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REMOVAL OF PHOSPHATE FROM RHIZOSPHERE SOIL USING *Bacillus subtilis* AND *Enterobacter aerogenes*

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Abstract: The addition of phosphorus is one of the major environmental problems because of its leading contribution to the increased eutrophication process of lakes and other natural waters. The eutrophication is the process where excessive nutrients in a lake or other body of water usually caused by runoff of nutrients (animal waste, fertilizers, and sewage) from the land which causes a dense growth of plant life, the decomposition of the plants depletes the supply of oxygen which leads to the death of animal life. Microbial process is widely used for the removal of phosphorus from soil and wastewater to avoid eutrophication. The most efficient phosphate reducers chosen were namely *Bacillus subtilis* and *Enterobacter aerogenes*. The Mineral Salt Medium and the carbon sources (glucose, sucrose, lactose and starch) at 0.5% and 0.7% were prepared. On the removal of phosphate by *Bacillus subtilis* and *Enterobacter aerogenes* it was found that the *Bacillus subtilis* was giving the maximum bacterial growth and was observed to be in lactose 0.107 OD at 0.7% concentration for 72th hour. In the case of *Enterobacter aerogenes* the maximum bacterial growth was found to be in sucrose 0.133 OD at 0.7% concentration at 72 hr. The pH change in the medium was found to be in both the isolates with different carbon sources but in overall the constant pH was at 7. Among the two organisms, *Bacillus subtilis* showed the maximum removal of phosphate 83% as starch as carbon source at 0.5% concentration whereas *Enterobacter aerogenes* showed 77.4% of phosphate removal at 0.5% concentration as glucose as carbon source. Therefore, these bacterial isolates can be used in the remediation of phosphate contaminated environments.

Keywords: Mineral Salt Medium; Phosphate reducers; Phosphate removal; Rhizosphere soil.

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INTRODUCTION

Phosphorus is a naturally occurring element that can be found in the earth's crust, water and all living organisms. Phosphorus is one of 16 elements that are essential for plant growth. Soils in Virginia are naturally low in phosphorus and most cropping systems on these soils require supplemental phosphorus to maximize their yield potential. Research has documented that applying fertilizer phosphorus increases crop growth and yields on soils that are naturally low in phosphorus and in soils that have been depleted through crop removal. Crop fertilization represents the greatest use of phosphorus in agriculture today. Excessive soil phosphorus is a potential threat to water quality (Cokgor EU *et al*., 2004).Phosphorus lost from agricultural soils can increase the fertility status of natural waters (eutrophication), which can accelerate the growth of algae and other aquatic plants. Phosphorus is usually the nutrient that controls eutrophication of fresh waters (Mullan *et al.,* 2002). The USEPA has recommended a limit for controlling eutrophication of 0.05 ppm for total phosphorus in streams that enter lakes and 0.1 ppm for total phosphorus in flowing streams. Acceptable levels of phosphorus in surface runoff from agricultural fields have not been established. Numerous water quality problems have been associated with eutrophication. Algal blooms can cause fish kills and may harm wildlife and livestock by reducing the oxygen content of water (anoxia) or

through the production of toxins (Reyes *et al.*, 1999). Lakes may become dominated by algae and coarse, rapidly-growing fish while high value edible fish, submerged macrophytes and benthic organisms disappear. Eutrophication can result in increased cost and difficulty of drinking water purification. Decaying algal biomass produces surface scums, odors, and increased populations of insect pests.

Phosphorus - An Overview

Phosphorus is recognized as one of the major nutrients required by the living organisms involved in various physiological processes. However, it can also be considered as pollutant if the concentrations are high under specific environmental condition 4 (Malacinski *et al.*,1967). The addition of phosphorus as phosphate ion is one of the most serious problems because of its contribution to the increased eutrophication process of lake and other natural waters. The possible entry of this ion into aquatic environment is through household sewage water and industrial effluents particularly fertilizers and soap industries (Mullan*et al.,* 2001). The main source of phosphorus into the environment includes fertilizers, detergents etc. Microbial strategies for the removal of environmental pollutants from waste streams or contaminated site can provide an alternative to traditional methods such as incineration or disposal in landfills (Usharani *et al.,* 2009). Phosphorus participates in many of the reactions that keep plants and animals alive and is essential for all living organisms. All living plants and animals require phosphorus. Phosphorus containing compounds are essential for photosynthesis in plants for energy transformations and for the activity of some hormones in both plants and animals (Tobin *et al.,* 2007). They occur in cell membranes and calcium phosphate hydroxyapatite, Ca_{10} (PO_4) OH is the principle component of bones and teeth. Phosphorous is found in two different forms in soil inorganic and organic phosphorus.

Inorganic Phosphorus: The main inorganic forms of phosphorus in soil are H_2PO_4- and HPO_4.This is the form in which phosphorus is used by plants. However, these ions can also absorb onto the surface (or adsorb into) solid matter in the soil. This phosphorus is then unavailable to plants (Asea*et al.,* 1988).

Organic Phosphorus: Between 50% and 80% of phosphorus in soil is organic phosphorus. This comes from the breakdown of dead plants etc., as phosphorus is found in cell membranes and DNA in living organisms. Phosphorus is thus naturally available in the soil (Van Loosdrecht *et al.,* 1997). However, there isn't usually enough available for plants to grow well. Phosphorus levels are reduced by animals eating the plants then dying elsewhere so that the phosphorus is removed, and also by phosphorus being absorbed into soil particles or washed away by excess rain. For this reason phosphate fertilizers are widely used (Filipe *et al.,* 2001).

Inorganic Phosphorus in Soil: Two types of inorganic reactions control the concentration of phosphate ions in solution and these are precipitation-dissolution and sorption-desorption processes. Precipitation dissolution reactions involve the formation and dissolving of precipitates. Sorption desorption reactions involve sorption and desorption of ions and molecules from the surfaces of mineral particles (Keasling *et al.,* 1996). The role of biological immobilization-decomposition will be dealt with in the section of organic phosphates. The movement of phosphate into plants also influences soil solution concentrations and promotes dissolution and desorption reactions. Soils contain a range of crystalline and near-amorphous minerals in clay-sized particles (<2 μm diameter). These are combined with an equally wide range of poorly characterized organic compounds which modify both the chemical and physical properties of the clays (Kim *et al.,* 1998). As a result, much research was done on the reactions between pure samples of individual soil components and phosphate solutions. Hydrous iron and aluminium oxides and alumino silicates occur widely in soils. They will react with phosphate solutions to produce an isomorphous series of iron and aluminium phosphates, strengite-barrandite-variscite $AlPO_4.2H_2O$- (AlFe) $PO_4.2H2O$ - $FePO_4.2H_2O$. While these materials have been identified by X-ray diffraction in laboratory experiments they have not been seen in natural soils. This was assumed to be due to the very small size of their crystals.

Organic Phosphorus in Soil: It consists largely of carbon, oxygen, hydrogen, nitrogen, sulphur and phosphorus. When soils develop or when virgin or arable soils are put under permanent pasture, their organic matter content generally increases. While organic nitrogen and sulphur components increase to equilibrium values within a relatively short period of time (5-20 years), organic phosphate compounds

appear to accumulate for much longer. As a result, the phosphorus content of soil organic matter is much more variable than its carbon, nitrogen or sulphur contents. This has led to the suggestion that organic phosphates can be divided into two fractions; one in association with carbon, nitrogen and sulphur in soil humus and the other as independent organic phosphate compounds. However it is likely that the individual organic phosphate compounds identified in extracts are combined into complexes of high molecular weights in soils and that a continuum exists over the range of organic phosphate compounds in soils.

Rhizosphere Soil- An Overview

The rhizosphere is an environment that the plant itself helps to create and where pathogenic and beneficial microorganisms constitute a major influential force on plant growth and health. Microbial groups and other agents found in the rhizosphere include bacteria, fungi, nematodes, protozoa, algae and micro arthropods. Many members of this community have a neutral effect on the plant, but are part of the complex food web that utilizes the large amount of carbon that is fixed by the plant and released into the rhizosphere. The microbial community in the rhizosphere also harbors members that exert deleterious or beneficial effects on the plant. Microorganisms that adversely affect plant growth and health are the pathogenic fungi, bacteria and nematodes, whereas microorganisms that are beneficial include nitrogen-fixing bacteria (Illmer *et al.,*1995). The number and diversity of deleterious and beneficial microorganisms are related to the quantity and quality of the Rhizosphere deposits and to the outcome of the microbial interactions that occur in the Rhizosphere (Kundu *et al.,* 1984). Understanding the processes that determine the composition, dynamics and activity of the rhizosphere micro flora has attracted the interest of scientists from multiple disciplines and can be exploited for the development of new strategies to promote plant growth and health. The rhizosphere environment generally has a lower pH, lower oxygen and higher carbon dioxide concentrations Rhizosphere microorganisms produce vitamins, antibiotics, plant hormones and communication molecules that all encourage plant growth (Illmer*et al.,* 1992).

Bacillus subtilis: *Bacillus subtilis*, also known as the hay bacillus or grass bacillus, is a Gram-positive, catalase-positive bacterium. *B. subtilis* is rod-shaped and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions (Zuber*et al.,*1998).Although this species is commonly found in soil, more evidence suggests that *B. subtilis* is a normal gut commensal in humans. A 2009 study compared the density of spores found in soil (~10^6 spores per gram) to that found in human feces (~10^4 spores per gram). The number of spores found in the human gut is too high to be attributed solely to consumption through food contamination (Liu*et al.*, 2007). Soil simply serves as a reservoir, suggesting that *B. subtilis* inhabits the gut and should be considered as a normal gut commensal.

Enterobacter aerogenes: *E. aerogenes* are smaller, rod-shaped cells that are motile and encapsulated compared to others in the same family of *Enterobacteriaceae*. *Enterbacter aerogenes* is a gram-negative, rod shaped bacterium that contains flagella surrounding its outer surface. *Enterobacter* are found in the soil, water, dairy products and in the intestines of animals as well as humans. They are most frequently found in the gastrointestinal tract and are studied in clinical sites in stool samples. The minimum, optimum and maximum pH for *E. aerogenes* replication is 4.4, 6.0-7.0, and 9.0. *E. aerogenes* is resistant to most antibiotics, including chloramphenicol, quinolones and tetracycline (Jeon*et al.,* 2000).

EXPERIMENTAL

Sample Collection: The samples were ordered from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology, Chandigarh which was isolated from Rhizosphere soil. The samples chosen were *Bacillus subtilis* (8558) and *Enterobacter aerogenes* (7193).

Preparation of Inoculum: Nutrient broth was prepared and samples of *Bacillus subtilis*, *Enterobacter aerogenes* were inoculated separately and incubated for 24th hour. Cells were recovered using centrifugation at 10,000 RPM for 15 min under aseptic conditions. After centrifugation the cell concentration were adjusted to 0.1 OD and the optical density was checked at 600nm using sterile saline.

Experimental Study:A Minimal Salt Medium (MSM) was prepared for further analysis of phosphate reducers. The media was prepared which contained 0.1g of KH_2PO_4 with two different concentrations

(0.5% and 0.7%) of carbon sources which included glucose, sucrose, lactose and starch. The medium was sterilized at 121ºCfor 15 min in an autoclave. The 0.1 OD adjusted isolates were inoculated in each flask and kept in shaker flask (150 RPM) at room temperature for the period of three days. For every 24th, 48thand 72th hour readings were taken on pH, bacterial growth and total phosphate concentration of the medium.

Analytical Methods

Growth of Bacteria and pH: The growth of the bacteria was monitored for every 24 hour by measuring OD at 600nm and the pH in the medium was also monitored for every 24 hour using pH strips.

Estimation of Phosphate: The phosphate uptake efficiency was quantified using stannous chloride colorimetric method. The soluble phosphate content was estimated after 24, 48 and 72 hour of incubation. After every 24th hour 10ml of agitated sample was taken in centrifuge tubes under aseptic condition. Then the tubes containing sample were centrifuged at 10,000 RPM for 15min and the clear supernatant was used for estimation. The estimation was done using optical density at 690nm.

Phosphate uptake efficiency was calculated using the following formula:

$$E = [(I-F)/ I] \times 100$$

Whereas, I and F are the initial and final concentrations of phosphorus respectively.

RESULTS AND DISCUSSION

The inoculated microbial cultures were chosen further with two different concentrations of 0.5% and 0.7%. The parameters used for the removal of phosphate included estimation of phosphate, pH, bacterial growth and phosphate removal percentage.

Table 1.Estimation of Phosphate in *Bacillus subtilis*

24th hour	0.686
48th hour	0.802
72th hour	1.104

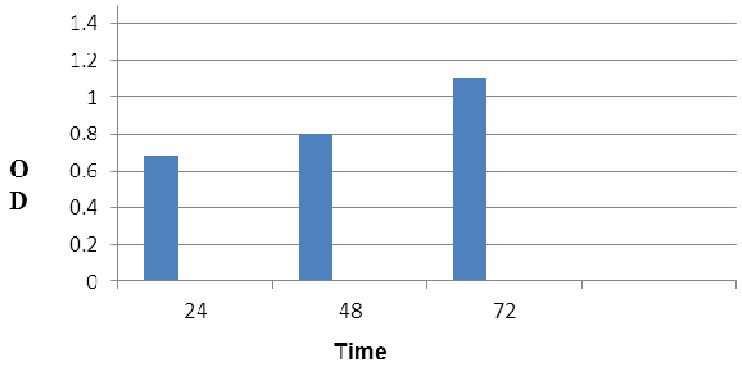

Figure 1. Estimation of Phosphate in *Bacillus subtilis*

The estimation of phosphate was performed for every 24 hours and an increase in OD was observed for every 24 hours. The maximum phosphate presence was found to be at 72nd hour (Table 1, Figure 1).

Table 2: pH at 0.5% concentration in *Bacillus subtilis*

Carbon sources	24th hour	48th hour	72th hour
Sucrose	7	7	7
Glucose	5	6	7

Lactose	6	7	7
Starch	7	7	6
Control	7	7	7

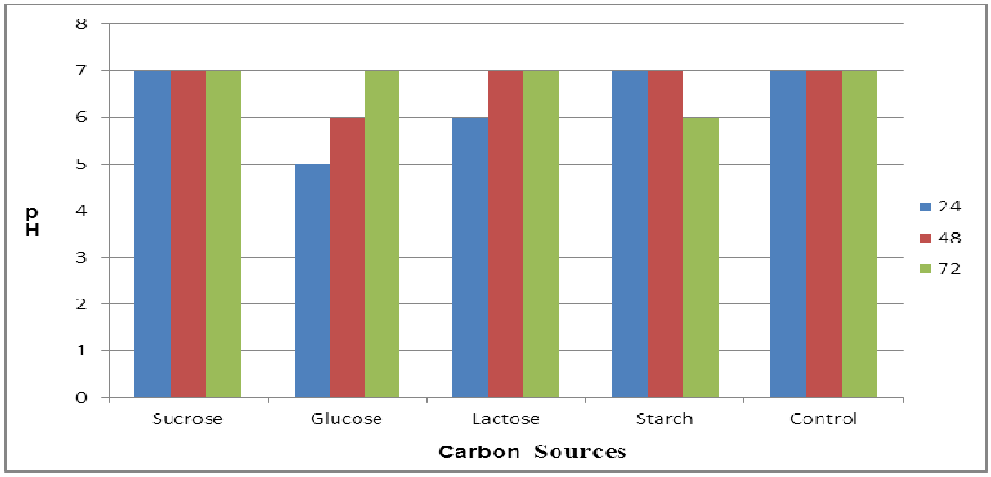

Figure 2: pH at 0.5% concentration in *Bacillus subtilis*

The changes were observed at different pH levels and with different carbon sources. At 0.5% concentration there was a drastic change in pH-5 for glucose at 24th hour, pH-6 was obtained for 48th hour and even in lactose the change was observed at pH- 6 for 24th hour and starch pH- 6 at 72nd hour. For carbon source the pH was remained constant at 7 (Table 2, Figure 2).

Table 3. pH at 0.7% concentration in *Bacillus subtilis*

Carbon Sources	24th hour	48th hour	72th hour
Sucrose	7	7	7
Glucose	5	6	7
Lactose	7	7	6
Starch	7	7	7
Control	7	7	7

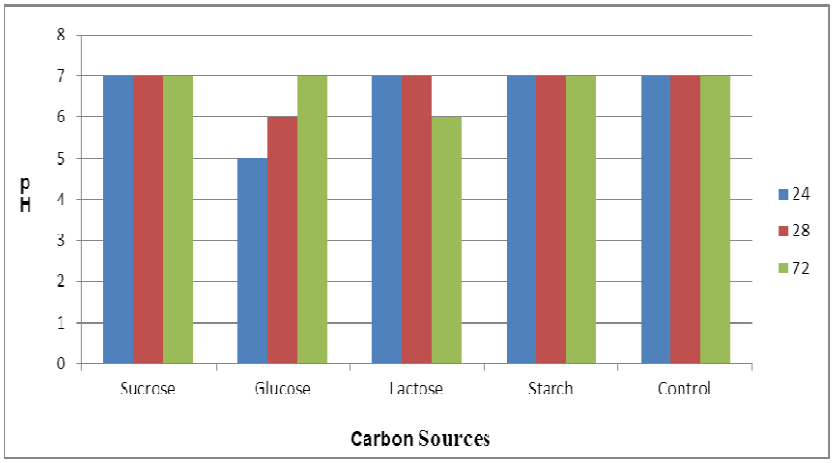

Figure 3. pH at 0.7% concentration in *Bacillus subtilis*

At 0.7% concentration, the change obtained for glucose was same as 0.5% concentration in *Bacillus subtilis*. At 0.5% concentration in *Bacillus subtilis*, starch showed the change at pH-6 in 72nd hour and in 0.7% concentration it showed pH-7. At 0.5% concentration in *Bacillus subtilis*, lactose showed the change at pH-6 in 24th hour and in 0.7% concentration it showed pH-7, whereas other carbon sources showed a constant pH-7 (Table 3, Figure 3).

Table 4. Bacterial growth at 0.5% concentration in *Bacillus subtilis*

Carbon Sources	24th hour (OD Concentration)	48th hour (OD Concentration)	72th hour (OD Concentration)
Sucrose	0.057	0.064	0.072
Glucose	0.063	0.077	0.089
Lactose	0.076	0.089	0.098
Starch	0.067	0.074	0.081
Control	0.004	0.012	0.023

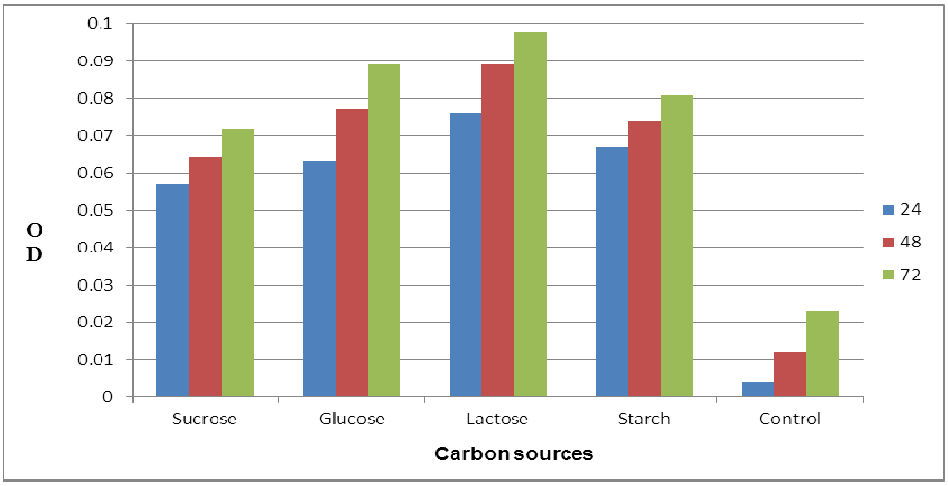

Figure 4. Bacterial growth at 0.5% concentration in *Bacillus subtilis*

For each 24th hour the growth was observed to be high. Among all the carbon sources, lactose showed the maximum bacterial growth of 0.098 OD at 72th hour (Table 4, Figure 4).

Table 5: Bacterial growth at 0.7% concentration in *Bacillus subtilis*:

Carbon Sources	24th hour (OD Concentration)	48th hour (OD Concentration)	72th hour (OD Concentration)
Sucrose	0.067	0.078	0.087
Glucose	0.070	0.083	0.094
Lactose	0.081	0.094	0.107
Starch	0.068	0.076	0.085
Control	0.010	0.017	0.023

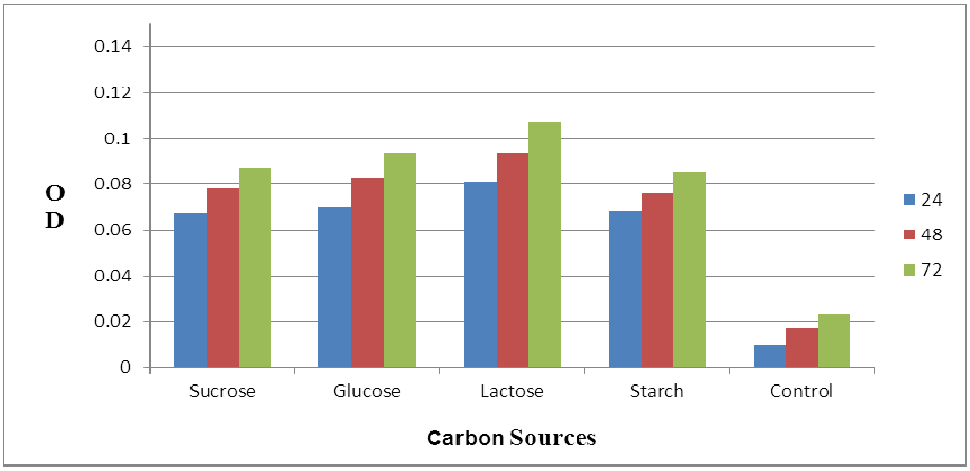

Figure 5: Bacterial growth at 0.7% concentration in *Bacillus subtilis*

At 0.7% concentration, the growth was going high for every 24th hr. Here lactose showed the maximum bacterial growth at 0.107 for 72nd hr (Table 5, Figure 5).

Table 6. Phosphate removal percentage at 0.5% concentration in *Bacillus subtilis*

Carbon Sources	24th hour (%)	48th hour (%)	72th hour (%)
Sucrose	75.8	77.8	83
Glucose	75.3	77.4	82.6
Lactose	71.2	73.8	80.5
Starch	74	76.5	82.1
Control	55	60	65.7

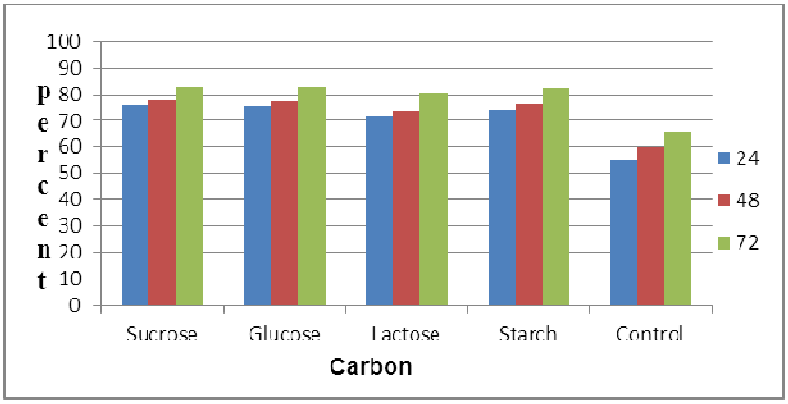

Figure 6. Phosphate Removal percentage at 0.5% concentration in *Bacillus subtilis*

In 0.5% concentration, phosphate removal percentage was the maximum in sucrose 83% in 72nd hr (Table 6, Figure 6).

Table 7. Phosphate Removal Percentage at 0.7% concentration in *Bacillussubtilis*

Carbon Sources	24th hour (%)	48th hour (%)	72th hour (%)

Sucrose	74.6	77.4	82.5
Glucose	74.0	76.4	82.2
Lactose	70.4	72.9	79.6
Starch	73.3	76.1	82.1
Control	39.2	47	60.9

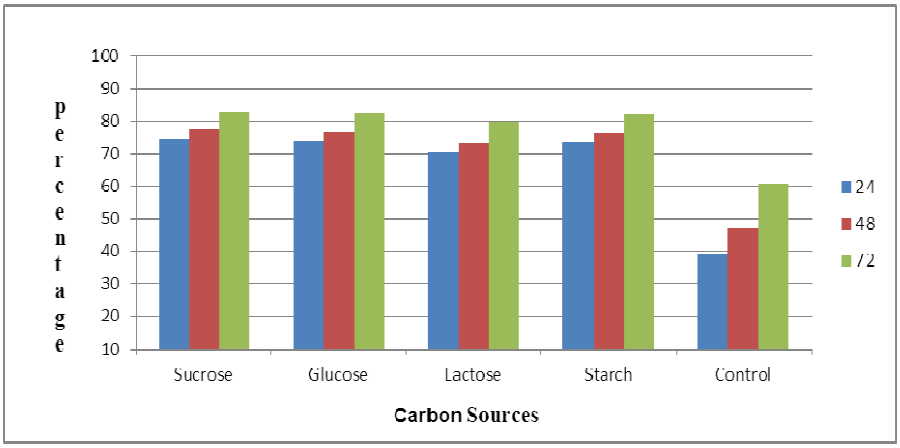

Figure 7. Phosphate Removal percentage at 0.5% concentration in *Bacillus subtilis*

In 0.7% concentration, phosphate removal percentage was the maximum in sucrose 82.5% in 72nd hour (Table 7, Figure 7).

Table 8: Estimation of Phosphate in *Enterobacter aerogenes*

24th hour	0.536
48th hour	0.603
72th hour	0.726

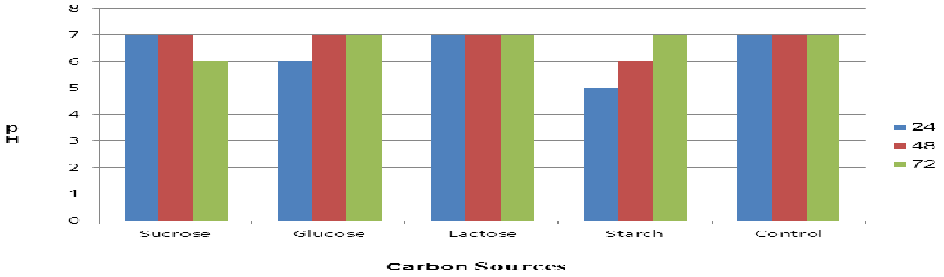

Figure 8. Estimation of Phosphate in *Enterobacter aerogenes*

The Estimation of Phosphate was performed for every 24th hour. The phosphate presence was increasing for every 24th hour. The maximum phosphate presence was found in 72th hour (Table 8, Figure 8).

Table 9. pH at 0.5% concentration in *Enterobacter aerogenes*

Carbon Sources	24th hour	48th hour	72th hour
Sucrose	7	7	6
Glucose	6	7	7
Lactose	7	7	7
Starch	5	6	6
Control	7	7	7

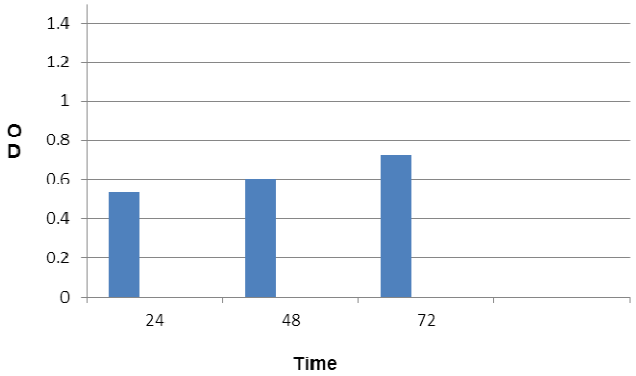

Figure 9. pH at 0.5% concentration in *Enterobacter aerogenes*

At 0.5% concentration, starch showed a minimum pH of 5 in 24th hour followed by pH-6 in 48th and 72th hour. Glucose showed pH-6 in 24th hour and sucrose showed pH-6 in 72th hour. The overall other carbon sources showed a constant pH-7 (Table 9, Figure 9).

Table 10. pH at 0.7% concentration in *Enterobacter aerogenes*

Carbon Sources	24th hour	48th hour	72th hour
Sucrose	6	7	7
Glucose	7	7	6
Lactose	7	6	7
Starch	7	7	7
Control	7	7	7

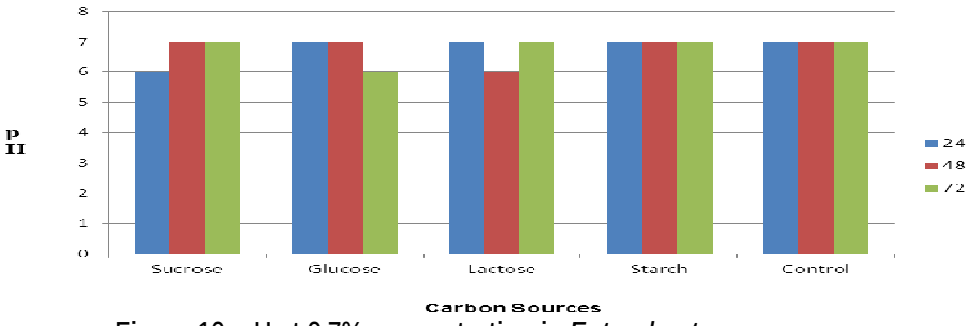

Figure 10. pH at 0.7% concentration in *Enterobacter aerogenes*

At 0.7% concentration, sucrose showed a change of pH in 24th hour, glucose in 72th hour and lactose in 48th hour, other carbon sources showed pH-7 (Table 10, Figure 10). In 0.5% concentration starch showed the maximum growth in 72nd hour (Table 11, Figure 11).

Table 11. Bacterial Growth at 0.5% concentration in *Enterobacter aerogenes*

Carbon Sources	24th hour (OD concentration)	48th hour (OD concentration)	72th hour (OD concentration)
Sucrose	0.078	0.083	0.097
Glucose	0.085	0.097	0.117
Lactose	0.067	0.077	0.085
Starch	0.091	0.104	0.123
Control	0.007	0.017	0.027

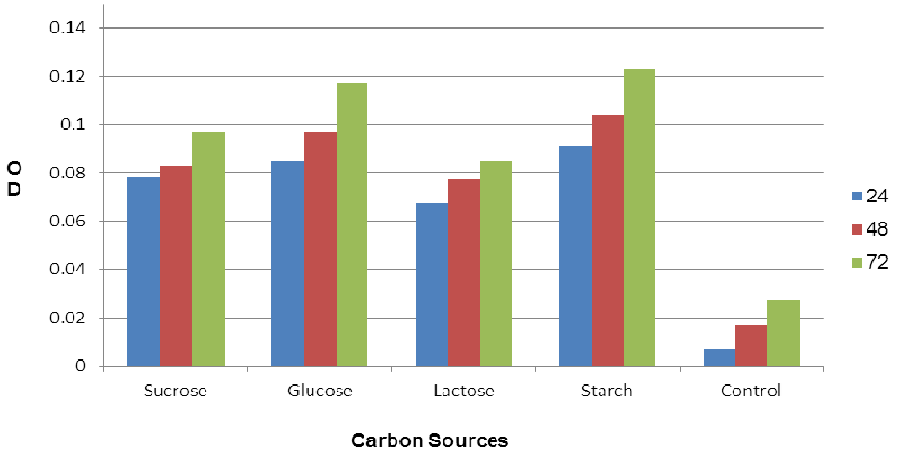

Figure 11. Bacterial Growth at 0.5% concentration in *Enterobacter aerogenes*

Table 12. Bacterial Growth at 0.7% concentration in *Enterobacter aerogenes*

Carbon Sources	24th hour (OD concentration)	48th hour (OD concentration)	72th hour (OD concentration)
Sucrose	0.081	0.092	0.133
Glucose	0.088	0.098	0.142
Lactose	0.073	0.087	0.093
Starch	0.095	0.113	0.127
Control	0.013	0.027	0.033

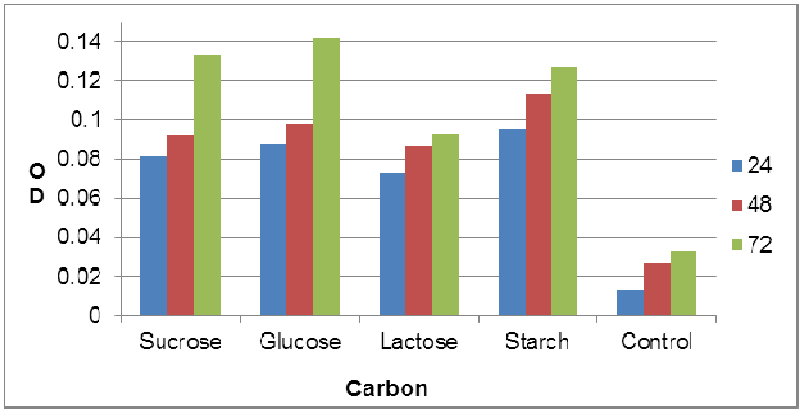

Figure 12. Bacterial Growth at 0.7% concentration in *Enterobacter aerogenes*

In 0.7% concentration glucose showed the maximum growth in 72th hour (Table 12, Figure 12). In 0.5% concentration, glucose showed the maximum phosphate removal of 77.4% (Table 13, Figure 13). In 0.7% concentration, glucose showed the maximum phosphate removal of 74.7% (Table 14, Figure 14).

Table 13. Phosphate Removal Percentage at 0.5% concentration in *Enterobacter aerogenes*

Carbon Sources	24th hour	48th hour	72th hour
Sucrose	67.7	69.9	72.8
Glucose	73.1	74.6	77.4
Lactose	69.5	70.4	73.9
Starch	63.9	66.3	70.1
Control	39.7	45.4	53.5

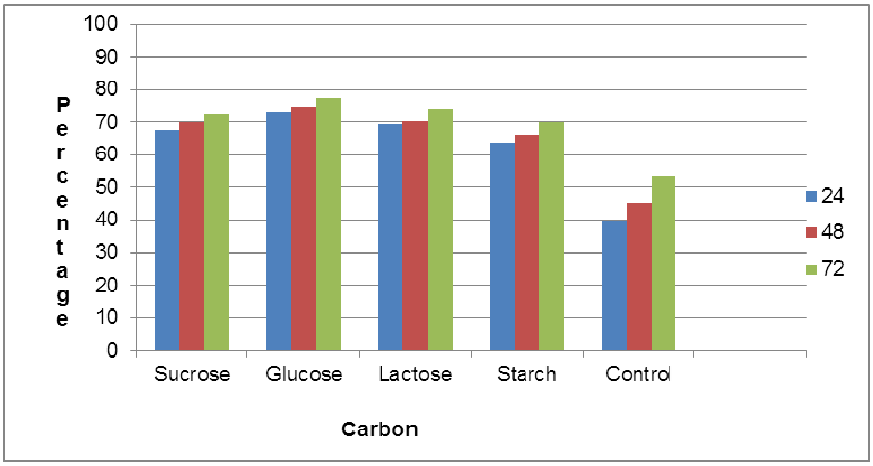

Figure 13. Phosphate Removal Percentage at 0.5% concentration in *Enterobacter aerogenes*

Table 14. Phosphate Removal Percentage at 0.7% concentration in *Enterobacter aerogenes*

Carbon Sources	24th hour	48th hour	72th hour
Sucrose	65.8	67.4	71.4
Glucose	71.4	73.3	74.7

Lactose	66.9	68.8	72
Starch	60.6	63.1	67.2
Control	37.1	43.6	41.3

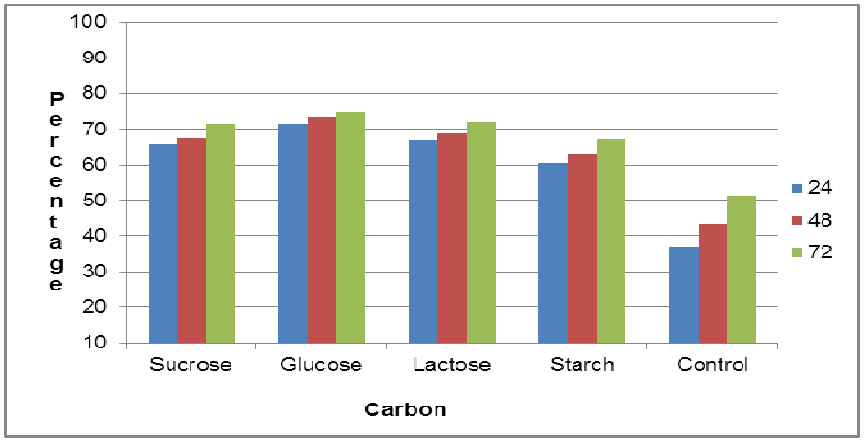

Figure 14. Phosphate Removal Percentage at 0.7% concentration in *Enterobacter aerogenes*

CONCLUSION

In present scenario Phosphorus is considered to be the one of the major environmental problems because of its leading contribution to eutrophication process. Microbial strategy was used to remove phosphorus from the rhizosphere soil. Totally two efficient phosphate reducers were ordered from microbial type culture collection which was isolated from rhizosphere soil. The two isolates were *Bacillus subtilis* and *Enterobacter aerogenes.* A Mineral Salt Medium was prepared along with the carbon sources (glucose, sucrose, lactose, starch) at 0.5% and 0.7% concentration. The pH change of the medium was found to be in both the isolates with different carbon sources, overall the constant pH was 7. Among the two organisms, *Bacillus subtilis* showed the maximum removal of phosphate 83% as sucrose as carbon source at 0.5% concentration whereas, *Enterobacter aerogenes* showed 77.4% of phosphate removal at 0.5% concentration as glucose as carbon source. From this study the maximum phosphate removal was found at 0.5% concentration. Sucrose as carbon source at 0.5% concentration in *Bacillus subtilis* showed a maximum removal of phosphate 83%. Glucose as carbon source at 0.5% concentration in *Enterobacter aerogenes* showed a maximum removal of phosphate 77.4%.Therefore, these bacterial isolates can be used for the removal of phosphate from rhizosphere soil.

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CONFLICT OF INTEREST : Nothing