

Effect of Physicochemical Parameters on Inorganic Phosphate Solubilisation by *Serratia marcescens* PH1 and Organic Acids Production

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PHOSPHATE solubilizing bacteria are capable to release inorganic phosphate and make it available to plants for growth enhancement. In this study, *Serratia marcescens* PH1, which has been isolated from tomato plant rhizosphere, has been subjected to different physical and chemical parameters for optimization of its phosphate solubilisation capacity. The best temperature, inoculum percentage, agitation rate, incubation time, and pH value were found to be 30°C, 1.5%, 150rpm, 5 days, and 7-8, respectively. The best carbon source for phosphate solubilisation among 5 different sources, glucose, sucrose, galactose, maltose, and arabinose, was glucose which allows for releasing 797mg/L of P. Besides, casein was the best nitrogen source for phosphorus release (853mg/L) and Ca₃(PO₄)₂ in Pikovskaya medium was the best P source (855mg/L). The key factors affecting P release in the modified Pikovskaya medium were glucose, casein and inoculum percentage. P concentration in the modified Pikovskaya culture medium was 853mg/ml comparing with only 749mg/L in the classical formula. For freeing of P, *Serratia marcescens* PH1 produces organic acids such as citric, lactic, malic, and benzoic acids. Phosphorus yield was increased gradually with organic acids production till reaching 861mg/ml in 5 days with pH drop to 1.1. *pqqC* (a gene responsible of PQQ production) gene fragment of approx. 568 bp was successfully amplified in phosphate solubilizing bacteria. Detection of *pqq* genes is an evidence of gluconic acid production capability. This acid is a well known biocontrol agent. *Serratia marcescens* PH1 is a strong phosphate solubilizer and we are planning to conduct more wide-scale experiments in pots to optimize the utilization of such bacterium before conducting agricultural applications as a plant growth promoter and a biocontrol agent.

Keywords: Gluconic acid, Phosphate solubilisation optimization, *pqqC*, *Serratia marcescens* PH1.

Introduction

The plant growth promoters (PGP) mediate many soil processes such as mineralization, nitrogen fixation, decomposition, denitrification, and others (Motsara et al., 1995; Whitelaw, 2000). In 1981, Kloepper & Schroth have used firstly the term Plant Growth Promoting Rhizobacteria (PGPR) for bacteria that are colonizing plant root and enhancing plant growth. One of these

rhizobacteria is phosphate-solubilizing bacteria which are known to enhance solubilisation of the fixed soil phosphorus resulting in higher crop yield (Abd-Alla, 1994; Jones & Darrah, 1994). Traditionally, phosphate solubilizers production is based on chemical processing of insoluble mineral phosphates. This includes energy intensive treatment. Besides, this process is environmentally undesirable and costly (Vassilev et al., 2006). On the other hand, phosphate

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Received 4/11/2019; Accepted 10/3/2020

DOI: 10.21608/ejm.2020.19042.1123

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solubilizing microorganisms can utilize root-born carbon compounds, sugars and organic acids, for their growth and solubilize inorganic phosphates for plant roots in return (Arcand & Schneider, 2006). The literature described many bacterial genera that have the ability to solubilize fixed phosphate such as *Serratia*, *Rhizobium*, *Pantoea*, *Enterobacter*, and *Pseudomonas* (Son et al., 2006; Buch et al., 2008; Sulbaran et al., 2009). Moreover, *Serratia marcescens* CTM50650 was the best phosphate solubilizer according to a study performed by Ben Farhat et al. (2009).

Many physical and chemical factors control the availability of inorganic phosphorus to plants. The physical factors include pH, temperature, aeration, cell density, and incubation time (Gaur, 1990). Growth medium composition also affects the efficiency of rhizobacteria to release phosphorus (Ben Farhat et al., 2009). This includes the type of insoluble inorganic phosphates, and different sources of nitrogen and carbon (Nautiyal, 1997; Ben Farhat et al., 2009). Moreover, nitrogen, phosphorus and carbon sources are control factors in soil. They influence microorganisms growth and consequently their phosphate solubilisation capacity (Ben Farhat et al., 2009).

Phosphate solubilizing bacteria produce variety of organic acids such as acetic, formic, lactic, gluconic, oxalic, succinic, malic and citric acids (Vyas & Gulati, 2009). Gluconic acid is the most effective acid in mineral phosphate solubilisation (Gulati et al., 2010; Ogut et al., 2010). This acid is further oxidized to 2-ketogluconic acid which is an effective calcium ions chelating agent (Werra et al., 2009). Gluconic acid is produced by direct glucose oxidation by glucose dehydrogenase equipped with PQQ (Pyrrolo Quinoline Quinone) as a cofactor (Kim et al., 2003). The *pqq* gene cluster producing PQQ is conferred with phosphate- solubilisation activity in most phosphate solubilizing bacteria (Krishnaraj & Golgstein, 2001; Kim et al., 2003).

In this study, a promising phosphate solubilizer, *Serratia marcescens* strain PH1 (Mohamed et al., 2018), was subjected to different physicochemical parameters to optimize its phosphate solubilisation efficiency and to detect its organic acids production ability. Besides, the presence of *pqq* C gene (which confers with phosphate solubilisation activity) was also tested.

Materials and Methods

Bacterial strains and solubilisation index determination

The bacterial strains used in this study, *Serratia marcescens* PH1, *Serratia marcescens* PH2, and *Bacillus subtilis* PH have been isolated from tomato plant rhizosphere and molecularly identified in our previous study (Mohamed et al., 2018). Sterilized Pikovskaya agar medium (Techno Pharm Chemicals, Haryana, India) was used to estimate the solubilisation index (SI) according to the following equation:

$$SI = \frac{\text{total diameter (colony + halozone)}}{\text{colony diameter}}$$

(Edi-Premono et al., 1996). SI was measured in 48hrs at 30°C (Mohamed et al., 2018).

Optimization of phosphate solubilisation in liquid cultures

Conical flasks containing Pikovskaya broth (in gm/L: Yeast extract 0.500, Dextrose 10.000, Calcium phosphate 5.000, Ammonium sulphate 0.500, Potassium chloride 0.200, Magnesium sulphate 0.100, Manganese sulphate 0.0001, and Ferrous sulphate 0.0001) or other described broth media were inoculated with *Serratia marcescens* PH1 in triplicates at different temperatures (25-40°C), Inoculum % (0.5- 2 v/v), agitation rates (50-180rpm), time intervals (1-5 days), and pH values (5-9). Besides, different carbon (glucose, sucrose, galactose, maltose, and arabinose), nitrogen (casein, peptone, NaNO₃, and NH₄SO₄), and inorganic phosphorous (Ca₃(PO₄)₂, AlPO₄, and FePO₄) sources were also used for optimization P release in Pikovskaya broth medium. Each single trial was performed in triplicates and mean values were considered. Besides, standard deviation values were estimated using Microsoft Excel.

Quantative estimation of phosphate solubilisation

Phosphate was estimated in clear supernatants (using Pikovskaya broth) by spectrophotometer at 475nm using phosphomolybdate method (Watanabe & Olsen, 1965). Total solubilized phosphorus was then calculated from a standard graph. Besides, bacterial growth was measured in the different liquid cultures spectrophotometrically at 550nm. pH values were also recorded in these liquid cultures (Mohamed et al., 2018). Pure and sterilized Pikovskaya broth (with no bacteria) was used as control and all other necessary reagent controls were used. The net results were recorded to be expressed in terms of net P solubilization.

Each single trial was performed in triplicates and mean values were considered. Besides, standard deviation values were estimated using Microsoft Excel.

Organic acid detection using HPLC

Pure liquid Pikovskaya cultures were centrifuged at 10000xg for 15min and supernatants were filtered and subjected to HPLC analysis using reversed phase HPLC Dionex Ultimate 3000 coupled with ultimate 3000 variable wavelength UV-VIS detector. Column C18 150X 4.6mm X 5 μ m (AOAC, 2000). HPLC was done at The Regional Center for Food and Feed (RCFF), Agricultural Research Center, Giza.

Detection of *pqqC* gene

Bacterial DNA was extracted using Insta Gene Matrix (Bio-Rad, Hercules, CA, USA). Pure DNA was used as a template for PCR amplification. The primers used for amplification of an internal fragment of *pqqC* genes are OIMBF3 (5' CCCGCGAGCAGATCCAGGGCTGGGT3') and OIMBF4 (5' TAGGCCATGCTCATGGCGTC3') (Ben Farhat et al., 2009). Amplification was done using 20ng DNA in 30 μ l reaction mixture containing 1U of Taq polymerase (Fermentas Life Sciences), 5 μ l of 10X PCR buffer, 1 μ l 10mM dNTP mix, 1.5 μ l of 50mM MgCl₂, 1 μ l of 10mM of each primer and 1 μ l of genomic DNA. The amplification program includes: denaturation step at 95°C for 1min, annealing step at 95°C for 1min, 59°C for 1min, 72°C for 2min (35 cycles) and final extension at 72°C for 3min (Stella & Halimi, 2015). The amplicons were separated with 1% (w/v) agarose gel.

Results

To detect the ability of *Serratia marcescens* PH1 to solubilize inorganic phosphate, the solubilisation index was measured, 3.1, using Pikovskaya agar medium (Fig. 1). Physical parameters have been tested for better phosphate solubilisation (calcium phosphate) in Pikovskaya broth. A temperature range from 25 to 40°C was tested using *Serratia marcescens* PH1, and free phosphorus was measured in mg/L (Table 1). The used inoculum percentage was 1% (v/v) at O.D.₅₅₀ = 1.1 in 5 days at pH= 7 and shaking of 150rpm (Mohamed et al., 2018). The optimum temperature for the highest phosphorus yield (735mg/L) was 30°C (Table 1).



Fig.1. A halozone around *Serratia marcescens* PH1 in Pikovskaya agar medium.

TABLE 1. Effect of different temperatures on phosphate solubilisation by *Serratia marcescens* PH1.

Temperature °C	[P] mg/L
25	711± 6.5
30	735± 7.7
35	668±2.3
40	722±5.3

For testing the effect of different inoculum percentages on phosphate solubilisation at 30°C, the physical parameters were fixed (agitation rate of 150rpm at pH=7 in 5 days). However, 1.5ml of seed culture (O.D.₅₅₀ = 1.1) per 100ml of Pikovskaya broth was the best inoculum percentage within the tested range (0.5-2% v/v) for the highest yield (722 mg/L) of free phosphorus (Table 2).

TABLE 2. Effect of inoculum percentage (v/v) on free phosphorus release by *Serratia marcescens* PH1.

Inoculum % (v/v) (O.D. 1.1) at 550nm	[P] mg/L
0.5	523±9.8
1	647±4.4
1.5	722±7.6
2	713±8.8

Different agitation rates (50-180rpm) were also experimented at fixed physical parameters (30°C, pH=7, inoculum percentages of 1.5, and 5 days of incubation). As shown in Table 3, the best phosphorus yield (733mg/L) was obtained under shaking at 150rpm.

TABLE 3. Effect of agitation rate (rpm) on phosphate solubilisation by *Serratia marcescens* PH1.

Agitation rate (rpm)	[P] mg/L
50	512± 8.1
100	679±5.7
150	733±5.4
180	699±6.2

A time intervals from 24hrs to 5 days were used to detect the best harvest of phosphorus concentration (Fig. 2). The highest yield (732mg/L) was noticed in 5 days and the lowest one (493 mg/ml) was detected in 24h, at fixed physical parameters (30°C, pH=7, inoculum percentage of 1.5, and agitation rate of 150rpm).

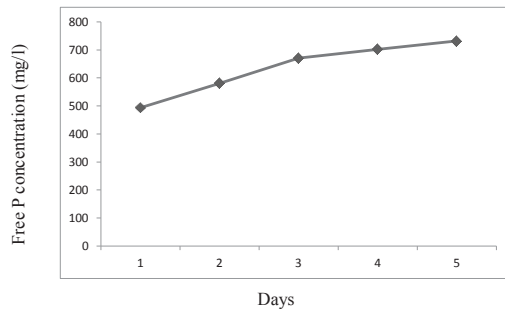


Fig. 2. Effect of time intervals on phosphate solubilisation by *Serratia marcescens* PH1.

Variations in free phosphorus concentration were detected using different pH values (5-9). The best pH values for highest phosphorus yield were 7 and 8. Lower phosphorus concentrations were recorded at higher and lower pH values (Table 4). The other physical parameters were fixed (30°C, inoculum percentage of 1.5, agitation rate of 150rpm, and incubation of 5 days).

TABLE 4. Effect of different pH values on free phosphorus release by *Serratia marcescens* PH1.

pH value	[P] mg/L
5	534± 7.8
6	603± 6.2
7	725±7.1
8	722±5.3
9	612±7.2

Three different chemical parameters were also examined for optimization of phosphate solubilisation ability by *Serratia marcescens* PH1 at fixed physical parameters (30°C, inoculum percentage of 1.5, agitation rate of 150rpm, and pH= 7 in 5 days incubation). Five different carbon sources were tested separately instead of dextrose in Pikovskaya medium (Fig. 3). The best phosphorus concentration (797mg/L) in liquid cultures was obtained using glucose as a sole carbon source followed by galactose (661mg/L) and the lowest value (406mg/L) was recorded using arabinose. Besides, cell growth was enhanced in the presence of glucose (O.D₅₅₀ = 1.29).

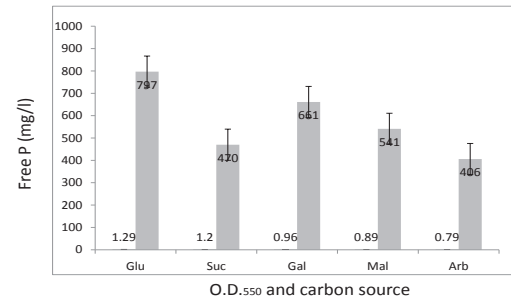


Fig. 3. Effect of different carbon sources on phosphate solubilisation by *Serratia marcescens* PH1.

Two different organic nitrogen sources (casein and peptone) and two inorganic nitrogen sources (NaNO₃ and NH₄SO₄) were used separately instead of yeast extract and ammonium sulphate in Pikovskaya medium. Phosphorus release was greatly enhanced (853mg/L) upon using casein and cell growth as well (O.D₅₅₀ = 1.44) (Fig. 4). Interestingly, red colour of *Serratia marcescens* PH1 was retained in the modified Pikovskaya agar medium and lost in the classical formula (Fig. 5). However, cells optical density, 1.38, and free phosphorus concentration, 797mg/L, were also enhanced in the presence of peptone as a sole nitrogen source.

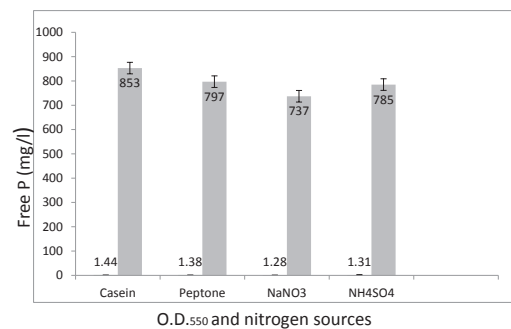


Fig. 4. Effect of different nitrogen sources on phosphate solubilisation by *Serratia marcescens* PH1.



Fig. 5. Red pigment of *Serratia marcescens* PH1 in the modified Pikovskaya culture medium (left) and white colonies in the Pikovskaya classical medium (right).

Finally, two different inorganic phosphate sources (AlPO_4 and FePO_4) were used separately and compared against $\text{Ca}_3(\text{PO}_4)_2$ in Pikovskaya medium. The results of cell growth and phosphorus release were illustrated in Fig. 6. Interestingly, $\text{Ca}_3(\text{PO}_4)_2$ gave the best yield of free phosphorus, 855mg/L, in 5 days with recognized growth enhancement ($\text{O.D.}_{550} = 1.4$).

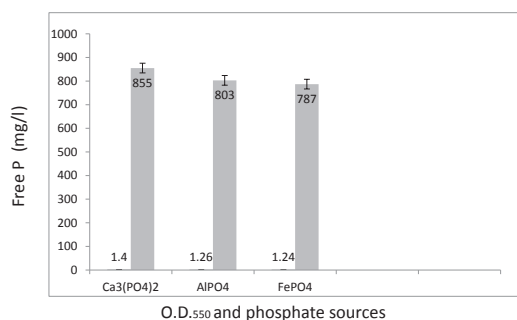


Fig. 6. Effect of different phosphorus sources on phosphate solubilisation by *Serratia marcescens* PH1.

A comparison between Pikovskaya medium with classical and optimized parameters is illustrated in Table 5. Three parameters differentiate between the two cases, inoculum percentage, nitrogen source and carbon source. Free phosphorus concentration was increased in case of using the optimized parameters (853mg/L) if compared with the classical ones (749mg/L).

Production of some organic acids (Table 6) by *Serratia marcescens* PH1 was tested using ingredients of the optimized medium throughout five days. Generally, organic acids production increased gradually by time till reaching the maximum values after 5 days. However, citric, lactic and malic acids recorded the highest values, 98.7, 91.17, and 90ppm, respectively. Free phosphorus was measured too in parallel to organic acids production throughout 5 days. Interestingly, phosphorus yield increased not only with time, but also with the increased production of the organic acids. Besides, acidity increased and pH decreased gradually with organic acids production till reaching 1.1 in 5 days.

For detection of *pqqC* (a gene responsible of PQQ production), a fragment of approx. 568bp was successfully amplified (Fig. 7) in three different strains, *S. marcescens* PH1, *S. marcescens* PH2, and *Bacillus subtilis* PH. The primers used for gene amplification were OIMBF3 and OIMBF4.

Discussion

The plant growth promoting rhizo-bacteria (PGPRB) are a group of bacteria that has the ability to solubilize phosphate to be available for plant growth. Therefore, they should have at least any two of the following criteria; 1- Stimulate plant growth, 2- Colonize around plant roots, 3- Control phytopathogens. (Vessey, 2003). The present study tested different physicochemical parameters effect on phosphate solubilisation by *Serratia marcescens* PH1. This bacterium has been isolated from tomato plant rhizosphere in our previous study (Mohamed et al., 2018). However, temperature, inoculum percentage, agitation rate, incubation time, pH, and different sources of carbon, nitrogen and phosphate are parameters that have different effects on phosphate solubilisation by *Serratia marcescens* PH1. The optimum temperature for the highest phosphorus yield (735mg/L) was 30°C and this result is in agreement with Mujahid et al. (2015), who revealed that at 30°C, 100% of their bacterial isolates (32 different bacterial rhizosphere isolates) had maximum P solubilisation. Besides, other studies also declared that 30°C is the optimum temperature for phosphate solubilisation (Fasim et al., 2002; Ben Farhat et al., 2009). Phosphorus is solubilized faster in warm humid climates and slower in cool dry climates and this temperature is optimum when combined with other factors with certain levels (Alori et al., 2017).

For higher solubilization of phosphatic compounds, high cell density is needed (Sardina et al., 1986). In this study, inoculum of 1.5% (v/v) is better than lower percentages for phosphate solubilisation. This high inoculum % provides high cell population for more phosphate solubilisation.

To suspend both of nutrients and cells evenly through the liquid medium, and to make oxygen more available for the growing cells, good agitation is required (Blanch & Eienlese, 1973). In our study, the best agitation rate for phosphate solubilisation by *Serratia marcescens* PH1 was 150rpm. For aerobic bacteria, efficient agitation is required for different bacterial activities including phosphate solubilisation. It was observed by Singha & Putatunda (2016), that *Pseudomonas aeruginosa* solubilized greater quantity of phosphorus under shaking condition as compared to stationary condition.

TABLE 5. Free phosphorus release in two different Pikovskaya formula.

Parameters	Inoc.% (v/v)	Agit. (rpm)	Incub. (days)	Incub. Temp. °C	pH	Nitrogen Source	Carbon source	Phosphate source	Free P (mg/L)
Pikovskaya with classical parameters	1	150	5	30	7	Yeast extract + ammonium sulphate	Dextrose	Calcium phosphate	749
Pikovskaya with optimized parameters	1.5	150	5	30	7	Casein	Glucose	Calcium phosphate	853

- The classical physical parameters were used according to Mohamed et al. (2018).

- Inoc., inoculation; Agit., agitation rate; Incub. Temp., incubation temperature.

TABLE 6. Organic acids production and P release through 5 days.

Days	Organic acids (mg/L)				P(mg/L)	pH
	Malic	Benzoic	Citric	Lactic		
1	9.92	3.25	59.5	1.5	557	6.0
2	29.6	6.65	70	25.6	583	4.5
3	60	10	70	26.31	644	3.7
4	70	20.26	90	55.89	775	2.5
5	90	37.21	98.7	91.17	861	1.1

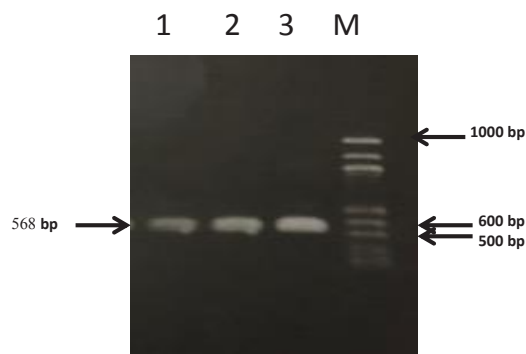


Fig. 7. Agarose gel electrophoresis of an amplified internal fragment of pqqC [Lanes 1, 2, and 3 are for *Serratia marcescens* PH1, *Serratia marcescens* PH2, and *Bacillus subtilis* PH, respectively. M is a 1kb DNA ladder].

In the current study, the incubation period was found to be a key factor for phosphate solubilisation. The highest yield of free phosphate (732mg/L) was noticed in 5 days and the lowest one (493mg/L) was detected in 24hrs, at fixed physical parameters. This result is in agreement with Singha & Putatunda (2016), who declared that *Pseudomonas aeruginosa* showed highest P solubilization (83ng/ml) after 168hrs (7 days). Singh & Ghosh (2012), also revealed similar results. However, in our case incubation time more than 5 days showed no significant increase in free P release.

pH plays an important role in phosphates solubilization (Nahas, 1996). According to Mujahid et al. (2015), the pH value for the best phosphate solubilisation in bacteria is between 5-7 and 8 for some strains as well. In our study, the best pH values for better P solubilisation is 7-8. A great percentage of phosphorus in chemical fertilizers is unavailable to plants after their application to the soil. This is because P is strongly bound to Ca and Mg in alkaline soil and to Fe and Al in acidic soils (Arpana et al., 2002). However, according to Singha & Putatunda (2016), the optimum conditions for P solubilisation using *Pseudomonas aeruginosa* PHSD5 is at pH 7. Moreover, Sagervanshi et al. (2012), declared that maximum P solubilisation observed at pH 7. The neutral pH is the optimum for P solubilisation in many studies (Chen et al., 2005; Zhu et al., 2011; Kuntia et al., 2014). In the other hand, Xiang et al. (2011), recorded that the maximum yield of free P was found at pH 8.

According to Mardad et al. (2014), glucose allows the production of 100% of orthophosphate by four different bacterial strains. Moreover, Mujahid et al. (2015), stated that phosphate solubilisation differs in the presence of different carbon sources according to the following order: glucose>fructose>mannitol>xylose>arabinose. However, *Serratia marcescens* CTM 50650 can utilize varieties of carbon sources with highest

solubilisation of phosphate in the presence of glucose (Ben Farhat et al., 2009). All of these findings are in parallel with our results. *Serratia marcescens* PH1 can use a variety of carbon sources, but phosphate solubilisation was greatly enhanced in the presence of glucose as a sole carbon source to reach 797mg/L. This is may be due to growth enhancement in the presence of this monosaccharide.

Among the tested nitrogen sources, organic nitrogen was more effective in releasing free P in the liquid medium. Casein and peptone released 853 and 797mg/L of free P, respectively. These results are in parallel with those of Singha & Putatunda (2016). They revealed that the best N source was found to be beef extract (162.33ng/ml) followed by peptone (78.66ng/ml). Moreover, Sagervanishi et al. (2012), reported that ammonium sulphate and casein recorded the maximum phosphate solubilisation. *Serratia marcescens* PH1 has gained its red color when casein is added to Pikoviskaya medium and lost it when ammonium sulphate is used. The red pigment, prodigiosin, is released as a secondary metabolite from *Serratia marcescens* when organic nitrogen is used (Helvia et al., 2010).

Three inorganic P sources were used separately, $\text{Ca}_3(\text{PO}_4)_2$, AlPO_4 and FePO_4 , in Pikoviskaya broth. The maximum P release (855mg/L) was found in case of $\text{Ca}_3(\text{PO}_4)_2$. This result is in agreement with Son et al. (2006), who recorded a valuable release of P when $\text{Ca}_3(\text{PO}_4)_2$ is used as a sole P source. Moreover, they observed the greatest phosphate solubilisation in 5 days of incubation. In addition, Nautiyal (1999), revealed that glucose and $\text{Ca}_3(\text{PO}_4)_2$ together were essential for maximum P production.

Phosphate solubilisation in two different Pikoveskaya formula revealed that glucose and casein together with inoculum percentage of 1.5% in the modified medium led to more efficient phosphorus release (853mg/L) than the classical conditions, 749mg/L. According to Mardad et al. (2014), monosaccharides are more efficient for phosphate solubilisation than disaccharides and polysaccharides. This may explain the high yield of phosphorus upon using glucose instead of sucrose. Besides, Sagervanishi et al. (2012), reported that casein recorded the maximum phosphate solubilisation than other used nitrogen sources.

In our study, increasing levels of organic acids production led to increasing levels of free phosphorus through time intervals. Organic acids excretion led to pH drop (Seshachala & Tallapragada, 2012) hence, phosphorus ions are released by substitution of H^+ for Ca^{2+} (Goldstein, 1994). Therefore, P is released from its chelator (Ca^{2+}) and become free. One of the most commonly produced organic acids for phosphate solubilisation is gluconic acid which is produced by direct glucose oxidation. The oxidation pathway is mediated by glucose dehydrogenase and its co-factor pyrroloquinoline quinone (PQQ) (Goldstein et al., 1999; Perez et al., 2007). In our study, *pqqC* gene was amplified and electrophoresed to detect its presence in three efficient phosphate bacterial solubilizers, *Serratia marcescens* PH1, *Serratia marcescens* PH2, and *Bacillus subtilis* PH, (Mohamed et al., 2018). *pqqC* is a member in a gene family which is involved in biosynthesis of PQQ cofactor (Kim et al., 1998; Kim, 2003). Detection of *pqq* genes in *S. marcescens* was reported by Ben Farhat et al. (2009). Therefore, detection of *pqq* genes is an evidence of gluconic acid production by *Serratia marcescens* (Ben Farhat et al., 2009). Beside its role in solubilizing mineral phosphate, gluconic acid is well known for its antifungal activity (Kaur et al., 2006). PQQ is also involved in the production of antifungal metabolites and enhancement of systemic resistance (Song et al., 2008).

Conclusion

In conclusion, carbon and nitrogen sources and inoculum percentage affect phosphate solubilisation greatly *in vitro*. Production of organic acids by phosphate solubilizing bacteria plays a key role in releasing free P by decreasing the pH. We strongly recommend the utilization of *Serratia marcescens* PH1 in further experiments to investigate its efficiency as a promising phosphate solubilizer and plant growth promoting agent in wider scales.

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