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Impact of heavy metals and initial pH variations on the phosphate solubilizing fungi (PSF) isolated from Nsukka pepper and garden-egg plants rhizosphere.

Onyia C.E.*[1], Anyanwu C.U.[2], Onuigbo A. C.[1] and Mbegbu E. C.[3]
South-East Zonal Biotechnology Centre, University of Nigeria, Nsukka, Enugu State, Nigeria[1]
Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria[2]
Department of Veterinary Physiology, University of Nigeria, Nsukka, Enugu State, Nigeria[3]

Abstract: *Effects of heavy metals and initial buffering variations on phosphate solubilization of insoluble phosphate were studied. Fourteen PSF obtained were quantitatively screened for phosphate solubilization. The best isolate in each rhizosphere were selected and subjected for further evaluation. The best two isolates were subjected to different buffering values and heavy metal concentrations. Results indicated that there were no significant differences between control of GF1 and 1ug/ml and control of PF7 upto 10 ug/ml copper concentrations. But the solubilization performances of each isolates in cadmium and zinc media indicated that there are significant differences between control and all the concentrations, except for GF1 were there was no significant differences ($P>0.05$) between controls and 1ug/ml of cadmium. The results on pH variation showed that isolate GF1 and PF7 were able to solubilize insoluble phosphate at various initial pH levels, but isolate GF1 can be used as better phosphate solubilizers at extreme environmental stress condition..But since these two fungal isolates tested have shown some level of resistance to heavy metal tested, this may make them potential candidates for further investigations for bioremediation of polluted environment and even solubilization at heavy metal environment.*

Keywords: *Buffering, Phosphate solubilization, Heavy metal, Environmental stress, Rhizosphere.*

I. Introduction

Plants absorb phosphorus in soluble form of inorganic phosphate which acts as an essential element for plant growth and development, making up about 0.2% of plant dry weight [1]. The availability of phosphorus in the soil is somewhat limited, notwithstanding the large amounts of soluble form of phosphate fertilizers that are applied to attain maximum crop production. However, the applied soluble forms of phosphate fertilizers are easily precipitated into insoluble forms such as tricalcium phosphate $Ca_3(PO4)_2$, Iron phosphate $FePO_4$ and Aluminum phosphate $AlPO_4$ [2]. It has been found that approximately 75–90% of applied phosphate fertilizers are precipitated by Ca, Fe and Al ions making phosphorus inaccessible by the plants [3], but some beneficial microorganisms in the soil are found to convert insoluble-phosphate into soluble form by the process of acidification, chelation and exchange reactions [4]. These reactions normally take place in the rhizosphere [5][6]. It has been observed that high proportion of phosphate solubilizing microorganisms is concentrated in the rhizosphere of plants [7]. [8], suggested that fungi are more active in solubilizing phosphate than bacteria.

Many researchers have attributed the low phosphate solubilization efficacy to some environmental factors [9], such factors that affect phosphate solubilization include; temperature, type of carbon and nitrogen sources, pH and presence of some heavy metals etc. Today, heavy metal pollution has become one of the serious issues of concern amongst all environmental crises. Heavy metals are one of the major sources of environmental pollutants and exist in soil as free metal ions, soluble metal complexes, exchangeable metal ions, organic bound metals, precipitated or insoluble compounds like oxides, carbonates and hydroxides [10]. They arise in soil by repeated applications of sewage sludge, municipal wastes and animal slurries, activity of mining and smelting industries, impurities in fertilizers and decomposition of air pollutants by burning of fossil fuels and various industrial activities [11]. Metals persist in soils and have a very slow leaching rate; hence, they tend to accumulate in soils making plants vulnerable to them. Heavy metals such as Cu, Fe, Mn, Ni and Zn are essential for plant growth at a certain threshold and are important constituents of many enzymes, whereas metals such as Al, As, Cd, Cr, Hg, Pb, Sb and Se are nonessential and are toxic above certain threshold levels [12]. The effect of heavy metals like mercury and zinc on the growth of phosphobacterial species reveals that the growth decreased with the increasing concentration of heavy metals [13].

The effective microbial phosphate solubilizations are severely affected by some environmental factors, especially under stress conditions. Most potent phosphate solubilizers when subjected to their natural habitat, losses its solubilization power due to some environmental factors present. However in other to increase the efficacy of phosphate solubilizing microorganisms, the factors that interfere with the ability of microorganism to solubilize insoluble phosphate must be considered. Therefore, the aim of this research is to isolate phosphate solubilizers that can thrive well under extreme pH and high heavy metal concentration.

II. Materials And Methods

Soil sample collection

Samples were collected from rhizospheres of Nsukka pepper plants and Nsukka-garden-egg plants at University of Nigeria, Nsukka agric farm. The soil samples were taken within the rhizospheric circumference of 1-10 cm radius by 5-10 cm depths. The soil samples were collected with a sterile container and were sent to laboratory immediately.

Isolation of indigenous rhizospheric fungi

From each soil sample, 10g were transferred into 250 ml Erlenmeyer flask each containing 90 ml of sterile distilled water. The flasks were shaken and 1 ml of the mixture were transferred to 9 ml of distilled water in test tube and serially diluted. The appropriate dilution is plated out using a pour plate method, where 0.1 ml aliquots of the dilutions are plated on potato dextrose agar (PDA) medium. Spraying method were also used, where soil samples were sprinkled directly on the plates and incubated.

Isolation of PSF using PVK agar

Each fungal isolate were aseptically transferred onto Pikovskaya (1948) medium supplemented with bromo-phenol-blue (BPB) and tri-calcium phosphate (TCP) using point inoculation and were incubated at 28 ± 2^0C for 7 days. Solubilization of phosphate was observed as a zone of clearance with a diameter that was measured in millimeters. The phosphate solubilization ability of the fungi was analyzed by determining the phosphate solubilization efficiency (E) of each isolate. [E = solubilization diameter / growth diameter x 100] by [15]. After confirming the phosphate solubilizing ability on solid medium, the phosphate solubilization were also carried out using National Botanical Research Institute's Phosphate - Bromo Phenol Blue (NBRIP-BPB) broth.

Confirmation of phosphate solubilizers using NBRIP-BPB broth

A 12mm cork bowered inoculums sizes of a 5day-culture grown in the PDA were used as the inoculums size. The phosphate solubilization activity of each of the isolates were determined by growing the isolates in NBRIP medium containing a pH indicator (Bromophenol Blue) for 12 day (taken reading at 4day interval) at 28 ± 2^0C. At the end of the incubation period, spectrophotometric readings were taken at OD600 and the final values were subtracted from the (control) initial values [15].

Preparation of stock solution of heavy metal salts

The heavy metal salts used in this study include: zinc sulphate heptahydrate, copper sulphate pentahydrate and cadmium sulphate octahydrate. Stock solutions of these heavy metal salts and their various concentrations were prepared. Solutions were adjusted to desired pH values (pH 7) with 0.1 M sodium hydroxide and 0.1 M hydrochloric acid.

Effect of heavy metals on fugal phosphate solubilization

To find out the effect of heavy metals (Zn, Cu and Cd) in the phosphate solubilizing activity of the best fungal species, NBRIP broth was prepared with different concentrations of heavy metals (0, 1, 10, 50, 100 ug/ml). They were conducted in triplicate with zero (0) concentration as the control.

Preparation of buffering solutions for various pH variations

To evaluate the effect of buffering on phosphate solubilizing activity of the PSF were grown on NBRIP-BPB broth containing $(NH_4)_2SO_4$ 0.1g/L, $Ca_3(PO_4)_2$ 5.0g/L, $MgSO_4.H_2O$ 0.25g/L, $MgCl_2.6H_2O$ 5.0g/L, KCl 0.2g/L, BPB 0.025g/L, Glucose 10g/L at pH 7.0 ± 0.2 [15] as control . The pH was adjusted using 0.1 M hydrochloric acid (HCl) and 0.1 M sodium hydroxide (NaOH) solution.

III. Results

Isolation of phosphate solubilizing fungi from Nsukka pepper and garden-egg rhizosphere

Out of the fourteen (14) fungi strains isolated from the rhizosphere of pepper and garden-egg, eleven (11) strains solubilized insoluble phosphate under PVK agar, isolate GF1 and PF7 were selected as the best phosphate solubilizers from each rhizosphere. GF1 has the solubilization efficiency of 160 mm which solubilizes 3.5 mg/ml of insoluble phosphate on day12, while PF7 has the solubilization efficiency of 240 mm which solubilizes 4.2 mg/ml of insoluble phosphate on day12.

Table 1: Phosphate solubilization efficiency using PVK agar

Fungal Isolates	GD (mm)	SD (mm)	SE (mm)
GF1	10.0	16.0	160.0
GF8	40.0	47.0	117.5
GF9	38.0	39.0	102.6
GF10	40.0	50.0	125.0
GF12	22.0	0.0	0.0
GF13	35.0	43.0	122.9
GF15	20.0	0.0	0.0
GF16	26.0	0.0	0.0
PF2	48.0	66.0	137.5
PF3	32.0	43.0	134.4
PF4	35.0	43.0	122.9
PF5	22.0	24.0	109.1
PF6	31.0	41.0	132.3
PF7	25.0	60.0	240.0

Effect of initial pH values on solubilization

Our result with initial pH 7±0.2 served as the control-measure, were GF1 and PF7 isolates solubilizes 3.78 and 4.02 mg/ml TCP respectively as its maximum solubilization on day12. The highest solubilizations of TCP at different initial buffering variations indicated that pH7> pH5> pH3> pH9> pH11. In **Table 2**, there is no significant difference between average control and average pH5 ($p<0.05$), while in **Table 3**, there are significant differences between control and all other initial pH variation although solubilization still occurred.

Table 2: Quantities of TCP solubilized (mg/ml) by GF1 at different pH variation with time

pH	Day4	Day8	Day12	Ave.solubilized
Control (7)	3.303^a	3.700^a	3.777^a	3.593^a
11	0.247^e	0.703^d	1.080^d	0.677^d
9	0.990^d	2.123^c	2.307^c	1.807^c
5	3.180^b	3.617^a	3.660^a	3.486^a
3	2.743^c	3.217^b	3.247^b	3.069^b

In each column, values with the same superscript alphabets are not statistically different ($p<0.05$), while values with different superscript alphabets are statistically different.

Table 3: Quantities of TCP solubilized (mg/ml) by PF7 at different pH variation with time

pH	Day4	Day8	Day12	Ave.solubilized
Control (7)	3.400^a	4.013^a	4.023^a	3.812^a
11	0.300^e	1.317^e	1.317^d	0.978^e
9	1.223^d	1.993^d	1.997^c	1.738^d
5	3.050^b	3.520^b	3.537^b	3.369^b
3	2.740^c	3.393^c	3.437^b	3.190^c

In each column, values with the same superscript alphabets are not statistically different ($p<0.05$), while values with different superscript alphabets are statistically different.

Effects of heavy metals (Cu, Cd and Zn) on solubilization

The effect of different concentrations (1, 10, 50 & 100 µg/ml) of heavy metals, namely copper, zinc and cadmium on fungal phosphate solubilization was studied. The result in **Figure 2** showed that, for GF1 in copper environment, there was no significant difference ($P>0.05$) between control (no heavy metal) and 1ug/ml and for PF7 there was no significant difference ($P>0.05$) between control and upto 10 µg/ml concentrations. Solubilization performances of these isolates in cadmium environment as was shown in figures 3 indicated that for GF1, there was no significant difference ($P>0.05$) between control and 1ug/ml except at day4 of 1ug/ml, while for PF7 there are significant differences between control and other cadmium concentration. At zinc treatment, there was no significant difference ($P>0.05$) between control and 1µg/ml for isolate GF1 but there is significant difference between control and other zinc concentration for PF7 as was shown in **Figure 4**.

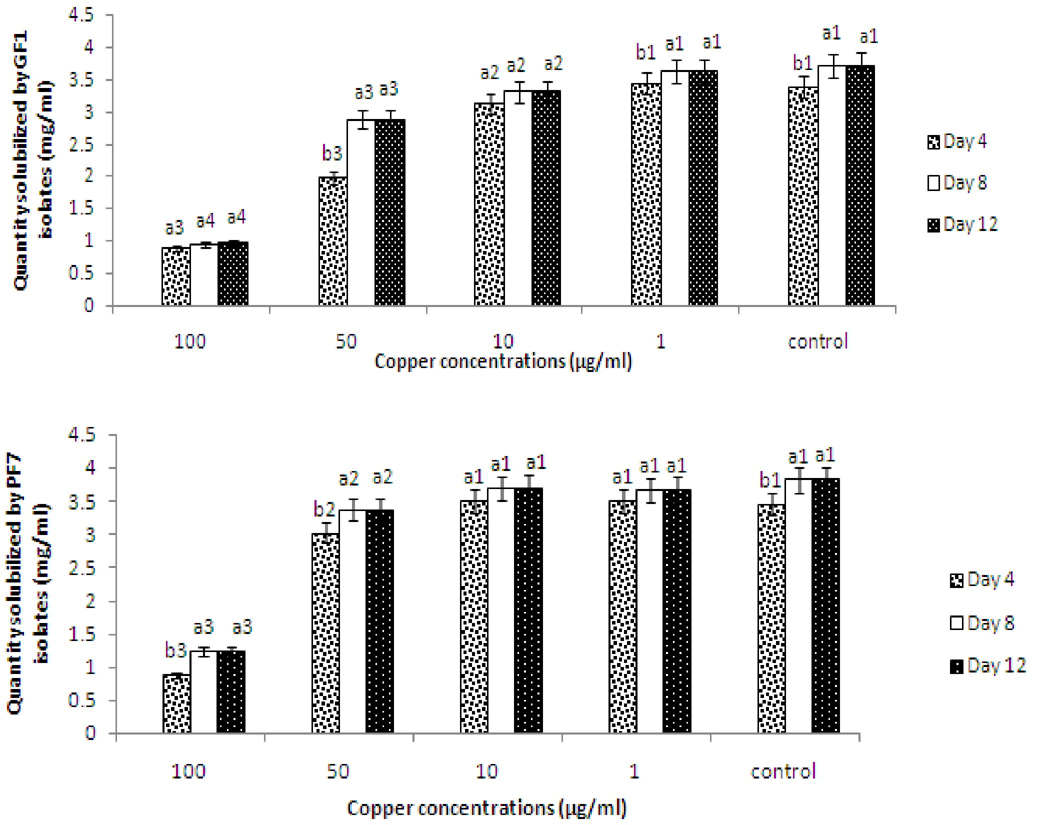


Figure 2: Solubilization of TCP in GF1 and PF7 culture media under copper heavy metal treatment with time

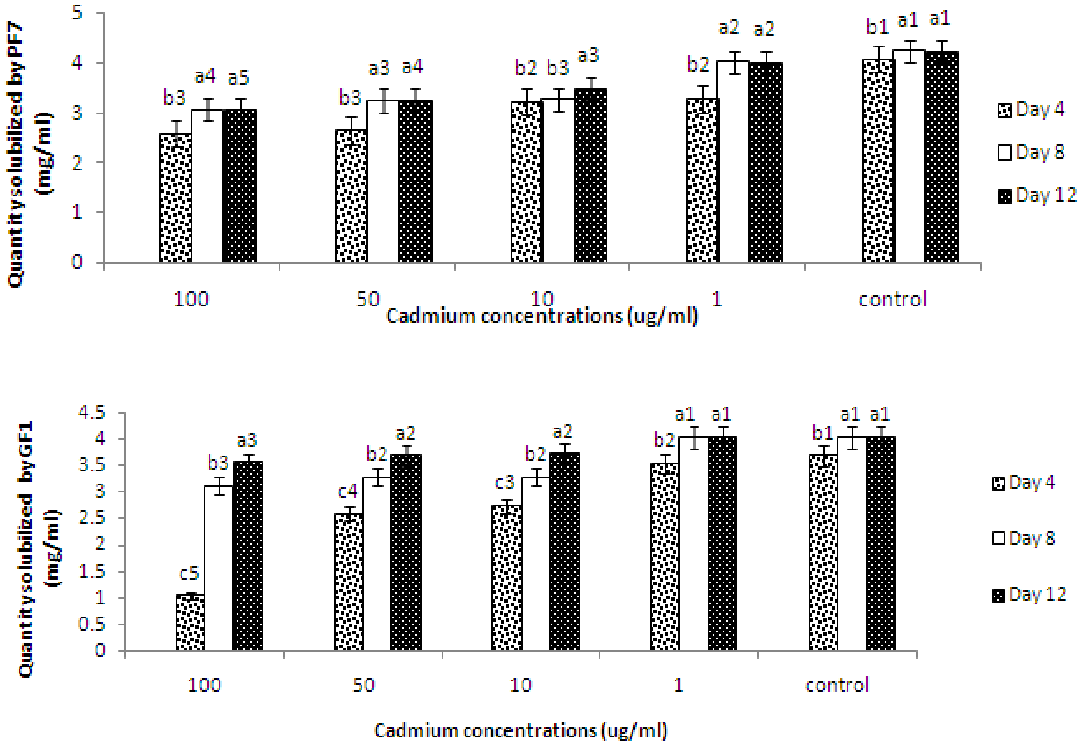


Figure 3: Solubilization of TCP in GF1 and PF7 culture media under cadmium heavy metal treatment with time

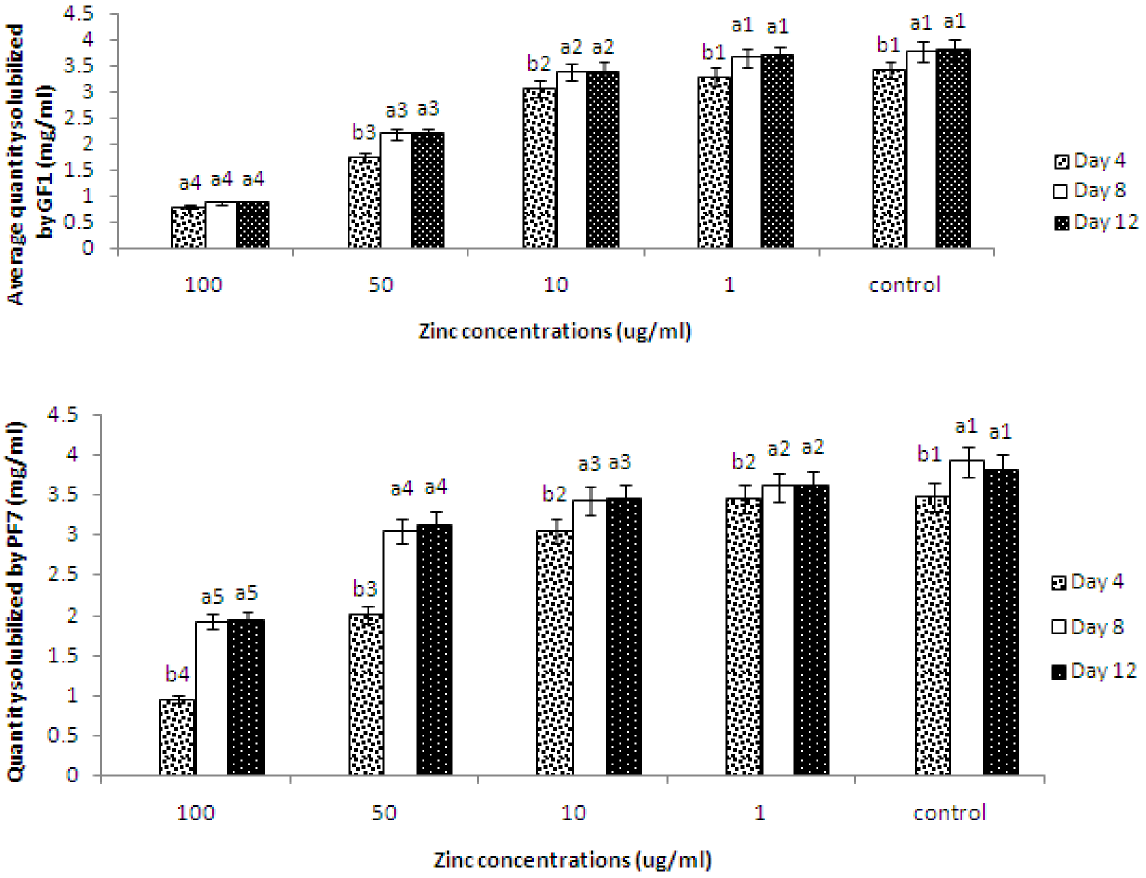
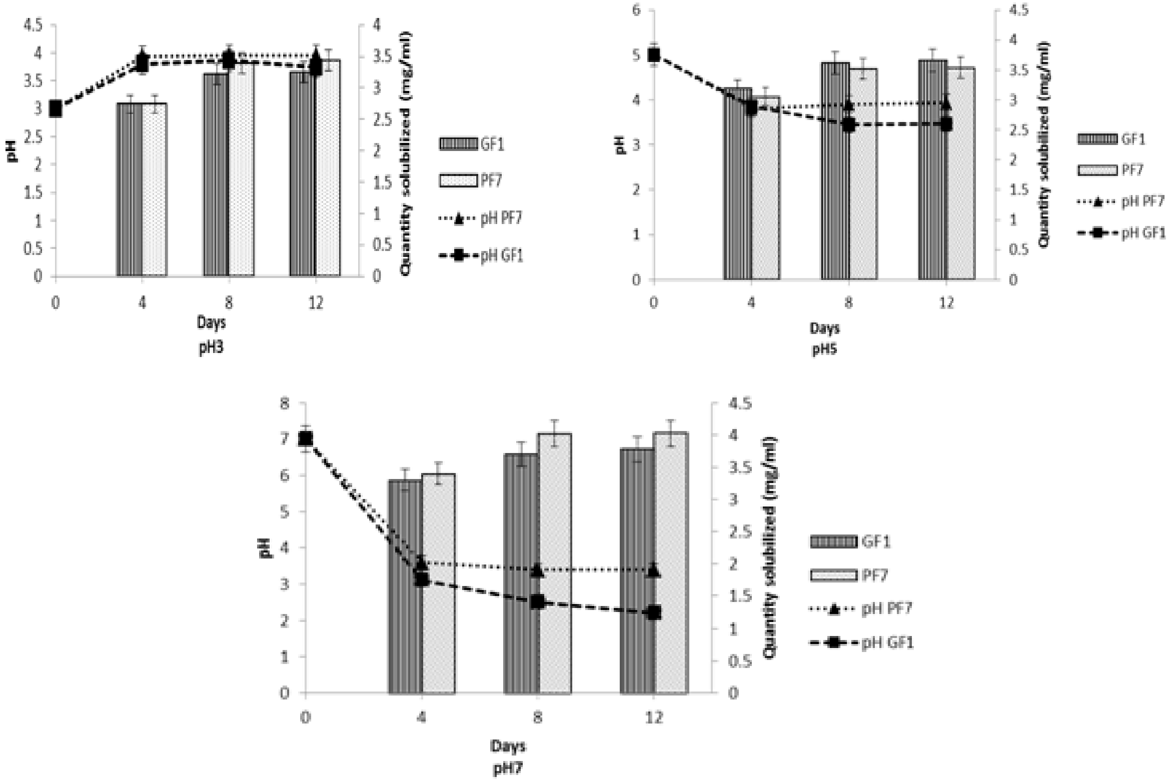


Figure 4: Solubilization of TCP in GF1 and PF7 culture media under copper heavy metal treatment with time



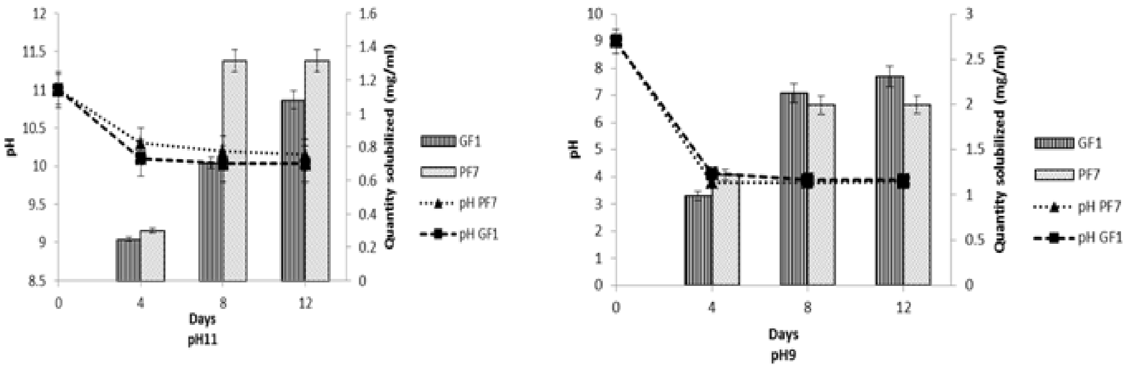


Figure 5: Changes in phosphate released and pH values during solubilization at different initial pH variation

IV. Discussion

Results obtained by Onyia and Anyanwu (2013) and Qurban et al., (2012) showed that microorganism from different rhizosphere solubilize insoluble phosphate mostly at favorable conditions which formed their bases of selecting potent phosphate solubilizers. But our results showed that presence of heavy metal like zinc, cadmium and copper and even pH variations needed to be considered before selecting potent phosphate solubilizers that will be most effective.

The results is consistent with the earlier observations made by Gyaneshwar et al., (2002) and Halder and Chakrabartty (1993), where the plant growth was limited by availability of phosphate despite the abundance of phosphate solubilizing bacteria in the rhizosphere. This problem was attributed to buffering (initial pH variation) of the environment.

Isolates GF1 and PF7 which thrive well at zero heavy metal concentration and neutral initial pH value were subjected to initial varying pH and heavy metal concentration to compare its solubilization strength. Our results showed that non induced medium solubilizes better than induced one, this was supported by Joseph and Jisha (2008) which proved that efficiency of phosphate solubilization was decreased in buffered media compared to non-buffered media. The buffering capacity of the medium reduced the effectiveness of phosphate solubilizing microorganism in releasing phosphate from tricalcium phosphates (TCP).

The initial pH7 (non induced pH) favors the growth of microorganism, which makes the organism to acclimatized faster to such environment, causing the final pH to be lower than the initial. The final pH of the medium is considered as one of the factors responsible for efficient solubilization of insoluble phosphate, but its initial pH on inoculation of the microorganism affects the microbial growth which invariably affects solubilization. Therefore phosphate solubilizers that can adapt to extreme stress condition are the better organism of choice.

On comparing our isolates to pH variations, isolate PF7 solubilizes more phosphate than GF1 at neutral pH, but GFI tolerate more initial acidic and alkaline pH variation than PF7. Our results showed that phosphate solubilizing organisms were able to adapt and reduce the pH of the medium in presence of induced buffer solution. The reduction in phosphate solubility has been shown to be due to initial high or low buffering capacity of soils which reduced the phosphate solubilizing efficiency of microorganism under buffered media conditions.

The significance and impact of this study, which is to isolate PSF that has the ability to solubilize insoluble phosphate even at extreme stress environment, were partially met. The solubilization of insoluble phosphate at extreme environmental stress conditions make the isolates potential isolates for further investigation towards bioremediation.

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