Iranian Journal of Environmental Technology
2nd Year, Vol. 2, No. 3, Spring & Summer 2016
Received: May 9, 2016/ **Accepted:** October 17, 2016. **Page:** 41- 48

The effect of isolated phosphorus solubilizing bacteria and molasses on Soluble and available P in wheat straw medium

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Abstract

Phosphorus solubilizing microorganisms are capable of transforming insoluble phosphorus (P) forms to soluble and bioavailable P. The aim of the present study was to isolate P solubilizing bacteria from forest soils around the rock phosphate (RP) mine of Koh-e-SepidLar in Kohgiluyeh and Boyer Ahmad province and evaluate their ability in Sperber culture medium and wheat straw medium in the presence of RP and molasses. Bacteria were isolated and conducted by serial dilution and culturedon Pikovskaya medium. Quantitative assessment of 6 isolated bacteria carried out in the Sperber medium with tricalcium phosphate with 3 replications. Soluble P was measured on 5, 10, 15 and 20 days. The ability of two most effective bacteria in the liquid culture, assessed in wheat straw medium in a completely randomized design. Treatments consisted of a factorial combination of three RP levels (zero, 1:8 and 1:4 RP: wheat straw) and molasses in three rates (zero, 0.5 and 1% w/w) and three bacteria levels (none inoculated, and inoculated with isolates 1 and 2, separately). Soluble P and available P were measured on 20 and 40 days with $CaCl_2$ 0.01 M and $NaHCO_3$ 0.5 M, respectively. Some bacteria with P solubilizing capability found in the Pikovskaya medium. Two isolates showed the highest ability in liquid culture. $CaCl_2$-P in the presence of isolates 1 and 2 was higher than blank in wheat straw medium. Molasses caused the increase of $CaCl_2$-P in both times. Presence of RP reduced $CaCl_2$-P.

Key words: Phosphate solubilizing bacteria, Rock phosphate**,** Molasses

Highlights

- Isolation of P solubilizing bacteria from forest soils around the rock phosphate (RP) mine in Kohgiluyeh and Boyer Ahmad
- Molasses caused the increase of $CaCl_2$-P in both time.
- Presence of RP reduced $CaCl_2$-P.

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Introduction

Phosphorus isone of the most essential elements for crop production. Chemical P fertilizers are common sources of available P (1). High rates of chemical fertilizers, in addition to high costs, have adverse effects on the plants including P toxicity because of high P sorption by plants, imbalanced nutrients equilibrium and yield reduction (2). Phosphorus solubilizing bacteria, as a permanent constitute of the soil, could transform the insoluble P fractions into soluble and available forms. Acidic exudations and pH reduction are responsible for this transformation and some of the bacteria excretions may substantially increase P availability by the chelates compounds formation. Phosphorus solubilizing microorganisms are one of the effective methods increasing available P in calcareous soils. Number and types of solubilizing bacteria depends on the soil conditions (3). Environmental quality problems and economic issues from P fertilizer and considering capability of microorganisms have resulted into the studies for bio-fertilizers production (4).

Molasses is a brown and sticky byproduct of sugar industry. Molasses quality depended on the maturity of the sugar beet or sugarcane, and the quantity of sugar. Molasses contains minerals, sugars, amino acids, and fatty acids (5), which could stimulate the bacteria growth. Molasses is among the low cost carbohydrates which produces the highest biomass (6). Some of the researchers have reported that the highest growth of *Bacillus* occurred at the presence of molasses as carbon source and yeast extract as the N source (7). Jahangirzadeh et al (6) observed that Molasses and N application could help P solubilization by *Aspergillus niger*. The present study conducted to isolate P solubilizing bacteria from forest soils around the rock phosphate (RP) mine of Koh-e-SepidLar in Kohgiluyeh and Boyer Ahmad province and to assess their ability in a natural culture medium and the effect of molasses on their P solubilizing capability.

Material and Method

Soil samples: Five soil samples were taken from surface horizon (0-30 cm) of the forest around Koh-e-SpeidLar mine RP. Soil characteristics including texture by hydrometer method (8), content of organic carbon by wet oxidation (9), content of calcium carbonate equivalent by neutralization with HCl (10), cation exchange capacity by replacing exchangeable cation with NaOAc (11) and Olsen-P (Table 1) were determined.

Table 1- Some physicochemical properties of soil samples.

Soil no.	Organic-P(mg/kg)	Olsen-P (mg/kg)	%CCE*	%OM	%Sand	%Silt	%Clay	Texture	Elevation (m)
1	9.27	14.6	36.2	5.88	25.8	35.1	39	Clay Loam	1830
2	13.5	17.9	46.2	9.97	7.4	59.2	33.4	Silty Clay Loam	1815
3	7.8	6.75	64.3	0.34	5.9	38.7	55.4	Clay	1798
4	21.3	10.7	55.6	2.74	27.2	29.2	43.4	Clay	1752
5	8.4	7.3	14.3	1.36	25.2	21.2	43.4	Clay	1744

*CCE and OM, stands for Calcium Carbonate Equivalent and Organic Matter, respectively.

Isolation of P solubilizing bacteria: Bacteria isolation carried out by serial dilution and culturing on Pikovskaya culture medium that contains the following component in 1 Liter: glucose 10 g, NH_4NO_3 5 g, KCl 0.2 g, $MgSO_4$ 0.1 g, $MnSO_4$ 0.0001 g, $Ca_3(PO_4)_2$ 0.5 g and agar 18 g . Bacteria were separated from soils by extraction of 5 g of moist soil with 40 ml sterile saline solution after 20 min of shaking following by centrifuging at 2000 rpm. Tenfold serial dilution of soil extracts were inoculated in plates containing Pikovskaya culture medium with three replications and incubated at 37 °C for 3 days. Plates were monitored each day and P solubilizing bacteria which showed clear zones around colonies, selected and inculcated in Nutrient Agar Medium (Merck, Germany). Strains of some bacteria with high clear zone were detected via *in vitro* tests (e.g. gram stain and some biochemical tests). Quantitative P solubilization capacity of the isolated bacteria measured in Sperber basic medium with three replications. Basic medium contained the following components in 1 Liter: $Ca_3(PO_4)_2$ 2.5 g, glucose 10 g, $CaCl_2$ 0.1 g and $MgSO_4$. $7H_2O$. Six isolations from previous step, used in the quantitative test. $1\text{-}5\times10^7$CFU bacteria were inoculated in the basic medium and incubated at 37 °C for 20 day. 10 ml of each sample were taken on 5, 10, 15 and 20 day, pH measured, centrifuged at 5000 g for 10 min, filtered by whatman42 filter paper and soluble P determined with molybdenum blue method with acid ascorbic as the reductant (12).

P solubilization in wheat straw medium: The experiment conducted with the factorial combination of 3 RP levels (zero, 1:8 and 1:4 RP: wheat straw) and molasses in three rates (0, 0.5 and 1% w/w wheat straw) and 3 levels of bacteria (non inoculated and inoculated with two of the isolates, isolate 1 and isolate 2) in a completely randomized design with three replications. Isolated bacteria used in this step were those showing the highest solubilization capacity in the liquid test. Wheat straw was ground and passed through 2 mm sieve. 30 g of wheat straw was placed in 250 ml Erlenmeyer and enriched with molasses and RP based on the experiment design. Samples were sterilized, in autoclave for 20 min at 121 °C and 1.5 kg/cm^3 pressure. One ml of saline solution added to each Erlenmeyer based on the experimental design. Samples were incubated for 40 day under the lab temperature and moisture content of 100% of water holding capacity of wheat straw. Moisture content was adjusted each three days. Soluble P and available P were sequentially extracted on 20 and 40 days with $CaCl_2$ 0.01 M and $NaHCO_3$ 0.5 M, respectively; and reactive P determined by molybdenum blue method with acid ascorbic as the reductant (12). pH of $CaCl_2$ extracts was also determined.

Results

The studied soils were heavy textured and their clay content ranged from 33 to 55%. Organic matter content was 0.34 to 9.97 %.Olsen-P ranged from 6.7 to 18 mg kg^{-1} and $NaHCO_3$ extractable organic P ($NaHCO_3$-P_o) was 7.8 to 21 mg kg^{-1}. The studied soils had a high content of available P and the sum of Olsen-P and $NaHCO_3$-P_o reached up to 30 mg kg^{-1} in some cases (Table 1). Generally, Olsen P and $NaHCO_3$-P_o is low in unfertilized soils. The content observed in the present study (Table 1) shows P enrichment and the presence of solubilization mechanisms in the studied soils. All of the soil samples

had phosphorus solubilizing bacteria (PSB). Twenty two PSB were isolated, 9 isolates from soil # 1, 4 isolates, from soil #2, 1 isolate from soil # 3, 3 isolates from soil # 4 and two isolates from soil # 5. Two isolates that showed the highest clear zone (isolates from soil # 1, 5 and soil # 1, 3) were examined to detect species. Isolates No.# 1,5: had a big-yellowish and creamy round colony with gram negative reaction, coccid form, without spore, catalase and oxidase positive; it seem to be *Solanum* sp. No.# 1,3: had a small-white and smooth colony with gram positive reaction, bacilli form, with spore, catalase and oxidase negative; it seem to be *bacillus* sp.

Results of tricalcium phosphate solubilization and pH (Table 2) showed the ability of the isolates in P solubilization. Average of soluble P in the absence of bacteria were 8.59, 15.27, 17.07 and 28.09 mg/l on 5, 10, 15 and 20 days; respectively which increased to 33.5, 72.8, 271 and 392 mg l^{-1} by isolate 3 and to 146, 210, 540 and 1076 mg $l^{-1}$by isolate 6. Soluble P in the presence of isolate 5 was lower than other isolates which show the incapability of isolate 5. The most efficient bacteria (bacteria #6) showed less pH variation than the others. Average of pH in control was 6.4, 5.52, 6.08 and 5.94 on 5, 10, 15 and 20 days; respectively (Table 2).

$CaCl_2$ extractable P: Analysis of variance (Table 3) showed that molasses, RP content, bacteria and their interaction had significant effect ($p<0.01$) on $CaCl_2$-P.

Table 2- Average of soluble P and pH by P solubilizing bacteria on 5, 10, 15 and 20 days.

Number of isolates	Soluble P (mg/l)				pH			
	Time (days)				Time (days)			
	5	10	15	20	5	10	15	20
Blank	8.59	15.2	17.07	28.9	6.4	5.52	6.08	5.94
1	10.10	16.9	24.8	76.5	5.51	6.1	4.93	5.04
2	11.8	15.06	21.9	24.09	5.91	5.53	5.35	5.78
3	32.5	72.8	271	392	4.97	5.29	4.95	5.32
4	43.08	66.09	74.8	83.2	5.44	5.74	5.41	5.46
5	9.12	8.97	18.18	22.7	6.02	5.93	5.84	5.72
6	146	210	540	1076	4.34	4.9	4.59	4.92

Table 3- Analysis of variance of $CaCl_2$ and $NaHCO_3$ extractable on different time.

Source of variation	Degrees of Freedom	$NaHCO_3$-P		$CaCl_2$-P	
		20d	40d	20d	40d
Bacteria	2	1553**	1776**	3207808**	58689**
Molasses	2	830**	894**	19914**	91277**
Rock phosphate	2	275**	841**	89980**	209033**
Bacteria*molasses	4	713**	136**	8266**	101879**
Bacteria*Rock phosphate	4	147**	108**	23292**	52587**
Molasse*Rock phosphate	4	603**	224**	25315**	50276**
Bacteria*Molasses* Rock phosphate	8	327**	210**	42908**	34213**
Error	54	19.66	0.757	235	311

**Significant at P<0.01.

Bacteria inoculation increased $CaCl_2$-P by time. $CaCl_2$-P in zero level of molasses and absence of bacteria was 436 and 469 mg/kg on 20 and 40 days which increased to 536 and 597 mg /kg by bacteria #1 inoculation (Table 4).In the present study, isolate #2 did not significantly increase $CaCl_2$-Pon 20 and 40 days (Table 4). Molasses significantly increased $CaCl_2$-P on 40 days in the inoculation of isolate #1 (Table 4). Soluble P (extracted by $CaCl_2$) was 536 mg/kg in blank (none inoculated and zero molasses level) and increased to 665 and 730 mg/kg in the presence of 0.5 and 1% level of molasses (Table 4). In the 1:8 RP: wheat straw, $CaCl_2$-P was 603, 547and 475 mg/kg in zero, 0.5 and 1 % molasses for isolate 1 and reached to 605, 446 and 562 mg/kg for isolate 2 inculcation. In the 1:4 RP: wheat straw, no significant changes observed for both isolates (Table 4).

Table 4- Average of soluble P (mg/kg) in $CaCl_2$ 0.01 M in various levels of RP, molasses and bacteria inoculation on different times.

RP	Molasses	Day 20			Day 40		
		Blank	isolate1	isolate2	Blank	isolate1	isolate2
zero	zero	436	536	722	469	597	536
1:8	0	426	809	454	214	603	605
1:4	0	232	263	375	339	412	532
0	0.5	514	354	396	438	524	665
1:8	0.5	412	363	340	525	547	446
1:4	0.5	354	291	311	335	432	608
0	1	737	499	858	499	760	730
1:8	1	799	494	696	334	475	562
1:4	1	513	508	566	469	463	531

LSD ($p<0.01$) 209 and 196 on 20 and 40 d, respectively.

$NaHCO_3$-P: Analysis, of variance (Table 3) showed that molasses, RP, bacteria and their interactions, significantly affected $NaHCO_3$-P_i($p<0.01$). $NaHCO_3$-P_i increased in the presence of bacteria and with application of molasses after 20 days; the average of $NaHCO_3$-P_i was 22.2 mg/kg in blank which were increased to 55 and 51.3 mg/kg by isolates 1 and 2 inoculation, respectively. Generally, bacteria inoculation increased $NaHCO_3$-P_i and the increase was higher in the presence of molasses. In 0.5% molasses and absence of bacteria $NaHCO_3$-P_i was 33 mg/kg which increased to 49 and 31.5 mg/kg by isolates 1 and 2 inculcation, respectively. In 1% molasses level and non-inoculated$NaHCO_3$-P_i was 38.8 mg/kg and reached to 61.7 and 60.5 mg/kg by isolates 1 and 2 inoculation, respectively. On 40 day, higher increase of $NaHCO_3$-P_i observed in the 1% molasses. The average of $NaHCO_3$-$P_i$was 22 mg/kg in zero level of molasses and increased to 49 and 51.3 by isolates 1 and 2 inoculation (Table 5).

In the 0.5% molasses level, and zero RP and non-inoculated, $NaHCO_3$-P_i was 33 mg/kg on 20 day and isolates 1 inoculation increased it to 55 mg/kg, but isolate 2 did not significantly affected it (Table 5). In 1% molasses level, similar trend was observed on 20 and 40 days. In1:8 RP: wheat straw, isolates 1 and 2 did not significantly affect $NaHCO_3$-P_i in different molasses levels (Table 5). In 1:4 RP: wheat straw similar trend for different molasses levels observed (Table 5).

Table 5- Average of soluble P (mg/kg) in $NaHCO_3$ 0.01 M in various levels of RP, molasses and bacteria inoculation on different times.

RP	Molasses	Day 20			Day 40		
		Blank	isolate1	isolate2	Blank	isolate1	isolate2
Zero	Zero	22.2	49	51.3	68	87.8	66.5
1:8	0	40.3	45.5	55.4	64	92.7	79.1
1:4	0	28.7	21.5	53.6	73.8	83.1	67.8
0	0.5	33	55	31.5	90.6	83.1	91.3
1:8	0.5	22.4	47.2	50.2	79	79.7	74
1:4	0.5	24.3	42.8	43	35.6	77.4	78.6
0	1	38.8	61.7	60.5	60.3	91.8	68.8
1:8	1	36	48.8	57.6	50.9	72.9	66.9
1:4	1	30.8	40.9	42.5	71	69.6	66.9

LSD ($p<0.01$) =209 and 196 on 20 and 40 d, respectively.

Table 6- Average of pH in $CaCl_2$ 0.01 M various levels of RP, molasses and bacteria inoculation on different times.

RP	Molasses	Day 20			Day 40		
		Blank	isolate1	isolate2	Blank	isolate1	isolate2
0	0	4.74	5.33	4.73	5.31	5.39	4.44
1:8	0	4.89	5.38	5.21	5.27	5.56	4.89
1:4	0	5.82	5.76	5.44	5.78	5.89	5.61
0	0.5	4.85	5.50	5.26	5.20	6.27	5.76
1:8	0.5	5.34	5.44	5.43	5.48	6.04	5.64
1:4	0.5	5.55	5.59	5.67	6.02	6.05	5.36
0	1	5.69	5.25	4.64	4.77	5.06	4.57
1:8	1	4.78	5.62	5.26	5.34	5.17	5.04
1:4	1	5.4	5.84	5.65	6.11	5.26	5.49

LSD ($p<0.01$) 209 and 196 on 20 and 40 d, respectively.

The pH is determined on 20 and 40 days using $CaCl_2$ 0.01 M (Table 6). pH of isolates 1 and 2 was around 5 and did not change by bacteria significantly in the incubation time. No significant correlation observed between $CaCl_2$-P increase and pH.

Discussion and Conclusion

The studied soils had a high content of available P and the sum of Olsen-P and $NaHCO_3$-P_o reached up to 30 mg kg^{-1} in some samples. Generally, Olsen P and $NaHCO_3$-P_o is low in virgin soils. Murphy and Riley reported that Olsen-P and $NaHCO_3$-P_o were less than 5 mg/kg (12). All of the soil samples had phosphorus solubilizing bacteria (PSB). Twenty two PSB was isolated. Vazquez et al. reported the presence of P solubilizing microorganisms in the rhizosphere of mango trees of the semiarid region of Mexico (13). They reported that the isolated bacteria were *Bacillus*, *Pseudomonas*, *Vibrio*, *Enterobacter*, *Rhizomonas*, *Clivera*, *Xanthobacter* and an unknown genus. Kucey (14) found that the number of P solubilizing bacteria was higher than fungi.

Soluble P in the presence of isolate 5 was lower than other isolates which show the incapability of isolate 5. It seemed that isolates 3 and 6 were the most efficient in P solubilization. Phosphorus solubilization increased gradually by time but the rate of solubilization was higher in the presence of isolate 6. Kucey (14) compared the ability of P solubilizing microorganisms in liquid

culture containing RP or tricalcium phosphate and found a significant relation between RP solubilization and formation of clear zones in the Pikovskaya medium. Generally, results showed that bacteria inoculation in liquid culture caused solubilization of insoluble phosphates but the effects were different; because of the biological differences among bacteria in organic acid production and H^+ release, there upon some bacteria shows higher capability in P solubilization. Many reports exist about the dissolution of tri-calcium phosphate in liquid culture (15, 16). Rodriguez et al. (16) reported that P solubilizing bacteria decrease pH and P solubilization by organic acid production and content of solubilized P was directly related to organic acid production. Esmaeelkho and Ali-Khani concluded that *Thiobacillus* spp. had high P dissolution potential (17). Chen et al reported that the highest P solubilization belonged to *Arthrobacter* (519 mg/l) and pH of 4.9 (18). In the present study, isolate #2 did not significantly increase $CaCl_2$-P on 20 and 40 days. Jahangirzadeh et al. (6) reported that $CaCl_2$-P increased by *Aspergillus niger* and a fungus isolated from Koh-SepidLar. Soluble P was 536 mg/kg in blank and increased to 665 and 730 mg/kg in the presence of 0.5 and 1% level of molasses. Jahangirzadeh*et al.* (6) reported that molasses and nitrogen addition could increase insoluble P solubilization by *Aspergillus niger*. In 1:4 RP: wheat straw, similar trend for different molasses levels was observed (Table 5). It seems that RP application reduced $NaHCO_3$-P_i. $NaHCO_3$-P_i is introduced as di-calcium phosphate (19, 20) and in a more precise description it could be defined as P forms more soluble than di-calcium phosphate which has showed close relation with Olsen-P in calcareous soils (19). Jorizi (21) reported that fungi inoculation significantly increased RP dissolution. Saha and Jan reported the increase of Olsen-P by *Bacillus* inoculation specially *B. Polymixa* (22).

pH of isolates 1 and 2 was around 5 and did not change by bacteria, significantly in the incubation time. Murphy *et al.* (12) reported that pH increase had a negative significant correlation with $CaCl_2$-P and pH decrease increased available P. some of the micro- organisms could increase available P by pH decrease, but it does not seem that always pH decrease cause available P increase (23).Results of the present study also showed the presence of P solubilizing bacteria in the studied region. Molasses application may accelerate solubilization of RP by PSB. Isolation of PSB and their usage as biofertilizer could be a substitution for chemical P fertilizers and consequently reduces environmental contamination.

Acknowledgement

This work was financially supported by Yasouj University. All authors had equal role in design, work, statistical analysis and manuscript writing. The authors declare no conflict of interest.

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