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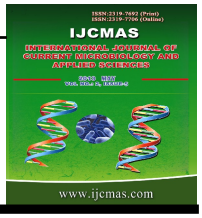

Original Research Article

Optimization of Culture Conditions of Phosphate Solubilizing Activity of Bacterial sp. Isolated from Similipal Biosphere Reserve in Solid-State Cultivation by Response Surface Methodology

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Keywords

Similipal Biophere Reserve, Phosphate Solubilization, Central Composite Design, Solubilizing index

A B S T R A C T

In this investigation, an attempt was made to determine the load of phosphosphate solubilizing bacteria in soil samples collected from Similipal Biosphere Reserve and to carry out the condition optimization of phosphate solubilizing activity of desirable isolates by solid state culture against physical parameters. Total bacterial load in the samples ranged between $1.15x10^5$ -$5.8X10^7$ CFU/gm of soil. Whereas, the density of phosphate solubilizing bacteria ranged between $3.0X10^4$ -$9.3X10^5$ CFU/gm of soil amounting to 20-60% of total bacterial populations. Five isolates S_{1-7} (*Bacillus subtilis*), S_{5-1} (*Bacillus megaterium*), S_{7-1} (*Pseudomonas* sp.-1), S_{10-1} (*Pseudomonas* sp.2) and S_{16-1} (*Bacillus* sp.) showed highest phosphate solubilizing activity among the isolates were selected for condition optimization for incubation period, temperature, pH and carbon sources. Further, experimentation based on Central Composite Design (CCD) were conducted to study the co-effect of incubation period, temperature and pH on activity of phosphatase were optimized using response surface methodology (RSM). The optimum values of incubation period, temperature and pH were found to be 3 days, 36°C and pH-7respectively for all the isolates. Maximum phosphatase activity (Solubilizing index) was recorded on the presence of dextrose 1% in the medium. The isolates S_{7-1} and S_{16-1} retained their phosphate solubilizing activities at 45°C with 10% NaCl in the medium.

Introduction

Phosphate is one of the important macronutrient present in soil as inorganic insoluble forms and that can be made biologically available by different biogeochemical cycle (Harris *et al*., 2006; Perez *et al*., 2007). The concentration of bio-available P in soil is very low reaching the level of 1.0 mg kg–1 soil (Ezawa *et al*., 2002). Some bacterial species have mineralization and solubilization potential for organic and inorganic phosphorus, respectively and made available for plant growth (Goldstein, 1994; Khiari and Parent, 2002). A special group of microorganisms known as plant growth promoting rhizobacteria that are present in

rizospheric region and they promote plant growth either by mineralization and production of growth factors or by controlling the plant pathogens available in soil by production of antimicrobial compounds (Latour *et al*., 2003). Response Surface Methodology (RSM) is an important statistical technique employed for multiple regression analysis by using experimental data obtained from properly designed experiments using Central Composite Design (CCD) (Ratnam *et al*., 2003). Similipal Biosphere Reserve (SBR) is a unique ecosystem, it's climatic and edaphic conditions are suitable for growth of different types of microorganisms. Similipal hills located in the midst of Mayurbhanj district of Orissa state in the Indian union and located in between $21^0 28'$ to 22^0 08'N latitude and 86^0 04' to 86^0 37' E longitude and covering 5,578 km^2 of forest land. To the best of our knowledge, this unique ecosystem (SBR), is not explored much for its microbial wealth, except few sporadic studies (Bahuti *et al.,* 2006 and Behera *et al*., 2009). This prompted us to undertake this study to isolate bacteria from SBR soil with reference to phosphatase activity and plant growth hormones producing capabilities for agricultural practices.

Materials and Methods

Sample collection and bacteriological enumeration

Random sampling was carried out following the method of Baruah and Barthakur (1998). Total fifty soil samples were collected from different locations of Similipal Biosphere Reserve especially from Rhizospheric regions. Enumeration of bacteria was carried out by standard plate count method (Cruickshank *et al*., 1972).

Phosphate solubilizing studies

Phosphate solubilization activity of the isolates was tested on Pikovskaya's agar (PA) containing tricalcium phosphate as insoluble phosphate source. Pikovskaya's agar plates were prepared as per manufacturer's (Hi-Media Pvt. Ltd. Mumbai, India) instructions. Freshly grown cultures of bacteria were spot inoculated on PA plates by the help of a sterile loop. Inoculated plates were incubated at 37^0C. Formation of a halo zone around the colonies is indicative of positive phosphatase activity. Solubilizing index (SI) of the isolates was determined against different incubation time, carbon source, pH and temperature. The maximum SI with growth period was recorded, and the solubilizing index was determined by using the formula (Arun and Sridhar, 2005) as follows:

$$SI = \frac{\text{Halo zone + Colony diameter}}{\text{Colony diameter}}$$

Measurement of pH and Titrable Acidity with Respect to Phosphate Solubilizing Activity

In order to measure the titrable acidity of the culture medium, the isolates were cultured in Pikovaskaya's broth for five days and the cultures were centrifuged at 10000 rpm for 10 min. Few drops of phenophthalin indicator was added to 5 ml. of supernatant and titrated against 0.01N NaOH. The tritrable acidity was expressed as ml of 0.01NaOH consumed per 5.0 ml of culture filtrate (Ponmurugan and Gopi, 2006).

Experimental Design and Optimization

Condition Optimization for different physical parameters of Phosphatase

activity in terms of solubilising index of selected bacterial species was studied using Central Composite Design (CCD) experiments (Stat-Ease, version 8 of Design-Expert). Incubation period (X_1, day), incubation temperature (X_2, °C) and pH (X_3,) were selected as the independent variables (Table 1). Phosphatase activity (Y, solubilising index) was used as the dependent output variable. The three independent factors were investigated at five different coded levels (-1.682, -1, 0, +1,+1.682) A 2^3 factorial Central Composite Experimental Design, with six axial points and six replications at the centre points leading to a total number of 20 experiments was employed for the optimization of parameters and given in Table 2. Equation-1: $Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{11}X^2_1+b_{22}X^2_2+b_{33}X^2_3+b_{12}X_1X_2+b_{23}X_2X_3+b_{13}X_1X_3$. Whereas, Y is the predicted response (solubilising index) of X_1, X_2 and X_3 are the coded levels of the independent variables, b_0 the offset term, b_1 ,b_2 and b_3 the linear effects, b_{11}, b_{22} and b_{33} the quadratic effects and b_{12}, b_{13} and b_{23} are the interaction effects. If the curve shape of the response surface plot is elliptical or circular then it is presumed that the interaction between the variables are significant (Manikandan and Viruthagiri, 2010).

Sodium Chloride Tolerance Test

Bacteria was cultured in nutrient broth at different concentration of NaCl (3%, 5%, 10%, 15%. 20%, 25% and 30%) in the medium. Tolerance was determined by subculturing one loop of sample onto NA plates and observed for growth after incubation at 37^0C.

Results and Discussion

Enumeration of bacteria was carried out on basal media like Nutrient agar through pour and spread plate method. The bacterial load on NA medium ranged between 10^5 -10^7 CFU/gm of soil. Whereas, the population density of Phosphate solubilizing bacteria when enumerated on Pikovaskaya's agar medium ranged between $3.X10^4$ - $9.3X10^5$ CFU/gm of soil. These findings corroborate with the result of several groups (Gledhill and Carida, 1969; Boer *et al*., 2003; Laturer *et al*., 2003; Vikram, 2007) who isolated bacteria from various forest soils.

However, our enumeration findings are in direct correlation with the results of (Bhahuti *et al*., 2006 and Behera *et al*., 2009). In total 160 bacteria were selected from 50 soil samples collected from SBR, based on their morphology on NA, Gram's reaction and an array of biochemical characters, were identified and assigned to different genera (*Bacillus subtilis* (28%), *Pseudomonas* spp. (25%), *B. magaterium* (16%), *B. coagulans* (6%), *B. thuringensis* (6%), *Staphylococcus* sp. (13%), and *Geomicroccus* spp. (6%)).

Further, plant diversity affects the bacterial community, more specifically soil bacterial genera such as *Bacillus* and *Pseudomonas* (Stephan *et al*., 2000). In this respect, Similipal Biosphere Reserve with its rich diversity of plant species reveals *Bacillus* as the dominant genera followed by *Pseudomonas.*

Table 1. Original and coded levels of the independent variable

Independent variables	Coded levels				
	-1.682	-1	0	+1	+1.682
Incubation Period (day)	-0.35	1	3	5	6.36
Incubation Temperature (^{o}C)	15.82	24	36	48	56.18
pH	1.95	4	7	10	12.05

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

Standard order	Incubation period (day)	Incubation Temp. °C	pH	Isolates S_{1-7}		Isolates S_{5-1}		Isolates S_{7-1}		Isolates S_{10-1}		Isolates S_{16-1}	
				AV	P V	AV	P V	AV	P V	AV	P V	AV	P V
1	1.00	24.00	4.00	00	0.10	00	-0.21	00	-0.14	-0.12	0.12	00	-0.092
2	5.00	24.00	4.00	0.00	0.22	1.10	0.96	1.1	0.96	1.10	0.92	0.000	0.25
3	1.00	48.00	4.00	0.00	-0.10	0.000	0.019	0.0	-0.11	0.000	-0.096	1.10	0.53
4	5.00	48.00	4.00	0.00	0.22	0.000	0.086	1.10	0.94	1.10	0.91	1.10	0.88
5	1.00	24.00	10.0	0.00	-0.10	0.000	-0.21	0.00	-0.13	0.000	-0.11	0.000	-0.11
6	5.00	24.00	10.0	0.00	0.22	1.10	0.96	1.20	1.02	1.18	0.97	0.000	0.23
7	1.00	48.00	10.0	0.00	-0.10	0.000	0.019	0.0	-0.14	0.000	-0.12	1.20	0.61
8	5.00	48.00	10.0	0.00	0.22	0.000	0.086	1.1	0.96	1.10	0.92	1.20	0.96
9	-0.36	36.00	7.00	0.00	0.31	0.000	0.17	0.00	0.18	0.000	0.12	0.000	0.65
10	6.36	36.00	7.00	1.33	0.86	1.20	1.21	1.8	2.03	1.57	1.88	1.40	1.23
11	3.00	15.82	7.00	0.00	-0.084	0.000	0.36	0.0	0.22	0.000	0.23	0.000	-0.33
12	3.00	56.18	7.00	0.00	-0.084	0.000	-0.18	0.0	0.19	0.000	0.21	0.000	0.80
13	3.00	36.00	1.95	0.00	-0.084	0.000	0.087	0.0	0.19	0.000	0.21	0.000	0.21
14	3.00	36.00	12.05	0.00	-0.084	0.000	0.087	0.0	0.22	0.000	0.23	0.000	0.26
15	3.00	36.00	7.00	1.40	1.42	1.50	1.49	1.92	1.90	1.66	1.65	1.50	1.52
16	3.00	36.00	7.00	1.43	1.42	1.50	1.49	1.90	1.90	1.67	1.65	1.50	1.52
17	3.00	36.00	7.00	1.42	1.42	1.50	1.49	1.91	1.90	1.66	1.65	1.50	1.52
18	3.00	36.00	7.00	1.40	1.42	1.50	1.49	1.90	1.90	1.66	1.65	1.50	1.52
19	3.00	36.00	7.00	1.40	1.42	1.52	1.49	1.92	1.90	1.68	1.65	1.68	1.52
20	3.00	36.00	7.00	1.45	1.42	1.45	1.49	1.90	1.90	1.67	1.65	1.50	1.52

*AV: actual Value and PV: Predicted value

Table.3 ANOVA for phosphatase activity of the isolates on solid state cultivation

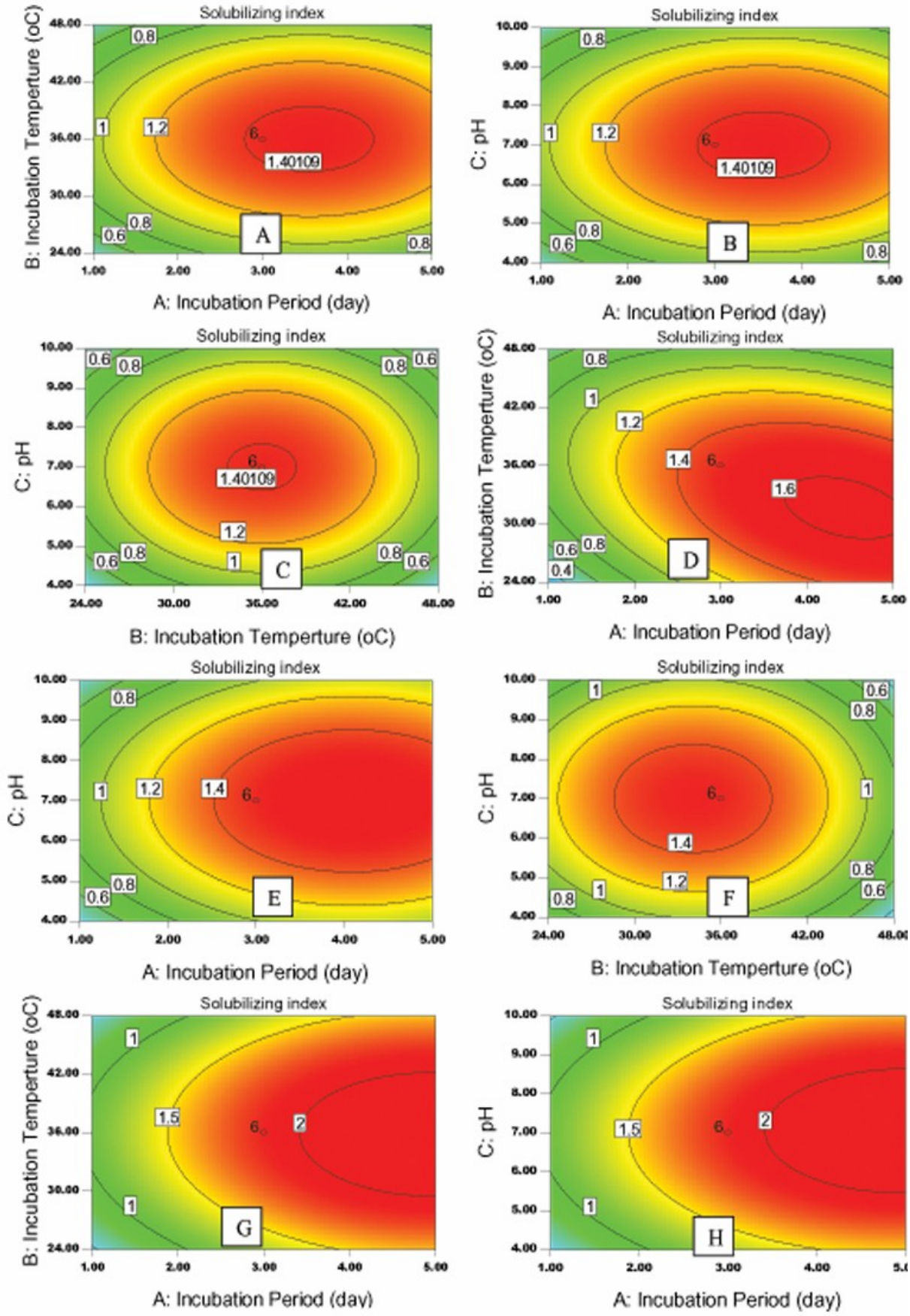
Isolates	R2	Adjusted R2	Predicted R2	Adequate Precision	Lack of Fit F-value	Sources	Sum of Squares	Degree of freedom	Mean square	F-value	p-value
S_{1-7}	0.93	0.8749	0.501	8.873	276.17	Model	8.39	9	0.93	15.77	< 0.0001
						Pure Error	2.13	5	4.26		
						Total	8.98	19			
S_{5-1}	0.96	0.9307	0.725	12.829	126.57	Model	9.27	9	1.03	29.36	< 0.0001
						Pure Error	2.75	5	5.50		
						Total	9.62	19			
S_{7-1}	0.97	0.9447	0.78	15.022	866.51	Model	13.99	9	1.55	37.07	< 0.0001
						Pure Error	4.83	5	9.66		
						Total	14.41	19			
S_{10-1}	0.95	0.9157	0.66	12.662	1500.38	Model	10.77	9	1.20	23.92	< 0.0001
						Pure Error	3.33	5	6.66		
						Total	11.27	19			
S_{16-1}	0.92	0.8557	0.42	9.711	645.02	Model	10.48	9	1.16	13.52	0.0002
						Pure Error	1.33	5	2.66		
						Total	11.34	19			

Desirable isolates were selected by plate assay method. All the 160 bacteria were assessed for phosphate solubilizing capacity on Pikovskaya's agar and it was found that 44.7% bacteria were positive for their phosphatase activity. In proper agreement to our findings earlier worker Behera *et al*., (2009) reported (36.73%) of the bacterial isolates with phosphatase activity, amongst which *Bacillus* and *Pseudomonas* spp. dominated the group. First, it was designed to evaluate the activity with variation of incubation period, temperature and pH. All isolates were subjected to evaluate the rise of phosphate solubilizing capacity with incubation time. Maximum phosphatase efficiency was observed on three days of incubation on pikovaskays agar medium (Fig.1). Phosphate solubilizing efficacy of microorganisms is influenced by medium composition, especially the N and C sources, and the pH of the medium used (Pradhan and Sukla, 2005 and Whitelaw 2007). Amongst all the phosphate solubilizing isolates, five isolates S_{1-7} (*B. subtilis*), S_{5-1} (*B. Magaterium*), S_{7-1} (*Pseudomonas* sp.) S_{10-1} (*Pseudomonas* sp.) and S_{16-1} (*Bacillus* sp.) that showed better phosphatase activity were considered further for condition optimizations. While studying, the impact of medium composition it was observed that the solubilizing index (SI) of the test isolates varied greatly with respect to carbon sources used (fig. 3). The bacterial isolates showed highest solubilizing index (SI) on Pikovaskaya's agar medium with 1% glucose. Isolate S_{7-1} showed highest phosphatase activities (SI, 1.93) followed by, S_{5-1} (SI, 1.26), S_{16-1} and S_{1-7} (SI,1.22). In a study (Sridevi *et al*., 2005) reported the effect of C in the medium on phosphatase activity of *Rhizobium* isolates. It was observed that glucose is the most suitable carbon source that enhances the phosphate solubilizing activity of *Rhizobia* species.

When the phosphate solubilizing index of these isolates was studied at different pH (pH 4-10), maximum solubilizing index (SI) was observed at neutral pH (pH 7.0) Fig. 2.

It is important to mention that all the bacterial isolates retained their phosphatase activities within a wide range of (pH 4-10), Growth, viability, and metabolism of microorganisms are directly related to the environmental factor from which they have been isolated. In contrast to this, we reported that our isolates (bacteria) retained phosphatase activity at alkaline pH, through they were isolated from acidic soils. Further, retention of phosphatase activity of these isolates at both acidic and alkaline pH range is suggestive of their applications, for amendment both in acidic and alkaline soils.

Production of organic acids is directly proportional to phosphatase activity of the isolates (Vikram *et al*., 2007). Therefore, an attempt was made during this investigation to measure the amount of titrable acid produced by the isolates in the medium, which results in change of pH. Maximum titrable acidity was recorded with bacteria S_{7-1} (3.5), followed by S_{5-1} (3.3). We observed the production of titrable acidity in the medium is directly correlated to the reduction in pH of the medium (Fig. 4). Phosphate solubilizing microorganisms produce a variety of organic acids from simple carbohydrates (Bajpai and Rao, 1971) by virtue of which they solubilize insoluble inorganic phosphates (Barik and Dey 1982 and Vazques *et al*., 2008). Acidification of the periplasmic space by direct oxidation


Solubilizing index
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Solubilizing index
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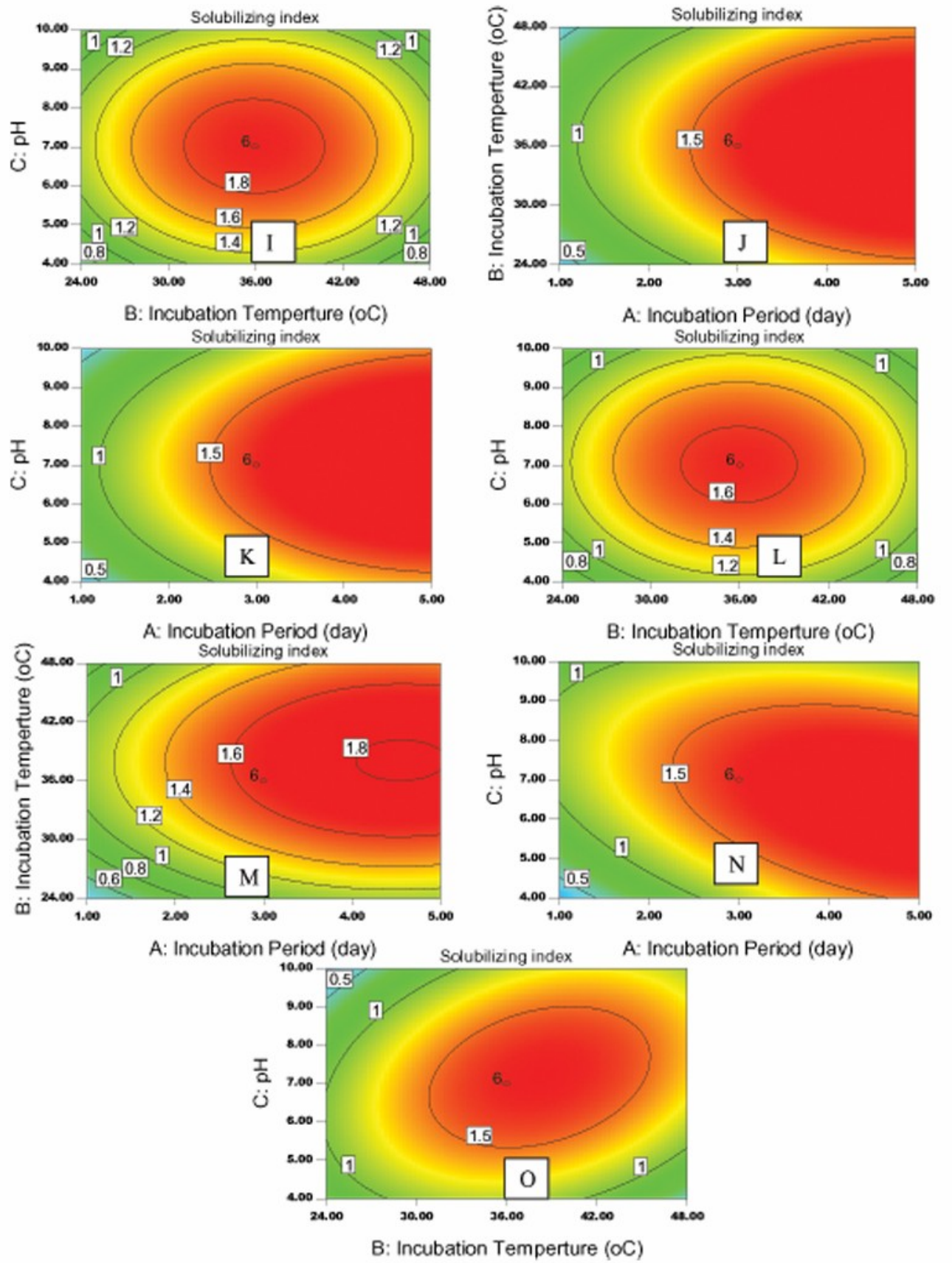


Fig.5. Response surface curve for phosphate solubilization by Bacterial Isolates: A,B,C of S1-7; D,E,F of S5-1; G,H,I of S7-1; J,K,L of S10-1 and M,N,O of S16-1

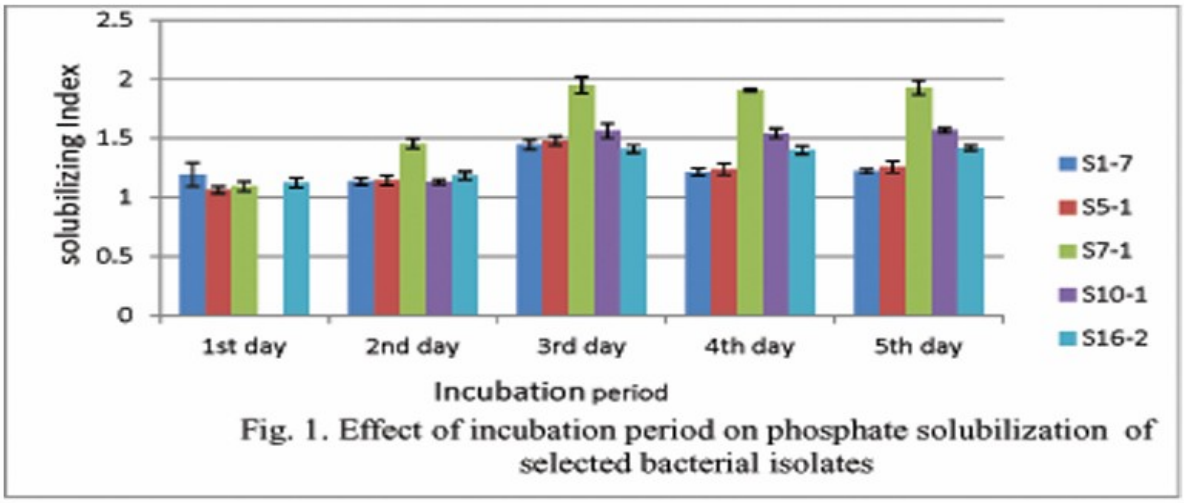


Fig. 1. Effect of incubation period on phosphate solubilization of selected bacterial isolates

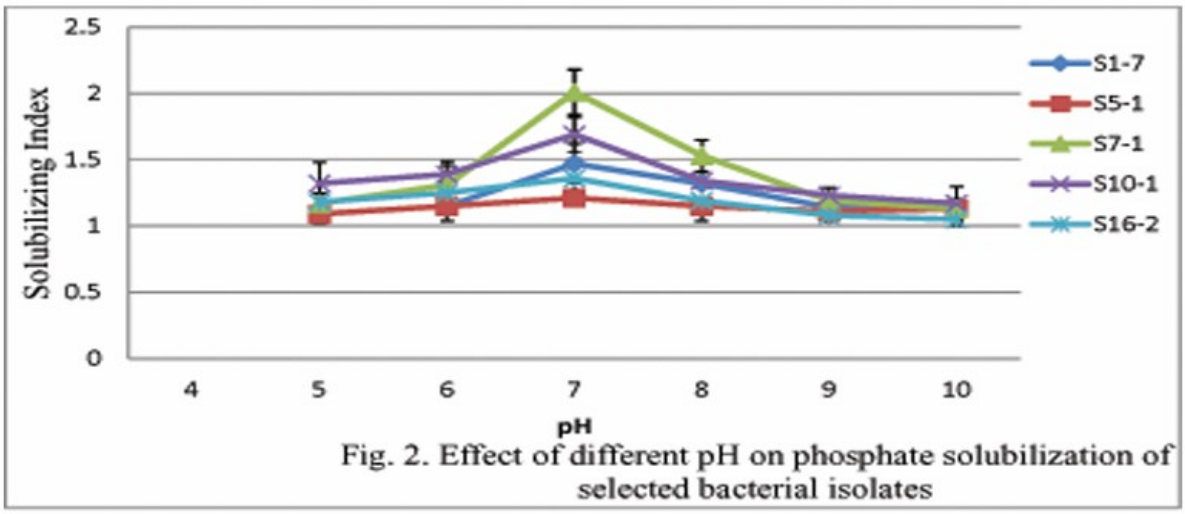


Fig. 2. Effect of different pH on phosphate solubilization of selected bacterial isolates

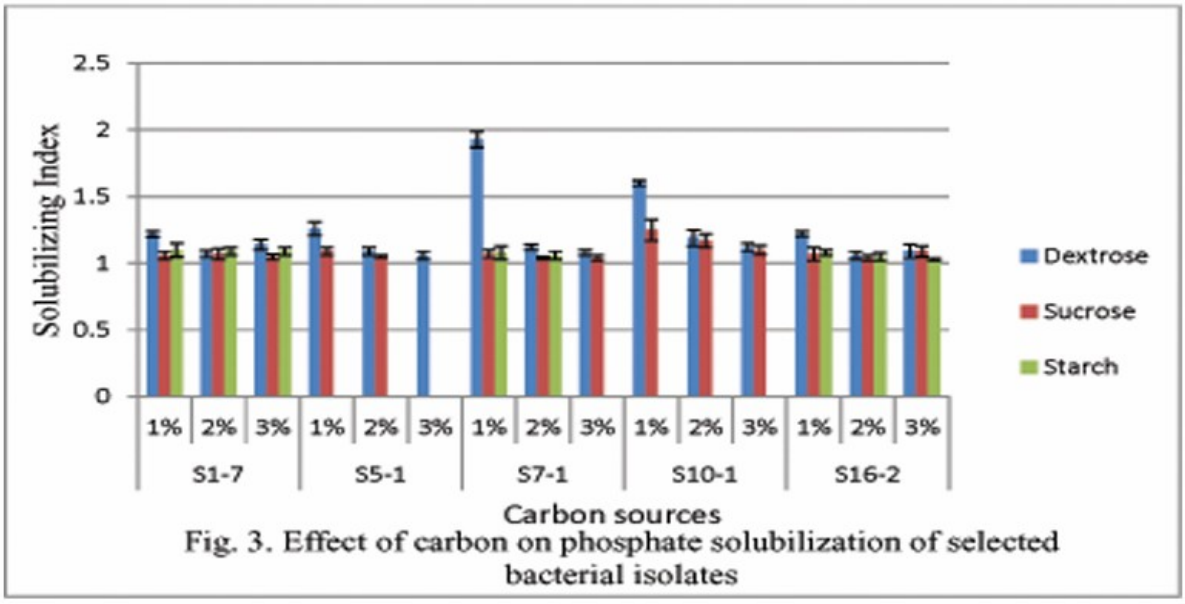
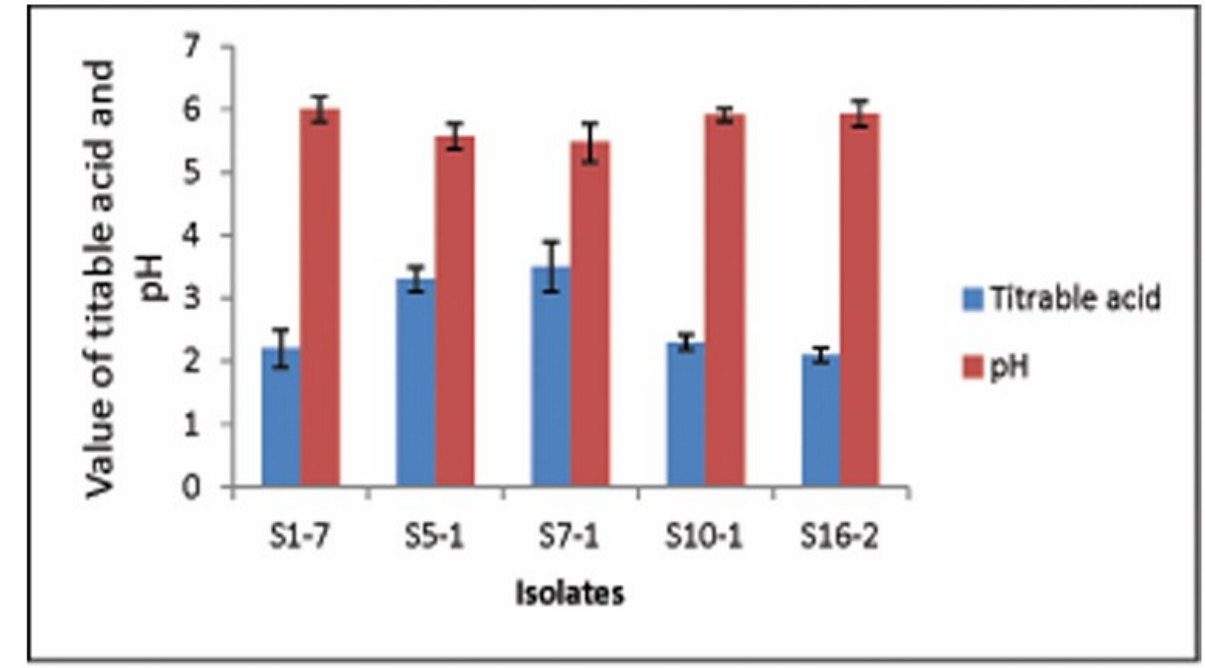


Fig. 3. Effect of carbon on phosphate solubilization of selected bacterial isolates

Figure.4 Titrable acid production and change in pH of the medium. .



pathway of glucose to gluconic acid as the major cause of mineral phosphate solubilization in gram-ve bacteria has also been demonstrated (Illmer and Schinner, 1995). In agreement to these points, in the present study the PSB produced organic acids in the medium indicating the ability of P solubilization. Production of organic acids by PSB have been reported earlier (Lal, 2002; Vikram *et al*., 2007) which are line with the results of the present investigation.

Effect of NaCl on phosphatase activity was studied by growing the isolates on PA medium, supplemented with different concentration of NaCl (5%, 10% & 15%) separately. Only two strains S_{5-1} (*B. magaterium*) and S_{7-1}(*Pseudomonas* sp.) retained phosphatase activities at 10% NaCl concentration. The tritrable acidity was expressed as ml of 0.01N NaOH consumed per 5.0 ml of culture filtrate.

Optimization of incubation period, temperature and pH by using RSM

The physical factors affecting phosphatase activity of five bacterial samples (S_{1-7}, S_{7-1}, S_{1-5}, S_{10-1} and S_{16-1}) was studied by using CCD experiments. The incubation period (X1, day), incubation temperature (X2, °C) and pH (X3) were chosen as the independent variables as shown in Table 2. Phosphatase activity in terms of solubilizing index was selected as the dependent response variable (Y). Twenty experiments for each isolate based on the CCD were carried out with different combinations of variables and the results were presented in Table 2. The actual activity (phosphatase) of isolates obtained in the experiments and the yields predicted by the model equation (1) are given in Table 2. The effect of all three parameters studied, interaction effects between the parameters concentration were found to be significant from the response surface plots

as shown in Fig. 5(A-O). The clear elliptical shape of the curve shown in Fig.5 indicates that the interaction effect between the incubation period (X1) and temperature (X2), pH and temperature are significant with a P value of 0.02 for S16-1 and 0.0001 for all the isolates. The ANOVA result of quadratic regression model for Y is described in Table 3, where Y is the enzyme activity in terms of solubilizing index.

The coefficient of determination (R^2) for all the isolates was found to be more than 0.92 for phosphatase activity, which implied the experimental yields fitted the second order polynomial equation well. The model F-values are 15.77, 29.36, 37.06, 23.99, 13.52 of the isolates S_{1-7}, S_{5-1}, S_{7-1}, S_{10-1}, S_{16-1}, respectively, indicating that the model is significant for all the isolates. The shape of the corresponding contour plots indicates whether the mutual interactions between the independent variables are significant or not. From the response surface plots, the optimal value of the independent variables and their interaction was observed. The orientation of the principal axes of the contour plots between incubation period and temperature, pH and temperature and temperature and incubation period indicated that the mutual interactions between these set of variables had a significant effect on response of dependent variable of phosphatase activity. The three contour plots for each isolates proved the significance of earlier response *i.e* incubation period with temperature, incubation period with pH and temperature with pH. Thus, incubation period of 3days, temperature 36^{o}C and pH (7.0) were adequate for attaining maximum enzymatic activity (Table 2) (Fan *et al*., 2011; Ray *et al*., 2010; Sogi *et al*., 2003; Rao and Satyanarayan, 2003).

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