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Enhanced Phosphorus Solubilization by *Bacillus licheniformis* CKA1 Using Central Composite Design and Response Surface Methodology

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In the present study phosphate (P) solubilizing strain *Bacillus licheniformis* CKA1 previously isolated from apple rhiosphere, was evaluated for mineral P-solubilization in Pikovskaya's (PVK) medium containing tricalcium phosphate (TCP) as insoluble P source. Highest P-solubilization (118.00 μg mL^{-1}) was achieved at 96 h of incubation and decreased thereafter. P solubilization potential of *B. licheniformis* CKA1 was further enhanced by statistical optimization of culture conditions and media components under *in vitro* conditions. Incubation temperature, inoculum size, glucose and yeast extract concentration were identified as significant factors affecting the P-solubilization in Plackett Burman design and were further selected for optimization in central composite design (CCD) of response surface methodology (RSM). The final medium optimized was (g L^{-1}): glucose, 10; yeast extract, 0.50, ammonium sulphate, 0.50; KCl, 0.20; TCP, 15; $MgSO_4.7H_2O$, 0.1; $MnSO_4$, 0.01; $FeSO_4$, 0.01; initial pH 7.00 with 1.5 O.D. inoculum size and incubated at 35 °C for 96 h, which give 400 μg mL^{-1} of soluble P with 3.39-fold increase in P-solubilization compared with that obtained in the unoptimized medium. The results in study suggested that *B. licheniformis* CKA1 could be used in a bioprocess for the manufacture of phosphatic fertilizer from phosphate calcium minerals.

Key words: *Bacillus licheniformis*, central composite design, phosphate solubilization, response surface methodology.

Phosphorus (P) is a key nutrient for biological and sustained agriculture productivity. Soils are generally low in P readily available for plant growth. Phosphate deficiency is thus widespread, and P fertilizers are almost universally required to maintain crop productivity. However, the applied P fertilizers are easily precipitated into insoluble forms—$CaHPO_4$, $Ca_3(PO_4)_2$, $FePO_4$ and $AlPO_4^-$, and are not efficiently taken up by the plants, which lead to an excess application of P fertilizer to crop land[1]. Overfertilization of P leads to pollution due to soil erosion and run-off water containing large amounts of soluble P. Therefore, the necessity to develop economical and eco-friendly technologies is steadily increasing. Soil–plant–microbe interactions are complex and there are many ways in which the outcome can influence the plant health and productivity[2]. The interaction may be harmful, beneficial, and neutral to the plants. However, our focus should be to exploit the beneficial interaction of plants and microbes. The use of microbial technologies in agriculture is expanding quite rapidly with the identification of new bacterial strains, which are more effective in promoting plant growth. The use of plant growth-promoting rhizobacteria (PGPR) including phosphate solubilizing bacteria (PSB) as biofertilizers was suggested as a sustainable solution for the improvement of nutrient

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availability, plant growth, and yields[3]. P-solubilizing soil microorganisms act by releasing P from inorganic and organic pools of total soil P through solubilization and mineralization resulting in higher crop yields[4]. Selection of P-solubilizing rhizobacteria requires an understanding of its mechanism of P-solubilization[5], and modulation of culture conditions and nutritional parameters of PSB help to give an insight into their mechanism behind phosphate solubilization[6]. Moreover, phosphorus solubilization activity has been strongly influenced by the culture conditions, presence or absence of nitrogen, phosphorus, carbon sources, salts or certain metal ions[7].

Strains of the genus *Bacillus* are among the most commonly reported PGPR[8, 9]. *Bacillus* strains have the advantage of being able to form endospores which warrant the prevalence of *Bacillus* under different environmental cues, its long-term storage and easy development of reliable formulations[10]. Numerous studies have highlighted the functions of phosphate solubilising *Bacillus* strains and their role in plant growth promotion[11-13]. However, to date little information exists on optimization of cultural conditions and nutritional media for characterization and enhanced P-solubilization by P-solubilizing *Bacillus* spp. Traditional optimization methods involve changing one independent variable while fixing others at a certain level. This single-dimensional approach is laborious, time-consuming and does not analyse the interactions between variables, so it is not appropriate to reach a true optimum. The response surface methodology (RSM) is an experimental strategy to find the optimum condition for a multivariable system[14], but so far, this methodology has not been well exploited to optimize bacterial P-solubilization process. Keeping this in view, response surface optimization technique was applied to maximize the P-solubilization efficiency of *Bacillus licheniformis* CKA1, a P-solubilizing strain originally isolated from apple rhizosphere. Significant variables were identified in Plackett-Burman design (PBD) and the effect of significant variables and their interactions was studied by employing central composite design (CCD) in response surface methodology for enhanced P solubilization.

MATERIAL AND METHODS

Bacterial strain

Bacterial strain, *Bacillus licheniformis* strain CKA1 (NCBI Accession number: GQ 980017) originally isolated from apple rhizosphere was used in the study. The strain was maintained at -20 °C in nutrient broth amended with 30% glycerol and streaked from glycerol stock onto nutrient agar plates, incubated at 35 ± 2 °C for 24–48 h. The strain was selected on the basis of marked P-solubilizing activity on PVK agar in terms of halo zone formation after 96 h of incubation.

Inorganic phosphate solubilization

Phosphate solubilization ability was evaluated in PVK medium with tricalcium phosphate (TCP) as insoluble source of phosphate. Strain CKA1 was first screened on PVK agar plates. The P-solubilization was exhibited with a clear halo zone formed around the bacterial colony. Further quantitative estimation of phosphorus was done in PVK broth amended with 0.5% TCP by the vanadomolybdate method[15].

Plackett-Burman design

Plackett-Burman design, an efficient way of screening multiple factors to find the most significant independent factors[16], was used to screen the important variables that significantly influenced phosphate solubilization. In PBD, nine variables were selected at two levels, high (+1) and low (-1) Table 1. Using the selected levels for each variable and two dummy variables a 12 runs experiment was generated using the "Design Expert 9.0" Stat-Ease, Inc., Mineapolis, USA and used to analyze the experimental Plackett-Burman design.

Response surface methodology

A central composite design of RSM was employed to optimize the four most significant factors screened by PBD; incubation temperature, inoculum size, glucose and yeast extract concentration for enhancing P solubilization by *B. licheniformis* CKA1. The four independent factors were investigated at five different coded levels (-2, -1, 0, +1, +2). The P-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:

$$Y = \beta_0 + \Sigma\beta iXi + \Sigma\beta iiXi^2 + \Sigma\beta ijXiXj \quad ...(1)$$

Where *Y* is the predicted response, β_0, βi, âii and âij are the constant regression coefficients of the model; Xi, Xj (i = 1,4; j = 1,4, i ≠ j) represent the independent variables (medium components) in the form of coded values. The accuracy and predictive ability of the polynomial model was evaluated using the coefficient of determination R^2. Each experimental design was carried out in triplicate, and the mean values were given.

Statistical analysis

The statistical software package Design-Expert 9.0 (StatEase, Minneapolis, MN) was used for regression analysis of experimental data to obtain working parameters and to generate response surface graphs. 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 *t*-test.

RESULTS

Characterization for mineral phosphate solubilisation by *Bacillus licheniformis* CKA1

The strain *B. licheniformis* CKA1 showed marked P solubilization potential with 3.60 phosphate solubilization index (PSI) in solid PVK medium after 96 h of incubation. Mineral phosphate solubilization by the *B. licheniformis* CKA1 was quantitatively measured in PVK medium containing 0.5% TCP at different time intervals (Fig. 1). The amount of soluble P in the medium showed a gradual increase and reached a concentration of

Table 1. Levels of the factors tested for the P-solubilization by *B. licheniformis* CKA1 using Plackett-Burman design

Factors	Code	Unit	Lower level (-1)	Higher level (+1)
Incubation time	X1	h	72.00	120.00
Incubation temperature	X2	°C	30.00	40.00
pH	X3	-	6.00	8.00
Inoculum size	X4	OD	1.00	2.00
Glucose	X5	g L^{-1}	8.00	12.00
Yeast extract	X6	g L^{-1}	0.30	0.70
Ammonnium sulphate	X7	g L^{-1}	0.30	0.70
KCl	X8	g L^{-1}	0.100	0.30
TCP concentration	X9	g L^{-1}	10.00	20.00

Table 2. The Placket-Burman design variables (in coded levels) with soluble phosphate as response

Run	Variable levels											Soluble P (μg mL^{-1}) actual	Soluble P (μg mL^{-1}) predicted
	X1	X2	X3	X4	X5	X6	X7	X8	X9	D1	D2		
1	1	-1	1	1	-1	1	1	1	-1	-1	-1	95.00	97.50
2	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	90.00	92.50
3	-1	1	-1	1	1	-1	1	1	1	-1	-1	240.00	242.50
4	-1	1	1	-1	1	1	1	-1	-1	-1	1	155.00	159.17
5	1	-1	1	1	1	-1	-1	-1	1	-1	1	150.00	154.17
6	1	1	-1	1	1	1	-1	-1	-1	1	-1	160.00	155.83
7	-1	1	1	1	-1	-1	-1	1	-1	1	1	175.00	172.50
8	1	-1	-1	-1	1	-1	1	1	-1	1	1	130.00	127.50
9	1	1	1	-1	-1	-1	1	-1	1	1	-1	165.00	160.83
10	-1	-1	-1	1	-1	1	1	-1	1	1	1	135.00	132.50
11	-1	-1	1	-1	1	1	-1	1	1	1	-1	115.00	110.83
12	1	1	-1	-1	-1	1	-1	1	1	-1	1	110.00	114.17

118.00 µg mL^{-1} at 96 h of incubation and decreased thereafter. Concomitant with the P increase, there was a decrease in the pH of the medium. The pH value dropped to 4.5 from initial pH of 7.0.

Selection of significant variables by Plackett-Burman design

In PBD a first order model was fitted to results obtained from twelve runs experiment. The *t*-test was used to identify the effect of each factor on P-solubilization. A twelve runs experiment was 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_2, X_4, X_5, X_7, X_8 and X_9 had positive effects on P-solubilization, whereas X_1, X_3 and X_6 had negative effects. Analysis of variance (ANOVA) for selected factorial models identified the significant variables for P-solubilization in PBD (Table 3). The variables X_2, X_4, X_5 and X_6, with $P < 0.05$ were considered as significant parameter while X_1, X_3, X_7, X_8 and X_9 with $P > 0.05$, were considered insignificant and were not included in the CCD experiments.

The model equation for phosphate solubilization could be written as:

$$Y = 143.33 - 8.33X_1 + 24.17X_2 - 0.83X_3 + 15.83X_4 + 15.00X_5 - 15.00X_6 + 10.00X_7 + 0.83X_8 + 9.17X_9 \quad ...(2)$$

Optimization of significant variables using RSM

Central composite design was employed to study the interactions between the significant factors and also to determine their optimal levels. The selected four significant variables (incubation temperature, inoculum size, glucose and yeast extract) and levels of these variables are given in Table 4. For RSM based on the CCD, used for the optimization of independent variables for P-solubilization, 30 experimental runs with different combinations of four factors were carried out. The design matrix with the corresponding results of CCD, as well as the predicted results is presented

Table 3. ANOVA for selected factorial model and identification of significant variables for P-solubilization by *B. licheniformis* CKA1 using Plackett-Burman design

Source	Sum of squares	df	Mean square	F value	*P* value (Prob > F)
Model	18475.00	9	2052.78	28.98	0.0338
X1	833.33	1	833.33	11.76	0.0755
X2	7008.33	1	7008.33	98.94	0.0100*
X3	8.33	1	8.33	0.12	0.7643
X4	3008.33	1	3008.33	42.47	0.0227*
X5	2700.00	1	2700.00	38.12	0.0252*
X6	2700.00	1	2700.00	38.12	0.0252*
X7	1200.00	1	1200.00	16.94	0.0543
X8	8.33	1	8.33	0.12	0.7643
X9	1008.33	1	1008.33	14.24	0.0636
Residual	141.67	2	70.83		
Corrected total	18616.67	11			

The Model F-value of 28.98 implies the model is significant. There is a 4.54% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant.

*, significant model terms at $P \leq 0.05$

Table 4. Coded values of independent variables at different levels used in central composite design

Independent variables	Code	Unit	Levels				
			-2	-1	0	+1	+2
Incubation temperature	A	°C	25.00	30.00	35.00	40.00	45.00
Inoculum size	B	OD	0.50	1.00	1.50	2.00	2.50
Glucose	C	g L^{-1}	6.00	8.00	10.00	12.00	14.00
Yeast extract	D	g L^{-1}	0.10	0.30	0.50	0.70	0.90

in Table 5. The experimental responses showed considerable variation in the concentration of soluble P depending on the four independent variables in the medium. The minimum and maximum concentration of soluble P was 140 µg mL^{-1} and 400 µg mL^{-1}, respectively. The highest soluble P was obtained from Run No. 2 while the lowest activity was obtained in Run No. 22. The regression equation coefficients were calculated, and the data were fitted to a first-order polynomial equation. The significant model terms were evaluated by ANOVA in the optimization study ($P < 0.05$) (Table 6) and were identified as B, A^2, B^2, C^2, D^2 and AC, AD, BC, BD and CD. Statistical analysis revealed that the linear effect of A, C, D and interaction effect of AB were found to be insignificant terms in the quadratic model. The polynomial model for soluble phosphate (Y) was constructed using the significant terms and is presented in terms of coded factors:

$$Y = 356.81 - 13.45B - 31.45A^2 - 44.51B^2 - 24.44C^2 - 14.71D^2 - 13.44AC - 12.19AD + 12.19BC + 8.44BD - 10.31CD \quad ...(3)$$

The statistical significance of polynomial equation was checked by F-test, and ANOVA for the response surface quadratic model is shown in Table 6. The model was highly significant as evident by the model F value and a low probability value (P model > F = 0.0001).

Table 5. Actual and predicted values of P-solubilization recorded in experimental setup of response surface methodology

Std	Run	Incubation temperature (A)	Inoculum size (B)	Glucose (C)	Yeast extract (D)	P-solubilization (µg mL^{-1}) actual	P-solubilization (µg mL^{-1}) predicted
13	1	-1	+1	+1	+1	280.00	273.67
12	2	0	0	0	0	400.00	356.81
19	3	0	0	0	+1	330.00	344.22
30	4	2	0	0	0	225.00	224.74
29	5	-2	0	0	0	230.00	237.24
25	6	-1	+1	+1	-1	255.00	248.81
4	7	+1	-1	-1	-1	285.00	283.87
6	8	0	0	2	0	265.00	265.64
9	9	-1	-1	+1	+1	255.00	257.43
23	10	0	0	0	-2	295.00	293.74
5	11	+1	-1	-1	+1	260.00	267.07
18	12	+1	-1	+1	-1	250.00	259.45
10	13	-1	-1	+1	-1	270.00	266.33
20	14	0	0	0	-1	330.00	339.98
26	15	+1	-1	+1	+1	205.00	201.81
1	16	+1	+1	-1	+1	235.00	230.81
21	17	-1	-1	-1	-1	230.00	236.59
3	18	0	0	0	+2	300.00	302.20
11	19	-1	+1	-1	+1	245.00	236.43
24	20	0	+1	0	0	290.00	298.86
14	21	+1	+1	-1	-1	215.00	213.45
2	22	0	+2	0	0	140.00	151.88
7	23	+1	+1	+1	-1	245.00	238.19
16	24	+1	+1	+1	+1	220.00	205.67
22	25	-1	-1	-1	+1	270.00	268.95
8	26	-1	+1	-1	-1	175.00	170.32
15	27	0	0	+1	0	320.00	335.67
17	28	0	0	-2	0	250.00	252.42
28	29	0	-2	0	0	215.00	213.80
27	30	0	-1	0	0	290.00	298.86

Comparison of observed and predicted values of P-solubilization

A regression model can be used to predict future observations for a response corresponding to particular values of the regression variables. In predicting new observations and in estimating the mean response at a given point, one must be careful about extrapolating beyond the region containing the original observations. It may be possible that a model that fits well in the region of the original data will no longer fit well outside the region. Figure 2 showed the observed P-solubilization (response) versus those obtained from the empirical model in Equation 3. Figure confirmed that the predicted data of response from the empirical model is in agreement with the observed values in the range of the operating variables.

Response surface graphs

Figure 3 depicts the 3D plot and its corresponding contour plot, showing the effect of incubation temperature and inoculum size on the P-solubilization, while the other two factors were fixed at their middle level. The figure indicates that the concentration of soluble P increased gradually with increase in incubation temperature and inoculum size upto their middle level and achieved a predicted concentration of 356.81 µg mL^{-1} and decreased thereafter. This suggests that increasing the inoculum size and incubation time for *B. licheniformis* CKA1 within the desired range was beneficial to achieve maximum P-solubilization.

Figure 4 presents the 3D plot and its corresponding contour plot showing the effect of incubation temperature and yeast extract concentration on P-solubilization, while the other two factors were fixed at their middle level. It is evident that the P-solubilization increased gradually with increase in incubation temperature upto the middle level (35 °C) and achieved a concentration of 342.00 µg mL^{-1}, but above 35 °C, a decrease in concentration of soluble P was observed at low yeast extract concentration. Whereas, P-solubilization was increased to 330 µg mL^{-1} with increase in concentration of yeast extract to the high level (0.70 g L^{-1}) at low level of incubation temperature (30 °C).

Figure 5 depicts the 3D plot and its corresponding contour plot showing the effect of inoculum size and glucose concentration on P-solubilization, while the other two factors were fixed

Table 6. ANOVA for response surface quadratic model (P-solubilization)

Source	Sum of squares	df	Mean square	F-value	*P* value (Prob > F)
Model	70152.50	14	5010.89	22.43	< 0.0001*
A	234.38	1	234.38	1.05	0.3220
B	4580.96	1	4580.96	20.50	0.004*
C	271.19	1	271.19	1.21	0.2880
D	116.35	1	116.35	0.52	0.4816
A^2	24296.76	1	24296.76	108.74	< 0.0001*
B^2	43612.92	1	43612.92	195.18	< 0.0001*
C^2	13859.16	1	13859.16	62.03	< 0.0001*
D^2	4722.17	1	4722.17	21.13	0.0003*
AB	14.06	1	14.06	0.06	0.8053
AC	2889.06	1	2889.06	12.93	0.0026*
AD	2376.56	1	2376.56	10.64	0.0053*
BC	2376.56	1	2376.56	10.64	0.0053*
BD	1139.06	1	1139.06	5.10	0.0393*
CD	1701.56	1	1701.56	7.62	0.0146*
Residual	3351.66	15	223.44		
Lack of Fit	3351.66	14	239.40		
Pure Error	0.000	1	0.000		
Corrected Total	73504.17	29			

Note: The Model F-value of 22.43 implies the model is significant. There is only 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant

at their middle level. The figure revealed that concentration of soluble P increased gradually with increase in inoculum size and glucose concentration upto the middle level (1.5 OD, 10.00 g L^{-1}) and achieved a P-solubilization of 356.81 µg mL^{-1} and decreased thereafter.

Figure 6 shows the 3D plot and its corresponding contour plot showing the effect of

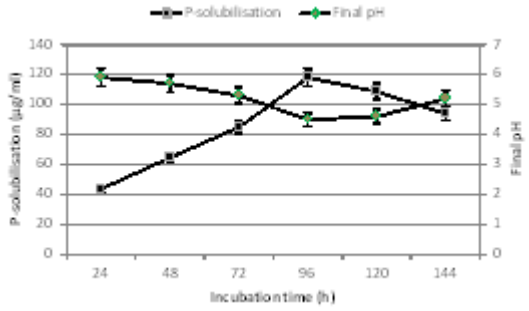

Fig. 1. Changes in concentration of soluble phosphorus and medium pH with time mediated by *Bacillus licheniformis* CKA1

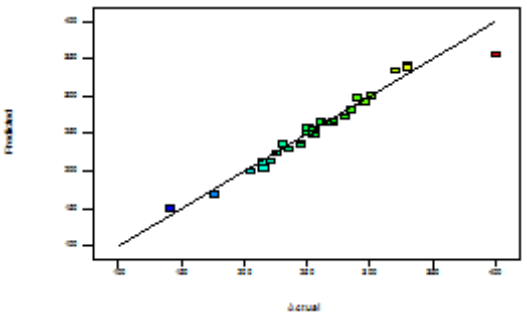

Fig. 2. Observed P-solubilization versus the predicted P-solubilization in response to selected variables

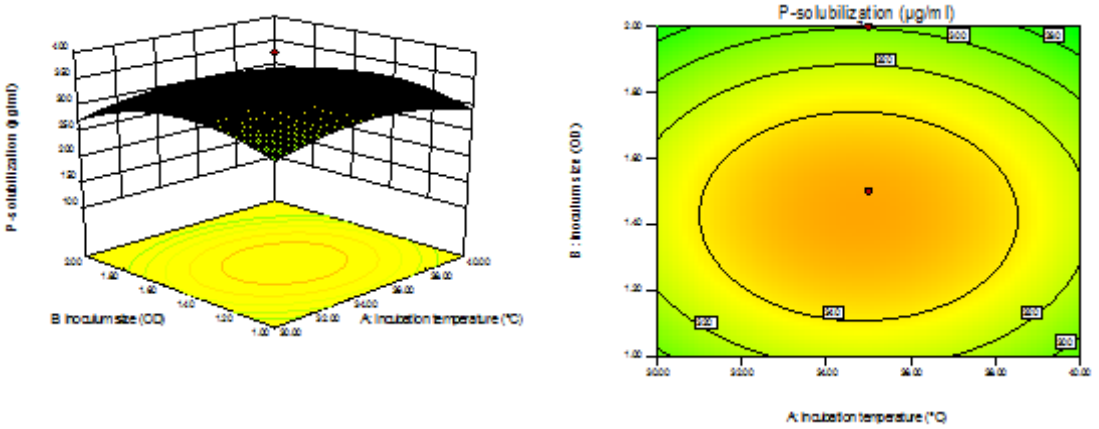

Fig. 3. Response surface plot (a) and contour plot (b) of the combined effect of incubation temperature and inoculums size on the P-solubilization by *Bacillus licheniformis* CKA1

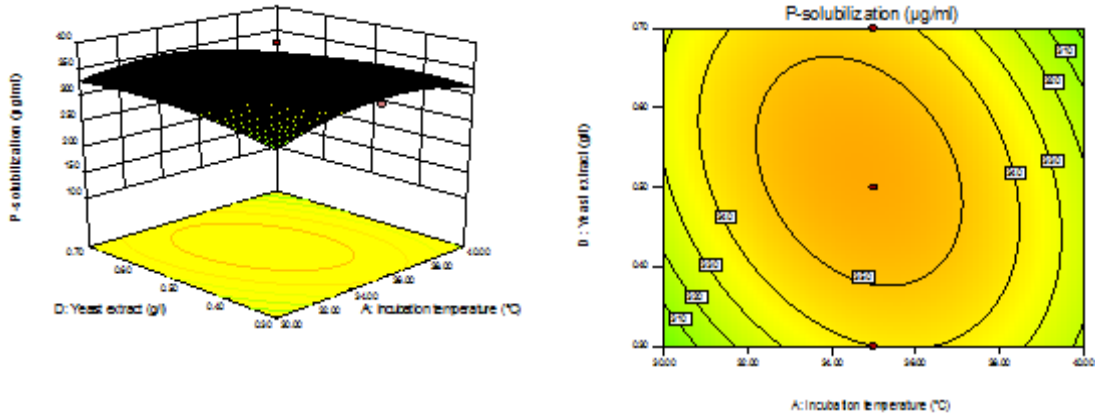

Fig. 4. Response surface plot (a) and contour plot (b) of the combined effect of incubation temperature and yeast extract on the P-solubilization by *Bacillus licheniformis* CKA1

glucose and yeast extract concentration on P-solubilization, while the other two factors were fixed at their middle level. It was revealed that, at low yeast extract concentration, with increase in concentration of glucose to a high level (11.00 g L^{-1}) concentration of soluble P also increased and achieved a concentration of 342.00 µg mL^{-1} and decreased thereafter. Similarly the P-solubilization was gradually increased when concentration of yeast extract was increased to high level (0.70 g L^{-1}) and achieved a concentration of 326.26 µg mL^{-1} at low glucose concentration (8.00 g L^{-1}).

Model validation

The precision of a model can be checked by the determination coefficient (R^2). The regression equation obtained from ANOVA showed that the value of R^2 was 0.954. This implies that the sample variation of 95.40% for P-solubilization was due to independent variables, and only 4.60% of the total variation cannot be explained by the model. The value of the adjusted determination coefficient (Adj-R^2 = 0.911) further confirms the significance of model. The value of the predicted determination coefficient (pred-R^2 = 0.879) is in reasonable agreement with value of the adjusted determination coefficient. Adequate precision measures the signal to noise ratio. An adequate precision ratio of 19.38 indicates that the model can be used to navigate the design space. The lower reliability of the experiment is usually indicated by the high value of the coefficient of variation (CV). Here a low CV (5.77) denotes that the experiments performed were highly reliable.

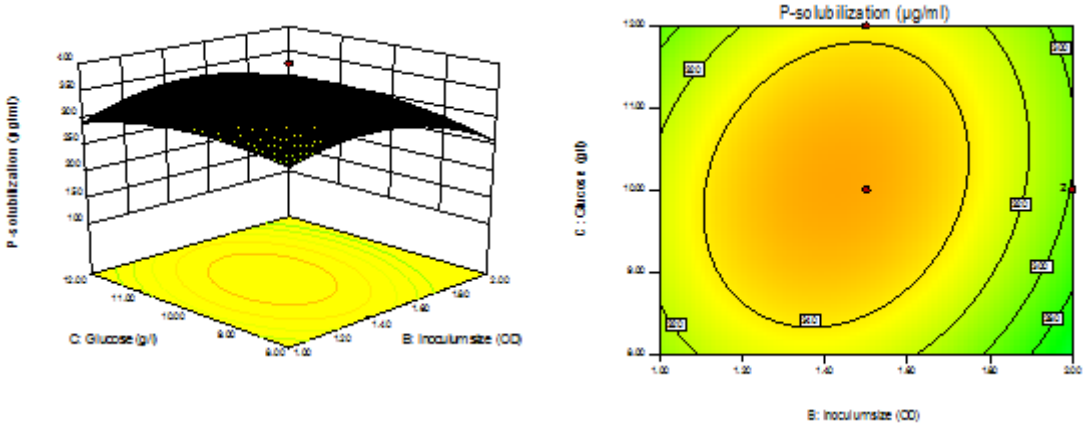

Fig. 5. Response surface plot (a) and contour plot (b) of the combined effect of inoculum size and glucose concentration on the P-solubilization by *Bacillus licheniformis* CKA1

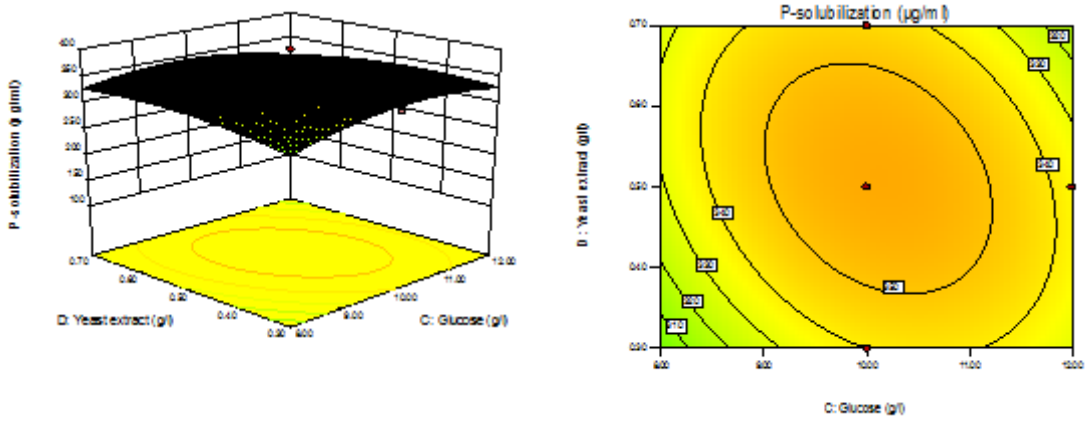

Fig. 6. Response surface plot (a) and contour plot (b) of the combined effect of glucose and yeast extract concentration on the P-solubilization by *Bacillus licheniformis* CKA1

DISCUSSION

Phosphorous nutrition influences overall plant growth and root development. In view of environmental concerns and current developments in sustainability, research efforts are concentrated on development of techniques involving the use of less expensive sources of plant nutrients such as rock and by application of PSB so that agronomic effectiveness can be enhanced[17]. In view of this the present study described the P-solubilizing efficacy of *B. licheniformis* CKA1 in PVK medium containing TCP as insoluble P source. *B. licheniformis* CKA1 showed marked P solubilization potential with 3.60 PSI in solid PVK medium. The amount of soluble P in the medium showed a gradual increase upto 96 h of incubation and decreased thereafter. Concomitant with the P increase, there was a decrease in the pH of the medium (Fig. 1). The results are in accordance with earlier reports, which have shown that the solubilization of mineral phosphate is accompanied by a decrease in pH[18, 19]. Reduction in amount of soluble phosphorous towards the later stages of incubation might be due to the depletion of nutrients in culture medium, in particular, carbon source needed for the production of organic acids[20].

P-solubilization potential of *B. licheniformis* CKA1 was further enhanced by optimizing culture conditions and media components under *in vitro* conditions. Optimization of single independent variable at a time approach is laborious, and time-consuming, moreover it does not analyze the interaction between different variables. The application of statistical design for screening and optimization of culture conditions for the P-solubilization allows quick identification of the important factors and the interactions between them[21]. The response surface methodology is an experimental strategy to find the optimum conditions for a multivariable system, but so far, this methodology has not been well exploited to optimize phosphorus solubilization by the P-solubilizing *Bacillus* strains. Use of Placket-Burman design to screen significant variables affecting P-solubilization by phosphate solubilizing *Acinetobacter calcoaceticus* has been reported by Fan *et al.*[22]. In the present study, out of total nine independent variables, 4 variables (incubation temperature, inoculum size, glucose and yeast extract) were identified as significant factors affecting the P-solubilization in PBD and were further selected for CCD experiment. The results revealed that there is no only factor in the P-solubilization, instead there are a complex of factors interact with each other in P-solubilization process as the consumption of glucose (converted into gluconic acid after oxidation and associated with drop in pH), organic and inorganic nitrogen sources (associated with the fall in pH), different growth conditions and inoculum size could affect the metabolism of the strain and change the organic acid secretion pattern and ultimately resulted in variable P-solubilization[7]. Our findings are in agreement with those of Nautiyal[23], who observed that glucose significantly affected P-solubilization. Detection of glucose as the essential component for P-solubilization by *B. licheniformis* CKA1 indicates that P-solubilization potential of the strain might be dependent on glucose metabolism involving enzyme glucose dehydrogenase involved in production of gluconic acid which might result in lower pH of the medium. In the present study yeast extract has negative but significant effect on P-solubilization. Our results coincide with those of Mehta *et al.*[24] who found that the presence of yeast extract in PVK medium may be inhibitory to the P-solubilization. RSM was further employed for enhanced P-solubilization by *B. licheniformis* CKA1 by optimizing four significant variables in CCD. The RSM applied to the optimization of P-solubilization in this investigation suggested the importance of a variety of factors at different levels. A high degree of similarity was observed between the predicted and experimental values, which reflected the accuracy and applicability of RSM for *in vitro* optimization of biological processes. The analysis of variance (F test) shows that the model was significant. CV indicates the degree of precision with which treatments were compared. Usually, the higher the value of CV, lower is the reliability of experiments[25]. In the present study, a lower value of CV (5.77) indicated a better precision and reliability of the experiments.

There have been reports on the optimization of culture media using statistical approaches for P-solubilization by fungi[26,7]; but not for P-solubilizing *Bacillus* strains. A response

surface method with four-factors-five-level in central compostite design has been successfully applied in the present study for P-solubilization by *B. licheniformis* CKA1 that have overcome the limitations of classical empirical methods. The statistical optimization resulted in maximum 400 µg mL^{-1} of soluble P after 96 h of incubation which was 2.86-fold higher than the lowest value of P-solubilization (140 µg mL^{-1}) (Table 5). In contrast to our results, Fan *et al*.[22] reported only 1.26-fold increase in P-solubilization over lowest value of released phosphorus after RSM. The results of CCD indicated the significance of inoculum size on P-solubilization by *B. licheiformis* CKA1 (Table 6), thus indicating that adding appropriate inoculum of PGPR is necessary to achieve significant P-solubilization and other PGP traits. Despite the single effect of other three variables, interactions within and between variables i.e. A^2, B^2, C^2, D^2, AC, AD, BC, BD and CD were found to be significant. Similar to our results Noppart *et al.* (2009) also found that interactions within the variables had more significant effect on P-solubilization by *Aspergillus japonicus*.

CONCLUSION

Statistical optimization of cultivation conditions using the central composite design appeared to be a valuable tool for the determination of enhanced P-solubilization by *B. licheniformis* CKA1. Incubation temperature, inoculum size, glucose and yeast extract concentration were identified as significant variables in Plackett Burman design and were further optimized for P-solubilization in RSM. The final medium optimized was (g L^{-1}): glucose, 10; yeast extract, 0.50, ammonium sulphate, 0.50; KCl, 0.20; TCP, 15; $MgSO_4.7H_2O$, 0.1; $MnSO_4$, 0.01; $FeSO_4$, 0.01; initial pH 7.00 with 1.5 O.D. inoculum size and incubated at 35 °C for 96 h, which resulted in overall 3.39-fold increase in P-solubilization compared with that obtained in the unoptimized medium. Model validation also confirms the accuracy of experiment and results showed that observed experimental values closely followed predicted experimental values.

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