Original research

Biological removal of phosphate from synthetic wastewater using Enterobacter cloacae

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Abstract:

Phragmites australis may be considered one of the plants used a lot in treating areas contaminated with phosphate, it contains a strong microbial community that can be used in treating areas contaminated with phosphates including *Enterobacter cloacae*, as used in this study. This study aims to use *Enterobacter cloacae* isolated during the summer of 2019 from *Phragmites australis* from the Kima drain located in Ezzbet El- Nahda in Kima, Aswan Governorate, for removing phosphate in synthetic wastewater. These bacteria were tested for their phosphate removal efficiency using the MIC assay. The highest growth of *Enterobacter cloacae* in the synthetic wastewater supplied with a concentration of 900 mg/l of phosphate after 72 hours of treatment was 0.350 OD, and the change in pH after 72 hours of treatment was 7.1, and the remaining phosphate in Synthetic wastewater after 72 hours of biological treatment with *Enterobacter cloacae* and using a carbon source such as glucose and pH 7.9 was 530.33 mg/l with a removal rate of 41.074%. Thus, we found that *Enterobacter cloacae* achieved high efficiency in removing phosphates from the synthetic wastewater, despite the presence of phosphates in high concentrations close to the MIC (Minimum Inhibitory concentration). Thus, *Enterobacter cloacae* use pollutants as nutrients and can be used to treat environments contaminated with phosphates.

Keywords: bioremediation of phosphate, *Enterobacter cloacae*, *Phragmites australis*, and synthetic wastewater.

1- Introduction

Phosphate is an important element for living organisms, but due to rapid industrialization, there has been a rise in the number of effluents containing high concentrations of phosphates disposed of in natural water bodies causing serious environmental damage such as eutrophication and the depletion of large amounts of oxygen in the aquatic environment (**Syahrul *et al*., 2015; Dipak and Sankar, 2015**), which affects the aquatic organisms and the nature of the water when the water is drained and mixed with these aquatic environments.

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The concentration of phosphate in water should not exceed 0.01-0.1 ppm, according to federal government regulations (**EPA, 1991**; **Usharani and Lakshmanaperumalsamy, 2010**). In Egypt, the pollution by Phosphate is one of our most serious environmental problems. Effluents resulting from daily domestic and industrial activities may induce considerable changes in the physical and chemical properties of the Nile water due to occur eutrophication, which leads to low dissolved oxygen, murky water, fish kills, and the death of desirable flora and fauna. In addition, the increase in algae and turbidity impedes the movement of ships in the Nile River, and thus affects the tourism and economic activity, and increases the need to chlorinate drinking water, which, in turn, leads to higher levels of disinfection by- products have been shown to increase the risk of cancer, and thus increasing the economic cost. In Egypt, the concentration of phosphate in water should not exceed 0.2 ppm, according to the Egyptian drinking water quality standards (**EWQS, 2007**; **Elham** *et al.***, 2020**; **Seliem** *et al.***, 2023**). Treating areas contaminated with high concentrations of phosphate is one of the most important things to do to preserve the environment and living organisms. Biological treatment may be considered one of the types of effective treatments for treating areas contaminated with phosphate (**Deborah and Raj 2016**; **Dan** *et al.***, 2020** ; **Desiderata, 2021**). Common reed (*Phragmites australis* Cav. Trin.) is one of the most extensively distributed plant species on earth and is restricted predominantly to marshy areas and swamps and may be considered one of the plants used a lot in treating areas contaminated with phosphate because it contains a strong microbial community that can be used in treating areas contaminated with phosphates (**Kobbing** *et al.***, 2013**; **Štrbac** *et al.***, 2014** ;**Desiderata, 2021**). **Yan** *et al.***(2010**) pointed out that the endophytic strains in reed roots play important roles in the removal of toxic material and phosphorus such as *Enterobacter cloacae*, as used in this study, and showed in their research that *Phragmites australis* contributed to removing approximately 56%, 48%, and 13% of the total N, P, and organic matter, respectively, in wetland systems in China. **Munazzam** *et al.***(2020**) explained the role of endophytes as the microorganisms residing in the roots of plants (such as *Phragmites australis*) have a major contribution to the uptake of metals from the contaminated media. These microorganisms encourage the breakdown of inorganic compounds and complex organic into simple nutrients, mobilize metal ions, and rise the bioavailability of plants. Endophytes may help the plant to relieve stress by the blended action of multiple mechanisms. Direct mechanisms include nitrogen-fixing abilities, phosphate-solubilizing compounds, siderophore production, phytohormones, and antimicrobial metabolites. The various awarded endophytic metabolic pathways increase the request of these special microbes in bioremediation and biocontrol. The present study aims to use endophytic bacteria such as *Enterobacter cloacae* isolated from *Phragmites australis*, for the removal of phosphate from synthetic wastewater and treat environments contaminated with phosphate wastewater and make phosphate within the permissible limits to produce water that can be reused in the field of life.

2.Materials and Methods

2.1. Collection of *Phragmites australis* samples:-

Plant samples were collected during the summer of 2019 in sterile plastic bags from the Kima drain located in Ezbet El-Nahda in Kima, Aswan Governorate (24° 04`35.0" N 32$^o$ 55` 15.8" E),

as shown in Figure 1, and including fresh and healthy roots. Then were transported to the lab for isolating endophyte bacteria.

2.2.Isolation of endophytes bacteria from plant *Phragmites australis*:-

P.australis roots were collected from the Kima drain located in Ezzbet El- Nahda in Kima, Aswan Governorate, for isolating endophytic bacterial isolates. healthy and fresh roots were washed for removing all soil exhaustively under running tap water and then surface sterilized (70 % C_2H_5OH, 3 min, 0.5 % NaOCl, 3 min, and 70 % C_2H_5OH, 30 sec) and washed three times by sterile distilled water. To test the efficiency of surface sterilization, surface sterilized root samples were inoculated on a nutrient agar plate (5g of peptone, 3g of beef extract, 15g of agar, 1000 ml of distilled water, pH 7.4), preceding inoculate endophyte bacteria. The surface sterilized roots were air dried with sterile air, then cut thin pieces, and aseptically put over an LB agar plate (10g of peptone, 5g of yeast extract, 5g of NaCl, 0.1g of glucose, 15g of agar, 1000ml of distilled water, pH 7.4), and incubated at 30°C for 24-72 h in incubator (**Sambrook *et al.*, 2001**). For selecting single endophytes, the colonies of bacteria around root pieces were picked and streaked freshly on LB agar. Throughout the isolating process, aseptic conditions were maintained. (**Abbas *et al.*, 2018**). Single endophytes that grew on plates were selected and streaked on the fresh LB agar plates repeatedly for making isolation and purification. These bacterial isolations were obtained on 17 isolations.

Figure 1: A satellite view of the site, Kima drain is located in Ezzbet El- Nahda in Kima, Aswan Governorate.

2.3. Selective and identification of phosphate-resistant and tolerant bacteria:-

Stock phosphate solution (1000 mg PO_4/l) was prepared by dissolving 1.834g dipotassium hydrogen phosphate in 1000 ml distilled water and sterilized by autoclave at 121^0C for 20 min. Then LB agar medium was prepared and sterilized by autoclave at 121^0C for 20 min, 20 ml LB agar medium was poured into each petri dish then was took a volume from the stock phosphate

solution to get 100 ppm of phosphate in each plate according to this equation: $C_1 x V_1 = C_2 x V_2$, where (C_1) was the concentration of phosphate in-stock solution, (V_1) was the used volume of stock solution, (C_2) was the concentration of phosphate in agar and (V_2) was the used volume of agar. Seventeen bacterial isolates separately were streaked on LB agar plates containing 100ppm of phosphate (PO_4) and incubated at 30^0C for 24-72 h to estimate the resistance and tolerance to phosphate. This procedure was carried out in three replications. The bacterial isolates were identified morphologically by using gram stain, motility test, and colony shape on LB agar medium. (**Amna *et al*., 2012**; **Arjun *et al*., 2019** ; **Seham *et al*., 2020**). Biochemically by using the Biomerieux Vitek 2 System at the Animal Health Research Institute in Dokki, Giza Governorate. **Maria Lina Mezzatesta *et al*., 2012**; **Hesham *et al*., 2020** and **Seham *et al*., 2020** were assigned symbols depending on their isolation source, *Phragmites australis* (Pa).

2.4. Determination of MIC of phosphate against bacterial isolates:-

MIC (Minimum Inhibitory Concentration) of phosphate in the form K_2HPO_4 in concentrations from 100 ppm to 1000 ppm against bacterial isolates (14Pa, 11Pa, 10Pa, 4Pa, and 12Pa) these were tolerant to phosphate in L.B agar plates containing 100 ppm of phosphate was determined by disk diffusion test. The initial phosphate concentration of 100 ppm was prepared by dissolving 0.0183g dipotassium hydrogen phosphate in 100 ml distilled water and sterilized by autoclave at 121^0C for 20 min. then the sterile disks were put in this concentration on L.B agar medium inoculated with (14Pa, 11Pa, 10Pa, 4Pa, and 12Pa) individually by the spread plate method and incubated at 30^0C for 24-72 h, The procedure was repeated with a 100 ppm increase in the range where the observable growth was absent around the disk and the zone of inhibition appeared clear of bacterial growth and this zone was measured its diameter to determine the MIC, this test was carried out in three replications.

2.5. Preparation of inoculum:-

A bacterial isolate (14Pa) was added into L.B broth and the mixture was incubated for 24 h at 30^0C in a rotating shaker incubator. This isolate had a Minimum Inhibitory concentration (MIC) at a concentration of 1000 ppm of phosphate higher than the other four isolates. Centrifugation was used to separate the cells for 15 minutes at 6000 rpm and washed twice with (0.85% NaCl) sterile solution. The cell concentration was set to an optical density at 600 nm (OD $_{600}$) to 0.1 and about 74×10^5 CFU/ml (1ml of 0.1 OD) of the cell was utilized as an inoculum (**Usharani and Lakshmanaperumalsamy, 2010**; **Usharani *et al*., 2011**; **Sayeda *et al*., 2012**; **Dipak and Sankar, 2015**).

2.6. Phosphate removal by bacteria in synthetic wastewater:-

The experiment was prepared using the bacterial isolate (14Pa). The experiment contained three flasks containing a synthetic wastewater to remove phosphate at a concentration of 900 mg/l inoculated with the bacterial isolate (14Pa), and three flasks containing only the synthetic wastewater as a control. The synthetic wastewater was prepared containing 900 mg/l phosphate concentration as K_2HPO_4 and KH_2PO_4 at a 1.2:1. (**Usharani and Lakshmanaperumalsamy, 2010**; **Usharani *et al*., 2011**; **Sayeda *et al*., 2012**). As shown in Table 1. Then the 250 ml flasks

containing180 ml of the synthetic wastewater were sterilized by autoclave at 121^0C for 20 min. The flasks were inoculated with inoculum {1ml of (0.1 OD) from the bacterial isolate (14Pa)} and incubated for three days at 30^0C in a rotating shaker incubator at 150 rpm. Samples were examined for pH change and bacterial growth (OD $_{600}$) at 0, 24, 48, and 72 h. The experiment was performed in triplicates. After the incubation period for three days, centrifugation was used to obtain the clear supernatant at 6000 rpm for 15 min for soluble phosphate estimation by a spectrophotometer at 660 nm by the colorimetric method (**APHA, 2005**), this analysis was carried out at the Unit of Environmental Studies and Development at Aswan University. This equation E= (I x F)/I x 100 was used to calculate the efficiency of phosphate uptake (E), (where (I) is the initial phosphate concentration, (F) is the final phosphate concentration). (**Usharani and Lakshmanaperumalsamy, 2010**; **Usharani** *et al.***, 2011**).

Table 1: Composition of synthetic wastewater for phosphate removal

Component	Amount (g/L)
K_2HPO_4	0.917
KH_2PO_4	0.5727
Na_2HPO_4	0.0334
$MgSO_4.7H_2O$	0.002
$CaCl_2$	0.004
NH_4Cl	0.003
NaCl	0.007
Glucose	0.1
pH	7.96

2.7.Statistical analysis:-

Data obtained were subjected to statistical analysis using Correlation analysis that was conducted on selected parameters based on average data to the Pearson correlation coefficient according to (**Gomez and Gomez, 1984; Lescesen** *et al.***, 2015**). Statistical analysis was performed using Statistix 8.1 program.

3. Result and Discussion

3.1. Isolation and identification of phosphate-resistant and tolerant bacteria:-

The results of the isolation and screening show that the bacterial isolates (14Pa, 11Pa, 10Pa, 4Pa, 12Pa) were tolerant to phosphate in L.B agar plates containing (100 ppm) of phosphate from 17 isolations. The bacterial isolates (14Pa, 11Pa, 10Pa, 4Pa, 12Pa) were identified morphologically by using gram stain, motility test, and the colony shape on LB agar medium as shown in Table 2, and Biochemically by using the (Biomerieux Vitek 2 System) at the Animal

Health Research Institute in Dokki, Giza Governorate according to **Hesham *et al*. (2020)**. These isolates were identified as shown in Table 2

3.2. Determination of MIC of phosphate against bacterial isolates:-

The results show that the MIC (Minimum Inhibitory concentration) for bacterial isolates (14Pa, 11Pa, 10Pa, 4Pa, 12Pa) was 1000 ppm by disk diffusion test the zone of inhibition appeared clear of bacterial growth as shown in Figure 2, and this zone was measured its diameter to determine the MIC according to **Usharani *et al*. (2011)**, as shown in Table 2. Thus, *Enterobacter cloacae* (14Pa) are highly efficient phosphate removers based on the diameter of the inhibition zone, and was 2 cm as in Figure 3.

Table 2: Morphological characteristics, Biochemical identification by using the Biomerieux Vitek 2 System, and MIC based on the diameter of the inhibition zone of bacterial isolates (14Pa, 11Pa, 10Pa, 4Pa, 12Pa)

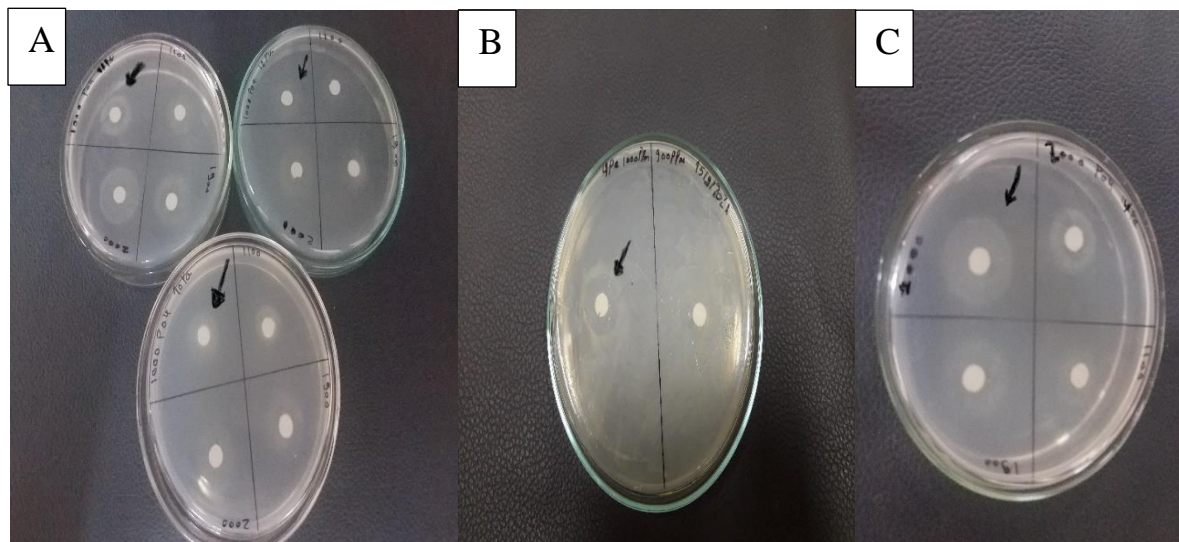| Bacterial isolates	14Pa	11Pa	10Pa	4Pa	12Pa
Morphological characteristics					
Colony color	White-cream	White-cream	White-cream	White-cream	yellow
Colony shape	Circular	Circular	Circular	Circular	Circular
Gram nature	-	-	-	+	+
Cell morphology	rod	rod	rod	rod	rod
Motility	+	+	+	+	+
MIC	2^*	1.8	1.7	1.6	1.6
Biochemical identification	*Enterobacter cloacae*	*Enterobacter cloacae*	*Enterobacter cloacae*	Unidentified organism	Unidentified organism

*maximum zone formation

Figure 2: The inhibition zone of phosphate for bacterial isolates (14Pa, 11Pa, 10Pa, 4Pa, 12Pa) at a concentration of 1000 ppm by disk diffusion test after 72h from incubation. Where A (11Pa, 10Pa, 12Pa), B (4Pa), C (14Pa).
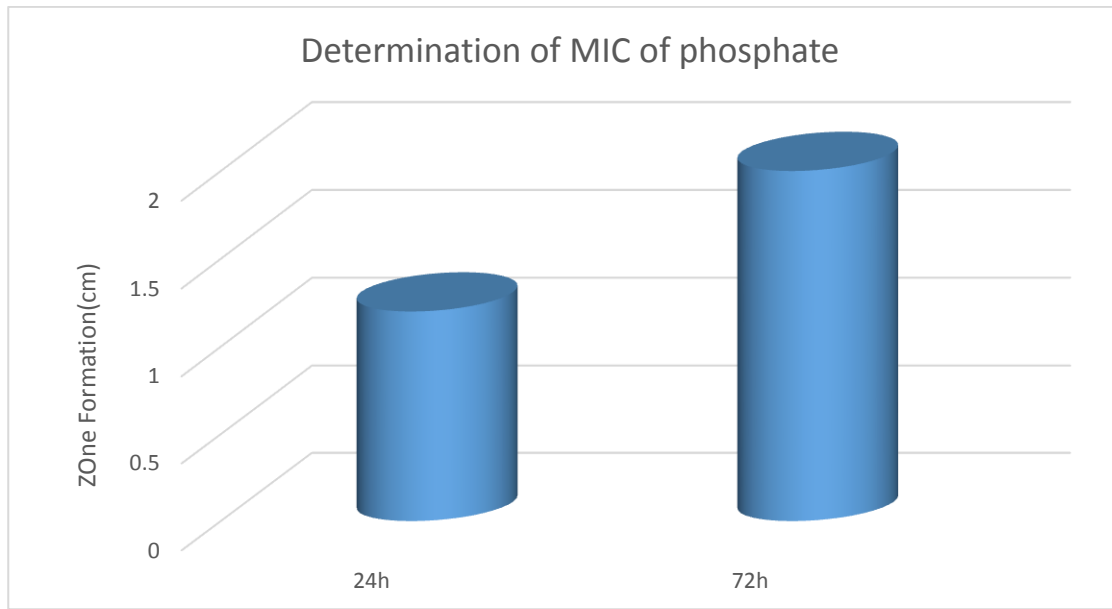
Figure 3: Determination of MIC of phosphate for bacterial isolate (14Pa) at a concentration of 1000 ppm by disk diffusion test after 24h and 72h from incubation.

3.3. Phosphate removal by bacteria in synthetic wastewater:-

The growth of bacteria in synthetic wastewater was increased and had an insignificant negative correlation with the pH change during the incubation period for three days (-0.05).

3.3.1.Estimation of pH change:-

After 72h, the pH in synthetic wastewater changed from (7.96 to 7.1), as in Figure 4. We found that the pH value in synthetic wastewater in the presence of glucose as a carbon source decreased at a constant rate during the 72-hour incubation period according to **Usharani and Lakshmanaperumalsamy (2010**) and **Usharani *et al.*(2011)**.
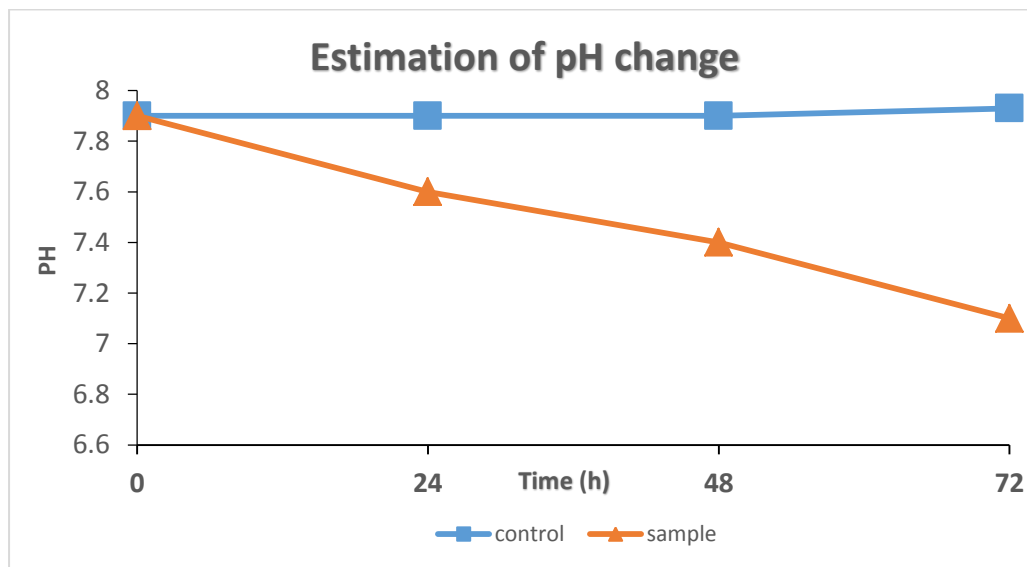

Figure 4: Estimation of pH change in synthetic wastewater for removing phosphate at a concentration of 900 ppm every 24h.

3.3.2. Estimation of growth of bacteria:-

The maximum growth of *Enterobacter cloacae* in synthetic wastewater after the incubation period for three days was (0.35) as in Figure 5. According to **Wan *et al*.(2017)** under aerobic conditions, cells used phosphate to grow and the reformation of polyphosphate. An increase in biomass concentration increased the efficiency of phosphate uptake and improved phosphate removal efficiency.
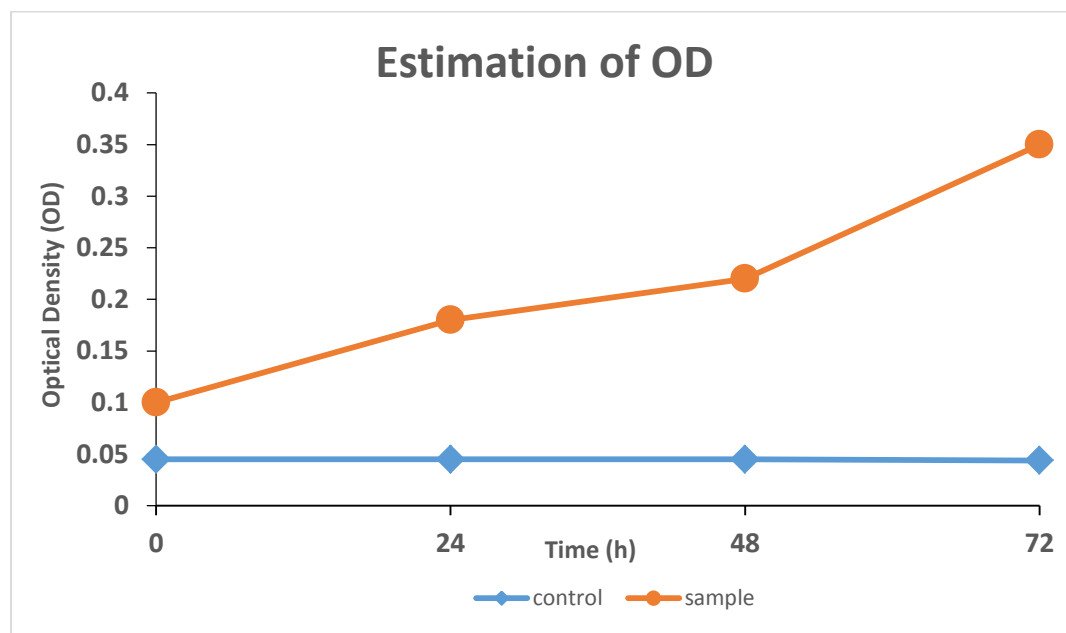

Estimation of OD

Figure 5: Estimation of growth of bacteria in synthetic wastewater for removing phosphate at a concentration of 900 ppm every 24h.

3.3.3. Estimation of percentage removal of phosphate:-

The residual phosphate in synthetic wastewater was 530.33 ppm after the incubation period for three days. *Enterobacter cloacae* in synthetic phosphate wastewater at a concentration of (900 mgl^{-1}) and with glucose as a carbon source at initial pH of 7.96 achieved a phosphate removal rate of (41.074 %) as shown in Figure 6. These results agree with **Usharani and Lakshmanaperumalsamy, 2010; Usharani *et al*., 2011)** which found that *Pseudomonas sp*. alone or by a bacterial consortium (*Bacillus*, *Pseudomonas*, and *Enterobacter*) in glucose carbon source achieved maximum removal of (68%) and maximum growth of 0.9886 OD at pH 7.0 $\pm$ 2 at the concentration of phosphate (100 mgl^{-1}). **Dipak and Sankar(2015)** reported that glucose could be a good booster for the performance of removal of phosphate biologically. Carbon source as glucose is oxidized to gluconate, which is transformed to other components, like 2-keto-3-deoxygluconate, pyruvate, or glyceraldehydes, and the mechanism such as the liberation of protons related to biological ammonium assimilation in the existence of organic acids that boosts the consumption of phosphate. The existence of organic acids leads to a decrease in the medium's pH. According to **Majid *et al*. (2009)** PAOs (Phosphorus accumulating Organisms) took up carbon sources and transformed them into carbon storage products that supply energy and growth, and too stored PHB (polyhydroxy butyrate) is metabolized, supplying energy from oxidation for novel cell growth. The energy released from PHB (polyhydroxy butyrate) oxidation

is utilized to compose polyphosphate bonds during cell storing so that soluble orthophosphate is eliminated from the solution and combined into polyphosphates within the bacteria cell. According to **Akpor and Muchie (2010)** the later studies reported the Proteobacteria to be the most dominant PAOs in synthetic wastewater and in clarified effluent of a conventional activated sludge system. Polyphosphate accumulating organisms (PAOs) are microorganisms that can store phosphate as intracellular polyphosphate, leading to phosphate removal from the bulk liquid phase in the waste-activated sludge. DNPAOs, or denitrifying phosphate-accumulating organisms, were described in a review study by **Shukla *et al.*(2020**) as a subset of PAOs. The most DNPAOs are *Dechloromonas* and *Zoogloea* and belong to the class Betaproteobacteria and share a metabolic pathway with PAOs. While DNPAOs can also use nitrite or nitrate to absorb phosphate via decomposing PHAs, PAOs can only employ oxygen as an electron acceptor for respiration. **DebRoy *et al.*(2012)** have shown the mechanism of phosphate removal in bacteria leads to the intracellular accrual of polyphosphate granules, these could be used as potency nominees for the seizure of phosphate from environmental sites. **Wan *et al.*(2017**) reported that *Enterobacter cloacae* may be a phosphorus-accumulating, denitrifying bacterium with the capacity to efficiently remove nitrogen and phosphorus from both synthetic and real wastewater.
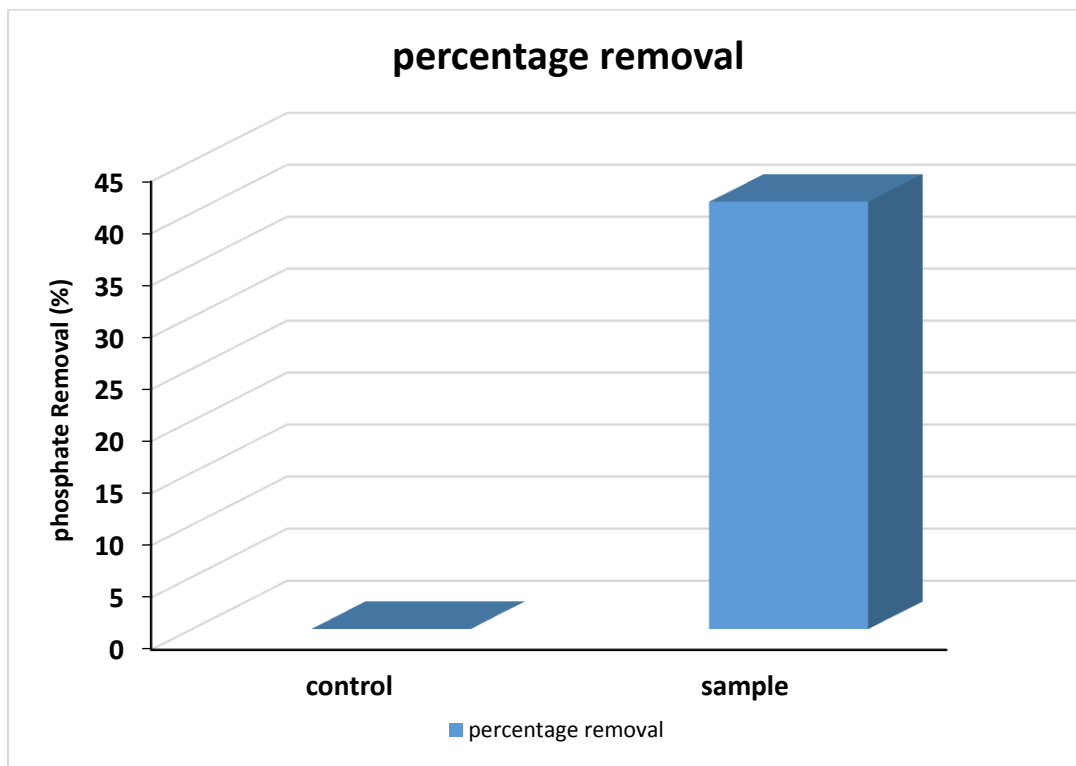

Figure 6: Estimation the percentage of phosphate removed from synthetic wastewater for removal phosphate at a concentration of 900 ppm after 72h of incubation.

Denitrifying phosphorus-accumulating bacteria (DPAB) are facultative anaerobic bacteria, which can use O_2, NO_3^--N, or NO_2^--N as final electron acceptors to take up phosphorus under aerobic conditions or to release phosphorus under anaerobic conditions, and according to **Wan *et al.*(2017**) DPAB may hydrolyze intracellular polyphosphate (poly-P) and glycogen to produce

energy during the anaerobic phase. It can also absorb volatile fatty acids (VFA) to create intracellular polyhydroxyalkanoates (PHAs) and release phosphate into the surrounding environment. The DPAB can absorb more soluble phosphorus into the cell in the ensuing aerobic phase in the form of poly-P than was released in the preceding anaerobic phase, so phosphorus is removed from the wastewater.

Conclusion

In this study, we found that *Enterobacter cloacae* achieved high efficiency in removing phosphate from the synthetic wastewater, despite the presence of phosphate in high concentrations close to the MIC. Thus, *Enterobacter cloacae* use pollutants as nutrients and can be used to treat environments contaminated with phosphates.

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