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PHOSPHATE SOLUBILIZING MICROORGANISMS ISOLATED FROM MANOA SOIL IN OAHU, HAWAI'I

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ABSTRACT

Phosphorus (P) is an important nutrient besides nitrogen. It is an essential element in the soil for plant development and growth of microorganisms. The common method to supply phosphate to plants is to add soluble mineral phosphate fertilizers into soil. However, this practice is not cost-effective. This study attempted to isolate phosphate-solubilizing microorganisms which can solubilize insoluble rock phosphate to a soluble form. Soil microorganisms were isolated from root and soil samples at the rocky site of Waahila Ridge, Manoa area. Large numbers of fungi and bacteria were recovered. The microorganisms were purified by transferring twenty-four of single colonies to YMA agar. The isolates were then examined for their ability in rock phosphate solubilization. All isolates showed the positive activity and generated different levels of soluble phosphate concentration associated pH drop in the liquid medium. Two out of nineteen isolates showed the highest activity in solubilizing North Carolina rock phosphate and the soluble phosphate concentration in the liquid medium were 19.33mg/L and 18.1mg/L. This study demonstrated that selected soil microorganisms could convert insoluble phosphate to soluble form generally through the process of acidification.

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1 INTRODUCTION

Phosphorus is one of the major nutrient elements that influences plant growth and production (Bi *et al*., 2011; Sharma, 2011). It is not acquired through the biochemical reactions in which nitrogenous compounds are derived. Plants or soil microorganisms utilize phosphate nutrient either from natural sources or fertilizer applications. The common sources of P nutrient for plant uptake are chemical fertilizers, animal manures, and plant residues. However, native phosphorus in both organic and inorganic compounds also present in soils and are mostly found in mineral binding forms (Rao, 1982 cited by Osorio and Habte, 2001).

According to Kim *et al*. 1997, the principal mechanism for mineral phosphate solubilization in soils is the production of organic acids which are synthesized by soil microorganisms. This mechanism was then confirmed by Osorio and Habte (2001). There was a little relationship between lowering pH and increasing insoluble phosphate solubilization (Wakelin *et al*., 2004). However, Osorio and Habte (2001) addressed that there was a strong relationship of pH reduction and increasing soluble phosphate concentration in the growing medium of microorganisms. Gluconic acid seems to be the most frequent agent of mineral phosphate solubilization (Whitelaw *et al*., 1999; Ogut *et al*., 2010). Other organic acids including formic acid, acetic acid, propionic acid, lactic acid, glycolic acid, fumaric acid, and succinic acid were also addressed (Rashid, 2004). In addition to acidification, phosphate solubilizing microorganisms convert insolu-

ble phosphates into soluble forms through the process of chelation and exchange reactions (Sharma, 2011). These acids lower the pH and dissolve the bound forms of phosphates in mineral rock phosphate.

Some soil microorganisms have an ability to bring phosphate from insoluble form to soluble form (Osorio and Habte, 2001). Previous researchers had pointed out that bacteria belonging to the genera *Bacillus* and *Pseudomonas*, and fungi belonging to the genera *Penicillium* and *Aspergillus* can solubilize insoluble phosphates in soil into soluble forms (Harris *et al*., 2006; Zhang *et al*., 2006; Xiao *et al*., 2009; Minghe *et al*., 2011; Yu *et al*., 2011). According to Osorio and Habte (2001), *Motierella sp* fungi also performed a high level of phosphate solubilization.

The rhizosphere is the zone surrounding the roots of plants in which complex relations exist among plants, soil microorganisms and the soil itself. This area is rich in root exudates which contain carbohydrates, organic acids, vitamins and many other substances essential for the life. Microorganisms play important roles in the soil environment in supplementing nutrients to plants. However, insoluble phosphate preserved in the minerals is not available sources in the natural environment for plant uptake unless it is solubilized. Fortunately, some particular species of soil microorganisms are able to convert phosphorous from the mineral binding forms to accessible compounds in the soil environment (Osorio and Habte, 2001; Wakelin *et al*., 2004; Hamdali *et al*., 2008; Yu *et al*., 2011).

Plants growing on rock may associate with phosphate-solubilizing microorganisms. The phosphate-solubilizing microorganisms may serve as an intermediary factor to solubilize insoluble phosphate from the rock phosphate into useable form that plants can uptake. We hypothesized that the phosphate-solubilizing microorganisms may colonize in soil and plant roots in the rocky sites where plants are growing. The insoluble form of phosphorous in rock phosphate is solubilized and released into soil by activities of phosphate-solubilizing microorganisms. The aim of this research was to isolate phosphate-solubilizing microorganisms associated with the plant roots and soil and to examine their phosphate solubilization activities.

2 MATERIALS AND METHODS

2.1 Sampling sites

Some rocky locations in Waahila Ridge, Manoa area were accessed to collect soil samples with plant roots. Stainless steel trowel was used to collect soil and plant roots. Approximately 200 g of soil and 3.0 g of roots were collected. Soil and plant roots were placed in plastic bags for microorganism isolation. Fine plant roots were gently separated from the soil. The soil and root samples were processed immediately in the laboratory.

2.2 Experimental procedures

The isolation process was presented in Figure 1. Basically, a series of dilutions were prepared for both soil samples and plant roots. One gram of soil was suspended in 99 mL of sterile deionized water to achieve 10^{-2} dilution and mixing well by vigorous hand shaking. A 1.0 mL of aliquot was subsequent to test tubes containing 9 mL of sterile deionized water for lower dilutions of 10^{-3}, 10^{-4} and 10^{-5}. A series of 0.2 mL of aliquot of suspension from the 10^{-3} to 10^{-5} is transferred to tubes containing 5.0 mL of a particular medium and vortex. The medium used for isolating phosphate solubilizing microorganisms was described by Osorio and Habte (2001) (Medium 1). The composition of medium 1 included 1.0 g NaCl, 0.2 g $CaCl_2.2H_2O$, 0.4 g $MgSO_4.7H_2O$, 1.0 g NH_4NO_3, 10.0 g dextrose, 7.0 g agar, and 3.5 g Carolina rock phosphate. All components were added to 1.0 liter of distilled water and then autoclaved at 120^0C for 30 minutes.

Likewise, another series dilution was prepared for root samples. One gram of fine roots was transferred into bottles containing 99 ml of sterile deionized water. A 1.0 mL of aliquot was subsequently added to test tubes containing 9 mL of sterile deionized water for a series dilution of 10^{-3}, 10^{-4} and 10^{-5}. An aliquot of 0.2 mL suspension from the 10^{-3} to 10^{-5} dilutions were transferred to tubes containing 5.0 mL of a particular medium as described above. The culture media containing microorganisms were poured into Petri plates containing solidified water agar (15g/L- agar has been prepared and solidified before pouring) and incubated the plates in an incubator at 27^0C for 15days.

After incubation, large colonies with a clear zone around them were selected and then aseptically streaked on Petri dishes containing yeast-mannitol-agar (YMA). The YMA contains 0.5 g of KH_2PO_4, 0.2 g of $MgSO_4.7H_2O$, 0.1 g of NaCl, 10.0 g of mannitol, 1.0 g of yeast extract, 15 g of agar per liter. The pH of the medium was adjusted to 6.8 by adding 0.05M HCl before autoclaving (Model: STM-EL, serial number 215220). The YMA agar plates were incubated at 27^0C for 4 days. Then, individual colonies were transferred to YMA slants and stored at 4^0C for later use.

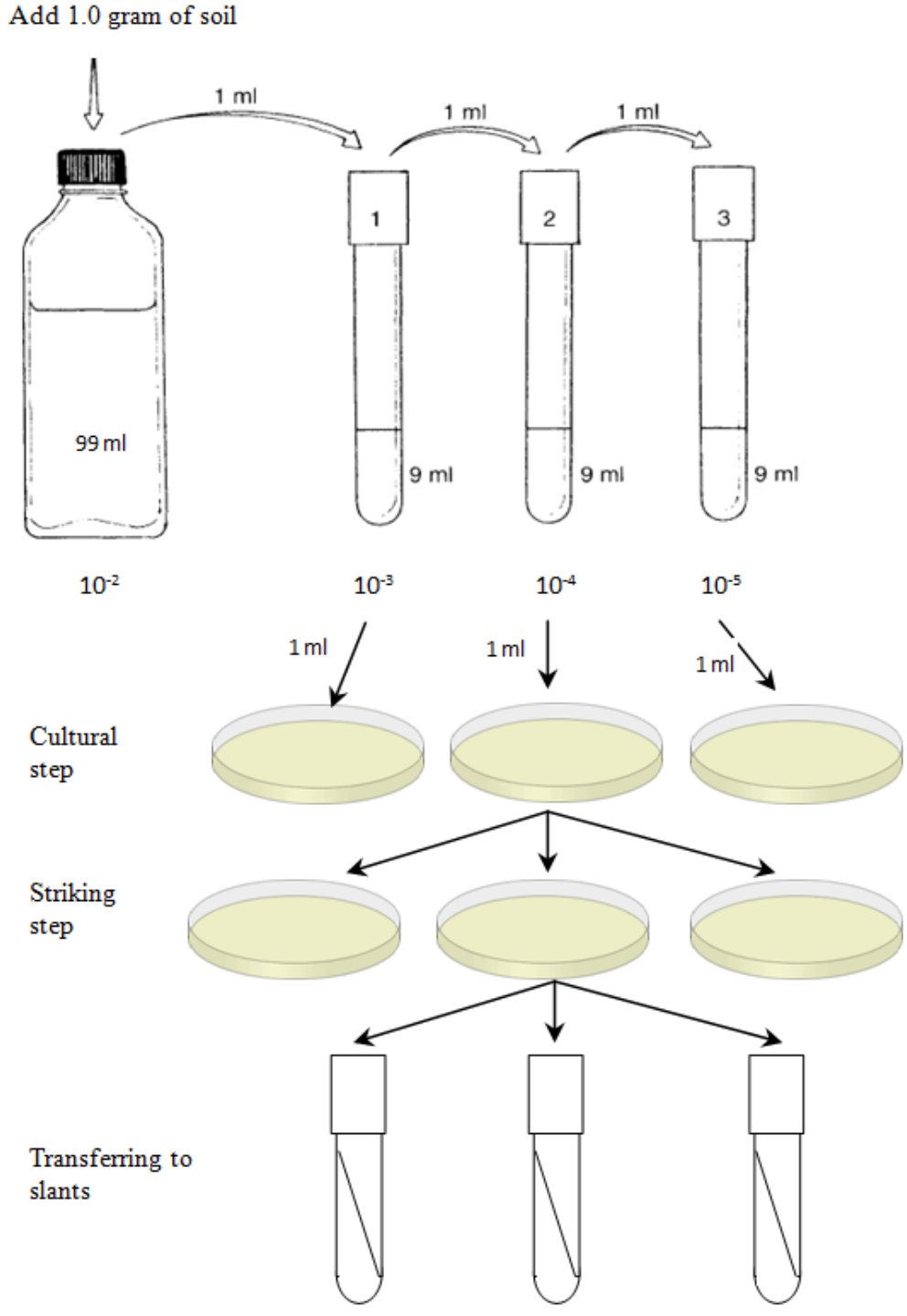

Fig. 1: Isolation process of microorganisms from soil and plant root

A loopful of each isolate from YMA slants was transferred into test tubes 20mL of above medium without agar to test the positive activity of isolated microorganisms in solubilization of Carolina rock phosphate. The culture tubes were then incubated in a continuous shaking incubator (Model: New Brunswick Incubator Shaker G24) for 7 days at room temperature. pH of the solution was measured by using a pH meter (Model: Orion 420A) after incubation. A 30 mL of the solution was then centrifuged at 10,000xG for 10 minutes and filtered the supernatant through Whatman#42 filter paper. The soluble phosphate remaining in supernatant

was then determined by using molybdate-blue method.

Isolates showing positive activity in Carolina rock phosphate solubilization was then examined with medium 2 containing 1.0 g/L of NaCl, 10 g/L of dextrose, 0.2 g/L of $CaCl_2.2H_2O$, 0.4 g/L of $MgSO_4.2H_2O$, 1.0 g/L of NH_4NO_3, and 6.4 g/L of North Carolina rock phosphate. The isolates in YMA slants were suspended in 10 mL sterile deionized water. 1.0 mL of suspension was transferred into tubes containing 25 mL of the liquid medium. The culture tubes were then incubated and shaken for 7 days at room temperature. pH of solution was measured after incubation. The soluble phosphate was eventually examined using the method described above.

3 RESULTS

High numbers of potential phosphate-solubilizing microorganisms were recovered from the soil as well as from the roots. Fungi was isolated from both soil and plant roots. The numbers of fungi that were recovered from soil and plant roots were $55x10^4$ CFU/g and $90x10^4$ CFU/g, respectively. Phosphate-solubilizing microorganisms recovered the soil with population of $235x10^4$ CFU/g were bacteria. There was no bacteria associated with the plant roots that were examined.

The experimental results of insoluble phosphate solubilization by selected phosphate-solubilizing microorganisms and the associated pH changes in the medium were presented in Figure 2. Twenty-four isolates were tested for their ability of rock phosphate solubilization. All isolates were positive in solubilization of Carolina rock phosphate. After 7day incubation, the soluble phosphate concentration in the liquid medium ranged from 1.49 to 31.7mg/L and there was a variation of soluble phosphate concentration among different microorganisms. It indicated that each isolate had different ability in solubilizing Carolina rock phosphate. The results also showed that the solubilization of rock phosphate in this medium by different microorganisms was accompanied by pH drop from initial 6.86 to the lowest value of 3.64 in culture inoculated by A2 microorganism. Moreover, the pH of supernatants was reduced from 6.86 to 4.17, 4.33, and 4.43 for microorganisms G, EE and B1, respectively (Figure 2). The regression analysis showed that the dissolved concentration of phosphate production from the rock associated by microbial activities was strongly correlated with pH in cultural media (Figure 3). The lowest amount of soluble phosphate concentration was obtained the culture containing microorganism N and the pH drop was 4.51.

Table 1: The number of fungi and bacteria obtained from soil and plant roots in Waahila Ridge, Manoa area

	Fungi, CFU/mL	Bacteria, CFU/mL
Soil	55×10^4	235×10^4
Roots	90×10^4	<1

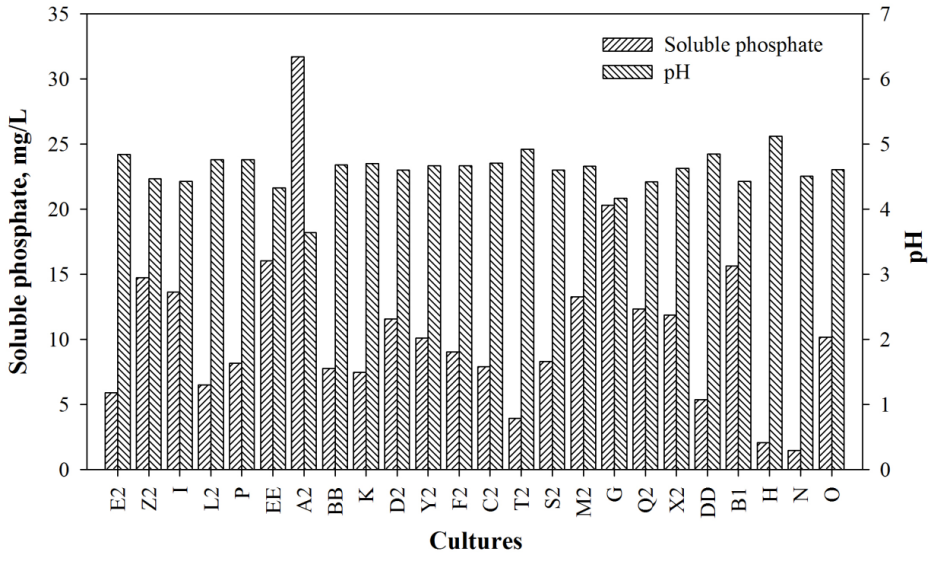

Fig. 2: Changes in pH and soluble phosphate concentration in liquid medium 1 (without agar) containing Carolina rock phosphate by phosphate-solubilizing microorganisms recovered from Waahila Ridge, Manoa

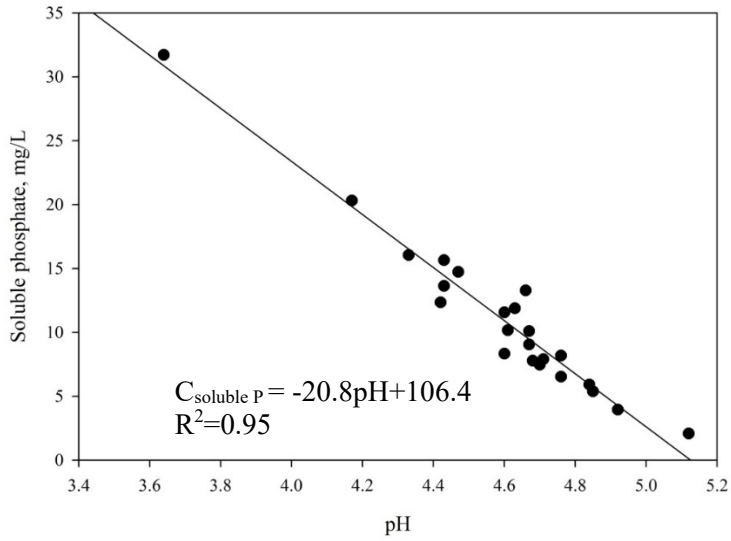

Fig. 3: Correlation between soluble phosphate production and pH

Once the test for positive response in rock phosphate solubilization by microorganisms isolated from Manoa area had been confirmed, another experiment with liquid medium containing North Carolina rock phosphate solubilization was then carried out. Nineteen out of 24 isolates were selected to perform insoluble phosphate solubilization. They were selected and grown in liquid medium containing North Carolina rock phosphate. The experimental result showed that all isolates have been shown to have insoluble phosphate solubilizing activity in comparison to the control except microorganism S2 (Figure 4). Particularly, there were two microorganisms having high insoluble phosphate solubilization capacity. The microorganisms A2 performed the highest ability in P solubilization with 19.33 mg/L of soluble phosphate in the liquid medium. The second species was G which generated 18.01 mg/L. Four microorganisms including A2, G, B1, and EE showed the highest capacity in solubilizing insoluble rock phosphate in liquid medium. The soluble phosphate concentrations that were achieved in the culture containing four microorganisms were from 12.38 mg/L to approximately 15 mg/L in corresponding by I, B1, EE, and P, respectively.

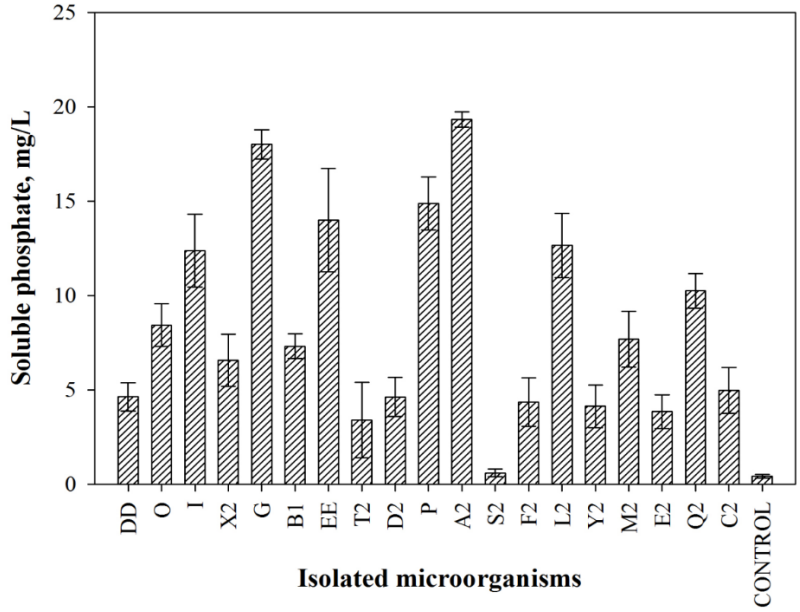

Fig. 4: North Carolina rock phosphate solubilization by selected phosphate-solubilizing microorganisms

4 DISCUSSION

Different levels of soluble phosphate concentration were obtained in this study. The production of soluble phosphate was associated with the pH drop in the liquid medium. The results suggested that there might be some acids released while microorganisms were growing in the liquid media. These experimental results had an agreement with previous study in which Kim *et al*. 1997 had addressed that acidification was one of the principal mechanisms for mineral phosphate solubilization in soils. Whitelaw *et al*. 1999 also reported that phosphate-solubilizing microorganisms dissolved the insoluble phosphate by production of inorganic or organic acids, which resulted in the drop of pH values. In this study, the culture that was obtained the highest soluble phosphate concentration had a significant drop in pH to 3.64. In addition, there was high correlation between pH and soluble phosphate production. According to Osorio and Habte (2001), there was strong relationship between pH drop in the growth medium and increasing soluble phosphate concentration in the medium. In contrast, a weak relationship between lowering pH and increasing insoluble phosphate solubilization had been reported (Wakelin *et al*., 2004). Although, the highest level of phosphate solubilization was accompanied by a maximum drop in pH. However, pH values in culture containing microorganism N were approximately equal some other cultures while there was small amount of soluble phosphate production (Figure 2). This might indicate that other mechanisms existed a long with acidification to solubilize the rock phosphate as addressed by Sharma (2011).

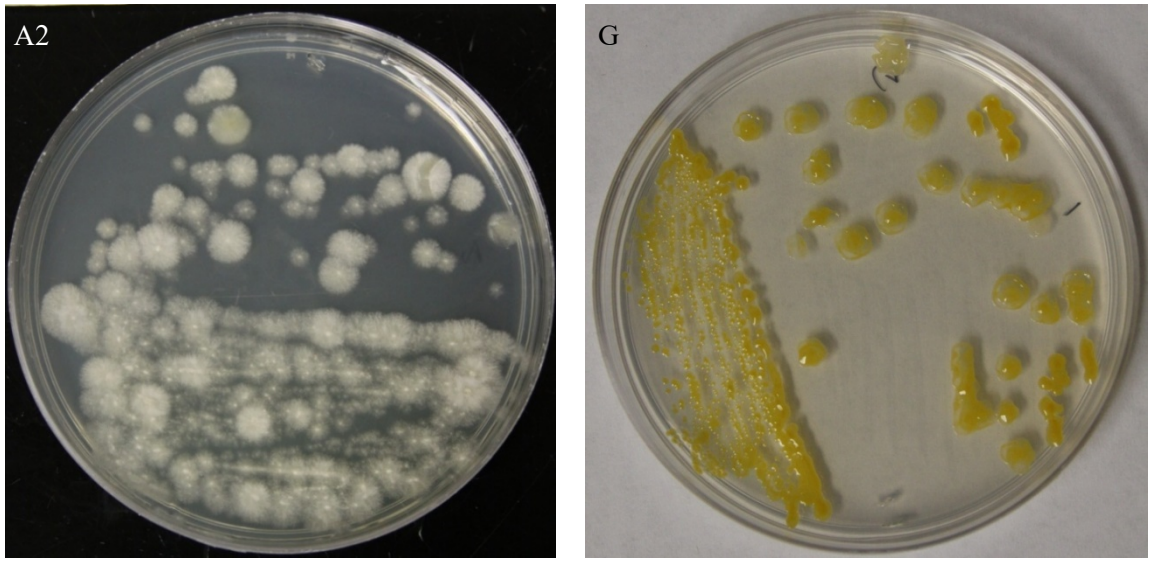

Fig. 5: Different morphological features of microorganisms with highest phosphate solubilization capacity recovered from Manoa soil

This study demonstrated that microorganisms isolated from Manoa area have ability in solubilizing the insoluble phosphate. Two microorganisms having the highest activity in solubilization of rock phosphate were fungi (A2) and bacteria (G) (Figure 5). Different soil microorganisms were reported to have ability in solubilizing insoluble phosphates (Wakelin *et al*., 2004; Hamdali *et al*., 2008; Yu *et al*., 2011). Both microorganisms showed different levels of phosphate solubilization activity in liquid broth culture. The highest phosphate solubilization activity of phosphate-solubilizing microorganism isolated from Hawaii soils was identified as fungi (Osorio and Habte, 2001). In comparison to earlier reported data, the highest soluble phosphate concentration obtained in this study was higher than that using rock phosphate solubilizing actinomycetes as inoculum to solubilize Khouribga phosphate mines (Hamdali *et al*., 2008). It was likely similar to the data that was obtained in the other studies using different strains of *Penicilium* sp fungi to solubilize Idaho rock phosphate (Wakelin *et al*., 2004) and *Mortierella* sp fungi to solubilize North Carolina rock phosphate (Osorio and Habte, 2001). However, the dissolved phosphate concentration was much lower in comparison to a report in which bacteria were used as inoculum in culture containing $Ca_3(PO_4)_2$, $AlPO_4$, and $FePO_4$ as insoluble phophate (Yu *et al*., 2011).

5 CONCLUSIONS

The overall results showed that the microorganisms isolated from Waahila Ridge, Manoa Valley have ability to solubilize insoluble rock phosphate with

the association of pH drop. The insoluble rock phosphate was converted to available forms, which are available for plant uptake and microbial utilization. One fungal strain and one bacterial strain had showed the highest ability in solubilizing insoluble rock phosphate.

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