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# Sulfur Oxidizing Bacteria from Sulfur Rich Ecologies Exhibit High Capability of Phosphorous Solubilization

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## Abstract

Sulfur oxidizing bacteria (SOB) oxidize elemental sulfur ($S^{\circ}$) and reduced S compounds to generate sulfuric acid which has the ability to solubilize and convert the insoluble phosphorous (P) compounds to simple plant available P compounds. In this study, SOB strains were isolated from the samples collected from ten different ecologies and then screened on the basis of pH reduction (in thiosulphate broth media) and phosphorous solubilization index (PSI). Phosphorous solubilization efficiency of the ten selected SOB was tested in 0.5% tricalcium phosphate (TCP) broth media. Results indicated that the strain IW16 released 954.2 mg $L^{-1}$ P (95.2%) during 32 days of incubation. Quantity of P dissolved had a significant positive correlation with the concentration of biologically produced sulfates by SOB ($r$ = 0.80, 0.89, 0.91 and 0.92 after 8, 16, 24 and 32 days, respectively). The most efficient SOB belonged to the sulfur rich ecologies such as industrial wastewater, sewerage water and sulfur mud due to the availability of reduced S compounds in large quantities in these ecologies. Existence of SOB isolates in paddy, wheat, sugarcane and maize rhizosphere was due to the presence of reduced S compounds in the soil. The selected SOB were characterized for different morphological, physiological and biochemical properties and were identified as the genus *Thiobacillus*. © 2014 Friends Science Publishers

**Keywords:** Sulfur oxidizing bacteria; *Thiobacillus*; Sulfur; Sulfur oxidation; Phosphorous solubilization

## Introduction

An adequate phosphorous (P) supply in rhizosphere is essential to activate plant roots for proper P uptake which contributes a lot in crop yield. In cultivated soils although the amount of total P is fairly high ranging from 163-1050 mg $kg^{-1}$ (Memon *et al.*, 2011) however, the bio-available P is as low as 1.0 mg $kg^{-1}$ (Vassilev *et al*., 2001; Solangi *et al.,* 2006). Furthermore, P in the form of fertilizers immediately get fixed in the soil after application and P fertilizer use efficiency ranges between 10 to 25% throughout the world (Khiari and Parent, 2005). Main factors for P fixation in acidic soils are oxides and hydroxides of iron, while in alkaline and calcareous soils major cause is the high amount of $CaCO_3$ in the soil (Pant and Warman, 2000). Majority of Pakistani soils are alkaline and calcareous in nature with pH> 7.5 and $CaCO_3$ > 3.0% (Sharif *et al.,* 2000). More than 90% soils have low available P status and are moderate to high P deficient (Rehman *et al.,* 2000; Solangi *et al.,* 2006). Phosphorous fixation is a matter of great concern in soils of Pakistan due to alkalinity and calcareousness (Sharif *et al.,* 2000).

Elemental $S^{\circ}$ is a fundamental substrate for sulfur oxidizing bacteria (SOB) which oxidizes to sulfates during oxidation process (Pokorna *et al.,* 2007) and there exists a close bacteria-substrate relationship for S oxidation (Briand *et al.*, 1999). Elemental $S^{\circ}$ along with sulfur oxidizing bacteria has been confirmed very effective in enhancing P bioavailability in soil through the process of S oxidation (Aria *et al*., 2010). The genus *Thiobacillus* among SOB is very important in biological S oxidation in soil (Yang *et al*., 2010). *Thiobacilli* generally enhance sulfur oxidation rate and it is further boosted by the addition of sulfur in soil. Sulfur oxidation improves soil fertility which is an important step in S cycle. The acidity thus produced as a result of biological S oxidation increases the solubility of plant nutrients including P (Stamford *et al*., 2003; Yang *et al*., 2010). Sulfur oxidizing bacteria (*Acidithio bacillus*) oxidize S which results in P release from RP due to bacterially produced sulfuric acid during S oxidation phenomenon (Chi *et al.,* 2007).

Information regarding the type of ecologies where the most efficient phosphorous solubilizing sulfur oxidizing bacteria can be found for practical use was lacking before this study. Further, mechanism and rate of phosphorous solubilization by sulfur oxidizing bacteria from tricalcium phosphate through sulfur oxidation was also not reported previously. Keeping these facts in view, the present study was planned to isolate, characterize and explore P solubilizing capabilities of sulfur oxidizing bacteria.

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## Materials and Methods

### Isolation of Sulfur Oxidizing Bacteria

Samples were collected from ten different ecologies viz., paddy fields (PF), wheat rhizosphere (WR), sugarcane rhizosphere (SR), maize rhizosphere (MR), industrial wastewater (IW), canal water (CW), sulfur mud (SM), sewage water (SW), industrial waste sludge (IS) and sewage sludge (SS). Isolation of SOB was carried out by using thiosulphate broth medium (Beijerinck, 1904). Its composition is: $Na_2S_2O_3$, 5.0 g; $K_2HPO_4$, 0.1 g; $NaHCO_3$, 0.2 g; $NH_4Cl$, 0.1 g dissolved in 1.0 L distilled water. The pH of the medium was adjusted at 8.0. The indicator used was bromo cresol purple. The medium was autoclaved for sterilization. From the collected samples, 1 g in case of solid sample and 1 mL in case of liquid sample was added to 20 mL of the sterilized broth medium poured in test tubes under aseptic conditions. Then the tubes were incubated at 30°C in Bio Chemical Oxygen Demand (BOD) incubator for 4-5 d. Change in colour from purple to yellow indicated the growth of SOB in the tubes.

### Purification of Sulfur Oxidizing Bacteria

Purification of isolates was undertaken by transferring the isolates to the fresh broth medium thrice at fortnightly intervals. Individual colonies were obtained by streaking isolates on thiosulphate agar plates. Fifty pure isolates obtained were labeled according to their sampling ecologies. The detail is given in Table 1. These pure isolates were preserved for their characterization and further tests (Smibert and Kreig, 1994).

### Screening of Efficient Sulfur Oxidizing Bacteria

Two tests viz., pH reduction test and phosphorous solubilization index were performed for getting the most efficient sulfur oxidizing bacteria.

### pH Reduction Test

Thiosulphate broth medium was prepared and its pH was adjusted at 8.0. One milliliter specimens ($10^6$ cells $mL^{-1}$ fresh culture) of previously obtained isolates were inoculated in flasks containing 20 mL thiosulphate broth media and incubated at 30°C for 16 days. The experiment was carried out in completely randomized design (CRD) with three replications. Screening of isolates was done on the basis of their efficacy to reduce pH of the media. The pH of the samples was determined through Metrohm High-precision 780 pH meter.

### Phosphorous Solubilization Index

Preserved culture of each SOB (0.1 mL) was placed on thiosulphate tricalcium phosphate (TCP) 0.5% agar plates and incubated for 8 days at 30°C. The TCP agar plates were arranged in completely randomized design (CRD) having three replications. Phosphorous solubilization zones were formed on the thiosulphate TCP agar plates. Phosphorous solubilization index (PSI) was measured by using the following formula (Edi-Premono *et al.*, 1996).

$$PSI = \frac{\text{Colony diameter + Halozone diameter}}{\text{Colony diameter}}$$

### Quantification of Phosphorous Solubilization through Bioleaching Test

Phosphorous solubilzation efficiency of the most efficient 10 SOB isolates selected through pH and PSI measurements was determined by Tricalcium phosphate (TCP) bioleaching test. The experiment was arranged in completely randomized design (CRD) with three replications. Thirty three conical flasks were used. Each flask contained 100 mL thiosulphate broth medium along with 0.5% tricalcium phosphate. The pH was adjusted at 8.0. After autoclave the flasks were inoculated with 1.0 mL broth culture of each of the 10 selected SOB isolates in 3 flasks, and 3 flasks were kept as un-inoculated control. The flasks were incubated (100 rev $min^{-1}$) at 30°C for 32 d. After 8, 16, 24 and 32 days of incubation aliquot samples (5 mL) were drawn and centrifuged. The supernatants were examined for pH, sulfate contents and P solubilization.

The amount of soluble P was determined through Mo-blue method (Watanabe and Olsen, 1965). Two reagents were used viz., reagent A (ammonium heptamolybdate 12 g in 250 mL distilled water + antimony potassium tartrate 0.2908 g in 100 mL distilled water. Both were added in 1-L 5 N $H_2SO_4$ in a 2-L volumetric flask and made the volume with distilled water) and reagent B (L-Ascorbic acid ($C_6H_8O_6$) 1.056 g + 200 mL of Reagent A). Took 5.0 mL aliquot sample in a 50-mL volumetric flask, added 8 mL of reagent B and made the volume to 50-mL with distilled water. Then absorbance of blank, standards and samples were read after 10 min at 882 nm wavelength in spectrophotometer and P concentration was read from the calibration curve.

Sulfates concentration in the leach solutions was determined by ion chromatography (conductivity detector L-2470, pump L-2130, column oven L-2350) as described by Oh *et al.* (2010).

Different morphological, physiological and biochemical characteristics were studied to identify the most efficient SOB isolates according to Bergey's Manual of Systematic Bacteriology (Brenner *et al.,* 2005).

### Statistical Analysis

Variance in pH, phosphorous solubilizing index, sulfate contents and quantity of P solubilized were statistically analyzed using MSTAT-C software (Steel *et al*., 1997) taking SOB as source of variance. Simple linear correlation and regression were determined through MS Excel to evaluate the extent of interrelationship and interdependence among various variables.

## Results

All the collected 160 samples were tested for the presence of SOB; among them 50 samples were SOB + ve. Data revealed that sulfur based ecologies viz., industrial wastewater, sulfur mud and sewerage water had the highest 80, 60 and 60% frequency of SOB occurrence, respectively (Fig. 1).

Fig. 2 shows pH reduction by 50 SOB isolates during 16 days of incubation according to which more significant decrease of pH was observed in case of isolate IW16, resulting to the value of 2.42 (net decrease of 5.58 points). While the minimum decrease was noted in isolate SM9 giving a pH value of 7.06 (net decrease of 0.94 points). Five SOB isolates IW1 (2.84), SW2 (2.63), IW13 (3.53), IW14 (3.08) and SM1 (3.74) depicted pH values between 2.60 to 3.75 (net decrease of 4.25 to 5.40 points) and pH of the 4 SOB isolates SS1 (4.46), WR12 (5.50), SM3 (5.31) and SW11 (5.12) remained in the range of 4.40 to 5.50 (net decrease of 2.50 to 3.60 points). Whereas the growth media of 39 isolates had the pH range between 5.50 to 7.00 (net decrease of 1.50 to 1.00 points) after 16 days. However, no change in pH was observed in case of control where no inoculation was done.

Amongst the 50 isolates, 27 SOB isolates (PF2, IW1, IW3, IW5, IS1, IS2, IS11, SW1, SW2, SW4, SS1, SW5, CW2, CW3, WR2, WR4, WR10, WR12, WR13, SM3, IW13, IW14, IW16, SM1, SW11, MR8, and SM11) were selected on the basis of pH reduction test. Then they were examined for phosphorous solubilization index (PSI) according to which only 7 SOB (IW1, SW2, SS1, IW13, IW14, IW16, and SM1) were able to make holozones with in 1 d, 12 made holozones on the 2$^{nd}$ day and 8 started making holozones between 3 to 4 days. The highest PSI 9.83 was recorded in case of isolate IW16 followed by SW2 with PSI 8.42, while the lowest PSI 0.86 was noted in case of isolate SW5 (Fig. 3). No holozone was observed in thiosulphate agar plates where no inoculation was done (control).

Ten selected SOB isolates IW1, SW2, SS1, IW13, IW14, IW16, SM1, WR12, SM3 and SW11 on the basis of pH reduction (Fig. 2) and PSI (Fig. 3) were further tested for quantitative estimation of phosphorous solubilization in thiosulphate tricalcium phosphate (TCP 0.5%) media containing 1000 mg L$^{-1}$ insoluble P. Data concerning pH change by SOB isolates in TCP broth media are presented in Fig. 4. All treatments showed significant reduction in pH in comparison with the control from the day 8$^{th}$ to the day 32$^{nd}$. However, maximum pH decline was observed in first 8 d which indicated that maximum S oxidation occurred in the first 8 days. The highest reduced values of pH was recorded as 3.23, 3.01, 2.72 and 2.46 (net reduction of 4.77, 4.99, 5.28 and 5.54 points) in IW16, while the lowest decreased values of pH was noted as 7.18, 7.00, 4.86 and 4.52 (net reduction of 0.82, 1.00, 3.14 and 3.48 points) in treatment SM3 after 8, 16, 24 and 32 d, respectively. The

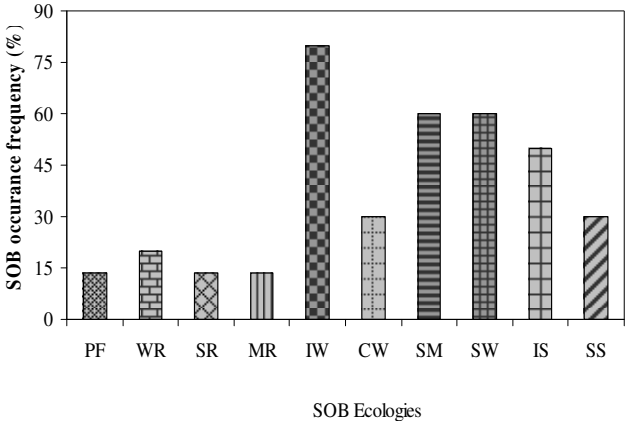


**Fig. 1:** Frequency of sulfur oxidizing bacteria in the sampling ecologies indicating highest number different SOB in industrial waste water

PF (paddy fields), WR (wheat rhizosphere), SR (sugarcane rhizosphere), MR (maize rhizosphere), IW (industrial wastewater), CW (canal water), SM (sulfur mud), SW (sewage water), IS (industrial waste sludge), SS (sewage sludge)

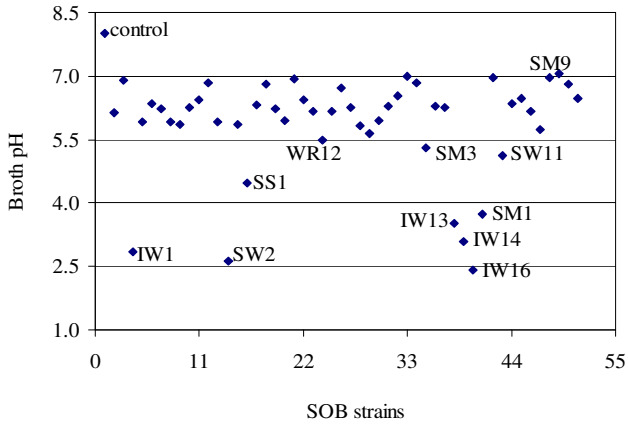


**Fig. 2:** Screening of SOB through pH reduction in thiosulphate broth

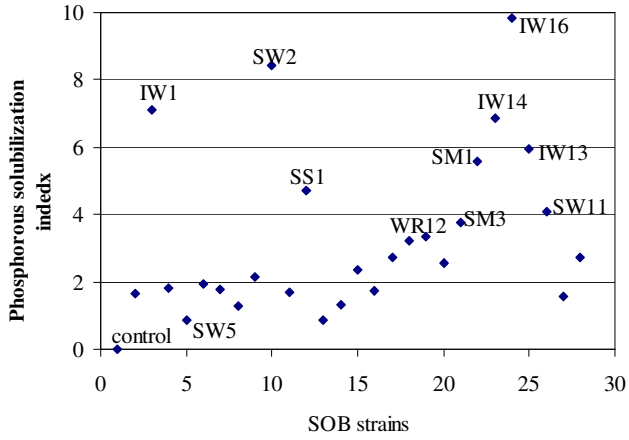


**Fig. 3:** Screening of SOB through phosphorous solubilization index

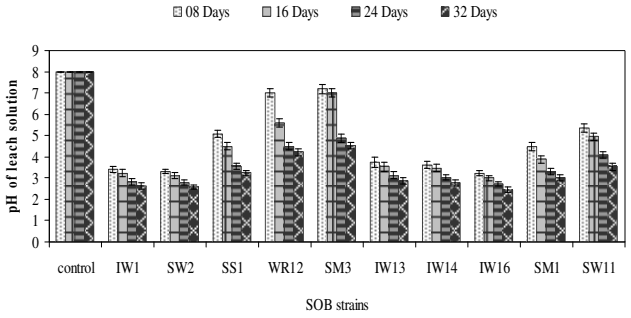


**Fig. 4:** pH reduction by SOB in thiosulphate tricalcium phosphate leach solution

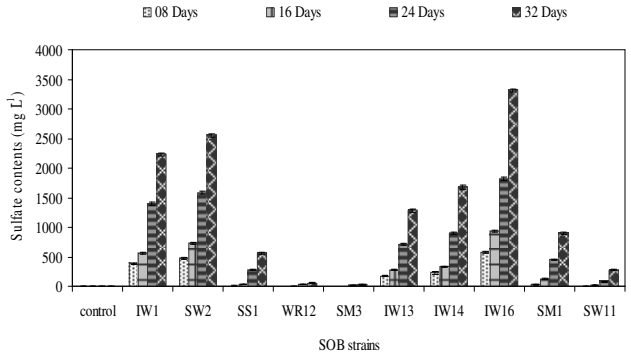


**Fig. 5:** Sulfates production by SOB in thiosulphate tricalcium phosphate leach solution

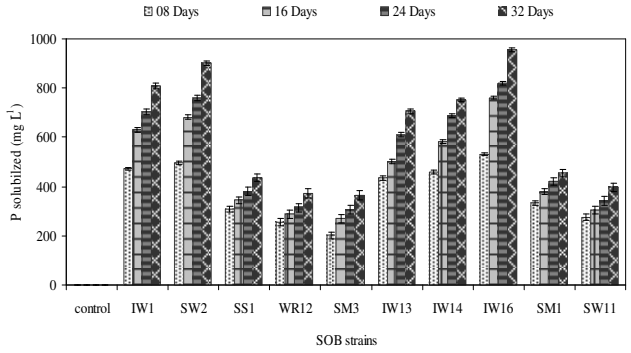


**Fig. 6:** Phosphorous solubilization by SOB in tricalcium phosphate leach solution

pH of the control flasks (without inoculation) remained unchanged during the whole period of incubation. Free sulfate contents, which remained unused in reaction with TCP gradually increased from 8 to 32 days of leaching time in all treatments and decreased pH except in control, where no change was observed (Fig. 5). Amongst the ten SOB isolates IW16 produced the highest amount of sulfate contents (571.6, 938.3, 1817.6 and 3315.9 mg $L^{-1}$ after 8, 16, 24 and 32 incubation days, respectively), while the lowest sulfate contents (nil, nil, 13.4 and 29.3 mg $L^{-1}$ after 8, 16, 24 and 32 days, respectively) were found in SM3.

The quantities of P solubilized by ten selected SOB isolates in TCP 0.5 % media are illustrated in Fig. 6. The strain IW16 dissolved 531.1, 761.2, 819.9 and 954.2 mg $L^{-1}$ of P in 8, 16, 24 and 32 days of leaching time, respectively and remained the highest amongst the ten SOB isolates, whereas the lowest performance was noted in case of SM3 that solubilized 202.3, 269.7, 306.1 and 364.7 mg $L^{-1}$ of P after 8, 16, 24 and 32 leaching days, respectively (Fig 7). Like sulfate contents the amount of P increased linearly from 8$^{th}$ to the 32$^{nd}$ day of incubation in all treatments except in control.

Morphological, physiological and biochemical characteristics of the best seven SOB isolates indicated that they were Gram negative and short rods (Table 2). Amongst the 7 isolates 4 isolates IW1, SW2, IW14 and IW16 utilized both elemental S and Thiosulphate, while 3 isolates SS1, IW13 and SM1 utilized only thiosulphate. Three SOB isolates IW1, SW2 and IW16 had smooth, round and yellow colored colonies, 2 SOB isolates SS1 and IW13 had smooth, round and pink colored colonies, while 2 SOB isolates IW14 and SM1 had smooth, round and white colored colonies. Other chemical tests showed variation due to strain type (Table 2).

## Discussion

Phosphorous solubilizing potential of sulfur oxidizing bacteria through bacterial sulfur oxidation mechanism has been illustrated in this study. Isolation data of SOB indicated that maximum percentage of SOB were found in sulfur rich ecologies viz., industrial wastewater, sulfur mud and sewerage water, because sulfur or reduced sulfur compounds are crucial for the existence of SOB as they totally depend on S oxidation for their energy requirements (Pokorna *et al.,* 2007). Presence of SOB in paddy, wheat, sugarcane and maize rhizosphere depicted the occurrence of reduced S compounds in the soil. Biological sulfur oxidation is a unique character of SOB through which they oxidize S or S compounds and produce sulfuric acid. Thus sulfur oxidation is a sulfuric acid generating process shown in the following chemical equation:

$$S^{\circ} + 1.5\,O_2 + H_2O \quad SO_4^{2-} + 2H \xrightarrow{\text{SOB}} (\Delta G^{o} = -587.1\text{kJ/reaction}).$$

The most efficient SOB isolates oxidize S compounds quickly and produce sulfuric acid in huge quantity and drop pH sharply like strain IW16 (Hassan *et al.,* 2010; Yang *et al.,* 2010). In the same way highly efficient SOB strains (IW16 and SW2) produced sulfuric acid rapidly and started making holozones from the days 1$^{st}$ of inoculation and consequently their PSI (9.83 and 8.42, respectively) was very high (Islam *et al.,* 2007). The strains having high PSI are reported to be the most efficient in solubilizing and enhancing P in different media (Hariprasad and Niranjana, 2009; Ahemad and Khan, 2010).

**Table 1:** Ecology-wise description of sulfur oxidizing bacteria

| Ecology | Total | SOB + ve | SOB -ve |
|---|---|---|---|
| | | ---------- No. of samples ---------- | |
| Paddy fields (PF) | 15 | 02 (PF2, PF3) | 13 |
| Wheat Rhizosphere (WR) | 50 | 10 (WR2, WR4, WR7, WR9, WR10, WR12, WR13, WR14, WR15, WR16) | 40 |
| Sugarcane Rhizosphere (SR) | 15 | 02 (SR2, SR8) | 13 |
| Maize Rhizosphere (MR) | 15 | 02 (MR6, MR8) | 13 |
| Industrial wastewater (IW) | 10 | 08 (IW1, IW3, IW4, IW5, IW7, IW13, IW14, IW16) | 2 |
| Canal water (CW) | 10 | 03 (CW1, CW2, CW3) | 7 |
| Sulfur mud (SM) | 15 | 09 (SM1, SM2, SM3, SM4, SM7, SM9, SM11, SM12, SM14) | 6 |
| Sewage water (SW) | 10 | 06 (SW1, SW2, SW4, SW5, SW11, SW14) | 4 |
| Industrial waste sludge (IS) | 10 | 05 (IS1, IS2, IS11, IS12, IS16) | 5 |
| Sewage sludge (SS) | 10 | 03 (SS1, SS4, SS6) | 7 |
| Total : | 160 | 50 | 110 |

**Table 2:** Morphological, physiological and biochemical characterization of sulfur oxidizing bacteria

| Characteristics | IW1 | SW2 | SS1 | IW13 | IW14 | IW16 | SM1 |
|---|---|---|---|---|---|---|---|
| Morphology | SR | SR | SR | SR | SR | SR | SR |
| Gram reaction | - | - | - | - | - | - | - |
| Elemental $S^{\circ}$ utilization | + | + | - | - | + | + | - |
| Thiosulphate utilization | + | + | + | + | + | + | + |
| Colony character | SRY | SRY | SRP | SRP | SRW | SRY | SRW |
| pH reduction | +++ | +++ | + | ++ | ++ | +++ | + |
| Sulfates production | +++ | +++ | + | ++ | ++ | +++ | + |
| Nutritional type | AT | AT | HT | HT | AT | AT | HT |
| Boitin Effect | + | + | + | + | + | + | + |
| Motility | M | M | NM | M | M | M | NM |
| Catalase | - | + | - | + | - | - | - |
| Oxidase | + | + | + | + | + | + | + |
| Nitrate reduction | + | + | - | + | + | + | + |
| Glucose | - | - | + | + | - | - | - |
| $H_2S$ production | - | - | - | - | - | - | - |
| Sucrose | - | - | - | - | - | - | - |
| MR | - | - | - | - | - | - | - |
| Citrate | - | + | - | - | + | - | - |
| Carbohydrate hydrolysis | - | - | - | - | - | - | - |

SR: short rod, SRY: smooth, round, yellow: SRP: smooth, round, pink: SRW: smooth, round, white, AT: Autotrophic, HT: Heterotrophic, M: motile, NM: non motile

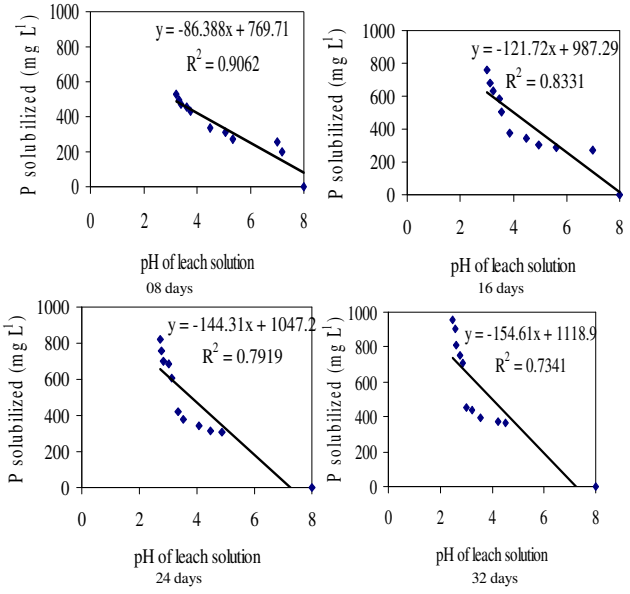


**Fig. 7a-d:** Relationship of P solubilization with pH in tricalcium phosphate leach solution

During tricalcium phosphate bioleaching (TCP) test one part of the bacterially produced sulfuric acid was used in solubilizing P from TCP, while the other part decreased pH of the media. The most efficient SOB (IW16 and SW2) produced rich amount of sulfuric acid and dropped pH drastically. Therefore, pH reduction in the media predicted the efficiency and capability of SOB isolates in P solubilization (Aria *et al.,* 2010; Oh *et al.,* 2010; Ullah *et al.,* 2013).

Likewise, the concentration of sulfates in the leach solutions depicted the efficiency of SOB isolates to oxidize S or S compounds. The most efficient SOB isolates rapidly oxidized S compounds into sulfates whereas less efficient SOB isolates did this slowly and consequently low quantity of sulfates were present in their leach solutions. Therefore, SOB isolates could be scrutinized on the basis of sulfate concentration detected from their leach solutions (Lee *et al.,* 2005; Yang *et al.,* 2010).

Phosphorous solubilzation data revealed that the isolates, which have the highest potential to produce sulfates (IW16 and SW2) solubilized maximum quantity of P from tricalcium phosphate, while the isolates

possessing less efficiency to oxidize S compounds (WR20) dissolved minimum amount of P in the leaching media. The chemical reaction for P solubilization is as under:

$$2Ca_3(PO_4)_2 + 4H_2SO_4 \longrightarrow 2Ca(H_2PO_4)_2 + 4CaSO_4$$

(Insoluble tricalcium phosphate) (Soluble calcium dihydrogen phosphate)

The above chemical reaction shows that bacterially produced sulfuric acid attacked on insoluble tricalcium phosphate and converted it into soluble and bio-available dihydrogen phosphate (Kumar and Nagendran, 2008; Bhatti and Yawar, 2010). It was noted that the soluble P contents were maximum in the first 16 days in all treatments which indicated that maximum S oxidation was upto the first 16 days of incubation (Aria *et al.,* 2010).

The correlation coefficient (*r*) values between pH and solubilized P contents (-0.95, -0.91, -0.89 and -0.86 after 8, 16, 24 and 32 days, respectively) and between sulfate concentration and solubilized P contents (0.80, 0.89, 0.91 and 0.92 after 8, 16, 24 and 32 days of leaching time, respectively) indicated that pH had a huge negative significant correlation with the quantity of P solubilzed and the concentration of sulfates predicted massive positive correlation with the amount of P solubilized. It showed that with the decrease in pH, sulfate contents increased and subsequently the amount of P increased in the leach suspensions due to enhancement in P dissolution phenomenon (Bhatti and Yawar, 2010). Moreover, Fig. 7 (a-d) presented simple regression analysis of pH with the amount of P solubilized. It showed that the relationship was linear and significant with the values of coefficient of determination ($R^2$) 0.91, 0.83, 0.79 and 0.73 after 8, 16, 24 and 32 days, respectively (Stamford *et al.,* 2003).

The selected SOB isolates were recognized as *Thiobacillus* spp. because they were Gram negative, short rods and possessed high ability to utilize S or thiosulphate as the only source of energy and carbon dioxide as a sole source of carbon. Furthermore, they showed great efficiency to produce sulfates and they also reduced pH of the growth media intensely. These characters showed that all these seven SOB isolates belonged to the genus *Thiobacillus* (Kelly and Wood, 2000; Vidyalakshmi and Sridar, 2007; Babana *et al.,* 2011).

It was concluded that sulfur rich ecologies have highly efficient sulfur oxidizing bacteria which are extremely and exceptionally competent in sulfates production and pH reduction in thiosulphate broth and thiosulphate tricalcium phosphate media. Moreover, these bacteria displayed high P dissolution capability and P solubilization rate was positively correlated with the rate of bacterially produced sulfates. Therefore, the sulfur oxidizing bacteria can be effectively utilized for solubilizing already present huge quantity of fixed P in alkaline and calcareous soils.

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