African Journal of Microbiology Research Vol. 5(20), pp. 3327-3333, 30 September, 2011
Available online http://www.academicjournals.org/ajmr
DOI: 10.5897/AJMR11.655
ISSN 1996-0808 ©2011 Academic Journals

Full Length Research Paper

Optimization of culture conditions for phosphate solubilizing by *Acinetobacter calcoaceticus* YC-5a using response surface methodology

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Accepted 26 August 2011

Response surface methodology was employed to optimize the composition of medium for phosphate solubilization by *Acinetobacter calcoaceticus* YC-5a, which is less well known as phosphate solubilizing plant-associated bacteria. $(NH_4)_2SO_4$, $Ca_3(PO_4)_2$ and KCl were found to have significant effects on phosphate solubilization by the Plackett-Burman design. The steepest ascent method was used to access the optimal region of the medium composition, followed by an application of response surface. The analysis revealed that the optimum values of the tested variables were $(NH_4)_2SO_4$ 0.2 g/L, $Ca_3(PO_4)_2$ 12.98 g/L and KCl 0.49 g/L, respectively. Phosphate solubilization of 747.72 mg/L, which was in agreement with the prediction, was observed in verification experiment. In comparison to the original level (405.4 mg/L), 1.83-fold increase had been obtained.

Key words: Phosphate solubilizing, *Acinetobacter calcoaceticus*, medium optimization, response surface.

INTRODUCTION

Phosphorus (P) is one of the major plant nutrients limiting plant growth. Most agricultural soils contain large reserves of phosphorus, a considerable part of which has accumulated as a consequence of regular applications of P fertilizers (Richardson et al., 1994). However, a large portion of soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants (Dey, 1988). Thus, the release of insoluble and fixed forms of P is an important aspect of increasing soil P availability. Phosphate-solubilizing bacteria (PSB) are well known to promote plant growth because of their ability to convert insoluble form of P to soluble form that can be readily taken up by the plant roots.

Various kinds of bacteria (Rodriguez and Fraga, 1999; Harris et al., 2006; Perez et al., 2007) and fungi (Whitelaw, 1999; Wakelin et al., 2007) have been isolated and characterized for their ability to solubilize unavailable reduced phosphorus to available forms. Nevertheless, very few studies have reported strains of *Acinetobacter* as phosphate solubilizing plant-associated bacteria (Kuklinsky-Sobral et al., 2004), *Acinetobacter* is a ubiquitous bacterial genus widely distributed in soil and water environments, however, their role in the soil P cycle is less well known.

In this study, *Acinetobacter calcoaceticus* YC-5a, which showed a strong phosphate solubilizing activity was isolated from rhizospheric soil of maize in Northwestern China. This work was to apply the Plackett-Burman design, followed by the paths of steepest ascent and response surface methodology to optimize the culture medium composition for phosphate solubilization by *A. calcoaceticus* YC-5a.The physiology of phosphate solubilization has not studied thoroughly (Rodriguez and Fraga, 1999) and the optimization of P solubilizing by *A.*

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Table 1. The Placket-Burman design variables (in coded levels) with soluble phosphate as response.

Run	Variable levels									Soluble P (mg/L)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	
1	+	-	+	-	-	-	+	+	+	624.3
2	+	+	-	+	-	-	-	+	+	256.3
3	-	+	+	-	+	-	-	-	+	453.6
4	+	-	+	+	-	+	-	-	-	492.3
5	+	+	-	+	+	-	+	-	-	336.4
6	+	+	+	-	+	+	-	+	-	506.1
7	-	+	+	+	-	+	+	-	+	484.8
8	-	-	+	+	+	-	+	+	-	550.3
9	-	-	-	+	+	+	-	+	+	368.6
10	+	-	-	-	+	+	+	-	+	439.7
11	-	+	-	-	-	+	+	+	-	393.7
12	-	-	-	-	-	-	-	-	-	373.8

calcoaceticus species has not yet been in full detail. Therefore, the aims were to gather some physiological information about the solubility mechanisms, thus hoping to throw some light upon the matter.

MATERIALS AND METHODS

Microorganism and medium

The strain *A. calcoaceticus* YC-5a maintained at -80℃ in peptone water medium containing 20% glycerol.

Analytical method

Solubilization of P by *A. calcoaceticus* YC-5a were estimated using insoluble $Ca_3(PO_4)_2$ in National Botanical Research Institute's Phosphate (NBRIP) broth medium (Nautiyal, 1999). The composition of the medium was (g/L): glucose, 10; $Ca_3(PO_4)_2$, 5; $MgCl_2{\cdot}6H_2O$, 5; $MgSO_4{\cdot}7H_2O$, 0.25 ; KCl, 0.2; $(NH_4)_2SO_4$, 0.1, pH 6.8-7.0. One milliliter bacterial suspension was transferred to 250 ml Erlenmeyer flask containing 80 ml of the NBRIP broth medium and incubated for 7 days. A separate NBRIP broth medium inoculated with sterile Milli-Q water served as the control treatment. The cultures were harvested by centrifugation at 10174 g for 10 min and soluble-P content of culture supernatant was estimated by the phosphomolybdate method (Murphy and Riley, 1962). The pH was determined with pH meter.

Experimental designs

Plackett-Burman design

Plackett-Burman design, a rapid screening multifactor to find the most significant independent factors (Plackett and Burman, 1946; Wang et al., 2007; Xiao et al., 2007), was used in the present study to screen the important variables that significantly influenced phosphate solubilization. In this study, a 12-run Plackett-Burman design (Table 1) with a first-order polynomial equation was applied to evaluate nine factors (including two dummy variables). Each variable was examined at two levels: -1 for the low level and +1 for the high level. The fitted first-order model is

$$Y = \beta_0 + \sum \beta_i x_i \qquad (1)$$

Y is the predicted response, β_0 and β_i are constant coefficients, and x_i is the coded independent factors.

Path of steepest ascent

The method of steepest ascent is a procedure for moving along the direction of the maximum increase in the response (He and Tan, 2006; Chen et al., 2009). The direction of steepest ascent is the direction in which the phosphate solubilization increases most rapidly by increasing or decreasing the values of the significant factors. The factors screened by the Plackett-Burman design were further optimized by this method. The path of steepest ascent was initiated from the center of the Plackett-Burman design. Experiments were performed along the steepest ascent path until the response did not increase any more. The experimental design and results of the steepest ascent method are shown in Table 3.

Response surface methodology

A central composite design (CCD) of RSM was employed to optimize the three most significant factors ($(NH_4)_2SO_4$, $Ca_3(PO_4)_2$, KCl) for enhancing P solubilization by *A. calcoaceticus* YC-5a, screened by Plackett-Burman design. The three independent factors were investigated at five different coded levels (-1.682, -1, 0, +1, +1.682) and the experimental design used for study is shown in Table 4. The variables were coded as in Equation 2:

$$x_i = (X_i - m_i)/l_i,\ i = 1,2,\ldots,k, \qquad (2)$$

Where Xi is the real value of the independent variable; x_i is the coded value; m_i is the value of Xi at the center point and l_i is the step change value. The phosphate solubilization was fitted using a second-order polynomial equation and a multiple regression of the data was carried out for obtaining an empirical model related to the most significant factors. The general form of the second-order polynomial equation is:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \qquad (3)$$

Where Y is the predicted response, x_i and x_j are independent factors,

Table 3. Experimental design and results of the steepest ascent path.

Run	$(NH_4)_2SO_4$ (g/L)	$Ca_3(PO_4)_2$ (g/L)	KCl (g/L)	Soluble P (mg/L)
	X_2	X_3	X_7	
1	0.3	5	0.2	405.4
2	0.24	6.5	0.24	427.7
3	0.18	8	0.28	596.5
4	0.12	9.5	0.32	675.3
5	0.06	11	0.36	659.7

Table 4. Experimental design and results of the central composite design.

Run	$(NH_4)_2SO_4$ (g/L)		$Ca_3(PO_4)_2$ (g/L)		KCl (g/L)		Soluble P
	x_2	X_2	x_3	X_3	x_6	X_6	mg/L
1	-1	0.06	-1	8	-1	0.28	624.87
2	1	0.18	-1	8	-1	0.28	613.24
3	-1	0.06	1	11	-1	0.28	736.76
4	1	0.18	1	11	-1	0.28	653.19
5	-1	0.06	-1	8	1	0.36	619.36
6	1	0.12	-1	8	1	0.36	668.23
7	-1	0.06	1	11	1	0.36	739.67
8	1	0.18	1	11	1	0.36	714.12
9	-1.682	0.02	0	9.5	0	0.32	668.26
10	1.682	0.22	0	9.5	0	0.32	641.28
11	0	0.12	-1.682	6.977	0	0.32	587.16
12	0	0.12	1.682	12.023	0	0.32	721.72
13	0	0.12	0	9.5	-1.682	0.25	635.81
14	0	0.12	0	9.5	1.682	0.39	676.80
15	0	0.12	0	9.5	0	0.32	672.74
16	0	0.12	0	9.5	0	0.32	671.16
17	0	0.12	0	9.5	0	0.32	677.57
18	0	0.12	0	9.5	0	0.32	683.66
19	0	0.12	0	9.5	0	0.32	681.03
20	0	0.12	0	9.5	0	0.32	687.54

β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient.

Statistical analysis

Minitab 15.0 (Minitab Inc., Pennsylvania, USA) was used for the experimental designs and subsequent regressional analysis of the experimental data. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equation was judged statistically by the coefficient of determination R^2, and its statistical significance was determined by an *F*-test. The significance of the regression coefficients was tested by a *t*-test.

RESULTS AND DISCUSSION

Plackett-Burman design

The Plackett-Burman design is a rapid method for screening significant factors. Twelve runs were carried out to analyze the effect of 9 variables on P solubilization and the results are demonstrated in Table 2. Analysis of the regression coefficients, *t*-values and *P*-values of 9 factors showed that X_1, X_3, X_5, X_6, X_7 and X_8 had positive effects on phosphate solubilization, whereas X_2, X_4 and X_9 had negative effects. The variable with $P<0.05$ is considered as significant parameter. It was clear that variables X_2, X_3 and X_7 were the significant factors, while X_1, X_4, X_5, X_6, X_8 and X_9, with $P>0.05$, were considered insignificant and were not included in the next path of steepest ascent and CCD experiments. The model equation for phosphate solubilization (Y) could be written as:

$$Y = 439.99 + 2.52\,X_1 - 34.84\,X_2 + 78.58\,X_3 - 25.21\,X_4 + 2.46\,X_5 + 7.54\,X_6 + 31.54\,X_7 + 9.89\,X_8 - 2.11\,X_9 \quad (4)$$

The prominent effects of $(NH_4)_2SO_4$ (X_2), $Ca_3(PO_4)_2$ (X_3)

Table 2. The Plackett-Burman design for screening variables in phosphate solubilization.

Factors (g/L)	Code	Experimental value		Effect	Coef*	*t*-value	P-value
		Low (-1)	High (+1)				
Intercept					439.99	60.89	0.000
Glucose	X_1	10	15	5.05	2.52	0.35	0.76
$(NH_4)_2SO_4$	X_2	0.1	0.3	-69.68	-34.84	-4.82	0.040
$Ca_3(PO_4)_2$	X_3	5	7.5	157.15	78.58	10.87	0.008
$MgCl_2·6H_2O$	X_4	5	7.5	-50.42	-25.21	-3.49	0.073
$MgSO_4·7H_2O$	X_5	0.25	0.5	4.92	2.46	0.34	0.766
NaCl	X_6	0	0.2	15.08	7.54	1.04	0.406
KCl	X_7	0.2	0.4	63.08	31.54	4.37	0.049
$MnSO_4·H_2O$	X_8	0	0.002	19.78	9.89	1.37	0.304
$FeSO_4·7H_2O$	X_9	0	0.002	-4.22	-2.11	-0.29	0.798

R^2=94.88%, R^2 (adj) =91.82%, *Coef: coefficient

and KCl (X_7) were likely due to the requirement of these medium components for phosphate solubilization. A critical K concentration is necessary for optimum solubilization rates (Beever and Burns, 1981; Illmer and Schinner, 1992).

Path of steepest ascent

Path of steepest ascent was based on the zero level of the Plackett-Burman design and moved along the direction in which $(NH_4)_2SO_4$ decreased and $Ca_3(PO_4)_2$, KCl increased. The experimental design and results were shown in Table 3. The highest response was 675.3 mg/L with $(NH_4)_2SO_4$ 0.12 g/L, $Ca_3(PO_4)_2$ 9.5 g/L and KCl 0.32 g/L. This point was concluded to be near the optimal point and was chosen for further optimization.

Optimization by response surface methodology

CCD was employed to study the interactions between the significant factors and also to determine their optimal levels. The design matrix of tested variables and the experimental results are represented in Table 4. Multiple regression analysis was used to analyze the data and thus a second-order polynomial equation was derived, as follows:

$$Y = 678.38 - 8.59X_2 + 39.86X_3 + 13.35X_7 - 4.87X_2^2 - 4.98X_3^2 - 4.32X_7^2 - 18.29X_2X_3 + 14.82X_2X_7 + 1.79X_3X_7 \quad (5)$$

The *t*-test and P-values were used to identify the effect of each factor on phosphate solubilization by *A. calcoaceticus* YC-5a (Table 5). A P-value of less than 0.05 indicates that the model terms are significant. In this case, $(NH_4)_2SO_4$, $Ca_3(PO_4)_2$ and KCl had a significant effect on phosphate solubilization, as well as the interaction terms. The fitness of the model was examined by the coefficient of determination R^2, which was found to be 0.9623. A regression model having an R^2-value higher than 0.9 was considered as having a very high correlation (Chen et al., 2009). Therefore, the present R^2-value reflected a very good fit between the observed and predicted response. The adjusted determination coefficient (*R*-Sq = 92.84%) was also satisfactory to confirm the significance of the model.

Furthermore, an analysis of variance (ANOVA) for the response surface quadratic model is presented in Table 6, which also proves that this regression was statistically significant at 95% of confidence level. The model also showed statistically insignificant lack of fit (P =0.053), so the model was supposed to be adequate for prediction within the range of variables employed. The response surface curves are plotted to explain the interaction of the variables and to determine the optimum level of each variable for maximum response. The response surface curves are shown in Figures 1 to 3. Each figure demonstrated the effect of two factors while the other factors were fixed at zero level. The model predicted the optimal values (coded) of the three most significant variables were X_2 = 1.339, X_3 = 2.320 and X_7 = 4.323. Correspondingly, the values of $(NH_4)_2SO_4$, $Ca_3(PO_4)_2$ and KCl were 0.2, 12.98 and 0.49 g/L, respectively. The maximum predicted phosphate solubilization was 747.72 mg/L.

Validation of the model

In order to confirm the optimized culture conditions, three additional experiments were performed using the predicted culture conditions. The mean values of phosphate solubilization was 743.5 mg/L, which agreed well with the predicted value. This result demonstrates the validity of the response model.

Conclusion

Fractional factorial design and response surface

Table 5. Regression coefficients and their significance for response surface model.

Term	Coef.	St. Dev. Coef.	T	P
Constant	678.38	4.45	152.397	0.000
X_2	-8.59	2.95	-2.908	0.016
X_3	39.86	2.95	13.496	0.000
X_7	13.35	2.95	4.519	0.001
X_2^2	-4.87	2.87	-1.693	0.121
X_3^2	-4.98	2.87	-1.733	0.114
X_7^2	-4.32	2.87	-1.504	0.164
X_2X_3	-18.29	3.85	-4.741	0.001
X_2X_7	14.82	3.85	3.839	0.003
X_3X_7	1.79	3.85	0.465	0.652

S = 10.9144, PRESS = 7761.46, R-Sq = 96.23%; R-Sq(adjust) = 92.84%.

Table 6. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of P solubilization.

Source	DF[a]	Seq. SS[b]	Adj.SS	Adj. MS[c]	F	P
Regression	9	30405.2	30405.2	3378.35	28.36	0.000
Linear	3	25136.5	25136.5	8378.84	70.34	0.000
Quadratic	3	809.0	809.0	269.65	2.26	0.143
Interactions	3	4459.7	4459.7	1486.56	12.48	0.001
Residual error	10	1191.2	1191.2	119.12		
Lack of fit	5	989.8	989.8	197.95	4.91	0.053
Pure error	5	201.5	201.5	40.30		
Total	19	31596.4				

[a] DF, Degree of freedom, [b] SS, sum of squares, [c] MS, mean square.

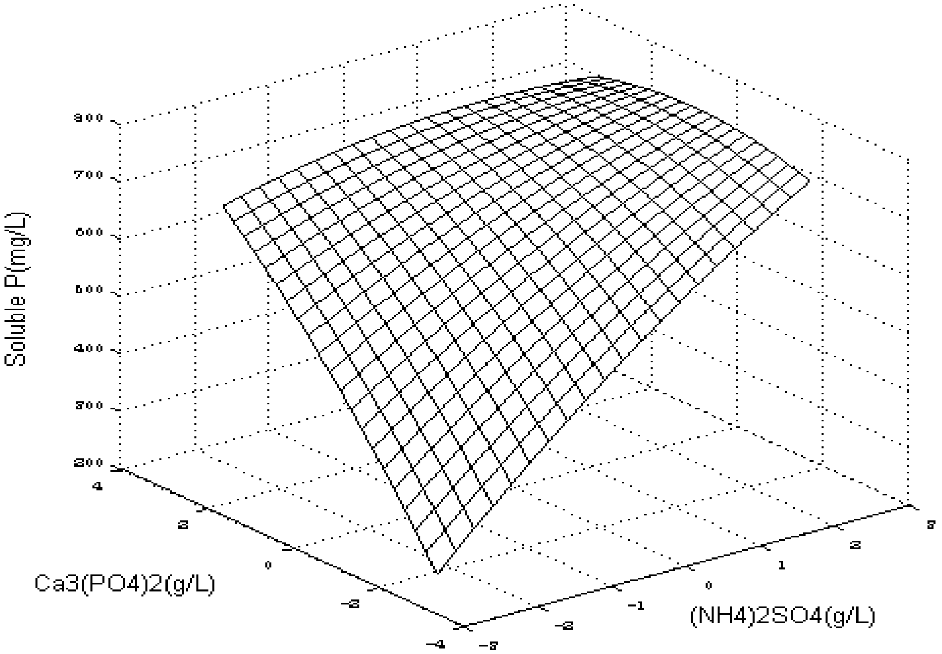

Figure 1. Response surface curve for phosphate solubilization by *A. calcoaceticus* YC-5a showing the interaction between $(NH_4)_2SO_4$ and $Ca_3(PO_4)_2$.

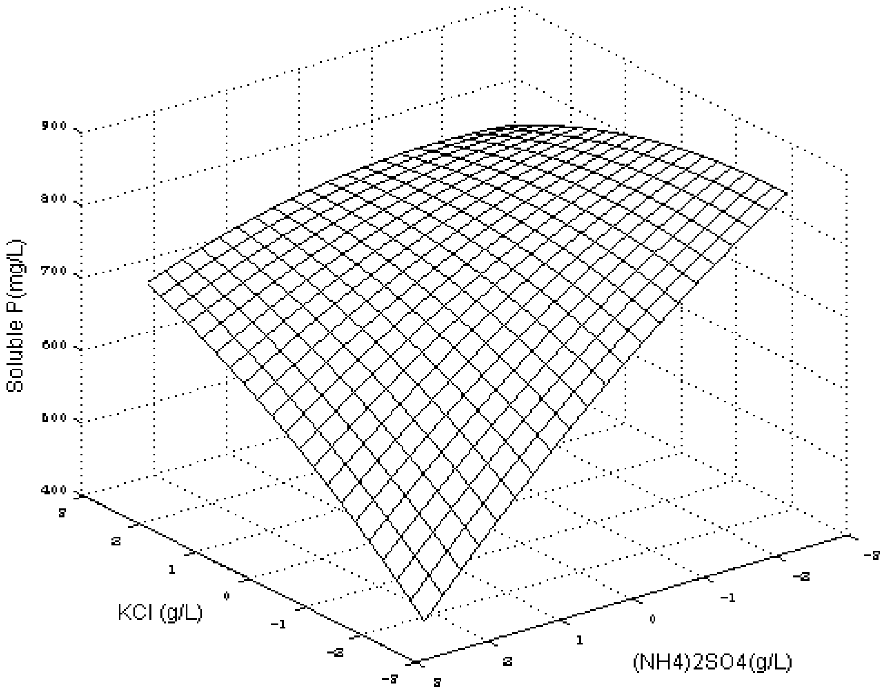

Figure 2. Response surface curve for phosphate solubilization by *A. calcoaceticus* YC-5a showing the interaction between $(NH_4)_2SO_4$ and KCl.

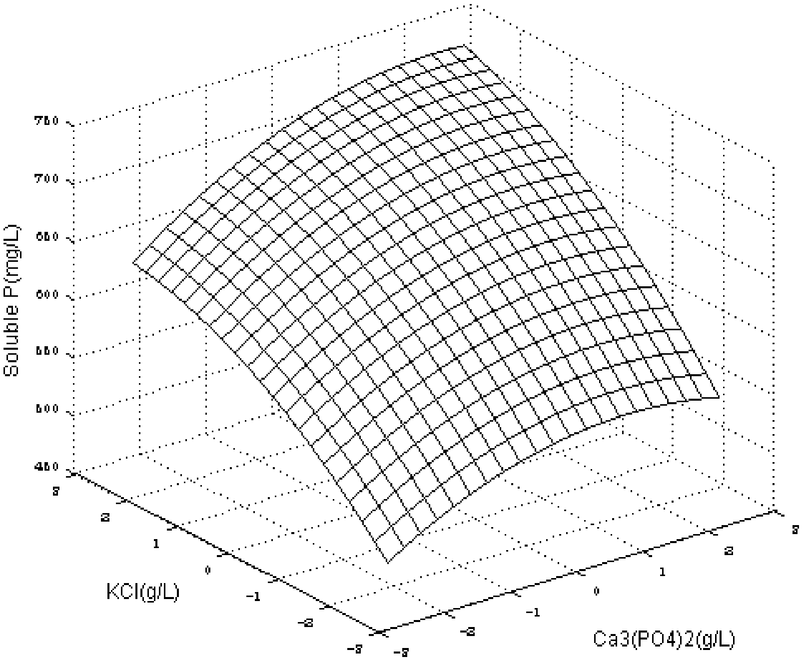

Figure 3. Response surface curve for phosphate solubilization by *A. calcoaceticus* YC-5a showing the interaction between $Ca_3(PO_4)_2$ and KCl.

methodology had been proved to be effective on optimizing phosphate solubilization by *A. calcoaceticus* YC-5a. The final medium composition optimized was (g/L): glucose, 10; $(NH_4)_2SO_4$, 0.2; $Ca_3(PO_4)_2$, 12.98; $MgCl_2.6H_2O$, 5; $MgSO_4{\cdot}7H_2O$, 0.25; KCl, 0.49 and initial pH, 6.8-7.0, which resulted in an overall 1.8-fold increase compared with that using the original medium. Validation experiments were also carried out to verify the adequacy and the accuracy of the model, and the results showed that the predicted value agreed with the experimental values well.

ACKNOWLEDGEMENTS

This study was financially supported by the National High Technology Research and Development Program of China [grant number 2007AA03Z456].

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