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Verification of Enhanced Phosphate Removal Capability in Pure Cultures of *Acinetobacter calcoaceticus* under Anaerobic/Aerobic Conditions in an SBR

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Abstract Laboratory experiments were conducted using pure cultures of *Acinetobacter* under anaerobic/aerobic cyclic conditions to explain the release and uptake of soluble phosphate in an activated sludge process showing enhanced biological phosphate removal (EBPR). Under anaerobic/aerobic cyclic conditions in a Sequencing Batch Reactor (SBR), COD uptake concurrent with soluble phosphate release by *Acinetobacter* was not significant during the anaerobic periods, indicating that EBPR would not be established in pure cultures. However, *Acinetobacter* cells accumulated higher phosphate content (5.2%) in SBR than that obtained (4.3%) from batch experiments. These results suggest that *Acinetobacter* sp. may not follow the proposed pattern of behavior of poly-P bacteria in EBPR activated sludge plants.

Keywords: Acinetobacter, EBPR, SBR, poly-P bacteria

INTRODUCTION

The enhanced biological phosphate removal (EBPR) process has been one of the major processes for the removal of phosphates from wastewater. This biological process includes soluble carbon substrate uptake under anaerobic conditions, while releasing soluble orthophosphate to provide energy for carbon substrate uptake. The soluble orthophosphate is released due to the hydrolysis of intracellular polyphosphates, which serve as internal energy under anaerobic conditions. In the subsequent aerobic stage, the stored carbon substrate is oxidized in the presence of the terminal electron acceptor, oxygen, and the metabolic energy used to take up soluble orthophosphate.

Currently, the EBPR phenomena have been observed in activated sludge systems, but in pure cultures this process has not yet been proved [1]. In some failed cases, a significant part of the soluble carbon substrate is taken up during the initial anaerobic stage without releasing soluble phosphates [2]. The principal organisms found to be responsible for EBPR have been identified as *Acinetobacter* sp. in most of the earlier studies [3-6]. These *Acinetobacter* sp. can survive under alternating anaerobic/aerobic conditions, with low dilution rates, in two stage chemostat systems [7]. However, more recently, it has been found that microorganisms other than *Acinetobacter* sp. may play a fundamental role in the EBPR systems [8]. Brodisch and Joyner [9] found low *Acinetobacter* populations (1-10%) in an EBPR activated sludge system and concluded that other microorganisms may be responsible for the EBPR. Hiraishi and Morishima [10] identified about 1% of the total viable population to be *Acinetobacter* sp. in the anaerobic/aerobic activated sludge systems, and concluded that the EBPR does not always depend on the presence of *Acinetobacter*. Other microorganisms, such as *Pseudomonas* sp., *Moraxella* sp. and *Aeromonas* sp., in AS have been found to show potential for the EBPR [11]. Hence, the objective of this research was to investigate if *Acinetobacter calcoaceticus* sp. shows the pattern of poly-P bacteria in pure cultures under anaerobic/aerobic conditions.

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MATERIALS AND METHODS

Culture and Growth Conditions

Acinetobacter calcoaceticus ATCC31012 strains were obtained from the American Type Culture Collection (Rockville, MD, USA). The cultures were grown in 1-L flasks with a working volume of 500 mL, provided with pure oxygen, and maintained in a temperature (25°C) controlled room. The growth media consisted of sodium acetate as the carbon source, and a mineral salts medium as follows: (made up to 1 liter with distilled water), $NaCH_3COO \cdot 3H_2O$, 5 g; KH_2PO_4, 0.15 g; NH_4Cl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $FeCl_3$, 0.02 g; $CaCl_2$, 0.02 g and 20 mL Hunter solution (per liter of Hunter solution contains: nitrilotriacetic acid, 10 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; and 50 mL Metals 44 solu-

tion). 100 mL of Metals 44 solution contains: EDTA, 250 mg; $ZnSO_4 \cdot 7H_2O$, 1095 mg; $FeSO_4 \cdot 7H_2O$, 500 mg; $MnSO_4 \cdot H_2O$, 154 mg; $CuSO_4 \cdot 5H_2O$, 39.2 mg; $Ca(NO_3)_2 \cdot 4H_2O$, 21.6 mg; and $Na_2B_4O_7 \cdot 10H_2O$, 17.7 mg. The growth media were sterilized in an autoclave at 121°C and 15 psi for 30 min. In order to maintain a healthy supply of colonies, agar plates were streaked every 2 to 3 weeks and incubated at 35°C. All of the agar plates were prepared under a laminar flow hood to prevent any contamination. The contents of the agar plate medium consist of yeast extract (3 g/L), peptone (5 g/L), and agar (15 g/L).

Sequencing Batch Reactor Experiments

Two liter fermenters (New Brunswick Scientific Co.) were used for this study. For the SBR runs, the maximum working volume was 1.5 L, with a minimum of 0.5 L. The dissolved oxygen (DO) concentration was maintained at 4-6 mg/L in the aerobic experiments and < 0.1 mg/L in the anaerobic experiments. The cyclic operation of the reactor was automated using a programmable timer (ChronTrol, XT model) and solenoid valves. The reactors were operated on 8-hour cycles, with each cycle including a 0.5 h fill time, a 2 h anaerobic time, a 5 h aerobic time and a 0.5 h withdrawal. Pure oxygen was provided only during the aerobic period, with only pure nitrogen during the anaerobic period. The cultures were mixed by a magnetic stirrer, except during the withdrawal period. One liter of sterilized medium was inoculated by suspending 2-3 loops of biomass from the agar plate cultures. The SBR was operated in batch mode until the optical density of the culture indicated the end of log growth phase, where no significant increase in cell culture concentration was observed. All experiments were performed at 25 ± 0.5°C in the temperature controlled room.

Analytical Methods

Total suspended solids (TSS) and volatile suspended solids (VSS) were used to monitor the biomass concentration. A known volume of sample was filtered through a pre-weighed Whatman GF/C filter with a 1.0 μm pore size. After filtration, each filter was dried for 1 h at 105°C, and weighed and the total suspended solids calculated from the difference. The dried filters were ignited in a furnace for 15 to 20 min at a temperature of 500 ± 50°C, and weighed again for the loss of volatile suspended solids. The COD of the culture broth was measured to determine the carbon substrate concentrations, as per the Standard Method procedure adapted by Hach Chemical Company [12]. The soluble orthophosphate was determined on samples filtered through a sandwich filter made up of a Whatman GF/C glass fiber filter (1.0 μm) and a Millipore filter (0.45 μm), and was measured by the vanadomolybdo-phosphoric acid colori-metric method, Section 4500-P C [12]. Total phosphorus was determined by the same method, on a sample prepared by the persulfate digestion method, Section 4500-P B [12].

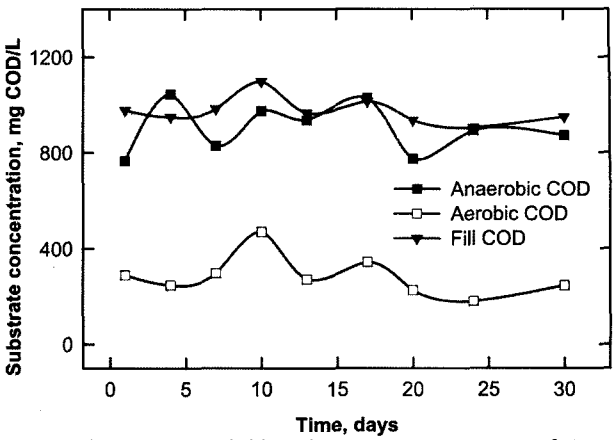

Fig. 1. The residual soluble substrate concentration of *Acinetobacter* under anaerobic/aerobic conditions in SBR.

RESULTS AND DISCUSSION

The residual substrate concentrations, at the end of anaerobic and aerobic stages, are shown in Fig. 1. Each data point in Fig. 1 represents the average of 9 measurements from 3 experiments. It can be seen that both the anaerobic and the aerobic COD concentrations were constant over the entire period of operation. The SBRs were fed with influent containing an initial COD concentration of 2,350 mg/L and were operated over a 30 day period. The average residual COD concentration after the aerobic stage was 280 mg/L, after the fill period was about 980 mg/L, and after the anaerobic period was 900 mg/L. The soluble COD mass balance was conducted at the fill stage to estimate if any soluble substrate was removed by uptake or dilution. The soluble COD balance was calculated as follows:

$$V_{Fill} \times COD_{Fill} = V_{Inf} \times COD_{Inf} + V_{Out} \times COD_{Out}$$

where

V_{Fill} = Volume of fill stage (L)
COD_{Fill} = COD concentration at the end of the fill stage (mg/L)
V_{Inf} = 0.5 L
COD_{Inf} = Influent COD concentration (mg/L)
V_{Out} = Volume of culture withdrawn (L)
COD_{Out} = COD concentration at the end of the withdrawal stage (mg/L)

Based on these results, the soluble COD removal during the anaerobic phase was reduced from about 980 mg/L to about 900 mg/L (about 8%) indicating that the anaerobic COD uptake by *Acinetobacter calcoaceticus* pure cultures was not significant. The aerobic stage reduced the soluble COD from about 900 mg/L to about 280 mg/L, a removal of about 63%. The overall soluble COD removal in the SBR during the anaerobic and aerobic phases is about 71% of the culture soluble COD concentration of about 980 mg/L at the end of the fill period.

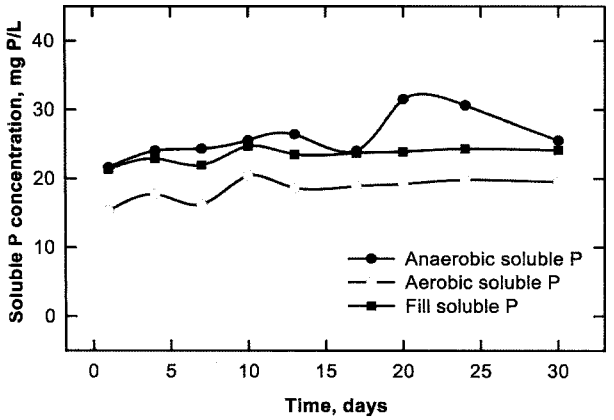

Fig. 2. Soluble P concentration of *Acinetobacter* under anaerobic/aerobic conditions in SBR.

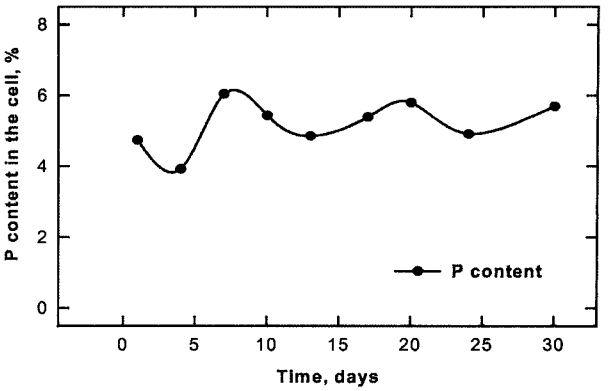

Fig. 3. P content of *Acinetobacter* cells under anaerobic/ aerobic conditions in SBR.

The anaerobic soluble phosphate, aerobic soluble phosphate, and the fill soluble phosphate concentrations of the *Acinetobacter* culture in SBR are shown in Fig. 2. It was observed that the average soluble phosphate concentration at the end of the anaerobic, the aerobic and the fill stages were 25.9 mg/L, 18.4 gm/L and 23.4 mg/L, respectively. About 44% of the soluble phosphate was taken up in this system with an influent soluble phosphate concentration of 33.0 mg/L. A soluble phosphate mass balance was conducted at the fill stage to determine the anaerobic soluble phosphate release. The soluble phosphate mass balance was calculated as follows:

$$V_{Fill} \times P_{Fill} = V_{Inf} \times P_{Inf} + V_{Out} \times P_{Out}$$

where

V_{Fill} = Volume of fill stage (L)
P_{Fill} = Soluble P concentration at the end of the fill stage (mg/L)
V_{Inf} = 0.5 L
P_{Inf} = Influent soluble P concentration (mg/L)
V_{Out} = Volume of culture withdrawn (L)
P_{Out} = Soluble P concentration at the end of the with-drawal stage (mg/L)

The average soluble phosphate release was about 2.6 mg/L during the anaerobic stage corresponding to a soluble COD removal of about 80 mg/L. The soluble COD uptake/phosphate release during the anaerobic stage was about 31 mg COD/mg PO_4-P. The average soluble phosphate release was about 6.1 mg P/g VSS which is much lower than the values (27-76 mg P/g VSS) found in EBPR activated sludge plants [13,14]. Even though several researchers have investigated the anaerobic phosphate release in pure culture batch experiments, the phenomena of soluble phosphate release concurrent with soluble COD uptake, during the anaerobic stage in continuous flow experiments, has not been reported. Tandoi and his colleagues [15] conducted two stage chemostat experiments with *Acinetobacter* under anaerobic/aerobic conditions, and found little or no soluble phosphate release under the anaerobic conditions. With pure cultures of *Acinetobacter* strains, Appeldoorn and his colleagues [16] obtained lower rates of phosphate release (less than 1 mg P/g dry cell weight) than from the tested phosphate-accumulating activated sludge. They stated that their enhanced biological phosphate removal sludge consists of a diverse group of *Acinetobacter* strains, which may be responsible for the higher phosphate release under the anaerobic conditions. Moreover, the possibility that bacteria other than *Acinetobacter* may be responsible for the enhanced biological phosphate removal has been proposed by several research groups [17-19]. Fig. 3 presents the phosphate content in the *Acinetobacter* cells during a 30-day period. The average phosphate content was 5.2% (52 mg P/g VSS). This value is higher than that (4.3% or 43 mg P/g VSS) obtained from batch experiments using the same *Acinetobacter* sp. [20]. Table 1 compares the phosphate content of the *Acinetobacter* pure cultures in this study with those obtained by Appeldoorn *et al.* under batch conditions. The results from this research compare well with those of Appeldoorn *et al.*, even though the SBR system was operated under anaerobic/aerobic conditions. The average performance of the SBR grown *Acinetobacter,* in terms of COD and phosphate removal, are shown in Table 2. Each of the parameters was determined, as shown below, and compared with typical values for a successfully operating laboratory scale EBPR activated sludge system obtained by Mamais [21].

$$\text{Overall COD removal efficiency} = \frac{COD_{Inf} - COD_{Out}}{COD_{Inf}} \times 100$$

$$\text{Overall P removal efficiency} = \frac{P_{Inf} - P_{Out}}{P_{Inf}} \times 100$$

$$\text{Anaerobic P release rate} = \frac{P_{An} - P_{Out}}{\text{Anaerobic VSS} \cdot \text{Anaerobic reacion time}}$$

$$\text{COD uptake/P release} = \frac{COD_{Fill} - COD_{An}}{P_{An} - P_{Fill}}$$

Table 1. Comparison of P content of *Acinetobacter* pure cultures.

	P content (mg P/g dry wt.)	Reference
Acinetobacter strain 1	52-62	[16]
Acinetobacter strain 210A	39-88	[16]
Acinetobacter strain B8	47	[16]
Acinetobacter strain P	56	[16]
Acinetobacter calcoaceticus, batch	43	From this study
Acinetobacter calcoaceticus, SBR	52	From this study

Table 2. Average performance of *Acinebacter* in SBR at steady state

	Performance	Reference [21]
COD removal efficiency		
in anaerobic period, %	8	65
total, %	71	83
Overall P removal efficiency, %	44	78
Anaerobic P release rate, mg P/g VSS · h	3.1	11
COD uptake/P release, mg COD/mg P	31	11
P content in the cell, %	5.2	4.5
Anaerobic COD uptake rate, mg COD/g VSS · h	83	110
Aerobic P uptake rate, mg P/g VSS · h	2.7	6.4

$$\text{P content in the cell} = \frac{P_T - P_{Ae}}{\text{Aaerobic VSS}}$$

$$\text{Anaerobic COD uptake rate} = \frac{COD_{Fill} - COD_{An}}{\text{Anaerobic VSS} \cdot \text{Anaerobic reacion time}}$$

$$\text{Aerobic P uptake rate} = \frac{P_{An} - P_{Ae}}{\text{Aaerobic VSS} \cdot \text{Aaerobic reacion time}}$$

Where

P_{An} = Soluble P concentration at the end of the anaerobic stage (mg/L)
P_T = Total P concentration at the end of the aerobic stage (mg/L)
P_{Ae} = Soluble P concentration at the end of the aerobic stage (mg/L)
COD_{An} = COD concentration at the end of the anaerobic stage (mg/L)

CONCLUSION

Pure cultures of *Acinetobacter* were grown in an SBR under alternating anaerobic/aerobic conditions to simulate a full scale EBPR system. However, these species were not able to take up soluble COD and release soluble phosphate under anaerobic conditions. The results obtained suggested that there might be some other mechanisms, other than that of EBPR, responsible for the proliferation of *Acinetobacter* under anaerobic/aerobic conditions in activated sludge EBPR systems. Alternatively, the features of the EBPR might not be able to be established in pure cultures due to the many environmental factors that exist in activated sludge systems. Even though EBPR mechanisms were not established in pure cultures of *Acinetobacter*, there is no doubt that the mechanisms in EBPR activated sludge systems play an important role in the removal of phosphate. These environmental factors, which affect selection in mixed populations, should be investigated further.

REFERENCES

[1] Jenkins, D. and V. Tandoi (1991) The applied microbiology of enhanced biological phosphate removal: Accomplishments and needs. *Wat. Res.* 25: 1471-1478.
[2] Cech, J. S. and P. Hartman (1993) Competition between polyphosphate and polysaccharide accumulating bacteria in enhanced biological phosphate removal systems. *Wat. Res.* 27: 1219-1225.
[3] Fuhs, G. W. and M. Chen (1975) Microbiological basis of phosphate removal in the activated sludge process for the treatment of wastewater. *Microb. Ecol.* 2: 119-138.
[4] Deinema, M. H., L. H. A. Habets, J. Scholten, E. Turkstra, and H. A. A. M. Webers (1980) The accumulation of polyphosphate in *Acinetobacter* spp. *FEMS Microbiol. Lett.* 9: 275-279.
[5] Ohtake, H., K. Takahashi, Y. Tsuzuki, and K. Toda (1985) Uptake and release of phosphate by a pure culture of *Acinetobacter calcoaceticus*. *Wat. Res.* 19: 1587-1594.
[6] Hao, O. J. and C. H. Chang (1987) Kinetics of growth and phosphate uptake in pure culture studies of *Acinetobacter* species. *Biotechnol. Bioeng.* 29: 819-831.
[7] Tandoi, V., M. Beccari, R. Ramadori, and L. S. Annicchiarico (1987) *Ascinetobacter* sp. growth in alternate anaerobic-aerobic conditions. pp. 305-308. In: R. Ramadori (ed.). *Advances in Water Pollution Control: Biological Phosphate Removal from Wastewaters.* Pergamon Press, Oxford, UK.
[8] Mino, T., M. C. M. van Loosdrecht, and J. J. Heijnen (1998) Microbiology and biochemistry of the enhanced biological phosphate removal process. *Wat. Res.* 32: 3193-3207.
[9] Brodisch, K. E. U. and S. J. Joyner (1983) The role of microorganisms other than *Acinetobacter* in biological phosphate removal in activated sludge processes. *Wat. Sci. Technol.* 15: 117-125.
[10] Hiraishi, A. and Y. Morishima (1990) Capacity for polyphosphate accumulation of predominant bacteria in activated sludge showing enhanced phosphate removal. *J. Ferment. Bioeng.* 69: 368-371.
[11] Lötter, L. H., M. C. Wentzel, R. E. Lowenthal, G. A. Ekama, and G. V. R. Marais (1986) A study of selected characteristics of *Acinetobacter* spp. isolated from activated sludge in anaerobic/anoxic/aerobic and aerobic systems. *Water SA* 12: 203-208.

[2] APHA, AWWA and WEF (1992) *Standard methods for the examination of water and wastewater*, 18th ed., American Public Health Association, Washington, DC, USA.
[3] Satoh, H., T. Mino, and T. Matsuo (1992) Uptake of organic substrates and accumulation of polyhydroxyalkanoates linked with glycolysis of intracellular carbohydrates under anaerobic conditions in the biological excess phosphate removal processes. *Wat. Sci. Technol.* 26: 933-942.
[4] Smolders, G. J. F., J. van der Meij, M. C. M. van Loosdrecht, and J. J. Heijnen (1994) Model of the anaerobic metabolism of the biological phosphorus removal process: stoichiometry and pH influence. *Biotechnol. Bioeng.* 43: 461-470.
[5] Tandoi, V., M. Majone, J. May, and R. Ramadori (1998) The behavior of polyphosphate accumulating *Acinetobacter* isolates in an anaerobic-aerobic chemostat. *Wat. Res.* 32: 2903-2912.
[6] Appeldoorn, K. J., A. J. Boom, G. J. J. Kortstee, and A. J. B. Zehnder (1992) Contribution of precipitated phosphates and acid-soluble polyphosphate to enhanced biological phosphate removal. *Wat. Res.* 26: 937-943.
[17] Gersberg, R. M. and D. W. Allen (1984) Phosphorus uptake by *Klebsiella pneumoniae* and *Acinetobacter calcoaceticus*. *Proc. IAWPRC Seminar on Enhanced Biological Phosphorus Removal from Wastewaters.* 1: 146-151.
[18] Suresh, N., R. Warburg, M. Timmerman, J. Wells, M. Coccia, M. F. Roberts, and H. O. Halvorson (1985) New strategies for the isolation of microorganisms responsible for phosphate accumulation. *Wat. Sci. Technol.* 17: 99-111.
[19] Nakamura, K., K. Masuda, and E. Mikami (1989) Polyphosphate accumulating bacteria and its ecological characteristics in activated sludge process. *Fifth International Symposium on Microbial Ecology.* Kyoto, Japan.
[20] Kim, H. and K. R. Pagilla (2000) Competitive growth of *Nocardia* and *Acinetobacter* under anaerobic/aerobic batch operation. *Wat. Res.* 34: 2667-2674.
[21] Mamais, D. (1991) *The effects of temperature and mean cell residence time on enhanced biological phosphorus removal by activated sludge*. Ph. D. Dissertation, University of California, Berkeley, CA, USA.

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