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Solubilization of Mazidagi Rock Phosphate by Locally Isolated *Aeromonas hydrophyla* MFB-25

Mehmet Nuri Aydogan* and Omer Faruk Algur

Department of Biology, Science Faculty, Ataturk University, Erzurum, Turkey.

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Total 670 bacteria isolated from various samples (soil, water and plant roots) were screened in terms of their capacities to solubilize $Ca_3(PO4)_2$ (tricalcium phosphate) or Mazidagi rock phosphate. At the end of two-step screening experiments, one isolate (MFB 25) having the best capacity to solubilize Mazidagi rock phosphate was selected and then used for the subsequent experiments. This isolate was identified as *Aeromonas hydrophyla* according to cellular fatty acid analysis (MIS) as well as some morphological and biochemical characteristics. The best carbon and nitrogen sources which *A. hydrophyla* MFB-25 needed to solubilize the rock phosphate in liquid medium were determined to be glucose and potassium nitrate, respectively. The other optimal parameters for rock phosphate solubilization were found as mazidagi rock concentration of 3 g/L, temperature of 25 °C and incubation time of 5 d. This is the first study on the potential of *A.hydrophyla* bacterium to solubilize mazidagi rock phosphate.

Key words: *Aeromonas hydrophyla,* Mazidagi rock phosphate, Phosphate solubilization.

Phosphorus (P) is one of the major nutrients required for plant growth. P plays important roles inplants in many physiological activities such as cell division, photosynthesis, and development of good root system and utilization of carbohydrate. P deficiency results in the leavesturning brown accompanied by small leaves, weak stem and slow development[1]. Therefore, large quantity of soluble forms of P fertilizers is applied to soils for achievementof maximum plant productivity. However, applied soluble forms of P fertilizers are easily precipitated into insoluble forms and are not efficiently taken up by the plants. This situation is known to cause many environmental problems like eutrophication and soil salinity. Besides, use of chemical phosphatic fertilizers has become a costly affair [2,3].

The biggest reserves of phosphorus are rocks and other deposits, such as primary apatites and other primary minerals formed during the geological age[4]. In recent years the possibility of practical use of rock phosphates as fertilizers has received significant interest. However, rock phosphate is not plant available in soils with a pH greater than 5.5–6.0 and, even when conditions are optimal, plant yields are lower than those obtained with soluble phosphate.In the recent years, it has been reported that phosphate solubilizing bacteria (PSB) convert insoluble rock phosphates[2,5] to soluble form. In the other words, the use of rock phosphate as phosphate fertilizer and its solubilization through PSB have become a valid alternative to expensive chemical fertilizers [6].It is generally accepted that the mechanism ofmineral phosphate solubilization by PSB strains isassociated with the release of low molecular weight organic acids,which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms[7]. On the other hand, PSB may have also potential to improve the plant productivity by producing other secondary metabolites such as indol acetic acid and siderophore[8,9].

* To whom all correspondence should be addressed. Tel.: +90 442 231 4330; Fax: +90 442 236 0948; E-mail:mnaydogan@atauni.edu.tr

Phosphate rock reserves are found in countires Algeria, Tunisia, Egypt, Israel, Jordan, Syria, Saudi Arabia, Turkey, and Iraq. They are made of deposits laid down in the ancient Tethy Sea of the Mesozoic and Tertiary ages. The importance of the phosphate rock deposits is that they form more than 70% of the total world phosphate reserves [10].

The Mazidag1 (Turkey) is situated near the border with Syria and it is very rich in rock phosphates[11].In Turkey, Mardin-Mazidagi rock phosphates are used as raw material for manufacturing of phosphate fertilizer. Commercial production of phosphate fertilizer fromMazidagi rock phosphates is currently performed by using chemical processes. However, to our best knowledge, there is no report on the use as natural phosphate fertilizer of Mardin-Mazidagi rock phosphates,which were solubilized by phosphate-solubilizing microorganism. Therefore, the present study aimed to investigate the potential of locally isolated *Aeromonas hydrophyla* bacterium to solubilize Mardin-Mazidagi rock phosphates.

MATERIALS AND METHODS

Isolation and screening of bacteria

Besides different soil and water samples, plant roots were also used as isolation source of phosphate solubilizing bacteria. Isolations were performed on *pseudomonas* isolation agar, tryptone-glucose-yeast extract agar and nutrient agar media at 30^0C for 3-5 days. Isolated bacteria were separately numbered and then screened for comparison of their phospahete solubizing activities. For this purpose, 50 µL seed culture (approximately 1-2x10^9cfu/ml) of bacterial strain was separately transferred in the glass tubes containing 10 mL of sterilized NBRIP-BPB broth medium (Brom phenol supplemented National Botanical Research Institues's phosphate growth medium). This medium contained (g/L) 10 glucose, 5 $Ca_3(PO4)_2$(tricalcium phosphate) or 10 Mazidagi rock phosphate, 5 $MgCl_2$, 0.25 $MgS0_4$, 0.2 KCl, 0.1 $(NH4)_2SO_4$ and 0.025 BPP (Bromphenol blue). Mazidagi rock phosphate was grounded before being added to the medium. The pH of the media was adjusted to 7.0 before autoclaving. Autoclaved and uninoculated medium served as controls. The tubes were incubated at 30^0C in a shaking incubator (New Brunswick Scientific, USA) at 180 rpm for 3 d. At the end of this incubation period, tubes were centrifuged at 5000 rpm for 10 min. Absorbances of obtained supernatants were assayed at 600 nm using a spechtrophotometer[12]. Soluble phosphate in culture supernatant was estimated by Vanadomolybdate method and expressed as equivalent phosphorus (mg/L)[13]. The best phosphate-solubilizing one isolate was determined and then used for the subsequent experiments.

Identification of phosphate solubizing bacteria

The first characterization of the most promising isolate was based on their morphological and biochemical characteristics. The color and shape of the colonies of bacterial isolate were determined on NA agar-containing petri dishes. Following this, gram-staining and endospore-forming properties as well as cellular morphology (rod,cocci, vibrio etc.) of isolate were investigated. For this purpose, endospore and simple staining methods as well as gram staining were applied to the isolate[14]. The isolate was then further characterized according to following variousbio chemical test including gelatin hydrolysis, catalase test, oxidase test and nitrate reduction. Biochemical properties of the isolates were evaluated according to Bergey's Manual of Systematic Bacteriology[15]. Finaly, three isolates was were identified based on whole-cell cellular fatty acids, derivatized to methyl esters, i.e. FAMEs and analyzed by gas chromatography (GC) using the MIDI system (MIDI, Newark, DE). The analysis was performed using the Sherlock Microbial Identification system TSBA 4.0 software and library general system software version 4.1. Qualitative and quantitative differences in the fatty acid profiles were used to compute the distance for each strain relative to the strains in the library[16-18].

Optimization of phosphate solubization by bacteria

Effects of some environmental and nutritional factors on the phosphate solubization ability of the bacterium were investigated in modified NIBRIP medium. In this context, different carbon (sucrose, glucose, maltose, fructose, galactose, lactose, sorbitol, xylose and mannitol) and nitrogen sources (ammonium sulphate, ammonium chloride, ammonium nitrate,ammonium iron sulphate**,** calcium nitrate, magnesium nitrate, potassium nitrate, sodium nitrate and urea) were tested as nutritional factor. Different

concentrations of mazidagi rock phosphate were tested in order to increase phosphate solubization efficiency of bacterium. As for environmental factors, incubation durations (1-10 d) and different temperatures (15-40 ^{0}C) were studied, respectively. Optimization studies were performed in 250-mL flasks containing 100 mL of modified NBRIP broth medium (This medium did not contain bromophenol blue during the optimization studies) (pH 7.0). The media were autoclaved and then cooled at the room temperature. The flasks inoculated with the fresh culture of the bacterium were incubated at 30 °C in a shaking incubator (New Brunswick Scientific, USA) at 180 rpm At the end of 3 d incubation period, 40 ml sample taken from flasks were centrifuged at 5000 rpm for 10 min. Soluble phosphate in culture supernatant was estimated by Vanadomolybdate method [13] and expressed as equivalent phosphorus (mg/L).

Statistically analysis

The experiments were conducted in 3 replications following a completely randomized block designand statistically analyzed. Mean values were pooled and standard deviation (S.D.) was calculated. The data represent mean values ±S.D. All data were subjected to an analysis of variance. The difference between the mean values of treatments was estimated using least significant difference (L.S.D.) at the 0.05 level of significance.

RESULTS AND DISCUSSION

Isolation, screening and identification of phosphate-solubilizing bacteria

In the first step of the study, a total of 670 bacteria strains were isolated from various samples such as soil, water and plant root. It has been well known that there has been a growing interest in isolation from various sources of plant - promoting microorganisms. For example, application of phosphate-solubilizing bacterial inoculants as biofertilizers has been reported to result in improved plant growth and increased yield [19-22].In this context, exploration of new bacteria isolates having phosphate-solubilizing activity may represent a significant contribution to agricultural studies. Therefore, 670 bacterial isolates were screened for determination of their phosphate solubilizing activitiesin NBRIP-BPB broth medium (Brom phenol supplemented National Botanical Research Institues's phosphate growth medium),which contained$Ca_3(PO4)_2$ or mazidag1 rock phosphate. Among these isolates, ones causing decolorizationin blue color of NBRIP-BPB broth medium were assumed to have phosphate-solubizing activity.In this regard, among 670 bacterial isolates, only 42 isolates were found to have a significant decolorization capacity and phosphate-solubizing activity. No significant visible decolorization was detected in the cultures where the remaining isolates were grown. Therefore, their phosphate-solubilizing activities were assumed to be low. It is well know that the produced organic acids drops medium pH, andblue color of the dye Brom phenol in NBRIP-BPB broth medium is opened as medium pH drops. In brief, there is a relationship between organic acids and Bromophenol blue's color density. Therefore, it was assumed that more organic acid-producing isolates had higher phosphate-solubizing activity. Besides, it is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by microorganisms. The organic acids secreted can either directly dissolve the mineral phosphate as a result of anion exchange of $PO4^{2-}$ by acid anion or can chelate both Fe and Al ions associated with phosphate [23]. Based on these results, the data for phosphate-solubilizing activities of only 42 isolates were given in Table 1. As seen from Table 1, lower absorbance values were attained in the media where more $Ca_3(PO4)_2$ and mazidag1 rock phosphate dissolved. The same table clearly shows that the most effective three isolates for solubilization of tricalcium phosphate were MFB 44 (125 mg/L), MFB 61 (123.33 mg/L) and MFB 72 (140.53 mg/L). Conversely, the most effective three isolates for solubilization of mazidag1 rock phosphate were found to be MFB 7 (76.96 mg/L), MFB 25 (91.33 mg/L) and MFB 61 (76.56 mg/L). Since the present study was mainly focused on determining mazidagi rock phosphate-solubilizing bacteria, the subsequent experiments were performed with only MFB-25 isolate.

Identification of MFB 25 isolate was performed as described in the material and methods section. This isolate was identified as *Aeromonas* at the genus level according to its morphological and biochemical characteristics. Colonies of the isolate had yellow color on nutrient agar. The cell shape of the isolate was slightly curved rod. Non-

endospore forming this isolate was gram-negative. The results on morphological and biochemical analyses were also summarized in Table 2.MFB-25 was then further identified as *Aeromonas hydrophyla* according to MIS analysis. High phosphate solubilization ability of the local isolate *A. hydrophyla* MFB-25 is very important. This is because, to our best knowledge, there is only one report investigating phosphate solubilization ability of*A. hydrophyla*[24]. Conversely, there are

Table 1. Screening of phosphate-solubilizing bacteria

Isolate code	Tri calcium phosphate medium		Mazidagi rock phosphate medium	
	Absorbance (600 nm)	Solubilized phosphate concentration (mg/L)	Absorbance (600 nm)	Solubilized phosphate concentration (mg/L)
MFB 1	0.633 ± .02	13.96 ± 1.23	0.980 ± .02	5.50 ± 1.70
MFB 2	0.516 ± .02	32.30 ± 3.06	0.666 ± .07	11.56 ± 2.20
MFB 3	0.546 ± .03	26.70 ± 2.81	0.900 ± .07	7.16 ± 3.38
MFB 4	0.720 ±. 06	10.66 ± 2.40	0.573 ± .04	16.06 ± 5.40
MFB 6	0.323 ± .03	71.23 ± 6.53	0.323 ± .04	37.10 ± 4.60
MFB 7	0.243 ± .01	95.66 ± 8.14	0.223 ± .02	76. 96 ± 3.16
MFB 9	0.463 ± .02	28.30 ± 4.25	0.470 ± .02	25.10 ± 2.59
MFB 14	0.493 ± .06	35.43 ± 2.05	0.346 ± .05	55. 83 ± 3.75
MFB 15	0.600 ± .04	16.03 ± 2.81	0.933 ± .07	6.33 ± 1.26
MFB 16	0.480 ± .02	29.66 ± 3.51	0.563 ± .05	18.70 ± 4.35
MFB 17	0.373 ± .03	53.63 ± 2.25	0.326 ± .03	61.80 ± 4.01
MFB 18	0.583 ± .01	25.20 ± 1.70	0.583 ± .01	17.53 ± 2.90
MFB 19	0.603 ± .04	16.60 ± 2.84	0.656 ± .07	12.63 ± 3.81
MFB 20	0.553 ± .02	33.66 ± 4.72	0.250 ± .05	66.20 ± 2.10
MFB 22	0.443 ± .02	44.23 ± 3.99	0.500 ± .06	22.30 ± 2.80
MFB 24	0.563 ± .05	23.66 ± 1.15	0.843 ± .12	8.46 ± 3.15
MFB 25	0.340 ± .04	88.60 ± 4.33	0.203 ± .03	91.33 ± 3.31
MFB 26	0.576 ± .04	17.33 ± 1.92	1.016 ± .16	5.63 ± 2.13
MFB 28	0.520 ± .02	33.93 ± 4.00	0.653 ± .02	13.86 ± 4.11
MFB 31	0.623 ± .03	15.36 ± 3.35	1.043 ± .22	5.30 ± 1.76
MFB 33	0.400 ± .06	55.23 ± 1.36	0.296 ± .01	48.10 ± 6.53
MFB 35	0.443 ± .02	41.20 ± 3.30	0.550 ± .07	19.06 ± 2.80
MFB 37	0.403 ± .04	52.76 ± 3.72	0.403 ± .04	37.76 ± 2.90
MFB 38	0.666 ± .03	13.96 ± 2.81	1.023 ± .16	7.70 ± 3.20
MFB 39	0.600 ± .04	16.56 ± 2.27	1.026 ± .16	5.50 ± 2.76
MFB 40	0.336 ± .03	66.40 ± 2.86	0.376 ± .03	47.30 ± 2.86
MFB 44	0.163 ± .02	125.00 ± 7.81	0.286 ± .02	68.43 ± 4.84
MFB 48	0.286 ± .02	72.53 ± 2.83	0.296 ± .03	40.66 ± 2.08
MFB 53	0.480 ± .03	32.73 ± 2.41	0.660 ± .07	11.80 ± 3.01
MFB 55	0.513 ± .04	34.00 ± 2.61	0.543 ± .09	21.30 ± 2.80
MFB 57	0.453 ± .00	44.42 ± 5.80	0.423 ± .05	27.76 ± 1.12
MFB 58	0.570 ± .04	16.50 ± 2.78	1.063 ± .21	5.10 ± 1.51
MFB 60	0.536 ± .04	22.63 ± 2.80	0.716 ± .03	12.03 ± 2.79
MFB 61	0.186 ± .04	122.33 ± 3.75	0.233 ± .03	76.56 ± 4.10
MFB 64	0.460 ± .06	44.00 ± 3.60	0.406 ± .02	33.06 ± 2.91
MFB 65	0.506 ± .06	27.03 ± 6.05	0.726 ± .10	12.73 ± 2.61
MFB 66	0.383 ± .04	63.76 ± 2.70	0.250 ± .02	72.83 ± 3.26
MFB 68	0.506 ± .03	25.10 ± 2.85	0.773 ± .07	13.50 ± 2.98
MFB 69	0.216 ± .05	113. 96 ± 3.95	0.264 ± .07	73.73 ± 2.19
MFB 71	0.283 ± .05	73. 36 ± 4.03	0.353 ± .05	46.43 ± 1.77
MFB 72	0.153 ± .01	140. 53 ± 6.81	0.300 ± .03	65.46 ± 3.93
MFB 81	0.236 ± .02	110. 56 ± 3.38	0.346 ± .06	49.26 ± 2.30
Control	1.066 ± .09	7.10 ± 1.57	1.093 ± .17	4.36 ± 0.90

many studies on phosphate solubilization ability of other bacteria such as Azotobacter, *Bacillus*, *Enterobacter*, *Rhizobium*, *Bradyrhizobium*, *Pseudomonas*, *Rhodococcus*,*Arthrobacter*, *Serratia* and *Klebsiella*[25-31].

Optimization of submerged culture conditions of *Aeromonashydrophyla*MFB-25for solubilization of mazidag1 rock phosphate

As described by Nautiyal[32], the influence of carbon sources for solubilization of mazidag1 rock phosphate was studied in NBRIP-BPB broth medium containing various carbon sources, where each carbon source wasadded to the medium at 10 g/L.Among the carbon sources tested, the highest phosphate solubilization (91.7 mg/L) was achieved with glucose. This result should not be so surprising, since many reports have demonstrated that glucose is the most favorable carbon source for phosphate solubilization in culture media of different microorganisms [33-36]. Obtaining of higher phosphate solubilization in glucose medium could be attributed to more organic acid production in the presence of glucose in comparison to the other carbon sources. Following glucose, galactose and xylose were found to be favorable carbon sources for solubilization of mazidagi rock phosphate. In contrast to glucose, galactose and xylose, no significant rock phosphate solubilization was observed when the other carbon sources (sucrose, maltose, fructose, lactose, sorbitol and mannitol) were used (Fig. 1). Based on these results, glucose as carbon source was selected for the subsequent experiments.

The previous studies reported that not only carbon sources but also nitrogen sources significantly affected phosphate solubilization by microorganisms [25,37]. Therefore, effect of different nitrogen sources on phosphate solubilization ability of *A. hydrophyla* MFB-25 was also tested at a concentration level of 0.1 g/L. The most extensive solubilization of rock phosphate was achieved with potassium nitrate (101.7 mg/L) among the tested nitrogen sources (Fig. 2).The second highest efficiency of rock phosphate solubilization was obtained with ammonium chloride (97.6 mg/L), followed by ammonium sulphate and urea, respectively. Conversely, the other nitrogen sources were identified to be unfovarable for phosphate solubilization. These results are in good agreemented with the fact that

Table 2. Morphological and biochemical characteristics of the isolate MFB-25

Morphological and biochemical characteristics	The isolate MFB-25
Colony color	Yellow
Cell shape	Rod
Gram reaction	-
Motility	+
Endospore	-
Catalase	+
Oxidase	+
Gelatine hydrolysis	+
Nitrate reduction	+

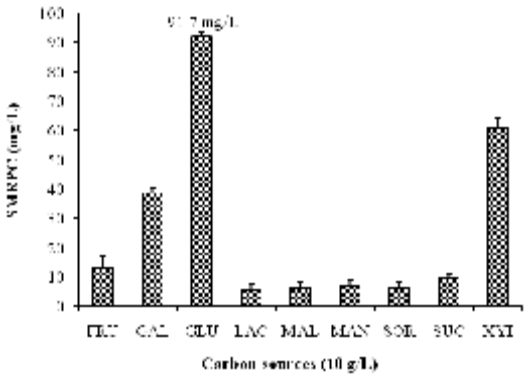

Fig. 1. The effect of carbon sources on phosphate-solubilizing potential of *A. hydrophyla* MFB-25. SMRPC, Solubilized mazidagi rock phosphate concentration. FRU, Fructose; GAL, Galactose; GLU, Glucose; LAC, Lactose; MAL, Maltose; MAN, Mannose; SOR, Sorbitole; SUC, Sucrose and XYI, Xylose

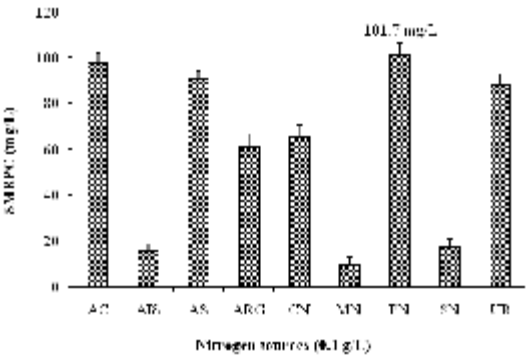

Fig 2. The effect of nitrogen sources on phosphate-solubilizing potential of *A. hydrophyla*MFB-25.SMRPC, Solubilized mazidagi rock phosphate concentration.

kind of the most favorable nitrogen source can vary from one microorganism to another. For example, Nautiyal et al. [33]reported that the most favorable nitrogen source for phosphate solubilization was potassium nitrate for two isolate among four bacterial isolates tested, however, the other two isolates preferred sodium nitrate and calcium nitrate as a sole nitrogen source.In the different study, Nautiyal[32] demonstrated that potassium nitrate was less effective carbon source compared to ammonium sulphate for phosphate solubilization abilities of different bacterial strains.Seshadri and Ignacimuthu[38] reported that nitrogen in the form of nitrate was very effective than ammonium form in microbial solubilizing of inorganic phosphates.

The present study also focused on investigating the effect of different concentrations of mazidagi rock phosphate on phosphate solubilization. *A. hydrophyla* MFB-25 showed the highest phosphate solubilization performance when grown in the medium containing 3 g/L mazidagi rock phosphate (Fig. 3). Mazidagi rock phosphate concentrations above 3 g/L significantly reduced the phosphate solubilization. This is an expected result, sinceit is well known that phosphate solubilization efficiencies of microorganisms were significantly affected by different concentrations of rock phosphate [5,39]. For example, Reddy *et al.* [5] informed that *Aspergillus tubingensis* showed the highest phosphorus solubilization when grown in the presence of 2% of rock phosphate.Xiao *et al.* [40] reported that four yeast strains, *Rhodotorula* sp., *Candida rugosa*, *Saccharomyces cerevisiae* and *S.rouxii*, showed the highest phosphate solubilization activity at the rock phosphate concentration of 5 g/L.

The experiments showed that phosphate solubilization activity of *A. hydrophyla* MFB-25 cells was sensitive to temperature changes in liquid medium. Above and below temperature of 25 ^{0}C, the phosphate solubilization activity of immobilized cells significantly decreased. This result is in good agreement with the fact that the medium temperature may affect microbial phosphate solubilization[33,41]. However, it has been seen that the bacterium has the potential to solubilize the mazidagi rock phosphate in the temperature range of 15-40^{0}C.

It has been extensively reported that the incubation time is one another critical factor affecting phosphate solubilization capacities of microorganisms[40,42,43].Similarly, the present results elucidated that the incubation period influenced phosphate solubilization in the culture of *A. hydrophyla* MFB-25. Solubilized concentration of

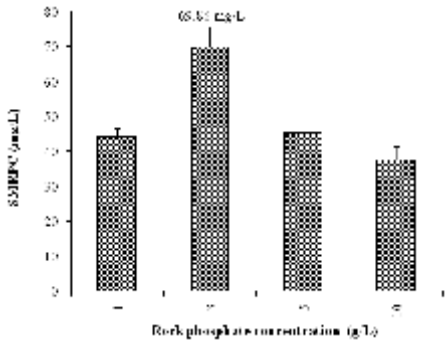

Fig. 3. The effect of mazidagi rock phosphate concentration on phosphate-solubilizing potential of *A. hydrophyla*MFB-25.SMRPC, Solubilized mazidagi rock phosphate concentration

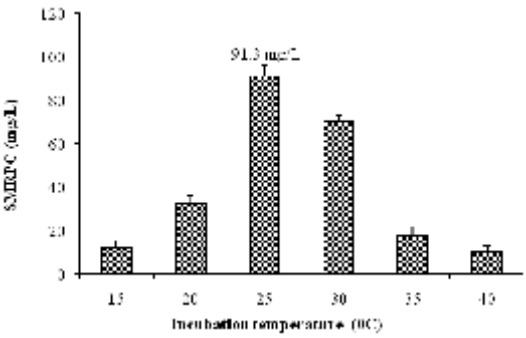

Fig 4. The effect of temperature on phosphate-solubilizing potential of *A. hydrophyla* MFB-25.SMRPC, Solubilized mazidagi rock phosphate concentration

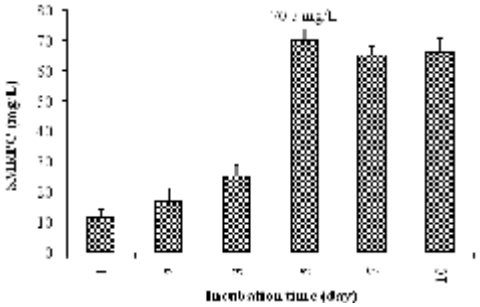

Fig 5. The effect of incubation time on phosphate-solubilizing potential of *A. hydrophyla* MFB-25.SMRPC= Solubilized mazidagi rock phosphate concentration

maz1dagi rock phosphate reached to the maximum value (70.3 g/L)at the end of 5-days incubation. A further increase in incubation period led to reductions in soluble phosphate concentrations. We were not able to provide definite explanation for this decrease. However, this decrease in the solubilized phosphate concentrations might be explained by the possibility that the bacterium used the solubilized phosphate as phosphorus source in culture medium in order to regulate its own metabolism.

In conclusion, this is the first report of solubilization of rock phosphates by *A. hydrophyla* and it shows that this bacterium may serve as an excellent rock phosphate solubilizerin the medium soils where mazidagi rock phosphate is used as P source. In this regard, it may be said that this bacterium may be cultivated in large-size ponds containing mazidagi-rock phosphates.At the end of appropriate cultivation period, P-rich water formed in these ponds may be used for irrigation of P-deficient agricultural soils. Furthermore, in the next studies, Mazidagi-phosphates rock to be inoculated with *A. hydrophyla* may be directly used as biofertilizer in agricultural studies. However, further studies are needed to prove this assumption.

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