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Phosphate removing using bacteria isolated from activated sludge for wastewater remediation

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Abstract

The objective of the current study is to isolate phosphate removing microbes from activated sludge for wastewater remediation in the area of Khenchela (Eastern Algeria) . Phosphate rate was determined using colorimetric method and batch tests were developed to evaluate the biomass composition of the sludge. Four efficient pure strains isolated from activated sludge samples and identified as *Acinetobacter junii*, *Pseudomonas aeruginosa*, *Moraxella lacunata*, *Alcaligenes denitrificans* were tested for their capacities of phosphate reduction. The best rate of phosphate removal by pure cultures is observed with *Acinetobacterjunii*, 76%, followed by *Alcaligenes denitrificans,* 70.42%, *Pseudomonas aeruginosa,* 61.78%, and *Moraxella lacunata,* 50.6%. Our results show that applying of mixed bacterial culture containing mostly isolated strains for bioremediation purpose can be used successfully for the elimination of phosphate from activated sludge of wastewater treatment plants.

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Introduction

Phosphorus is one of the major components of all living cells. The control of the amount of phosphates in water or wastewater became an important key to improving the quality of our ecosystems. At high concentrations, the phosphorus in ecosystems affects the quality of freshwater resources and the practice of reusing wastewaters (Anderson *et al.*, 2002).

The method most commonly used for disposal of wastewater phosphates is the physico-chemical phosphate removal in which metal salts (iron, aluminum, or calcium) were used to precipitate phosphate. However, for his significant costs associated with additional chemical reagents and a significant sludge production have limited its widespread use (Greaves *et al.*, 1999; Wrigley *et al.*, 1992). Biological processes appear now to be the most competitive and best adapted to the treatment of phosphate present in wastewater.

Phosphate can be efficiently removed from wastewaters by the activated sludge process which incorporates alternating anaerobic and aerobic periods (Serafim *et al.*, 2002; Kuba *et al.,* 1997). In their work Mielcarek *et al.* (2015) evaluate the feasibility of citric acid as external carbon source for biological phosphorus removal. A successful enhanced biological phosphorus removal was studied in both anaerobic-aerobic sequencing batch reactor to induce growth of phosphate accumulating organism and anaerobic-anoxic (Lee and Yun, 2014).

The Eastern Algeria is an agricultural area and the contamination of water by phosphates and nitrates is significant. The aims of our work is to study the kinetics of phosphate removal by mixed and pure cultures of activated sludge taken from the wastewater treatment plant of Khenchela (Eastern Algeria) and the possibility of applying these bacterial cultures for bioremediation purpose to reduce environmental pollution.

Materials and methods

Sampling and composition medium

The activated sludge samples were taken from the aeration tanks of wastewater treatment plant of Khenchela which mainly receives domestic wastewater. Samples were collected in sterilized glass bottles and transported on ice to laboratory for analysis.

The synthetic medium used in this study contains the minimum of nutrients indispensable for phosphate removal bacteria growth (Saito *et al.,* 2004). The composition of medium diluted in distilled water was summarized in table 1.

Table 1. Composition of the synthetic medium (Saito *et al.*, 2004).

Components	Concentration ($g.L^{-1}$)
$CH_3COONa.\ 3H_2O$	0.4
K_2HPO_4	0.049
KH_2PO_4	0.028
NH_4Cl	0.107
$MgSO_4.7H_2O$	0.180
$CaCl_2.2H_2O$	0.0003
*Trace solution	0.3 $mL.L^{-1}$

* 10$g.L^{-1}$ EDTA, 1.54 $g.L^{-1}FeSO_4.7H_2O$, 0.15 $g.L^{-1}$ H_3BO_3, 0.03 $g.L^{-1}$ $CuSO_4.5H_2O$, 0.12 $g.L^{-1}$ $MnCl_2.4H_2O$, 0.18 $g.L^{-1}KI$, 0.06 $g.L^{-1}Na_2.MoO_4.2H_2O$, 0.12 $g.L^{-1}$ $ZnSO_4.7H_2O$, 0.15 $g.L^{-1}CoCl_2.6H_2O$.

Phosphate removal by mixed culture

Inoculum preparation

Activated sludge samples were treated before inoculation to fermentors. First, the samples were diluted in 0.9% sterile physiological salt solution with stirring for 2 min by vortex mixer (Pauli and Kaitala, 1997) and the suspended activated sludge was *centrifuged* at 300 rpm for 2 min. Then, the decanted supernatant was enriched in 100mLof prepared medium and incubated with shaking during 24 h at 30°C. After, the culture was centrifuged at high speed (10000 rpm) for 15 min and the pellets were washed in a physiological salt solution. Finally, the suspended pellet was added to the reactors.

Culture conditions

Mixed culture was inoculated to 500 mL fermentors contained 300 mL of medium supplemented with different concentrations of sodium acetate as carbon source and K_2HPO_4/KH_2PO_4 as phosphorus source. The fermentors were operated in batch mode and incubated under two alternating phases and aerobically in stirred bain-marie (GFL1083) for 2h, then aerobically at 30°C for 4 h in horizontal stirred operating at 150 mpm. Experiments were performed in triplicata.

Isolation of bacteria

During phosphate reduction by mixed culture, isolation was carried out from Fermentors inoculated with activated sludge and aliquot of culture were streaked onto minimal agar medium. Then, the inoculated plates were incubated at 30°C for 24 h to 72 h. Isolated Bacteria with distinct colony morphologies were selected and sub-cultured onto the same medium until achievement of growing in pure cultures. The isolated bacteria were grown in nutrient broth, stored in 10% (v/v) glycerol at -20°C and used as stock cultures in subsequent analysis.

Bacterial identification

Morphology and biochemical characteristics of isolates

The cell morphology was determined microscopically after Neisser stain and Gram stain preparation. The biochemical characteristics were determined using tests including *Catalase, Oxidase, Nitrate reductase, Tryptophane desaminase, Decarboxylase, Urease, Indole production, Carbohydrate fermentation and Mixed acid fermentations*. Growth of strains on King A and King B media was tested too.

Identification of bacteria by API system

Pure bacterial strains were identified using API 20NE systems, according to the manufacturer's instructions (BioMérieux). The API strips were examined after 24 h and 48 h at 30°C and the identifications were carried out by identification database APixl.

Kinetics of phosphate removal by pure cultures

Inoculum preparation

20 µL of stock cultures were grown on nutrient agarduring 18 h at 30°C. Colonies of pure cultures were transferred in 250mLErlenmeyers containing 100mL of synthetic medium. Erlenmeyers were incubated in aerobic conditions at 30°C for 24h in 150 mpm horizontal shaking (Zafiri, 1999). After 24h cultures were centrifuged at 10000 rpm for 15 min and the pellets were suspended in a physiological salt solution. The optical density of inoculums were measured using UV-Vis spectrophotometer (Shimadzu) at 600 nm (Krishnaswamy *et al.,* 2009).

Culture conditions

Batch cultures were performed in 500 mL fermentors cantaining 300mL of synthetic medium supplemented with 5g.l^{-1} sodium acetate as sole carbon source and KH_2PO_4 as phosphorus ($P\text{-}PO_4^{-3}$=18 mg.L^{-1}) source. In each fermentor, 2.5% of different pre-cultures were added and incubated at 30°C with horizontal shaking of 150 mpm.

Sampling and Analytical methods

To estimate phosphate concentration, biomass growth and pH, 8 mL of liquid samples were withdraw aseptically from each fermentor through a sampling port using sterile syringe, at regular time intervals.

Optical density of suspensions was measured at 600 nm using SHIMADZU spectrophotometer and pH was measured using PHYWE pH meter. To determine the concentration of phosphate in cultures, ascorbic acid method was used.

Results

Identification of isolates

Different types of bacterial culture were isolated from activated sludge using streak plate method. Four strains were selected on the basis of morphological and biochemical characteristics, bacterial culture was tentatively identified as *Acinetobacter junii*, *Pseudomonas aeruginosa*, *Moraxella lacunata*,

Alcaligenes denitrificans. These results were confirmed using API 20NE system and the numerical profile is presented in Table 2.

Table 2. Identification of isolates using API 20NE system.

	S1	S2	S3	S4
Numerical profile	1001051	1354575	1010204	1000467
Strain Name	*Acineto-bacterjunii*	*Pseudomonas aeruginosa*	*Moraxella lacunata*	*Alcaligenes-denitrificans*

Culture medium optimization

Optimization of culture medium was performed to find the optimal initial concentration of sodium acetate, the optimal initial concentration of phosphate and the appropriate carbon source. The values of the kinetic of optimization parameters are summarized in Table 3.

As shown in (Table 3), 99.23% of phosphate was reduced when 5000 ppm of sodium acetate was supplemented to the medium, which provide the best rate of dephosphatation compared to the other carbon sources.

Table 3. Optimization of culture parameters.

Parameters	*Phosphate removal* rate (%)	Phosphate concentration ($mg.L^{-1}$)
Initial concentration of sodium acetate		
1000 ppm	83.38	2.71
2000 ppm	93.08	1.16
3000 ppm	93.80	1.03
4000 ppm	96.19	0.64
5000 ppm	99.23	0.13
Initial concentration of phosphate		
0 ppm		
5 ppm	99.68	0.027
10 ppm	98.87	0.14
30 ppm	93.91	0.24
50 ppm	71.09	14.98
Carbon sources		
Acetate	99.23	0.13
Glucose	71.22	3.82
Lactose	61.05	6.8
Lactate	78.51	4.22

Phosphate removal by mixed culture

For mixed culture, bacteria involved in the phosphate accumulation process present a latency phase of about 8 h. This *short* adaptation period could be the result of synergistic interrelation ships between co-cultured species (Cohen, 2011). After latency phase, phosphate accumulating starts with an accumulating rate of about 0.31 $mg.L^{-1}.h^{-1}$ during the first 50 h, exponential phase. Finally, the stationary phase was reached after 72 h of mixed culture treatment with a dephosphatation rate of about 92.87%.

Bacterial growth and Phosphate removal by pure cultures

The bacterial growth kinetic and phosphate concentration in the fermentors inoculated with pure cultures in the presence of 5 $g.L^{-1}$ of sodium acetate as carbon source are presented in Fig. (2, 3, 4 and 5).

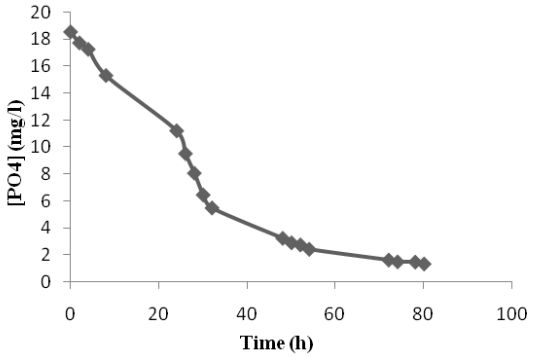

Fig. 1. Kinetic of Phosphate removal using mixed bacterial culture.

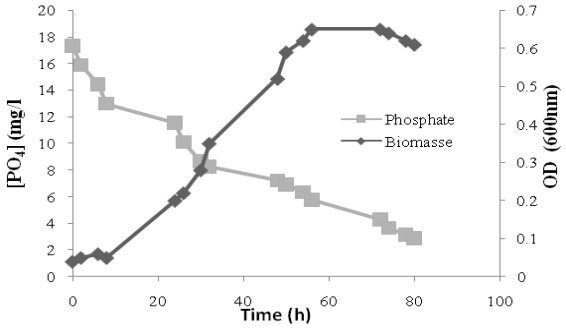

Fig. 2. Kinetic of bacterial growth and Phosphate removal using *Acinetobacter junii*.

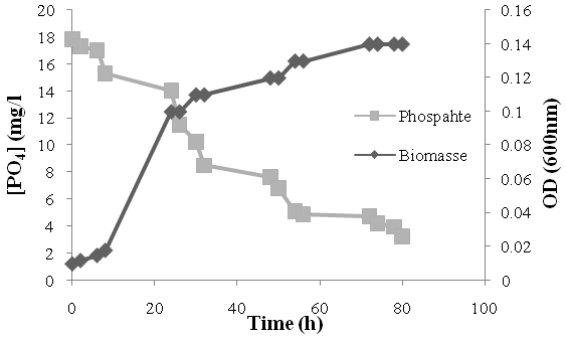

Fig. 3. Kinetic of bacterial growth and Phosphate removal using *Pseudomonas aeruginosa*.

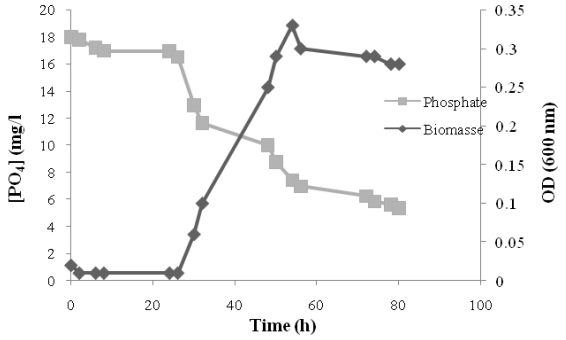

Fig. 4. Kinetic of bacterial growth and Phosphate removal using *Moraxella lacunata*.

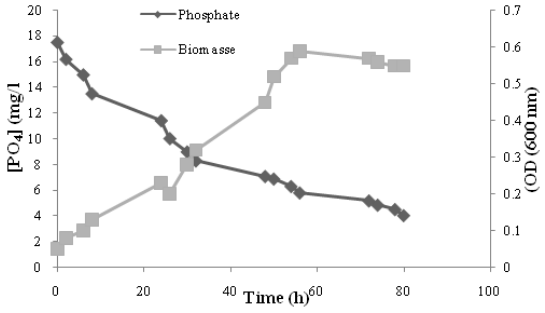

Fig. 5. Kinetic of bacterial growth and Phosphate removal using *Alcaligenes denitrificans*.

Bacterial growth and Phosphate removal by Acinetobacter junii

During the first hours, phosphate elimination was started quickly (0.21 $mg.L^{-1}.h^{-1}$), where 72.53% of initial concentration of phosphate was consumed (Fig. 2). This period corresponds to the exponential phase of growth. During this phase the optical density reaches its maximum value of 0.65 after 50 h of incubation. Beyond 60 h, bacteria enter in stationary phase during in which the rate of phosphate accumulation decreased to be 0.13 $mg.L^{-1}.h^{-1}$.

Bacterial growth and Phosphate removal by Pseudomonas aeruginosa

As shown in Fig. 3, no latency phase was observed which lead especially to the pre-enrichment of bacteria on the same medium used in batch culture. During the exponential phase, the maximum rate *of* phosphate removal was obtained, 60.02%. This significant reduction is accompanied by an important bacterial growth which reaches an optical density of 0.14.

Dephosphatation kinetic using *Pseudomonas aeruginosa* shows that removed phosphate concentration is estimated to be 12.59 $mg.L^{-1}$. Our results are better than those obtained by Momba and Cloete (1996) with the same strain, 28.50 $mg.L^{-1}$.

Bacterial growth and Phosphate removal by Moraxella lacunata

As shown in Fig. 4, a latency phase of about 26 hours was observed with a variation of optical density between 0.02 and 0.04. During this phase, the value of dephosphatation rate was estimated to be 8.32%. After 54 h, a maximum optical density of 0.33 was obtained with 46% of phosphate removing. Finally, stationary phase was reached after 72 h of incubation.

This result shows that, *Moraxella lacunata* capable to degrade about 50% of phosphate which is in agreement with previous researches (Bao *et al.*, 2007; Bitton, 2005; Merzouki *et al.*, 1999). They reported that different species of *Moraxella* can be used to remove phosphate

Bacterial growth and Phosphate removal by Alcaligenes denitrificans

During the first twenty hours, 6.08 $mg.L^{-1}$ of phosphate was removed. This concentration is approximately the same found by Bao *et al.* (2007), 6.43 mg.L-1. Bacterial growth start with an optical density of 0.05 to reach its maximum value of 0.59 after 60 h of incubation (Fig.5). During this phase,

66.68% of phosphate was eliminated with an accumulating rate of 0.2 $mg.L^{-1}.h^{-1}$. After 72 h of incubation, the optical density stabilizes and the dephosphatation rate decreases to 30.13%.

Discussion

In the current study, the biodegradation capacity of phosphate in liquid medium (batch culture) by mixed bacterial culture and four pure bacterial cultures isolated from activated sludge sample was analyzed. Neisser stain was used for detecting poly-P granules in bacteria. Poly-P has been observed in metacromatic granules (volutin) situated in the cytoplasm (Serafim *et al*., 2002).

The results described in this paper show that 5000 $mg.L^{-1}$ sodium acetate provide the best yield of phosphate elimination with an average rate of accumulation of 0.19 $mg.L^{-1}.h^{-1}$. The phosphate concentration value obtained after treatment was 0.13 $mg.L^{-1}$ which reflecting a satisfactory treatment; the phosphate concentration is known as polluting beyond 1 $mg.L^{-1}$.

The four selected species tested for their capacities of phosphate accumulating allowed a good growth in the minimum -culture medium. During exponential phase of *Acinetobacter junii*, *Alcaligenes denitrify-cans* and *Pseudomonas aeruginosa,* the phosphate concentration increases in parallel with bacterial growth and the best rate of dephosphatation was obtained during this phase, which is in agreement with the results obtained by Florentz and Hartemann (1984) and Morohoshi *et al.* 2003.

According to obtained results, the best rate of phosphate removal by pure cultures is observed with *Acinetobacterjunii*, 76%, followed by *Alcaligenes denitrificans,* 70.42%, *Pseudomonas aeruginosa,* 61.78%, and *Moraxella lacunata,* 50.6%. %. Similar results, demonstrating a successively decrease in phosphorus removal rate for *Acinetobater junii*, *Alcaligenes denitrifican*, *Pseudomonas aeruginosa*, *Moraxella lacunata*, have been reported in several studies (Sidat *et al*., 1999; Zarifi, *et al*., 1999; Sarioglu, 2005; Mubyana-John and Letsamao, 2007). Furthermore, using *Pseudo-monas aeruginosa* in synthetic medium supplemented with 1 $g.L^{-1}$ of sodium acetate Sarioglu (2005) found a less significant rate of about 25%. Whereas using *Pseudomonas putida*, Mubyana-John and Letsamao (2007) reported a rate of 34% phosphate removal. After 24 h of incubation, Sidate *et al*. (1999) showed that phosphate uptake provides varying yields using *Alcaligens denitrificans, Moraxella phenylpyruvica* and *Moraxella* spp. cultivated in synthetic medium containing 5% of sodium acetate and 38 mg L^{-1} of final phosphate concentration.

Conclusions

The objective of our work consist to isolate phosphate removing microbes from activated sludge for wastewater remediation in the area of Khenchela (Eastern Algeria). For this, four efficient pure strains isolated from activated sludge samples and identified as *Acinetobacter junii, Pseudomonas aeruginosa, Moraxella lacunata and Alcaligenes denitrificans* were tested for their capacities to remove phosphate. Our results show that the best rate of phosphate removing by pure cultures is observed with *Acinetobacter junii*, 76%, and mixed bacterial culture containing mostly isolated strains can be used successfully for removing phosphate from activated sludge of wastewater treatment plants.

Finally, the application of strains isolated from activated sludge of the wastewater treatment plant can be used for phosphate remediation and as a bioelement in biosensors in real water. For this, four cities of eastern Algeria will be selected for our future studies (Khenchela, Annaba, Guelma and Soukahras).

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