where X1 is Glucose, X2 is Ammonium sulphate and X3 is tri-calcium phosphate.

The t-test and p-value were used to identify the effect of each factor on phosphate solubilization by *Aspergillus niger* is given in Table 4.

Table 4. Regression coefficient results from the data of central composite designed experiments for P solubilization

Values	Coefficients	Standard error	t-value	p-value
Constant	0.5472	1.0653	0.5136	0.6259
Glucose	0.0737	0.9587	0.0769	0.9412
Ammonium sulphate	-11.2371	9.5865	-1.1722	0.2856
Tri-calcium phosphate	1.3906	1.9173	0.7253	0.4956
Glucose X Ammonium sulphate	4.4385	4.7201	0.9403	0.3833
Glucose X Tri-calcium phosphate	1.7823	0.9440	1.8880	0.1080
Ammonium sulphate X Tri-calcium phosphate	3.6150	9.4402	0.3829	0.7150
Glucose X Glucose	-0.2302	0.3338	-0.6899	0.5161
Ammonium sulphate X Ammonium sulphate	26.9750	33.3760	0.8082	0.4498
Tri-calcium phosphate X Tri-calcium phosphate	-2.4208	1.3350	-1.8133	0.1197

The fitness of the model was examined by the coefficient of determination R^2, whose value is 0.9024. A model having an R^2 value higher than 0.9 was considered as having a very high correlation (Chen *et al*, 2009). Fan *et al*. 2011 also used CCD-RSM model for optimization of phosphate solubilization and they reported R^2 value of 0.96. Hence, the R^2 value reflected a very good fit between the predicted and experimental response. P-value of less than 0.05 indicates that the models terms are significant. The model was highly significant with the p value of 0.0191.

The interaction effects of variables on phosphate (P) solubilized were studied by plotting 3D surface curves against two independent variables and keeping other variable at their central (0) level. The 3D curves and contour plots from the interactions between variables of the calculated response are shown in the Figure 1.

Figure 1a depicts the 3D Response surface plot and contour plot showing the relative effect of glucose and tri-calcium phosphate on phosphate solubilization while keeping ammonium sulphate concentration at its central level.Glucose concentration had less effect over Phosphate solubilized at lower tri-calcium phosphate concentration on the other hand, Phosphate solubilization increased with increase in tri-calcium phosphate at high glucose concentration.

Figure 1b depicts the 3D Response surface plot and contour plot showing the relative effect of tri-calcium phosphate and ammonium sulphate

on phosphate solubilization while keeping glucose concentration at its central level.The Phosphate solubilization varied with the increase in tri-calcium phosphate concentration. Phosphate solubilization increased till the concentration of tri-calcium phosphate was 0.6g, but decreased slightly followed by a slight increase at 1g of tri-calcium phosphate. Ammonium sulphate also had a varying effect on Phosphate solubilization, but not much significant.

From Figure 1c it was observed that at high glucose concentration, the Phosphate solubilization increased with decrease in ammonium sulphate concentration. With the increase in the concentration of ammonium sulphate the Phosphate solubilization increased with increase in glucose concentration.

From the Table 5 and Figure 2, the highest soluble Phosphate was observed in the presence of oxalic acid and lactic acid. Oxalic acid content was high (21.15mg/ml) and detected in 10^{th} run which consists of glucose-2g, ammonium sulphate-0.1g and tri-calcium phosphate-0.5g. It was also observed that in the absence of ammonium sulphate the production of oxalic acid decreased as observed in Figure 2c. Along with oxalic acid, lactic acid an unkown acid with retention time of 3.91 was observed at lower quantities. Other acids observed were succinic, citric, formic, maleic, malic and acetic acid. Alam *et al*. (2002) have reported that *Aspergillus niger* and *Aspergillus calvatus* produce oxalic acid, citric acid and gluconic acid during P solubilization. Oxalic acid as major organic acid produced from different *Aspergillus* species, an unknown species of *Penicillium* and *Sclerotium rolfsii* were reported by Banik and Dey (1983), Gupta *et al*. (1994), and Illmer and Schinner (1995). Kim *et al*. (1998) reported oxalic acid production by *Enterobacter agglomerans* (PSB). Akintoku *et al*, 2007 have reported that lactic acid, succinic acid, gluconic acid, fumaric acid were release when tri-calcium phosphate was used with different fungal species. Rashid *et al*. 2004 reported that the fungal strain 8RF- *A. niger* produced oxalic acid and citric acid as the major acids during phosphate solubilization. Venkateswarlu *et al*, 1994 detected lactic acid from *A. niger*, it was responsible for P solubilization. This coincides with the present study where it was observed that lactic acid was also detected during P solubilization. Phosphorous solubilization is carried out by a large number of fungi and bacteria, mainly by chelating-mediated mechanism (Rathore 2014; Whitelaw 2000). Inorganic phosphate was solubilized by the action of organic and inorganic acids secreted by Phosphate solubilizing microorganisms where hydroxyl and carboxyl groups of acids chelate cations like Ca, Al and Fe. They are also responsible for decreasing the pH in basic soils (Kang *et al*. 2002). The Phosphate solubilizing microorganisms dissolve the inorganic phosphorus by production acids such as acetate, lactate, oxalate, tartatate, succinate, citrate, gluconate, ketogluconate and glycolate (Baig *et al*. 2010; Puente *et al*., 2004; Song *et al*., 2008; Trivedi and Sa, 2008; Goldstein, 1995; Gyaneshwar *et al*. 1999; Deubel *et al*. 2000).

Table 5. Organic acid detection by HPLC

Runs	Phosphate estimated mg/ml	pH	Oxalic acid mg/ml	Citric acid mg/ml	Succinic acid mg/ml	Lactic acid mg/ml	Formi c acid mg/ml	Maleic acid mg/ml	Malic acid mg/ml	Acetic acid Mg/ml
1	0.3875	5.26	0.2	-	1.65	-	-	-	-	-
2	0.8026	2.68	-	12.5	-	-	0.07	0.02	-	-
3	0.1315	5.30	-	0.65	-	-	-	0.049	-	-
4	0.9999	2.54	-	-	0.32	-	-	-	0.316	-
5	0.3157	4.88	0.02	-	-	-	0.6	-	-	-
6	1.6314	3.83	-	17.59	-	-	-	0.0003	-	-
7	0.2499	5.20	-	0.53	-	0.025	-	-	-	0.0011
8	2.0000	4.19	1.99	-	-	2.74	-	-	-	-
9	0.2894	5.91	0.14	-	2.15	-	-	-	-	0.0006
10	1.9077	2.84	21.15	-	-	0.558	-	-	-	-
11	1.5788	3.16	1.5	-	-	2.7	-	-	-	-
12	1.6183	3.71	21.1	-	-	0.55	-	-	-	-
13	0.0789	1.86	-	-	9.36	-	-	-	-	-
14	1.3683	3.66	0.56	-	3.22	-	-	0.0029	-	-
15	1.3683	4.38	0.5	-	3.73	-	-	0.003	-	-
16	1.2893	4.15	0.4	-	2.3	-	-	0.0004	-	0.0004

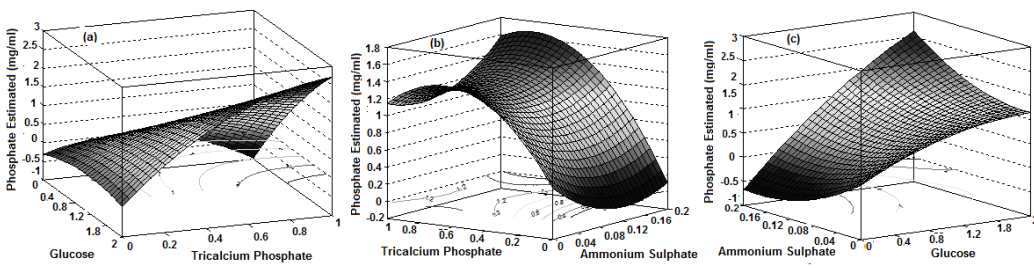

Figure 1a, b, c: The 3D curves a r plots

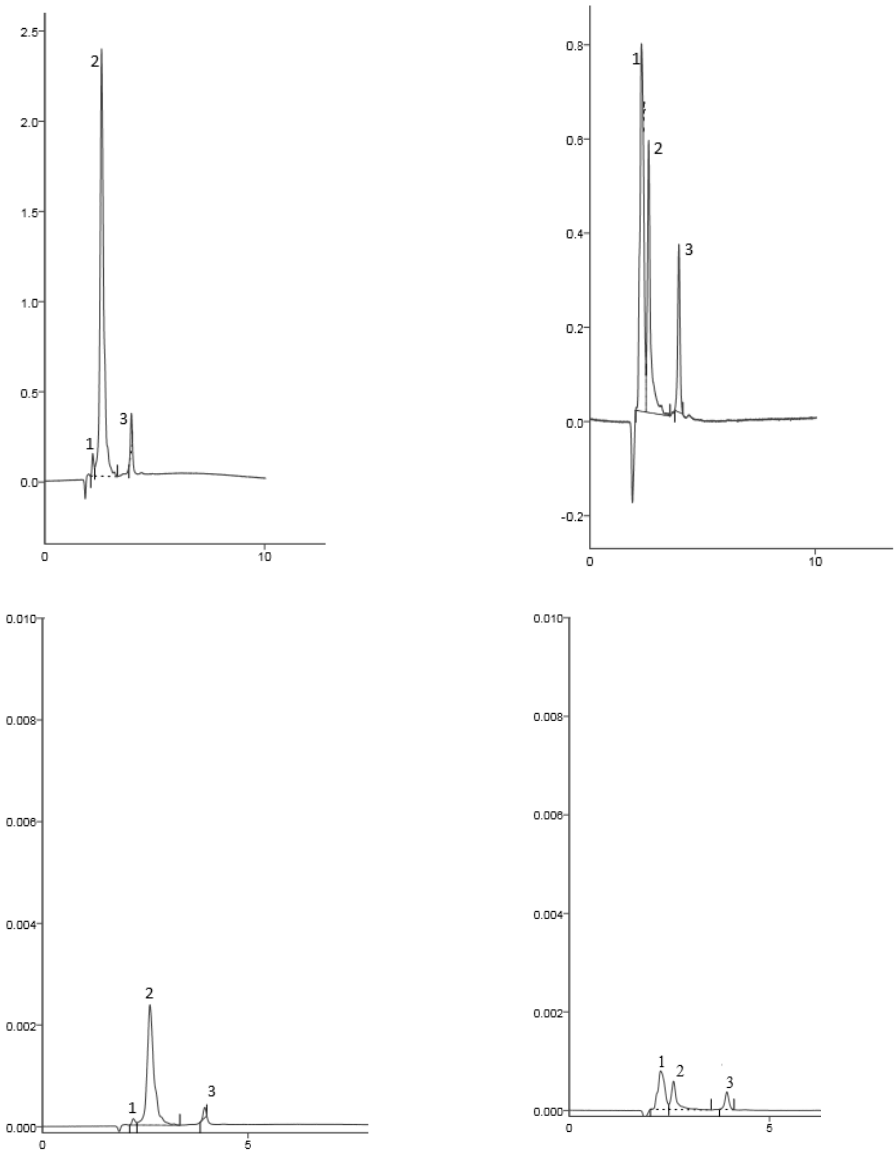

Figure 2a. HPLC chromatogram of 8th run having glucose-1.5g, ammonium sulphate-0.15g and tri-calcium phosphate-0.75g produced. oxalic acid, lactic acid and unknown acid.

b. HPLC chromatogram of 10th run having glucose-2g, ammonium sulphate-0.1g and tri-calcium phosphate-0.5g produced. oxalic acid, lactic acid and unknown acid.

c. HPLC chromatogram of 11th run having glucose-1g, ammonium sulphate-0 g and tri-calcium phosphate-0.5g produced. oxalic acid, lactic acid and unknown acid.

d. HPLC chromatogram of 12th run having glucose-1g, ammonium sulphate-0.2g and tri-calcium phosphate-0.5g produced. oxalic acid, lactic acid and unknown acid.

3.3. Validation of the model

The RSM (response surface methodology) was used as a tool to optimize Phosphate solubilization. Through CCD from RSM it was found that the optimum conditions for phosphate solubilization by *Aspergillus niger* was 3.64 mg/ml of soluble phosphate at concentration of 2g of glucose, 0.2g of ammonium sulphate and 1g of tri-calcium phosphate per 50ml of the medium.

4. Conclusions

The optimized medium resulted in 1.9-fold increase in phosphate solubilization compared with that of original medium. Not much work has been carried out for the optimization on phosphate solubilization process. Hence the present study reports the optimization of phosphate solubilization process using statistical tools Plackett-Burman and Response surface methodology. The major acids that have contributed for high P solubilization were oxalic acid and lactic acid.

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References

Akintokun,A.K., Akande, G.A., Akintokun, P.O., Popoola, T.O.S., Babalola, A.O. 2007. Solubilization of Insoluble Phosphorus by organic acid-producing fungi isolated from the Nigerian soil. Int. J Soil Sci., 2(4), 301-307.

Alam, Sadia., Samina, Khalil., Najma, Ayub ., Maliha, Rashid. 2002. In vitro solubilization of inorganic phosphate by phosphate solubilizing microorganisms (PSM) from maize rhizosphere. Int. J. Agr. Biol.,1560–8530.

Baig, K.S., Arshad, M., Zahir, Z.A., Cheema, M.A. 2010. Comparative efficacy of qualitative and quantitative methods for rock phosphate solubilization with phosphate solubilizing rhizobacteria. Soil & Environ. 29, 82 – 86.

Banik, S., Dey, B.K. 1983. Alluvial soil microorganisms capable of utilizing insoluble aluminium phosphate as a source of phosphorus. Zentral blatt Mikrobiol. 138, 437–42.

Chalit, Nopparat., Marisa, Jatupornpipat., Aree, Rittiboon. 2009. Optimization of the Phosphate-Solubilizing Fungus, *Aspergillus japonicus* SA22P3406, in Solid-State Cultivation by Response Surface Methodology. Kasetsart J. (Nat. Sci.). 43, 172 – 181.

Chen, X.C., Bai, J.X., Cao, J.M., Li, Z.J., Xiong, J., Zhang, L., Hong, Y., Ying, H.J. 2009. Medium optimization for the production of cyclic adenosine 3', 5'-monophosphate by *Microbacterium* sp. no. 205 using response surface methodology. Bioresour. Technol. 100, 919-924.

Cunningham, J.E., Kuiack, C. 1992. Production of Citric and Oxalic Acids and Solubilization of Calcium Phosphates by *Pencillium bilaii*. Appl. Environ. Microbiol. 58,1451-1458.

Deubel, A., Granse., Merbach, W. 2000. Transformation of organic rhizodeposits by rhizoplane bacteria and its influence on the availability of tertiary calcium phosphate. J. Plant Nutr. Soil Sci., 163, 387-392

Goldstein, A.H. 1995. Recent Progress in Understanding the Molecular Genetics and Biochemistry of Calcium Phosphate Solubilization by Gram-negative Bacteria. BAH. 12(1), 185-193.

Gupta, R., Rekha, S., Aparna, S., Kuhad, R.C. 1994. A modified plate assay for screening phosphate solubilizing microorganisms. J. Gen. Appl. Microbiol., 40, 255–60.

Gyaneshwar. P., Naresh, Kumar G., Parekh, L.J. 1998. Effect of Buffering on the Phosphate Solubilizing Ability of Microorganisms. World J. Microbiol Biotechnol. 14, 669-673.

Illmer, P., Schinner. F. 1995. Solubilization of inorganic calcium phosphate solubilization mechanisms. Soil Biol. Biochem, 27, 257– 63.

Isaie. M., Padmavathi, T. 2015. Statistical Optimization of Cellulase and Xylanase Enzyme Production by Penicillium Crustosum Using Sugar Beet Peel Substrate by Response Surface Methodology. Res. J. Pharm. Biol. Chem. Sci. 6, 1144-1151.

Kang, S.C., Ha, C.G., Lee, T.G. Maheshwari. D.K. 2002. Solubilization of insoluble inorganic phosphates by a soil-inhabiting fungus *Fomitopsis* sp. PS 102. Curr. Sci. 82, 439-442.

Minaxi, Saxena, J., Chandra, S., Nain, L. 2013. Synergistic effect of phosphate solubilizing rhizobacteria and arbuscular mycorrhiza on growth and yield of wheat plants. Journal of Soil Science and Plant Nutrition. 2013, 13 (2), 511-525

Morales. A., Alvear, M., Valenzuela, E., Castillo, C.E., Borie, F. 2011. Screening, evaluation and selection ofphosphate-solubilising fungi as potential biofertilizer. Journal of Soil Science and Plant Nutrition. 11 (4), 89-103.

Puente, M.E., Bashan, Y., Li, C.Y., Lebsky, K. 2004. Microbial populations and activities in the rhizoplane of rock-weathering desert plants. I. Root colonization and weathering of igneous rocks. Biology of Plants. 6, 629–642.

Rajendran, A., Meikandhan, Thirugnanam., Viruthagiri, Thangavelu. 2007. Statistical evaluation of medium components by Plackett-Burman experimental design and kinetic modeling of lipase production by *Pseudomonas fluorescens*. Indian J. Biotechnol. 6, 469- 478.

Rashid, Maliha., Samina, Khalil., Najma, Aryub., Sadia Alam., Farooq, Latif. 2004. Organic acid production and phosphate solubilization by phosphate solubilizing microorganisms (PSM) under in-vitro conditions. Pakistan J. Biol. Sci. 7, 187-196.

Schoebitz, M., Ceballos, C., Ciampi, L. 2013. Effect of immobilized phosphate solubilizing bacteria on wheat growth and phosphate uptake. Journal of Soil Science and Plant Nutrition. 13 (1), 1-10.

Song, O.R., Lee, S.J., Lee, Y.S., Lee, S.C., Kim, K.K., Choi, Y.L. 2008. Solubilization of insoluble inorganic phosphate by *Burkholderia cepacia* DA23 isolated from cultivated soil. Braz. J. Microbiol. 39, 151-156.

Subhagar, Seraman., Aravindan, Rajendran., Viruthagiri, Thangavelu. 2010. Statistical optimization of anticholesterolemic drug lovastatin production by the red mold Monascus purpureus. Food Bioprod Process. 88, 266- 276.

Sudhakara Reddy, M., Surender Kumar, K., Babita Reddy, M.S. 2002. Biosolubilization of poorly soluble rock phosphates by *Aspergillus tubingensis* and *Aspergillus niger*. Bioresource Technol., 84,187–189.

Swetha, S., Varma, A., Padmavathi, T. 2014. Statistical evaluation of the medium components for the production of high biomass, a-amylase and protease enzymes by *Piriformospora indica* using Plackett–Burman experimental design. 3 Biotech, 4, 439–445.

Trivedi, P., Sa, T.M. 2008. *Pseudomonas corrugata* (NRRL B-30409) mutants increased phosphate solubilization, organic acid production and plant growth at lower temperatures. Curr. Microbiol. 56, 140-144.

Venkateswarlu, B., Rao, A.V., Raina, P. 1994. Evaluation of phosphorus solubilization by microorganisms isolated from arid soils. J. Indian Soc. Soil Sci. 32, 273–277.

Wang, Guang-Hua., Jian, J., Mei-Na, X., Xiang- Wen, P., Tang, C. 2007. Inoculation with phosphate-solubilizing fungi diversifies the bacterial community in rhizospheres of maize and soybean. Pedosphere. 17, 191-199.

Whitelaw, M.A., Harden, T.J., Helyar, K.R. 1999. Phosphate solubilization in solution culture by the soil fungus *Penicillium radicum*. Soil Biol Biochem. 31, 655-66.

Whitelaw, M.A. 2000. Growth Promotion of Plants Inoculated with Phosphate Solubilizing Fungi. Adv Agron., 69, 99-151.