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Biological Removal of Phosphate Using Phosphate Solubilizing Bacterial Consortium from Synthetic Wastewater: A Laboratory Scale

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Abstract

Biological phosphate removal is an important process having gained worldwide attention and widely used for removing phosphorus from wastewater. The present investigation was aimed to screen the efficient phosphate solubilizing bacterial isolates and used to remove phosphate from synthetic wastewater under shaking flasks conditions. *Pseudomonas sp. JPSB12, Enterobacter sp. TPSB20, Flavobacterium sp. TPSB23* and mixed bacterial consortium (*Pseudomonas sp. JPSB12+Enterobacter sp. TPSB20+Flavobacterium sp. TPSB23*) were used for the removal of phosphate. Among the individual strains, *Enterobacter sp. TPSB20* was removed maximum phosphate (61.75%) from synthetic wastewater in presence of glucose as a carbon source. The consortium was effectively removed phosphate (74.15-82.50%) in the synthetic wastewater when compared to individual strains. The pH changes in culture medium with time and extracellular phosphatase activity (acid and alkaline) were also investigated. The efficient removal of phosphate by the consortium may be due to the synergistic activity among the individual strains and phosphatase enzyme activity. The use of bacterial consortium in the remediation of phosphate contaminated aquatic environments has been discussed.

Keywords: phosphate removal; synthetic wastewater; phosphate solubilizing bacteria; bacterial consortium

1. Introduction

Phosphorus is one of the most essential nutrients biological growth and development (Hu *et al*., 2006). This element is an important component of different cellular structures, including cell membranes and nucleic acids (Razzaque, 2011). However, their presence in excess amount in receiving water bodies such as lakes and other natural waters is the leading cause of eutrophication (Lau *et al*., 1997; Trépanier *et al*., 2002). Eutrophication is one of the most serious environmental problems, resulting in depletion in dissolved oxygen, excessive growth of phototrophs, and appearance of foul tastes in drinking water and death of aquatic life due to the production of phycotoxins (Steyn *et al*., 1975; USGS, 1996; De-Bashan and Bashan, 2004).

Due to rapid industrialisation there has been an increase in the amount of effluent being disposed to natural water bodies. Major contaminants found in wastewater include toxic metals, suspended solids, volatile, biodegradable and recalcitrant organic compounds, plant nutrients (nitrogen and phosphorus), microbial pathogens and parasites (Bitton, 1994). The main sources of phosphorus released into the environment include household wastes, fertilizers, detergents, cleaning preparations, and boiler waters to which phosphates are added for treatment (Patnaik, 1997). It is postulated that only orthophosphate can be chemically precipitated. At the time of biological treatment, most of the organic phosphorus and polyphosphates are converted to the orthophosphate form (Krishnaswamy *et al*., 2009; Olaolu *et al*., 2013). Phosphate, which in water is generally present as polyphosphate and orthophosphate, is reported to cause digestive problems in humans when present in extremely high concentrations. The concentration of phosphate in water bodies vary from 0.005-10 mg/L depending on the source of phosphate near the water body. At concentrations above 1.0 mg/L, it is indicated to interfere with coagulation in water treatment plants (DebRoy *et al*., 2012). Phosphate toxicity causes a wide range of complications such as dehydration, hypotension, tachycardia, hyperpyrexia, cardiac arrest, coma and accelerated aging (Loughnan and Mullins, 1977; Sotos *et al*., 1977; Kuro-o, 2001).

Conventionally, several chemical processes have been employed for the removal of phosphates from wastewaters (Donnert and Salecker, 1999; Penetra *et al*., 1999). Biological treatment is a cost-effective, eco-friendly method and efficiency at lower levels of contamination for wastewater before being discharged into the streams and rivers (Van Loosdrecht *et al*., 1997; Srivastava and Majumder, 2008; Krishnaswamy *et al*., 2011). Currently, phosphates are biologically removed by wastewater treatment facilities by

absorption of dissolved organic phosphate, orthophosphate and polyphosphate by microorganisms, such as bacteria, yeast, protozoa, microalgae, and fungi which is generally known as Enhanced Biological Phosphorus Removal (EBPR) (Melasniemi and Hernesmaa, 2000; Ogbonna *et al*., 2000; Srivastava and Srivastava, 2006; Akpor and Momba, 2010; Adelani-Akande *et al*., 2014). 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 wastewater.

2. Materials and Methods

2.1. Sampling site

Water samples were collected from near the effluent discharge point of different industries, Hooghly district, West Bengal of the river Ganga.

2.2. Isolation, characterization and identification of phosphate solubilizing bacteria (PSB)

Water samples were aseptically transferred to the laboratory and serially diluted water samples were plated on Petridishes containing Pikovskaya's (PKV) agar medium consisting of ingredients in g/L: Glucose 10g; tri-calcium phosphate (TCP) 5g; sodium chloride 0.2g; potassium chloride 0.2g; ammonium sulphate 0.5g; magnesium sulphate 0.1g; yeast extract 0.5g; ferrous sulphate trace; manganese sulphate trace; agar agar 15 g; the pH was adjusted to 7.0±0.2 before sterilization (Pikovskaya, 1948) by pour plate technique and incubated at 28±2°C for 48-96h. The bacterial colonies showing clear zone around them were considered as phosphate solubilizing bacteria (PSB) (De Freitas *et al*., 1997). Physiological, morphological and biochemical tests of the selected PSB isolates were carried out for their identification as per the procedures outlined in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

2.3. Preparation of bacterial inoculum for phosphate removal experiment

Nutrient broth was prepared and selected bacterial isolates were inoculated separately and incubated for 24 h at 27°C. The cells were recovered by centrifugation (10,000 rpm for 15 min) and were transferred to sterilized 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 solubilizing bacterial strain (A, B and C) were used for the removal of phosphate. The mixed bacterial consortium (A+B+C) were prepared in combination from the three isolates, by adjusting the cell concentration of A, B and C to 0.1 of OD_{600}. The phosphate solubilizing bacteria isolated from the different sample source were given symbols based on the source of isolation; Jute mill effluent-*JPSB*, Thermal power plant effluent-*TPSB*.

2.4. Effect of carbon source on the growth of bacteria and measurement of pH of the medium

Phosphate removal was carried out in synthetic wastewater solution containing 0.5 g/L phosphate concentration and 0.5% of different carbon substrates such as glucose, sucrose, fructose and lactose were prepared. Then the medium containing Erlenmeyer's flasks were sterilized at 121°C and at 15 lbs for 15 min in an autoclave. One ml of inoculum (0.1 OD_{600}) from the selected phosphate solubilizers A, B, C and consortium of combination A+B+C were inoculated in the individual flasks and incubated at 27°C in a rotary shaker maintained at 120 rpm for a period of 72 h. Aliquot samples were aseptically taken at 0, 24, 48, 72 h and analyzed for growth of bacteria by measuring optical density (OD_{600}) by UV-visible spectrophotometer (Shimadzu UV-1601PC) and pH change using pH meter of synthetic wastewater. All experiments were carried out in triplicates.

2.5. Estimation of phosphate removal efficiency

The phosphate removal efficiency of different strains and consortium sample in the synthetic wastewater were measured after 72 h of incubation by ascorbic acid method (APHA, 2005). After 72 h, 10 ml of sample was taken from individual flask and centrifuged at 10,000 rpm for 15 min and the transparent supernatant was used for phosphate estimation by using UV-Vis spectrophotometer (Shimadzu UV-1601PC).

Phosphate uptake efficiency (E) was calculated using the following formula (Krishnaswamy *et al*., 2009),

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

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

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

2.6. Measurement of phosphatase activity

The individual bacterial strains and consortium sample were inoculated into Pikovskaya's broth containing 5 g/L of calcium phosphate as a sole P source

in Erlenmeyer's flasks. After inoculation, each flask were incubated for 72 h, the cultures were centrifuged at 10000 rpm for 15 min. The supernatants were used to measure extracellular phosphatase activity. Acid and alkaline phosphatases activities were analyzed (Leelahawonge and Pongsilp, 2009; Pantujit and Pongsilp, 2010). One ml of reaction buffer (citric acid/ sodium-citrate buffer, pH 5.0 for acid phosphatase estimation and Tris-HCl buffer, pH 9.0 for estimation of alkaline phosphatase) was mixed with 100 µl of p-nitrophenyl phosphate solution (0.6 mg/ml in diethanolamine), then 3 ml supernatant was added. Reaction mixtures was incubated at 37°C for 20 min and terminated by adding 1 ml of 3M NaOH. Distilled water was used in reaction instead of supernatants for the preparation of blanks. p-nitrophenyl phosphate hydrolysis was estimated by measuring the concentration of p-nitrophenol with a spectrophotometer at a wavelength of 405 nm. The concentration of p-nitrophenol was determined by comparison with a standard curve. The amount of enzyme (mg) required to liberate 1µ mole of p-nitrophenol per minute indicates one unit of enzyme activity (Tso and Chen, 1997).

2.7. Statistical analyses

All experiments were carried out in triplicate, and the results were expressed as the mean. Experimental data were analyzed by using the SPSS 13.0 software package.

Table 1. Morphological, physiological and biochemical characteristics of the isolates

Characters/tests	Bacterial isolates		
	Pseudomonas sp. JPSB12 (A)	*Enterobacter sp. TPSB20* (B)	*Flavobacterium sp. TPSB23* (C)
Cell shape	Rod	Rod	Rod
Gram reaction	-	-	-
Motility	+	+	+
Growth at 5% NaCl	+	+	-
Catalase	+	+	+
Oxidase	+	-	+
IMViC test			
Indole production	-	+	-
Methyl red	-	-	-
Voges-Proskauer	-	+	-
Citrate	+	+	-
Urease	-	-	-
H_2S production	-	-	+
NO_3^- reduction	-	+	+
Gelatine liquefaction	+	-	+
Starch hydrolysis	-	-	-
Hugh-Leiffson (O/F) reaction	O/F	O/F	-
Utilization of carbon source			
Glucose	+	+	+
Fructose	+	-	-
Sucrose	+	+	-
Raffinose	-	-	-
Cellobiose	-	-	-
Xylose	+	-	-
Mannitol	-	-	-
Sorbitol	-	-	-
Dulcitol	-	+	-

+ indicates presence or positive; - indicates absence or negative; O = Oxidation; F= Fermentation

3. Results and Discussion

The three potential PSB isolates were characterized with the help of morphological, physiological and biochemical tests. Based on these characters, the isolates were identified as genus *Pseudomonas sp., Enterobacter sp. and Flavobacterium sp.* (Table 1). These bacteria were well known identified as phosphate solubilizer by many researchers (Kim *et al*., 1998; Paul and Sinha, 2013).

The growth of the bacterial species and the phosphate removal efficiency was analyzed using different carbon sources in phosphate medium up to 72 h of incubation period (Fig. 1). The individual strain of *Pseudomonas sp. JPSB12* was observed a growth of 0.456 OD in lactose and minimum of 0.073 OD in fructose, *Enterobacter sp. TPSB20* showed a maximum growth of 0.176 OD in sucrose and minimum of 0.058 OD in lactose and *Flavobacterium sp. TPSB23* have shown a maximum growth of 0.162 OD in lactose and minimum of 0.084 OD in glucose. But the bacterial consortium showed maximum growth of 0.269 OD in the presence of lactose. The metabolism of phosphate by *Pseudomonas sp. JPSB12, Enterobacter sp. TPSB20, Flavobacterium sp. TPSB23* and bacterial consortium were indicated by a visible increase in growth (OD) with time. Initially, the growth was suppressed in presence of phosphate but after adaptation to phosphate it was grown rapidly exhibiting high growth rate.

In order to find out the relationship between metabolic activities and phosphate reduction, pH of the culture medium was monitored. The pH changes in culture medium with time were shown in Fig 2. In synthetic wastewater, the maximum reduction of pH by individual PSB strain and consortium was seen in the medium in presence of glucose. The *Pseudomonas sp. JPSB12* showed maximum reduction of pH from 7 to 3.68, in case of *Enterobacter sp. TPSB20* maximum reduction of pH was from 7 to 3.29, in case of *Flavobacterium sp. TPSB23* maximum pH reduction was from 7 to 3.75 whereas in case of consortium maximum pH reduced was from 7 to 3.33. Decreases

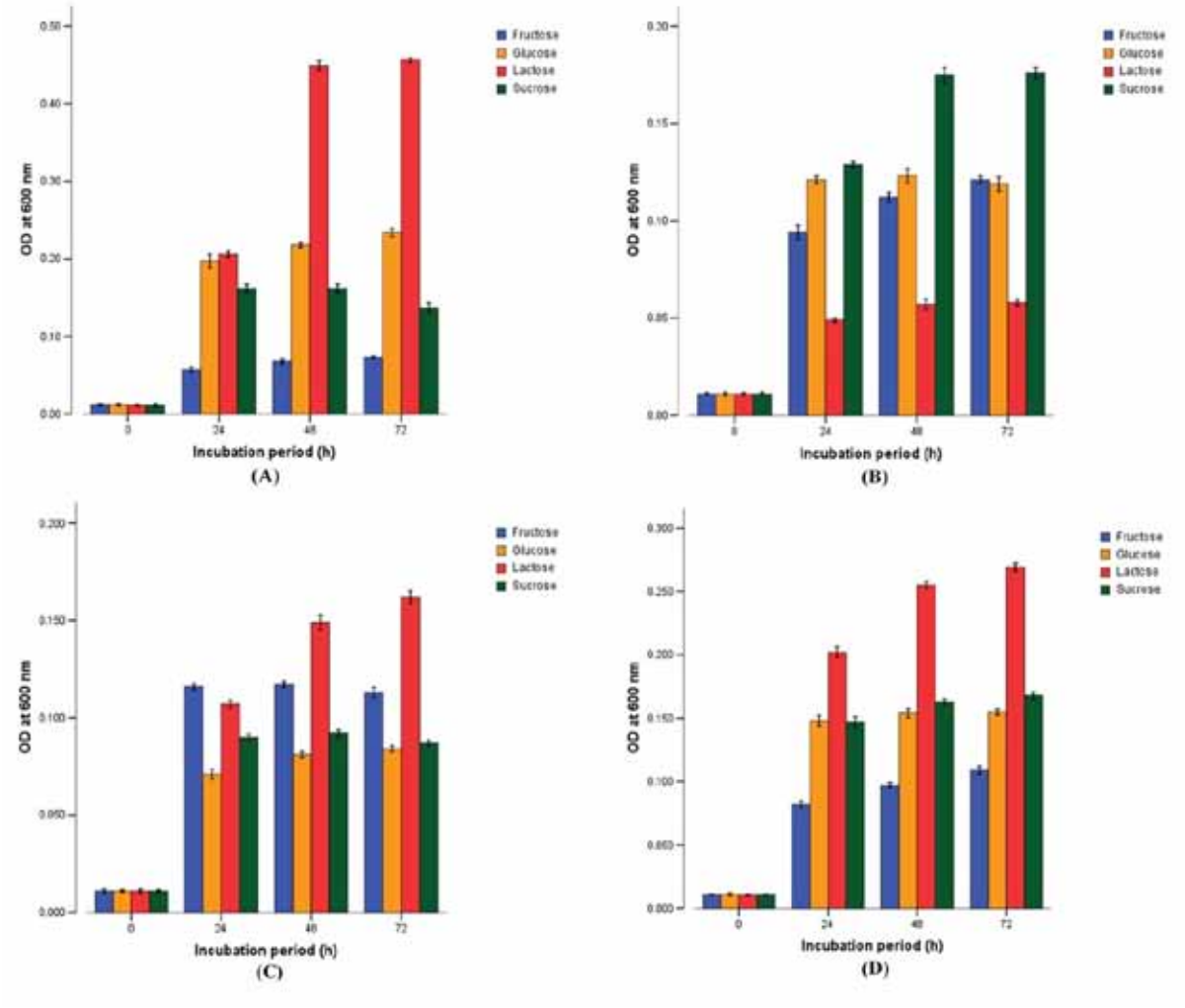

Figure 1. Effect of carbon sources on the growth of individual PSB strain and bacterial consortium in synthetic wastewater. (A) *Pseudomonas sp. JPSB12*; (B) *Enterobacter sp. TPSB20*; (C) *Flavobacterium sp. TPSB23*; (D) Bacterial consortium

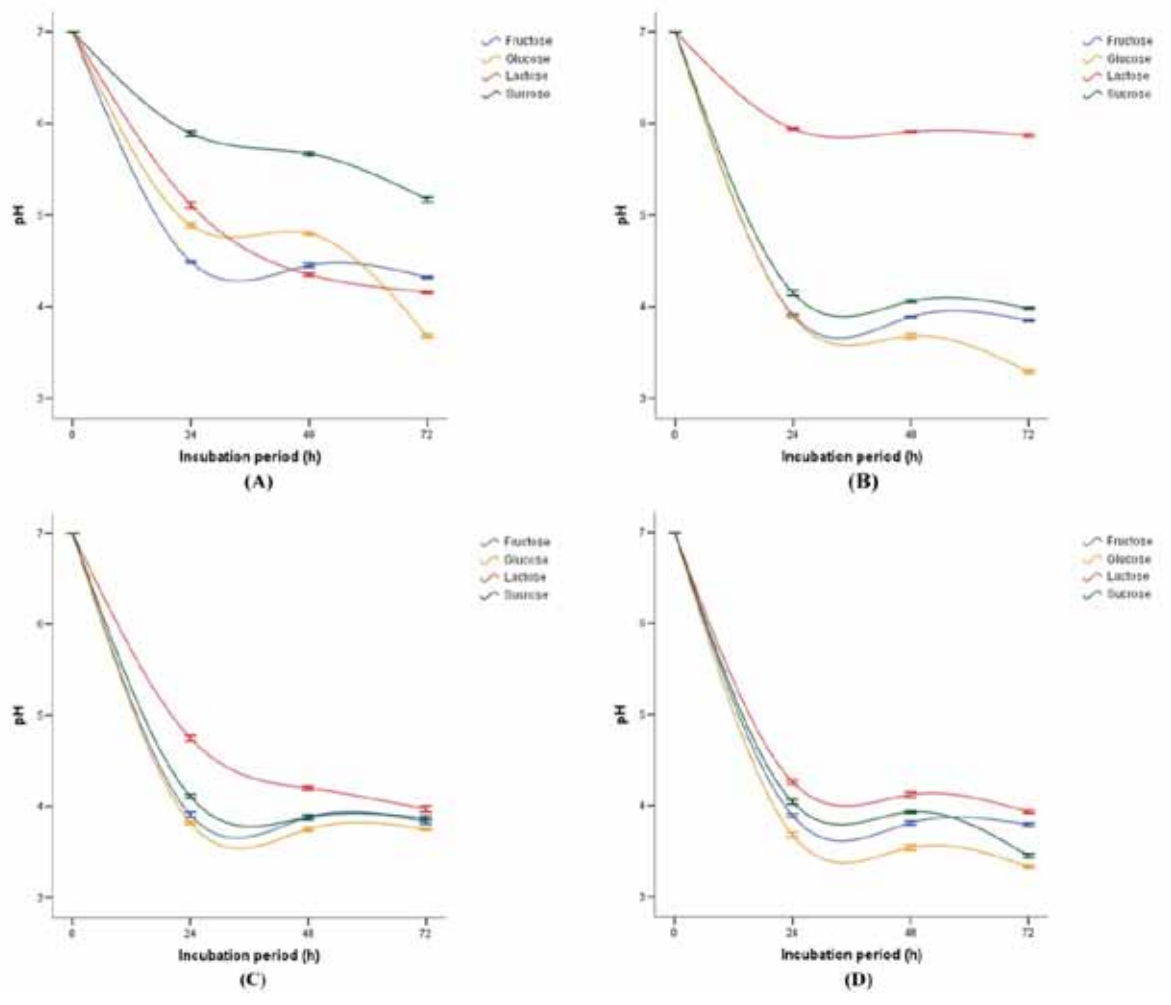

Figure 2. Change in pH of the culture medium during phosphate removal by individual PSB strain and bacterial consortium. (A) *Pseudomonas sp. JPSB12*; (B) *Enterobacter sp. TPSB20*; (C) *Flavobacterium sp. TPSB23*; (D) Bacterial consortium

in pH during nutrient studies have been reported by some investigators (Mullan *et al*., 2002; Krishnaswamy *et al*., 2011). The reduction in pH occurs may be due to production of various organic acids by phosphate solubilizers in the culture medium. Similar observations of the previous reports mentioned that phosphate solubilizing microbes produced various organic acids and consequent fall in pH of the medium (Whitelaw, 2000; Rashid *et al*., 2004; Chen *et al*., 2006). Reports suggested that the presence of organic acids released protons which involve biological ammonium assimilation that enhances utilization of phosphates (Illmer *et al*., 1995).

In synthetic wastewater, it was found to be maximum phosphate removal of 82.50% by the consortium where lactose was used as a carbon source and minimum of 74.15% phosphate removal in fructose's presence as shown in Table 2. But in the individual strain, maximum phosphate removal was 61.75% by *Enterobacter sp. TPSB20* where glucose was the carbon source. Carbon source enriched wastewater at the experimental concentration enhanced the phosphate removal and greatly influenced the bacterial growth. Carbon source like glucose is oxidized to gluconate, which is converted into other compounds, such as 2-keto-3-deoxygluconate, pyruvate or glyceraldehydes and the mechanism such as the release of protons associated with biological ammonium assimilation in the presence of organic acids that enhances the utilization of phosphates (Kim *et al*., 1998; Reyes *et al*., 1999). Several studies reported that glucose could induce good enhanced biological phosphate removal performance (Mino *et al*., 1998; Jeon and Park, 2000; Wang *et al*., 2002). The analysis of variance test indicated that the phosphate removal efficiency percentage among the bacterial species varied significantly (Table 3).

Phosphatase activities were measured in cell-free supernatants (extracellular enzyme) and it was calculated in mU/ml. Activities of individual strains and consortium are shown in Table 4. The individual strains produced extracellular acid phosphatases ranged between 6.86-8.32 in mU/ml and the alkaline phosphatase activity ranged between 4.54-5.64 mU/ml,

Table 2. Effect of carbon source on phosphate removal percentage by individual PSB strain and bacterial consortium

Carbon source	Phosphate removal efficiency (%)			
	Pseudomonas sp. JPSB12	*Enterobacter sp. TPSB20*	*Flavobacterium sp. TPSB23*	Bacterial consortium
Glucose	59.10	61.75	55.30	77.20
Fructose	51.65	53.45	57.60	74.15
Lactose	57.55	50.10	61.30	82.50
Sucrose	50.70	58.20	60.45	79.25

Table 3. Analysis of variance of phosphate removal percentage by individual PSB strain and bacterial consortium

Source of variation	df	SS	MS	F_{obs}	F_{tab}
Between carbon source	3	41.11	13.70	0.82	3.86
Between bacteria	3	1464.18	488.06	29.21	3.86
Error	9	150.38	16.71		
Total	15				

whereas the consortium produced much higher acid phosphatase (9.02 mU/ml) and alkaline phosphatase (7.86 mU/ml) activities than individual strain. It is reported that acidic pH is favourable for phosphate removal, which is based on the fact that acidic pH seems to be favourable to acid phosphatase, which aids in the removal of phosphate (Boquet *et al*., 1987; Krishnaswamy *et al*., 2011).

In the present study, a positive significant correlation between phosphate removal efficiency and phosphatase activity was found. The correlation coefficients (r) between phosphate removal efficiency percentage and acid as well as alkaline phosphatase activity were *0.734* and *0.897* respectively. Similar types of results also reported by Kong *et al*. (2009).

4. Conclusion

In conclusion, the shake flask culture study performed in the present investigation 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 solubilizing bacterial cultures. The results from this study indicated that the synthetic wastewater with different carbon sources showed significant phosphate removal efficiency and the bacterial consortium (*Pseudomonas sp. JPSB12, Enterobacter sp. TPSB20, Flavobacterium sp. TPSB23*) used in this study efficiently removed the phosphate. The phosphate could be reduced below the permissible limit within 72 h using lactose carbon source and could be useful to remediate wastewater containing phosphate. The efficient removal of phosphate by the consortium may be due to the synergistic activity among the individual strains and phosphatase enzyme activity. The various mineral salts present in the synthetic wastewater might influence the growth of the phosphate solubilizers and utilized the phosphate compound. Therefore, the synthetic wastewater with carbon source supported the removal of phosphate at higher level. Thus, the simple method of phosphate removal is possible by employing phosphate solubilizing bacterial strains (viz., *Pseudomonas sp. JPSB12, Enterobacter sp. TPSB20, Flavobacterium sp. TPSB23*) and they might use the contaminants as nutrient and as energy source or it might be degraded by co-metabolism. Hence, the bacterial consortium could be used in the remediation of phosphate contaminated environments.

Table 4. Measurement of extracellular phosphatase activity (mU/ml ± SE)

Isolates	Acid phosphatase activity	Alkaline phosphatase activity
Pseudomonas sp. JPSB12	8.32 ± 0.07	4.54 ± 0.04
Enterobacter sp. TPSB20	6.86 ± 0.05	6.16 ± 0.03
Flavobacterium sp. TPSB23	7.58 ± 0.04	5.64 ± 0.01
Bacterial consortium	9.02 ± 0.03	7.86 ± 0.03

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