

Biological removal of phosphate from synthetic wastewater using bacterial consortium

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Abstract

The biological phosphorus removal is a microbial process widely used for removing phosphorus from wastewater to avoid eutrophication of water bodies. The study was aimed to screen the efficient phosphate reducing isolates and used to remove phosphate from synthetic wastewater using batch scale process. The three most efficient phosphate reducers were isolated and screened from eutrophic lake water and forest soil samples. The total heterotrophic bacterial analysis of the samples showed the presence of about 38 phosphate reducers based on the minimum inhibitory concentration (MIC) test. Among them, *Bacillus* sp RS-1, *Pseudomonas* sp. YLW-7 and *Enterobacter* sp KLW-2 were found to be efficient in phosphate reduction. Among the individual strains, *Pseudomonas* sp YLW-7 was noticed to be 68% removal in MSM with glucose at neutral pH. The consortium with combination of *Bacillus* sp. RS-1, *Pseudomonas* sp. YLW-7 and *Enterobacter* sp KLW-2 was effectively removed the phosphate in the synthetic medium when compared to individual strains. The phosphate removal was observed to be maximum of 92.5% in mineral salts medium (MSM) at pH 7 and 5, and 63.4% in synthetic phosphate solution at neutral pH with lactose as a carbon source by the consortium after 72 h. Thus the microorganisms may use the contaminants as nutrients and as energy sources or it may be utilized by cometabolism. Therefore, these bacterial isolates might be used in the remediation of phosphate contaminated environments.

Keywords: Phosphate removal; Synthetic waste water; Consortium- *Bacillus* sp RS-1; *Pseudomonas* sp YLW-7; *Enterobacter* sp KLW-2

INTRODUCTION

Phosphorus is recognized as one of the major nutrients required by living organisms involved in major physiological processes. However, it can also be considered a pollutant if the concentrations are high under specific environmental conditions. The addition of phosphorus as phosphate ion is one of the most serious environmental problems because of its contribution to the increased eutrophication process of lakes and other natural waters. It occurs in natural water, wastewater, sediments and sludges. The possible entry of this ion into aquatic environment is through household sewage water and industrial effluents-particularly fertilizer and soap industries. The main sources of phosphorus released into the environment include fertilizers, detergents, cleaning preparations, and boiler waters to which phosphates are added for treatment (Pradyot, 1997). It exists in three forms: organic phosphorus (associated with organic molecules), orthophosphate (exists as an anion) and polyphosphates (from detergents). Only Orthophosphate can be chemically precipitated, however, most of the organic phosphorus and polyphosphates are converted to the orthophosphate form during biological treatment. Biological treatment is a cost-effective method for wastewater before being discharged into the streams and rivers. Microbial strategies for the removal of environmental pollutants from waste streams or contaminated sites can provide an attractive alternative to traditional methods such as incineration or disposal in landfills. Currently, phosphates are biologically removed by wastewater treatment facilities by absorption of dissolved orthophosphate, polyphosphate and organic

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phosphate by living microorganisms, such as bacteria, microalgae, yeast, protozoa, fungi, and macrophytes.

Biological phosphate removal from wastewater is a generally accepted; less costly alternative to chemical phosphate removal (Van Loosdrecht *et al.*, 1997). Phosphorus is used by the microorganisms for their cellular maintenance, synthesis of nucleic acids, construction of cell membranes (as phospholipids) and chemical energy transfer reactions within cells (as ATP molecules). Some phosphorus is also stored for future use by the cells. The quantity of eliminated phosphate depends on the net production of living biomass. Enhanced Biological Phosphate Removal (EBPR) is an alternate biotechnological as well as eco-friendly approach to both the environment and its living beings. The process of EBPR uses the metabolism of specific bacteria, which under certain conditions accumulate large amounts of phosphate as intracellular polyphosphate. These bacteria use the degradation of polyphosphate to produce energy under anaerobic conditions with the release of phosphate into the wastewater (resulting in a transient increase in the phosphate content of the waste water). The waste water is then treated in an aerobic basin. Here the polyphosphate bacteria use stored carbon reserves to produce energy for growth and to replenish their stores of polyphosphate. The result is a net removal of phosphate from the waste water (due to replenishment of polyphosphate in extant cells and de novo polyphosphate reserves in divided cells).

An important group of contaminants for which the efficient treatment methods needed are phosphates, since they may adversely affect and pose a threat to aquatic ecosystems. According to federal government standards, phosphate levels in water should not exceed 0.01-0.1 mg/l (EPA, 1991). Therefore it is essential to control the emission of phosphates from discharge of wastewater and reducing phosphorus concentrations to the lowest possible level is vital to the maintenance of unpolluted water supplies. Hence the objective of the present study was to examine the efficiency of bacterial species individually and in consortium for the removal of phosphate from synthetic phosphate solution and mineral salts medium (MSM).

MATERIALS AND METHODS

Sample Collection: The eutrophic lake waters were collected in sterile glass bottles from four different sampling stations of Yercaud and Kodaikanal Lakes

(South India). The soil samples from the rhizosphere and non-rhizosphere region of teak trees from the Siruvani Forest (located in Western Ghats of Coimbatore District, South India) were collected in sterile polyethylene bags.

Isolation and identification of phosphate reducers:

Pour plate technique was employed to enumerate total heterotrophic bacteria using Nutrient Agar (Hi-Media, Mumbai, India). Minimum inhibitory concentration (MIC) test with plate screening method was carried out to screen phosphate reducers using phosphate agar. The bacterial cultures isolated from nutrient agar and phosphate agar plates were classified to various genera based on their morphological and biochemical characters as given in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1994).

The phosphate reducers screened from the different sample source were given symbols (code name) based on the source of isolation; Kodaikanal Lake Water-KLW, Yercaud Lake Water-YLW, Rhizosphere Soil-RS, Non-Rhizosphere Soil-NRS.

Preparation of inoculum: Nutrient broth (Hi-Media, Mumbai, India) was prepared and selected bacterial isolates were inoculated separately and incubated for 24 h at room temperature. The cells were recovered by centrifugation (10,000 rpm for 15 min) and were transferred to sterile saline. The cell concentration of each strain was adjusted to an optical density at 600 nm (OD_{600}) of 0.1 and used as an inoculum. Three efficient phosphate reducers (*Bacillus* sp. RS1, *Pseudomonas* sp. YLW7 and *Enterobacter* sp. KLW2) were used for the removal of phosphate.

The mixed bacterial consortium (A+B+C) were prepared in combination from the three isolates *Bacillus* sp. RS1-(A), *Pseudomonas* sp. YLW 7-(B) and *Enterobacter* sp. KLW 2-(C), by adjusting the cell concentration of A, B and C to 0.1 of OD_{600} . About 97 (RS-1), 105 (YLW-7) and 92 (KLW- 2) $\times 10^4$ CFU/ml (1ml of 0.1OD) of the cells were used as an inoculum. Growth yield determinations were calibrated by gravimetric determination of dry cell mass grown in 1-liter cultures.

Phosphate removal by bacteria in synthetic wastewater (MSM and synthetic phosphate solution):

Effect of carbon source in medium: Shake flask batch culture experiments were performed. Phosphate removal was carried out by adding phosphate to syn-

thetic wastewater (mineral salts medium and plain distilled water). The synthetic phosphate solution (distilled water with phosphate concentration of 100 mg/l using Potassium dihydrogen phosphate; pH-7.2) and in mineral salts medium containing 100 mg/l phosphate concentration and 0.5% of different carbon substrates such as sucrose, starch, glucose and lactose were prepared. Then the medium containing flasks were sterilized at 121°C and at 15 lbs for 15 min in an autoclave. One ml of inoculum (0.1 OD) from the selected phosphate reducers A, B, C and consortium of combination A+B+C were inoculated in the individual flasks. They were incubated at room temperature in a shaker maintained at 150 rpm for a period of three days. Samples were collected at 0, 24, 48, 72 h and analyzed for growth of bacteria, pH change and change in total phosphate concentration of the medium. All experiments were performed in triplicates.

Effect of change in initial pH: The experiments were carried out by changing the initial pH to 5, 7 and 9 of the culture medium. The pH of synthetic wastewater was adjusted by hydrochloric acid (1N) and sodium hydroxide (1N) solution using pH meter. For each experiment, wastewater samples after treatment and control were analyzed for the efficiency of phosphate removal and biomass of the inoculums in terms of optical density (OD).

Growth of bacteria: The increase in growth of bacteria for every 24 h was monitored by measuring optical density (OD) at 600nm on a UV-Visible Spectrophotometer (UV-VIS Hitachi-U3210). The pH change in the culture medium after treatment was measured using a pH meter.

Estimation of phosphate removal: The phosphate uptake activities of different strains were quantified by stannous chloride reduced molybdophosphoric acid blue method. The soluble phosphate content in the cul-

ture medium was estimated after 24, 42, and 72 h of incubation by using the stannous chloride calorimetric method (Saxena, 1994) and (APHA, 1998). After every 24 h, 10 ml of the agitated sample was drawn from the series of individual flask and transferred into the centrifuge tubes of 15ml capacity under aseptic conditions. Then the tubes containing samples were centrifuged at 10,000 rpm at 15 min and the clear supernatant was used for soluble phosphate estimation at 690 nm by using spectrophotometer (UV-VIS Hitachi-U3210).

Phosphate uptake efficiency (E) was calculated using the formula,

$$E = [(I-F)/I] \times 100 / \text{OD growth of bacteria or g/l of dry biomass}$$

Where,

I and F are the initial and final concentrations of phosphorous respectively.

OD is the optical density at 600nm for the growth of bacteria

An efficiency value of 100% was obtained when no phosphate appeared in the water sample (i.e., F = 0).

RESULTS

Isolation and identification of phosphate reducers:

The maximum population of total heterotrophic bacteria (THB-64.7 CFU \times 10³ /g of soil) and phosphobacteria (4.7 CFU \times 10³ /g of soil) were observed in soils collected from rhizosphere soil region of teak trees from Siruvani forest, whereas in water samples, the maximum THB population of 28.5 CFU \times 10³ /ml and phosphate reducing bacterial population of 3.3 CFU \times 10³ /ml was noticed in Yercaud lake water sample as shown in Table 1.

It was found that 38 isolates were phosphate reducers. Among the 38 isolates A-*Bacillus* sp. (RS-1), B-*Pseudomonas* sp. (YLW-7) and C-*Enterobacter* sp. (KLW-2) were screened and identified as predominant

Table 1. Enumeration of Total heterotrophic bacteria (THB) and Phosphate reducers present in different samples.

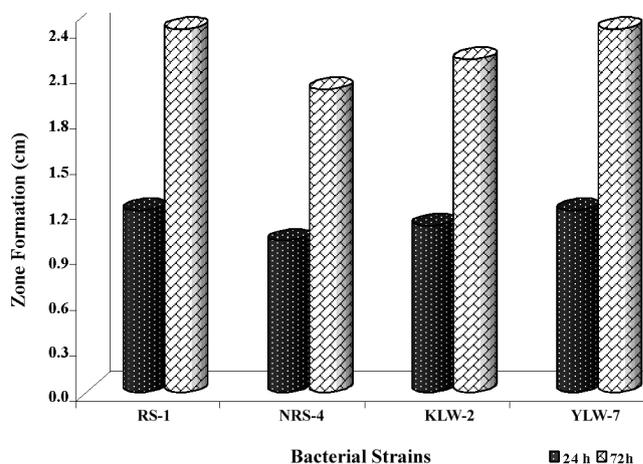
Source of Isolation	THB Population	Phosphate reducers
Rhizosphere Soil [RS] (Siruvani forest soil)	64.7 cfu \times 10 ³ /g	4.7 cfu \times 10 ³ /g
Non- Rhizosphre Soil [NRS] (Siruvani forest soil)	53.8 cfu \times 10 ³ /g	3.8 cfu \times 10 ³ /g
Kodikanal Lake Water [KLW]	17.8 cfu \times 10 ³ /ml	2.2 cfu \times 10 ³ /ml
Yercaud Lake Water [YLW]	28.5 cfu \times 10 ³ /ml	3.3 cfu \times 10 ³ /ml

Table 2. Screening of potential phosphate reducers based on the Minimum Inhibitory Concentration test.

Strain No.	Bacterial genera	Zone Formation after 72 h of incubation
RS-1	<i>Bacillus</i>	2.4*
RS-2	<i>Bacillus</i>	1.8
RS-3	<i>Pseudomonas</i>	1.5
RS-4	<i>Pseudomonas</i>	1.8
RS-5	<i>Bacillus</i>	1.8
RS-6	<i>Bacillus</i>	1.7
RS-7	<i>Bacillus</i>	2.1
RS-8	<i>Bacillus</i>	1.8
RS-9	<i>Pseudomonas</i>	2.0
RS-10	<i>Pseudomonas</i>	1.7
NRS-1	<i>Bacillus</i>	1.8
NRS-2	<i>Bacillus</i>	1.7
NRS-3	<i>Micrococcus</i>	1.3
NRS-4	<i>Bacillus</i>	2.0
NRS-5	<i>Pseudomonas</i>	1.9
NRS-6	<i>Pseudomonas</i>	1.6
NRS-7	<i>Bacillus</i>	1.5
NRS-8	<i>Bacillus</i>	1.7
NRS-9	<i>Bacillus</i>	1.9
KLW-1	<i>Pseudomonas</i>	1.6
KLW-2	<i>Enterobacter</i>	2.2*
KLW-3	<i>Enterobacter</i>	1.7
KLW-4	<i>Bacillus</i>	1.6
KLW-5	<i>Pseudomonas</i>	1.8
KLW-6	<i>Pseudomonas</i>	1.7
KLW-7	<i>Staphylococcus</i>	1.3
KLW-8	<i>Pseudomonas</i>	1.8
YLW-1	<i>Enterobacter</i>	1.5
YLW-2	<i>Pseudomonas</i>	1.5
YLW-3	<i>Pseudomonas</i>	1.5
YLW-4	<i>Pseudomonas</i>	1.5
YLW-5	<i>Pseudomonas</i>	1.9
YLW-6	<i>Enterobacter</i>	1.6
YLW-7	<i>Pseudomonas</i>	2.5*
YLW-8	<i>Micrococcus</i>	1.9
YLW-9	<i>Enterobacter</i>	1.8
YLW-10	<i>Pseudomonas</i>	1.8
YLW-11	<i>Pseudomonas</i>	1.9

*maximum zone formation.

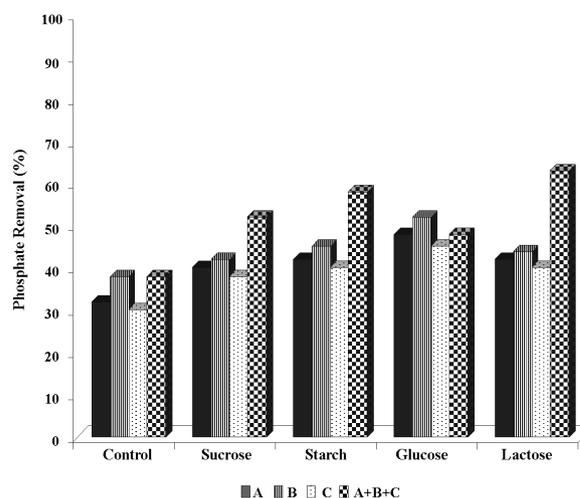
phosphate utilizers based on the minimum inhibitory concentration (MIC) test after 72 h of incubation in nutrient medium amended with phosphate (Table. 2 and Fig. 1). These isolates were used in this study to remove phosphate from two different medium, as in synthetic phosphate solution and mineral salts medium with 100 mg/l of phosphate concentrations.

**Figure 1.** Screening of efficient phosphate reducers based on the Minimum Inhibitory Concentration test (RS-1- *Bacillus* sp.; NRS-4 - *Bacillus* sp.; KLW-2- *Enterobacter* sp.; YLW-7- *Pseudomonas* sp.).

Phosphate removal by bacteria in MSM and synthetic phosphate solution:

Effect of carbon source in medium: Carbon sources-enriched synthetic medium at the experimental concentration greatly influenced the growth and phosphate removal efficiency of the bacteria. The results obtained from different bacterial species and its combinations were plotted in Figures 2-3 and 10-11.

In synthetic phosphate solution with 0.5% carbon source, lactose was observed to yield maximum phosphate removal (63.4%) and bacterial growth in terms of dry biomass (0.21 g/l) by the consortium (Figs. 2 and 10 D). In the individual strains, *Pseudomonas* sp. showed maximum phosphate removal (52.3%) and

**Figure 2.** Effect of carbon source on the removal of phosphate by the bacterial species in synthetic phosphate solution ('A' - *Bacillus* sp RS-1, 'B'-*Pseudomonas* sp. YLW-7, 'C' - *Enterobacter* sp. KLW-2 and 'A+B+C' -Consortium).

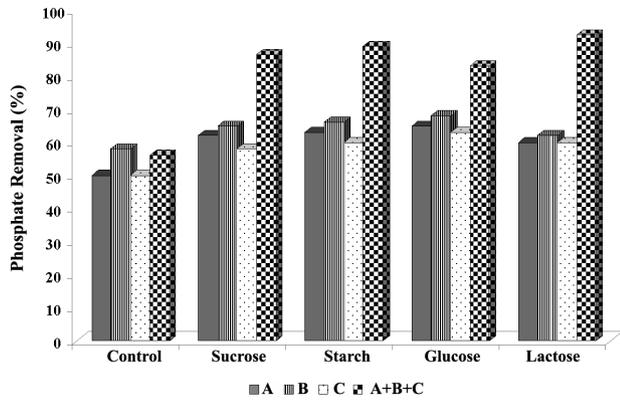


Figure 3. Effect of carbon source on the removal of phosphate by the bacterial species in Mineral salts medium (MSM) ('A'-*Bacillus* sp RS-1, 'B'-*Pseudomonas* sp. YLW-7, 'C'-*Enterobacter* sp. K LW-2 and 'A+B+C'-Consortium).

growth (0.21 g/l) in glucose as a carbon source followed by starch, lactose and sucrose as shown in Figures 2 and 10B.

In MSM with lactose carbon source, the phosphate removal and growth of the phosphate reducers were higher when compared to synthetic phosphate solution. From the Figures 3 and 11D, it was observed that, in MSM with 0.5% carbon source, the lactose carbon source showed a maximum phosphate removal of 92.5% and growth in terms of dry biomass (0.34 g/l) by the consortium after 72 h. But in the individual strains (*Pseudomonas* sp. YLW-7), the glucose showed a maximum phosphate removal (68.2%) and growth

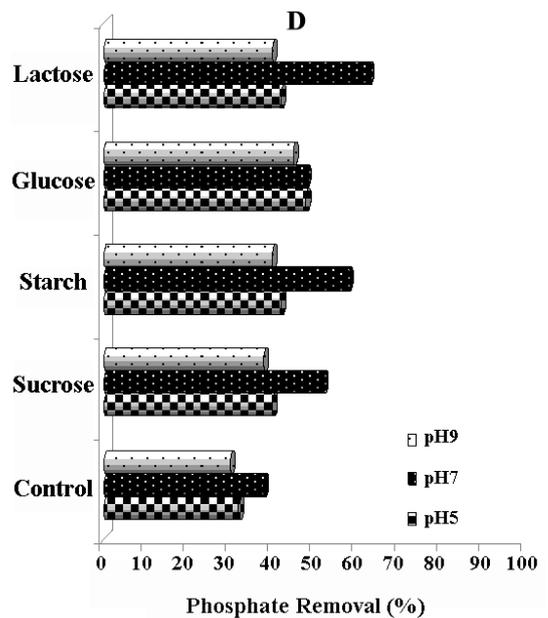
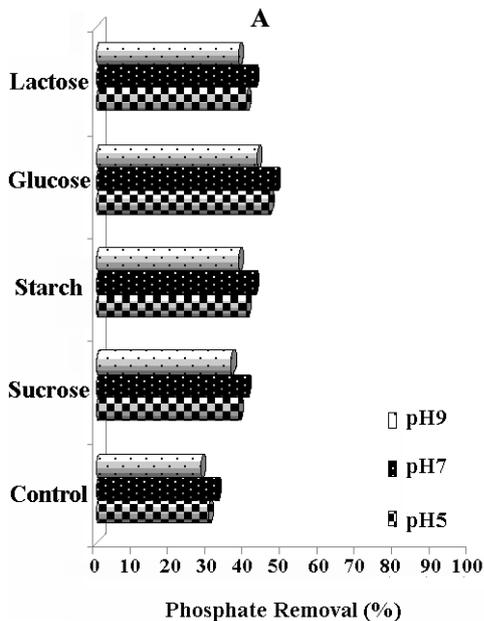
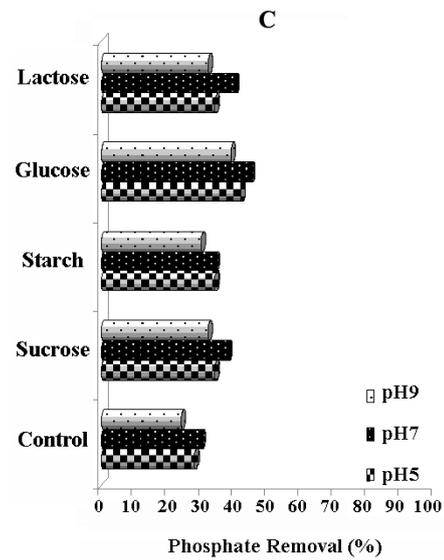
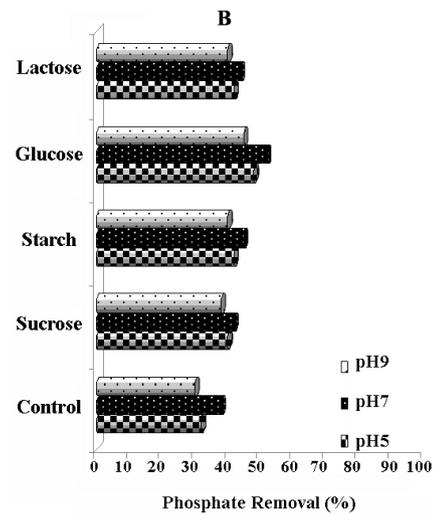


Figure 4. Change in initial pH of the medium (synthetic phosphate solution) during phosphate removal by bacteria (A: '*A*'-*Bacillus* sp. RS-1, B: '*B*'- *Pseudomonas* sp YLW-7, C: '*C*'-*Enterobacter* sp. K LW-2 and D: 'A+B+C'-Consortium).

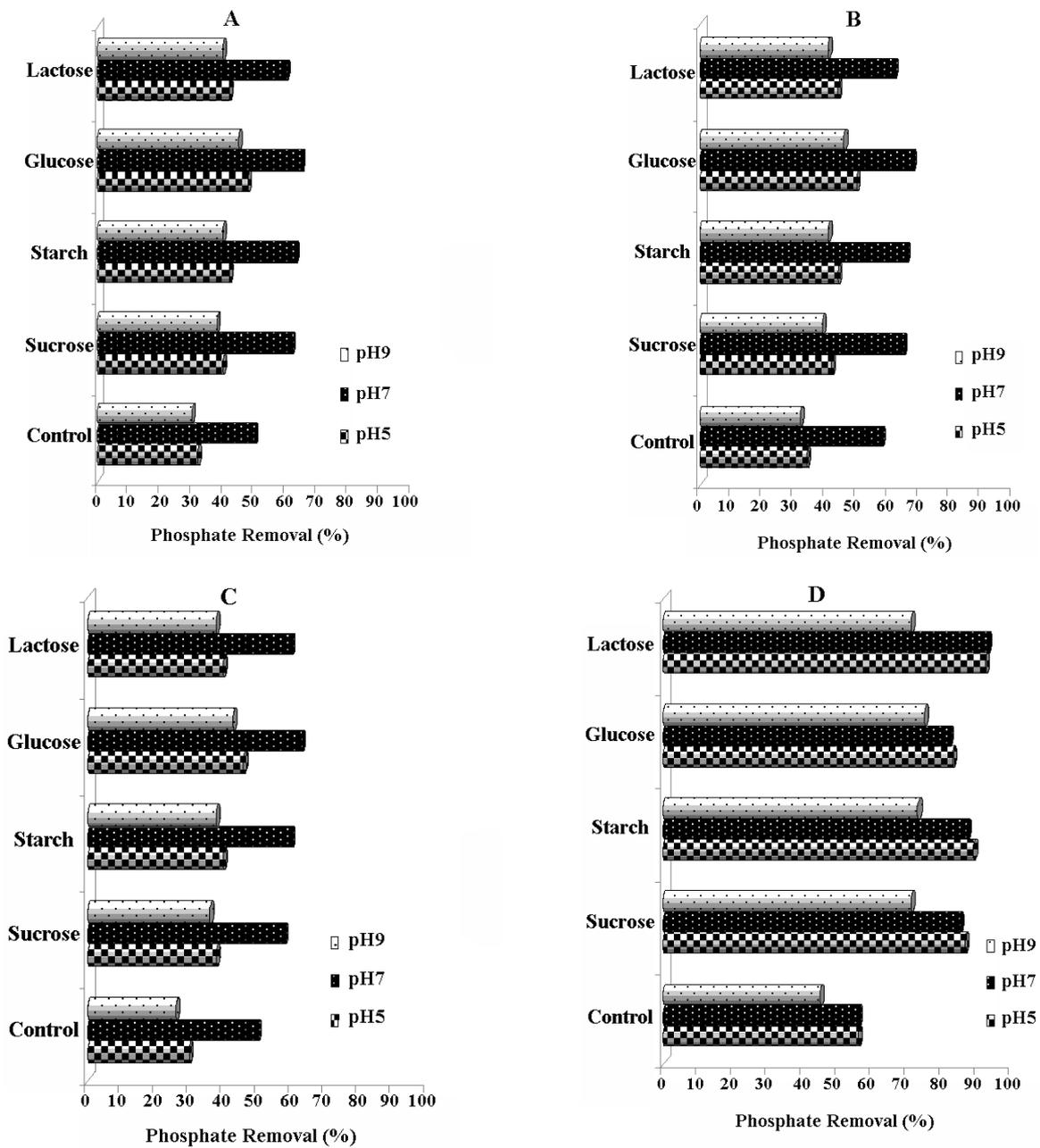


Figure 5. Change in initial pH of the medium (MSM) during phosphate removal by bacteria (A: 'A'-*Bacillus* sp. RS-1, B: 'B'- *Pseudomonas* sp YLW-7, C: 'C'-*Enterobacter* sp. K LW-2 and D: 'A+B+C'-Consortium).

(0.3 g/l) (Figs. 3 and 11B). The results of this study showed that the synthetic medium without carbon sources (control) showed less removal when compared to medium with carbon sources (Figs. 3 and 11B).

Effect of change in initial pH: The effect of change in initial pH of the culture medium (synthetic phosphate solution and MSM) with different carbon sources were shown in Figures 4A-D and 5A-D. In synthetic phosphate solution, the removal was noticed to be 52% by

Pseudomonas sp. YLW-7 and 63% by consortium at pH 7 in glucose carbon source (Fig. 4B). The pH 7 favored for maximum phosphate removal (68%) by the individual strain of *Pseudomonas* sp. YLW-7 in MSM medium enriched with glucose carbon source. But in the consortium, both pH 5 and 7 favored for maximum removal of phosphate (93%) in MSM with lactose carbon source (Fig. 5D).

Growth of bacteria: The initial optical density of 0.10

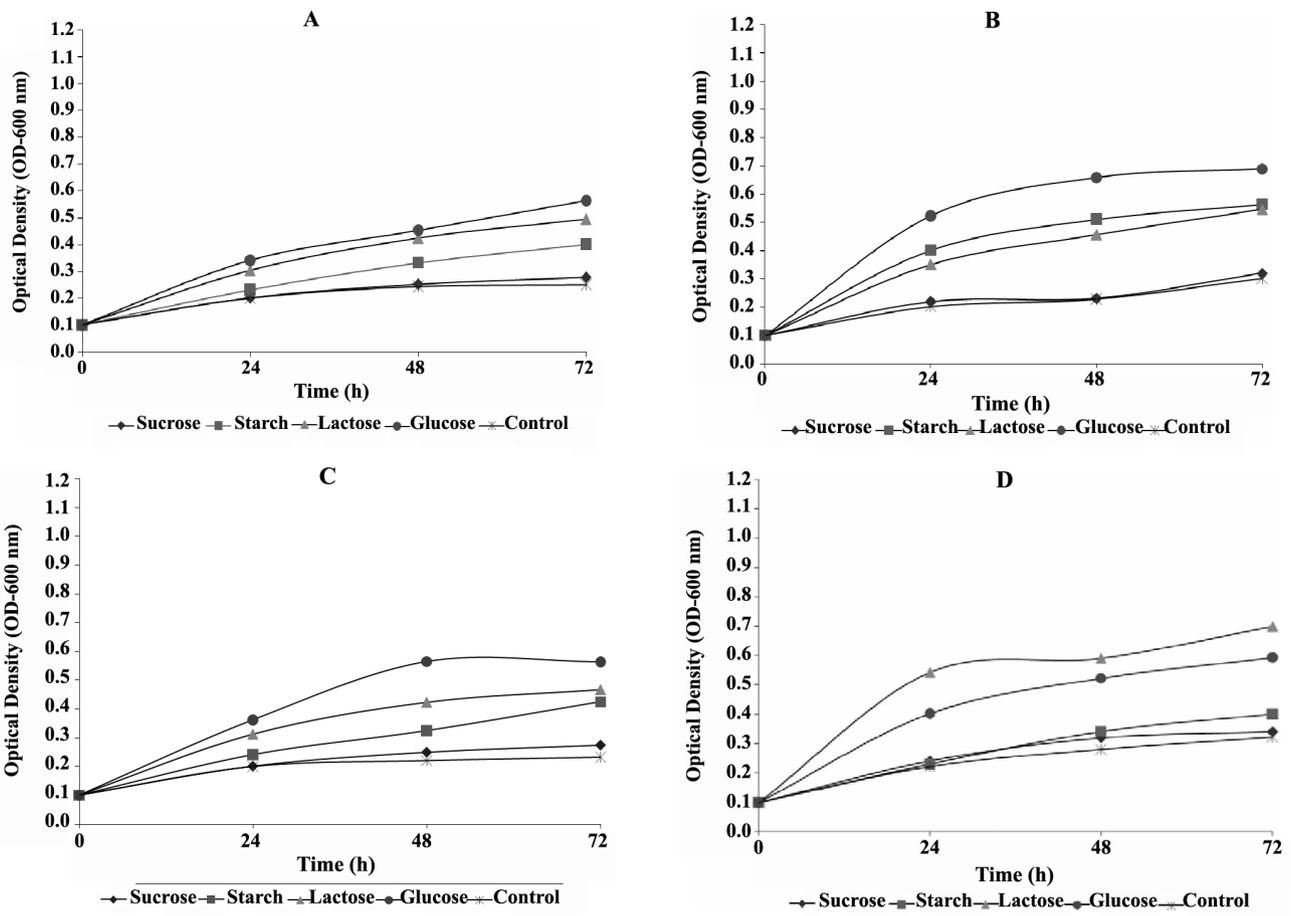


Figure 6. Effect of carbon source on the growth of bacteria in synthetic phosphate solution. (A: 'A'-*Bacillus* sp. RS-1, B: 'B'- *Pseudomonas* sp YLW-7, C: 'C'-*Enterobacter* sp. KLV-2 and D: 'A+B+C' -Consortium).

OD was measured at 600 nm in a spectrophotometer and their dry biomass was calculated. A linear relationship existed between dry cell mass and OD; each increase of 0.1 OD corresponded to an increase of 0.03 mg of dry biomass per ml. The calibration factors were identical also after growth with different substrates because the cell shape of these bacteria is very constant. Conversion factors for calculation of cell yields from optical density (OD) values were determined in 1-liter cultures. The conversion factors obtained were very similar for all bacteria used. An OD value at 600 nm (OD₆₀₀) of 0.1 measured against a medium blank corresponded to 29.8 mg (dry weight) of cells per liter with *Bacillus* sp RS1, 30 mg (dry weight) of cells per liter with *Pseudomonas* sp. YLW7 and 29.75 mg (dry weight) of cells per liter with *Enterobacter* sp KLV2. These values were used for calculations of cell yields in the subsequent experiments.

The effect of carbon source on the growth of bacteria in phosphate medium was analyzed after 72 h of incubation period. The results were shown in Figures

6A-D and 7A-D. In synthetic phosphate solution with 0.5% carbon sources, the individual strain of *Pseudomonas* sp. was observed a maximum growth of 0.6886 OD (dry biomass- 0.21 g/l) in glucose and minimum of 0.2752 OD (dry biomass-0.09 g/l) in sucrose by *Enterobacter* sp. as shown in Figures 6A, B and 10. But the consortium showed maximum growth of 0.6997 OD and growth in terms of dry biomass (0.21 g/l) in the presence of lactose.

In MSM with 0.5% carbon source, the individual strains of *Pseudomonas* sp. was observed a maximum growth of 0.9886 OD (dry biomass-0.3 g/l) in the presence of glucose and minimum of 0.3280 OD (dry biomass-0.09 g/l) in sucrose by *Bacillus* sp. as shown in Figures 7A, B and 11. Whereas in case of consortium, the maximum growth was found to be 1.1428 OD and growth in terms of dry biomass (0.34 g/l) in lactose source.

The metabolism of phosphate by *Bacillus* sp. (RS-1), *Pseudomonas* sp. (YLW-7) and *Enterobacter* sp (KLV-2) were indicated by a visible increase in growth (OD) with

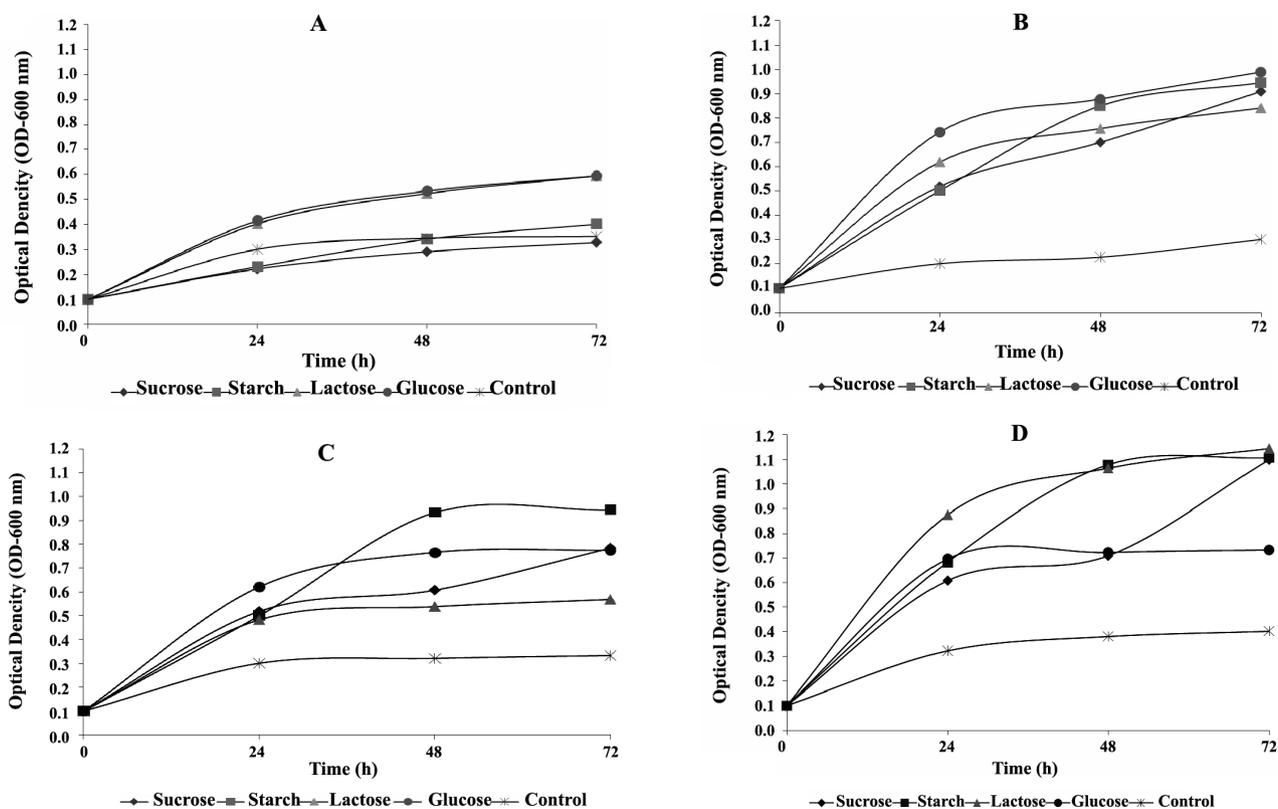


Figure 7. Effect of carbon source on the growth of bacteria in Mineral salts medium (MSM). (A: 'A'-*Bacillus* sp. RS-1, B: 'B'-*Pseudomonas* sp. YLW-7, C: 'C'-*Enterobacter* sp. KLV-2 and D: 'A+B+C'-Consortium).

time. Initially, the growth was suppressed in presence of phosphate, but after adaptation to phosphate it was grown rapidly exhibiting high growth rate. In later stage the amount of growth produced in the medium containing phosphate was much higher as compared to the growth in medium without carbon sources. This could be due to the availability of additional carbon source upon reduction of phosphate in the medium.

pH change in culture medium after biological treatment: The pH changes in culture medium with time were shown in Figures 8A-D and 9A-D. The pH value of the culture medium with 0.5% of carbon source was reduced during the process. In synthetic phosphate solution, the maximum reduction of pH from 7.2 to 6.0 was recorded in consortium with various carbon sources (Fig. 8D). Whereas in MSM, the reduction was from 7.2 to 5.7 in sucrose and glucose carbon sources by *Enterobacter* sp. after 72 h (Fig. 9C). The maximum reduction of pH (7.2 to 4.5) was recorded in the treatment by consortium where the glucose was used as a carbon source (Fig. 9D). In contrast, there is no significant change of pH was monitored in the medium without carbon source.

Phosphate removal: Among the medium, the MSM with lactose as a carbon source showed maximum phosphate removal when compared to synthetic phosphate solution with and without carbon sources. In synthetic phosphate solution at 100 mg/l of phosphate concentration in 0.5% carbon source, it was found to be maximum removal of 63% by the consortium with 0.6997 OD (dry biomass-0.21 g/l) where lactose was used as a carbon source and minimum of 48% with 0.5945 OD (dry biomass 0.18 g/l) in glucose source as shown in Figures 2, 4D and 10. But in the individual strains, 52.3% removal by *Pseudomonas* sp with 0.6886 OD (dry biomass-0.21 g/l) in glucose carbon source and minimum of 38% removal by *Enterobacter* sp. with 0.2752 OD (dry biomass-0.09 g/l) in sucrose carbon source were observed (Figs. 2, 4 and 10).

In MSM medium (at 100 mg/l of phosphate concentration with 0.5% carbon source) showed the maximum phosphate removal of 92.5% with 1.1428 OD (dry biomass-0.34 g/l) in lactose and minimum 83.2% with 0.7875 OD dry biomass-0.24 g/l) was observed in glucose as a carbon source by the consortium (Figs. 3, 5 and 11). The MSM with individual strains, the phos-

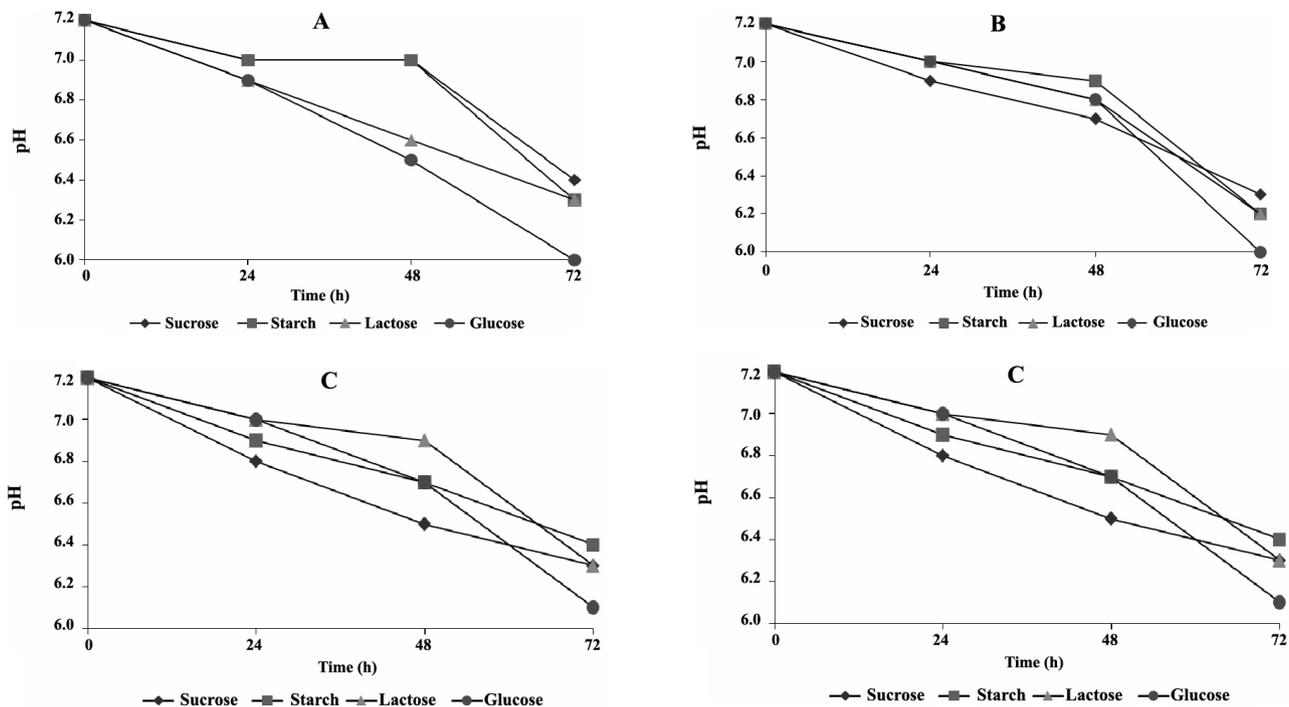


Figure 8. Change in pH of culture medium (synthetic phosphate solution) during phosphate removal by bacteria (A: 'A'-*Bacillus* sp. RS-1, B: 'B'-*Pseudomonas* sp. YLW-7, C: 'C'-*Enterobacter* sp. K LW-2 and D: 'A+B+C' -Consortium).

phate removal was found to be 68.2% by *Pseudomonas* sp with 0.9886 OD (dry biomass-0.3 g/l) in glucose carbon source and minimum of 58.3% by *Enterobacter* sp with 0.7839 OD (dry biomass-0.24 g/l) in sucrose as carbon source (Figs. 3, 5 and 11). The control was recorded very less removal when compared to other carbon sources as shown in Figures.

The results of this study showed that the synthetic phosphate solution without carbon sources showed less removal when compared to synthetic phosphate solution and MSM amended with carbon sources. Carbon source enriched medium was observed to enhance the phosphate removal and influenced the growth of bacteria.

DISCUSSION

Isolation and identification of phosphate reducers:

The strains of *Bacillus* sp. RS-1, *Pseudomonas* sp. YLW-7 and *Enterobacter* sp. K LW-2 were isolated from Rhizosphere Soil, Kodaikanal Lake Water and Yercaud Lake Water respectively. The removal efficiency of soluble phosphates varied with strains. Phosphate utilizing bacteria were known to be present in various environments (Illmer and Schinner, 1992) and (Illmer *et al.*, 1995). Various microorganisms are

capable of utilizing phosphate as a sole carbon source of phosphorus (Malacinski, 1967) and these microbial transformations have been proposed as key steps in the phosphorous cycle in nature. Bitton, (1994) reported that the *Pseudomonas* sp., *Aerobacter* sp., *Moraxella* sp., *Escherichia coli*, *Mycobacterium* sp., *Beggiatoa* sp. and *Klebsiella* sp. have the ability to accumulate phosphorus at approximately 1 to 3% of the cell dry mass reported.

Phosphate removal by bacteria in MSM and synthetic phosphate solution:

The carbon source was provided in the medium in order to enrich synthetic medium which in turn enhance the growth and phosphate uptake capacity of bacteria. Bacteria which can accumulate phosphate in the aerobic conditions and their internal phosphate had been depleted under anaerobic conditions. Among the carbon sources, the glucose source showed maximum phosphate removal of 68.2% by the *Pseudomonas* sp. in MSM, the glucose may be oxidized to gluconate which is further converted to other compounds. Glucose carbon source could induce good enhanced biological phosphate removal performance reported (Jeon, 2000). The carbon, i.e. glucose is oxidized to gluconate, which is converted into other compounds, such as 2-keto-3-deoxygluconate, pyruvate or glyceraldehydes was

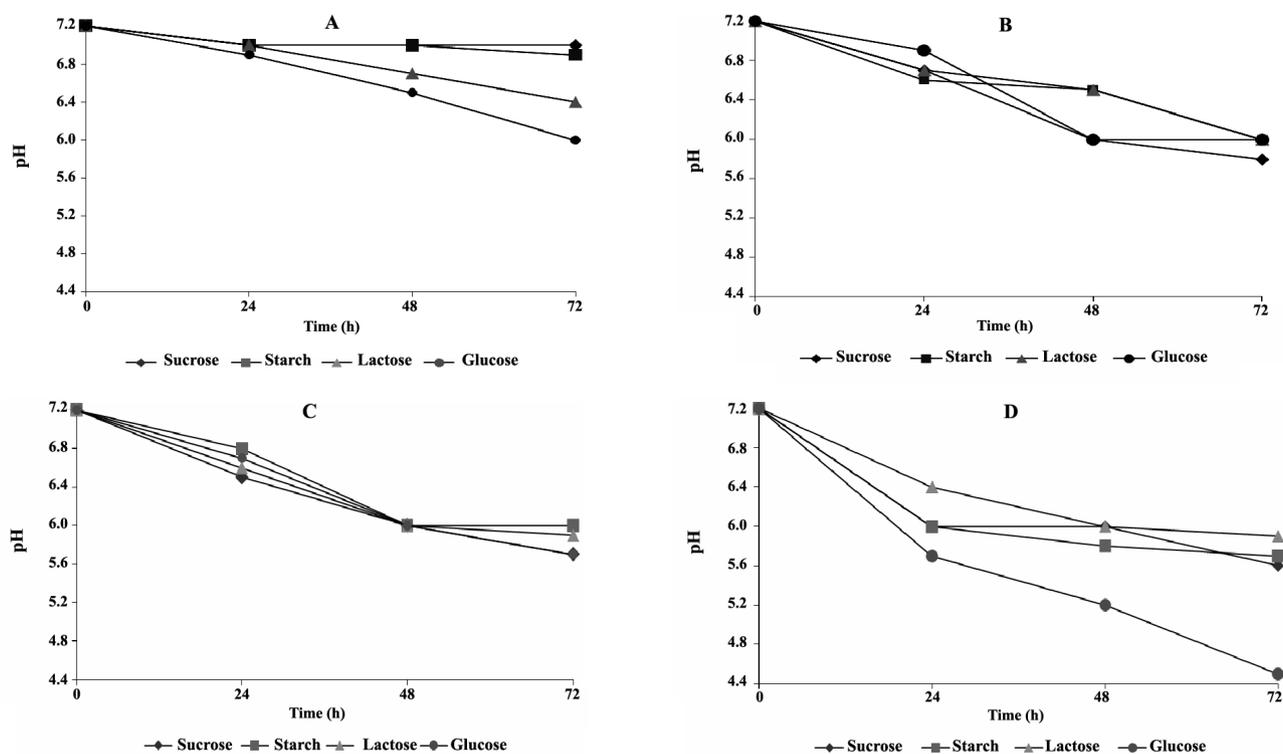


Figure 9. Change in pH of culture medium (MSM) during phosphate removal by bacteria (A: 'A'-*Bacillus* sp. RS-1, B: 'B'-*Pseudomonas* sp. YLW-7, C: 'C'-*Enterobacter* sp. KLW-2 and D: 'A+B+C'-Consortium).

reported (Kim *et al.*, 1998) and (Reyes *et al.*, 1999). Kim *et al.* (1998) suggested that the presence of organic acids (formate) and the mechanism such as the release of protons associated with biological ammonium assimilation that enhances the utilization of phosphates.

The initial pH at neutral and acidic conditions was favourable for the optimum removal of phosphate by the individual strain of *Pseudomonas* sp. and consortium. Bouquet *et al.* (1987) and Mullan *et al.* (2002) suggested that the acidic pH favoured for acid phosphatase. Acid phosphatase helps in the removal of phosphate. Cokgor *et al.*, 2004; Filipe *et al.*, (2001) suggested that a low pH value (pH \leq 7.25) was favourable for the growth of glycogen accumulating organism. Mc Garth *et al.* (2001) confirmed that a 50% enhancement in uptake of phosphate from sewage by an activated sludge inoculum grown at pH 5.5 with glucose as a carbon source and in only aerobic conditions. Concurrent accumulation of polyhydroxyalkanoates with polyphosphate was observed in *Pseudomonas* strains by Tobin *et al.* (2007).

In order to find the relationship between metabolic activities and reduction of phosphate, pH of the culture medium was monitored. The consortium showed max-

imum phosphate removal of 92.5% with pH change of the culture medium from 7.2 to 5.9. The reduction in pH may be due to the production of various organic acids by the phosphate reducers in the culture medium. Similar observations of the previous reports mentioned that the phosphate utilizing microorganisms produced various organic acids and consequently a fall in pH of the medium (Kundu, 1984) and (Satar and Gaur, 1984). Reports suggested that the presence of organic acids (formate) release protons which involves in biological ammonium assimilation that enhances the utilization of phosphates (Kim *et al.*, 1998). The individual strain of *Pseudomonas* sp. showed maximum phosphate removal of 68.2% with pH change from 7.2 to 6.0 after 72h in 0.5% glucose as the carbon source. The culture medium, pH 6.0 was favored for acid phosphatase secretion was reported (Bouquet *et al.*, 1987). *Burkholderia cepacia* maximum phosphate removal and accumulation of polyphosphate at pH 5.5 reported (Mullan *et al.*, 2002). Liu *et al.* (2007) reported that the optimal initial pH for higher soluble ortho-phosphorus removal efficiency was controlled between 6.4 and 7.2.

From the experimental study, the maximum growth was observed in consortium (1.1428 OD) after 72 h in

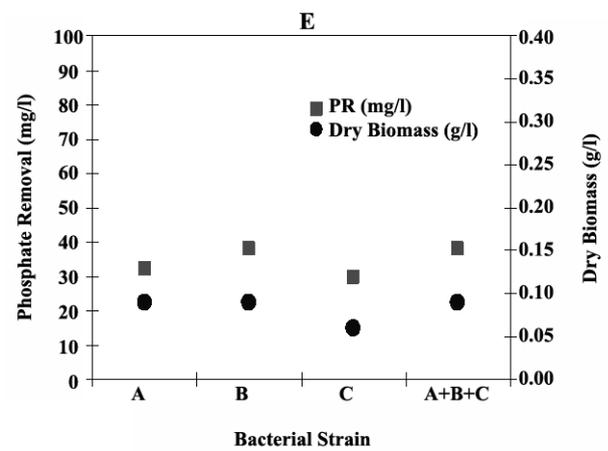
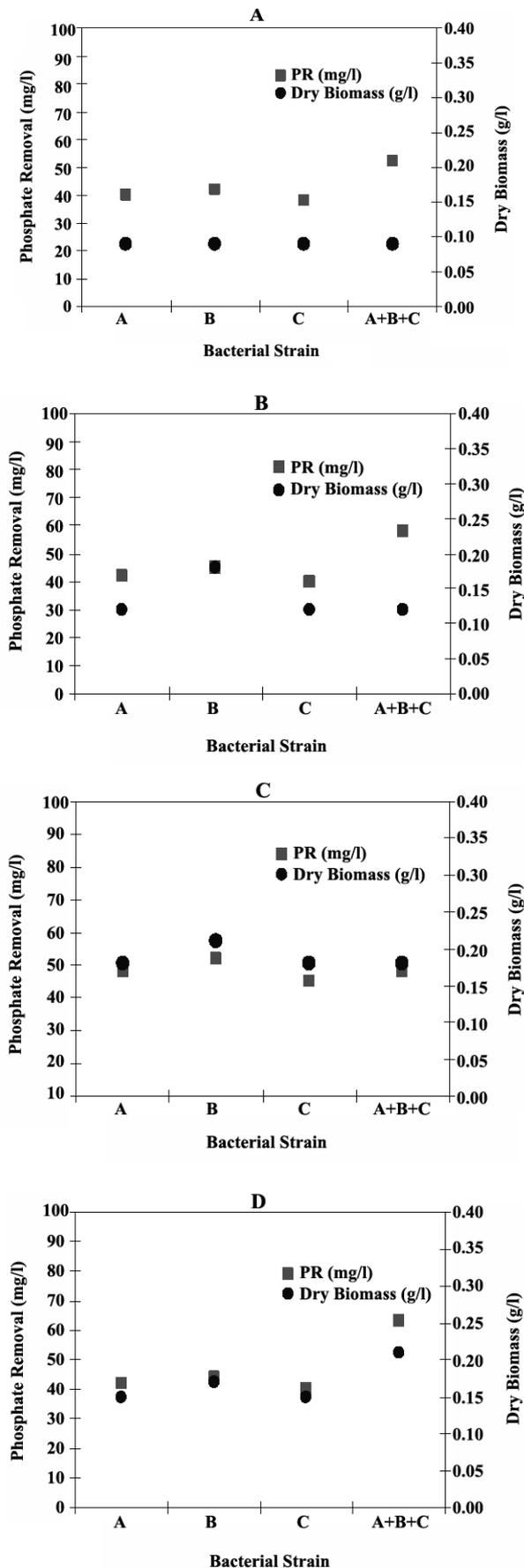


Figure 10. Phosphate removal versus growth of bacteria in synthetic phosphate solution ('A' - *Bacillus* sp. RS-1, 'B' - *Pseudomonas* sp. YLW-7, 'C' - *Enterobacter* sp. K LW-2 and 'A+B+C' - Consortium) [A: sucrose, B: starch, C: glucose, D: lactose and E: control].

MSM with 0.5% lactose. The bacterial consortium of A+B+C combination showed maximum phosphate removal of 92.5%. Phosphate removal was observed to be higher when the bacterial biomass (OD) increased from 24-72h after that the exponential growth phase of bacteria was started and there was no increase in the phosphate removal. The phosphate was taken up by cells for growth and to reform polyphosphate under aerobic condition. Increase in biomass concentrations showed a greater phosphate uptake capacity. This was attributed to an increase in the nutrient utilization rate of the polyphosphate organisms. Torriani-Gorini (1987) observed that the genes and proteins of microbial cells involved in the hydrolysis of organic phosphates. Some microorganisms can accumulate phosphate as polyphosphate (Kornberg, 1995) and (Keasling and Hupf, 1996). These microbes play a central role in the natural phosphorus cycle on a global scale. Biological phosphorus removal is based on the principle that, given optimal conditions, some heterotrophic bacteria are able to remove solubilized phosphates by accumulating them intracellularly in the form of polyphosphates. These bacteria use the stored carbon reserves to produce energy for growth and to replenish their stores of polyphosphate. The result is a net removal of phosphate from the wastewater.

The results showed that the strain could grow rapidly and remove phosphate efficiently in MSM when compared to synthetic phosphate solution with 0.5% carbon source. This may be the influence of various mineral salts (magnesium sulphate, sodium acetate, potassium nitrate) present in MSM when compared to

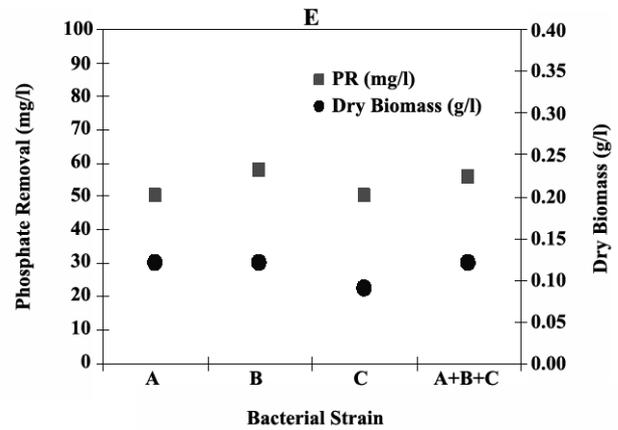
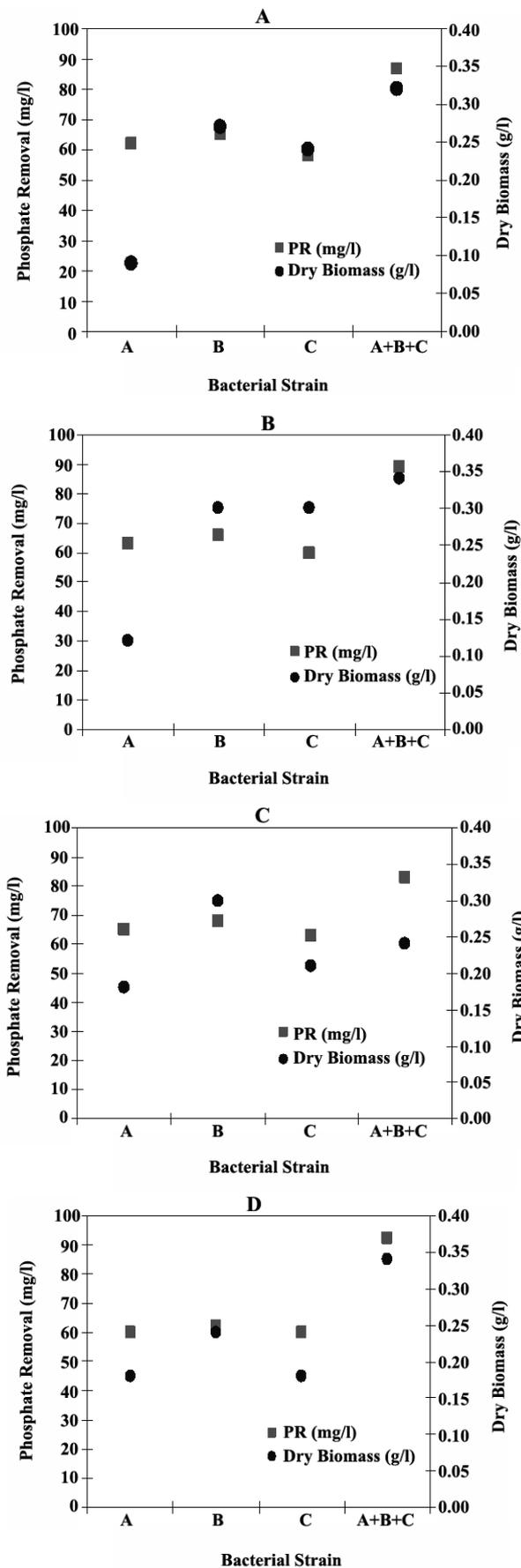


Figure 11. Phosphate removal versus growth of bacteria in Mineral salts medium (MSM). ('A'-*Bacillus* sp. RS-1, 'B'-*Pseudomonas* sp. YLW-7, 'C'-*Enterobacter* sp. K LW-2 and 'A+B+C' -Consortium) [A: sucrose, B: starch, C: glucose, D: lactose and E: control].

synthetic phosphate solution. The MSM medium enriched with carbon source at the experimental concentration greatly influenced the growth of bacteria and enhances the efficiency of phosphate removal.

CONCLUSIONS

In conclusion, the plate screening method of minimum inhibitory concentration (MIC) test and shake flask culture study performed for analysing growth, pH change and change in total phosphate concentration after biological treatment were proved to be effective, easy and reliable method of screening the phosphate reducing cultures. The results from this study indicates that the mineral salts medium with carbon sources showed maximum phosphate removal when compared to synthetic phosphate solution (with and without carbon and other nutrient sources). The bacterial consortium (*Bacillus* sp., *Pseudomonas* sp. and *Enterobacter* sp.) used in this study efficiently removed the phosphate. The phosphate could be reduced below the permissible limit as prescribed by Environmental Protection Agency (EPA, 1991) within 72 h using lactose carbon source and could be useful to remediate waste water containing phosphate. The efficient removal of phosphate by the consortium may due to the synergistic activity among the individual strains. The various mineral salts present in the MSM may influence the growth of the phosphate reducers and utilize the phosphate compound when compared to synthetic phosphate solution. Therefore, the mineral salts medium with carbon source support the removal of

phosphate at higher level. Thus, the simple method of phosphate removal is possible by microbial strains (viz., *Bacillus* sp. RS-1, *Pseudomonas* sp. YLW-7 and *Enterobacter* sp. KLV-2) and they may use the contaminants as nutrient and as energy source or it may be degraded by co-metabolism. Hence the bacterial consortium could be used in the remediation of phosphate contaminated environments.

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