Kinetic and metabolic aspects of *Defluviicoccus vanus*-related organisms as competitors in EBPR systems

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ABSTRACT

A reactor was successfully enriched (90% as shown by Fluorescence *in situ* Hybridization) in *Defluviicoccus vanus*-related organisms presenting a Glycogen Accumulating Organisms (GAO) phenotype. Initial batch tests were performed using anaerobic/aerobic conditions to assess the capacity of different carbon sources utilization frequently abundant in wastewater: acetate, propionate, butyrate, valerate and glucose. Acetate and propionate were totally consumed in the anaerobic phase as well as butyrate and valerate, though these last ones with a very low consumption rate. All substrates were converted to polyhydroxyalkanoates (PHA). Glucose had a very slight anaerobic consumption but failed to disclose a typical GAO phenotype. In aerobic conditions, again all carbon sources were readily consumed except for glucose, with acetate and propionate having the higher consumption rates. Therefore, glucose seems not be used by this type of organisms. Acetate and propionate consumption rates indicated that these GAOs could reveal good competition advantages in EBPR systems where these carbon sources are available, especially propionate. Volatile Fatty Acid (VFA) uptake in aerobic phase and consequential PHA production indicate these organisms as possible candidates for PHA production.

Key words | carbon substrate, EBPR, GAOs, *in vivo* NMR

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INTRODUCTION

Eutrophication has been considered a serious problem since it affects directly and indirectly water quality, landscape preservation and especially ecosystems. Since the 1970s a biological system, Enhanced Biological Phosphorus Removal or EBPR, has been developed and implemented. This system allows phosphorus removal from wastewater in a more sustainable alternative to chemical processes. However, dealing with a biological system implies some variability that will result from differences in wastewater composition, environmental and climatic conditions. Therefore, a large interest has arisen in studying the process variables as well as the microbiology dynamics involved.

EBPR systems function through a mixed cultures process mainly enriched in a group of organisms known as Polyphosphate Accumulating Organisms (PAOs). However, some system's failures have been reported where phosphorus removal capacity has decreased or ceased. These events have often been ascribed to another group of microrganisms known as Glycogen Accumulating Organisms (GAOs) that have a similar behaviour as PAOs, namely in the carbon cycling. GAOs can, therefore, compete with PAOs for carbon sources, but without storing internal polyphosphate reserves, and if the system's conditions favour them, they will strive and deteriorate the EBPR capacity. Several types of GAOs have been identified although none has been isolated. From these, two major groups seem to play a possible leading role in this PAO-GAO competition: *Candidatus* Competibacter phosphatis and *Defluviicoccus vanus*-related organisms (clusters 1 and 2).

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Defluviicoccus vanus-related organisms have been reported as presenting a GAO phenotype and have been found in some EBPR full scale systems, although their role seems to be less preponderant than the one of *C. phosphatis* (Burow *et al.* 2007). So far, several Fluorescence *in situ* Hybridisation (FISH) probes have been developed for the two main clusters identified (Wong *et al.* 2004; Meyer *et al.* 2006). Some other phylogenetic studies proposed other FISH probes to differentiate subgroups in each cluster (Wong & Liu 2007). Some FISH-MAR studies reveal some information on their plausible carbon sources (Burow *et al.* 2007; Wong & Liu 2007) and a recent study indicates their possibility to denitrify (Wang *et al.* 2007). Some kinetic data has also been made available using acetate and propionate as carbon sources (Dai *et al.* 2007). However little information is yet available about this group, especially concerning their metabolic and kinetic parameters.

This work intends to evaluate kinetic parameters of *D. vanus*-related organisms as a way to assess what type of conditions would influence these GAOs over PAOs. Also, metabolic pathways are being analysed by *in vivo* NMR in order to understand their energetic balance in the process and to determine whether they fit into the models proposed for GAO's metabolism.

METHODS

Reactor and batch experiments setup

A 1.5 L lab-scale reactor was inoculated with a GAO enriched sludge. Operating conditions comprise a five-sequence phase system of 2 hour anaerobic, 4 hour aerobic, 1 hour decanting, 0.5 hour purging exhaust medium and 0.5 hour feeding fresh medium. Hydraulic Retention Time (HRT) was 1 day and Sludge Retention Time (SRT) was kept at 10 days with manual sludge purging every other day. A synthetic effluent with propionate as carbon source (960 ppm COD) was used. pH was controlled at 7, reactor temperature was maintained at 30°C with a water jacket and phosphorus was kept at low concentration (approximately 1:120 P:C molar ratio). The reactor was regularly sampled to check for propionate, glycogen, PHA, ammonia, phosphorus and cell dry weight. Fixation for FISH analysis was done at least one every 3 SRT. The reactor was operated for 3 months until it reached a pseudo-steady state.

Batch experiments were held in a 600 mL reactor. Sludge for the essay was collected at the end of the aerobic phase from the mother reactor. Cells were washed and re-suspended in 400 mL of mineral medium. A separate solution containing carbon, ammonia and phosphorus was prepared using 200 mL of mineral medium. The start of the batch was determined by the addition of the last solution.

Synthetic medium

The reactor was fed with a synthetic mineral medium described in Lemos *et al.* (2003) with slight modifications: carbon source was propionic acid 0.63 g/l (960 ppm COD), a nitrification inhibitor *N*-allylthyorea 0.01 g/L was used. All batch experiments used the same mineral medium composition except for the ammonia that was added only to the carbon solution. A phosphorus solution (0.023 g/L K_2HPO_4 and 0.11 g/L KH_2PO_4) was autoclaved and later added to the main solution. All full cycle kinetic studies and anaerobic kinetic studies had a carbon concentration in the medium of 960 ppm COD. Aerobic studies had a carbon concentration of 20 C-mM.

Analytical methods

Volatile Fatty Acids (VFA) were analysed by High Pressure Liquid Chromatography using a BIORAD Aminex HPX-87H column and a UV detector at 210 nm. Samples were run using H_2SO_4 0.01 N as eluent (0.6 mL/min, 50°C). Glycogen was extracted from lyophilised cells (approximately 2 mg) by an acidic digestion (1 mL HCl 0.6 M, 2 hours, 100°C). Samples were analysed by HPLC using a BIORAD Aminex HPX-87H column and a Refractive Index detector, using H_2SO_4 0.01 N as eluent (0.6 mL, min 50°C). PHAs were analysed using the method described in Lemos *et al.* (2006). Dry cell weight was assumed as being equal to Volatile Suspended Solids (VSS). It was determined according to Standard Methods (APHA 1995). All data was calculated using specific cell dry weight (X) and converting it to C-mmol X assuming a global biomass equation of $CH_{1.89}O_{0.62}N_{0.18}$ (Oehmen *et al.* 2006). Phosphorus was determined using the ascorbic acid method and

using a segmented flow apparatus from SKALAR (SKALAR-5100, The Netherlands) at 880 nm. Ammonia was quantified using an ammonia gas sensing combination electrode ThermoOrion 9512.

FISH

FISH was performed according to the method described in Amann (1995). Probes were always used in pairs, specific probe (in CY3) against general probe (EUBmix, in FITC), except for controls using only EUBmix and no probe at all to detect auto fluorescence. General probes used were EUBmix targeting most bacteria and composed of probes EUB338, EUB338-II and EUB338-III in FITC (Stahl & Amann 1991, Daims *et al.* 1999), and ALF1b in Cy3 targeting Alpha-Proteobacteria (Manz *et al.* 1992). Specific probes for PAOs formed PAOmix composed by PAO462, PAO651 and PAO846 in Cy3 (Crocetti *et al.* 2000) and specific probes for *C. phosphatis* formed GAOmix composed of GAOQ431, GAOQ989 and GB_G2 in Cy3 (Crocetti *et al.* 2002; Kong *et al.* 2002). Specifically for *D. vanus* related GAOs, two groups were used: TFOmix targeting cluster 1 composed of TFO_DF218 and TFO_DF618 in Cy3 (Wong *et al.* 2004) and DEFmix targeting cluster 2 composed of DEF988 and DEF1020 in Cy3 (Meyer *et al.* 2006).

In-vivo NMR

This technique was used as described in Lemos *et al.* (2007) using 3-^{13}C propionate.

RESULTS & DISCUSSION

Microbial characterisation

Operating the SBR during 3 months led to an enrichment of 90% in *D. vanus*-related organisms, from which 70% belong to cluster 1 and 30% to cluster 2 as shown by FISH (Figure 1). No PAOs have ever been detected in the system when using PAOmix probes. *Competibacter phosphatis*, detected by GAOmix probes, was present at the seeding but totally disappeared in 3 weeks.

The system had a typical GAO phenotype where glycogen and PHA cycling and anaerobic propionate consumption was observed as shown in Figure 2. No overall phosphorus removal was observed.

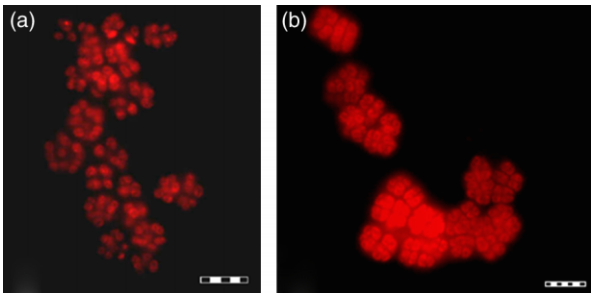

Figure 1 | FISH image of *Defluviicoccus vanus* related GAOs found in the system. a—cluster 1 detected by TFOmix (Cy3); b—cluster 2 detected by DEFmix (Cy3). (scale bar = 5 μm).

Batch tests

Several batch tests have been conducted using propionate, acetate, butyrate, valerate and glucose to determine whether this population would be able to use them without acclimatisation. Conditions for batch tests were anaerobic/aerobic, to simulate natural conditions. However, apart from acetate and propionate, no other carbon source could easily be taken up in anaerobic conditions. Hence, aerobic batch tests were conducted in order to assess whether the substrates can be used.

In anaerobic conditions, acetate was rapidly taken up, although at half the consumption rate for propionate. A slower acetate consumption rate was also reported by Dai *et al.* (2007) using a *D. vanus* related cluster 1 culture enriched with acetate as the carbon source. The other carbon sources had a very slow uptake in anaerobic

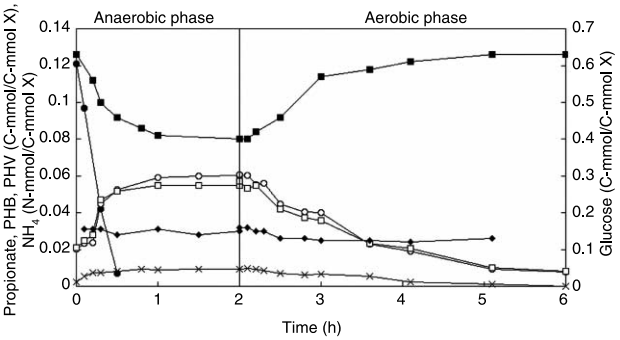

Figure 2 | Typical reactor cycle. ●—Propionate; □—PH2MV; ○—PHV; ×—PHB; ■—Glycogen; ◆—Ammonia.

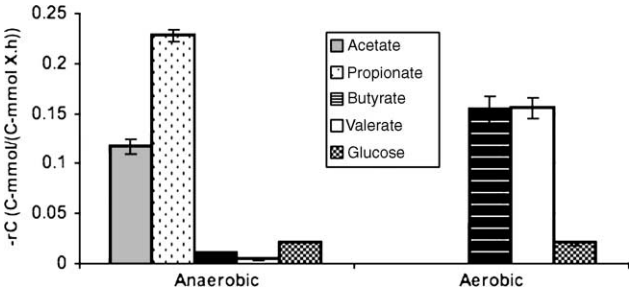

Figure 3 | Uptake rates of the several carbon sources tested for the system both in anaerobic and aerobic conditions.

conditions as shown in Figure 3. Despite their slow consumption rate, butyrate and valerate were clearly consumed. For butyrate a mixture of polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) was produced while for valerate the polymer was mainly composed by PHV and at less amount Polyhydroxy-2methylvalerate (PH2MV). The energetic effort of carbon uptake is translated in much higher glycogen/substrate yields for valerate and butyrate than for acetate and propionate. For glucose no PHA resulted from this slight consumption.

Considering these low anaerobic carbon consumption rates for butyrate, valerate and glucose, aerobic batches were conducted. As also shown in Figure 3, butyrate and valerate showed a significant consumption rate, similar to what was obtained in anaerobic batches with acetate and propionate. Aerobic carbon consumption led to PHA production. This population was unable to effectively use glucose, which agrees with what was reported by Burow *et al.* (2007) using FISH-MAR, but opposed to what Wong & Liu (2007) observed. Aerobic batch tests with acetate and propionate are still being conducted.

In vivo NMR

In-vivo NMR has successfully been able to present results concerning metabolic pathways used by mixed cultures (Lemos *et al.* 2003, 2007). Concerning the metabolic pathways used, preliminary *in vi*vo NMR was performed using labelled propionate (3-^{13}C-Propionate). This technique allows on-line monitoring of the evolution of the label throughout the different metabolites providing information not only on the final products but also on the metabolic pathways involved. The general metabolic inter-conversion of carbon for the GAO phenotype under anaerobic/aerobic conditions was observed (substrate to PHA and then to glycogen) as shown in Figure 4. A large resonance peak that could be attributed to carbon 5 of PHV was observed, but also may indicate the presence of other polymers such as PH2MV. Chloroform extracts of the polymer need still to be analysed in order to determine the labelling pattern in each carbon for metabolic pathway elucidation. In the aerobic phase, PHAs were clearly converted into glycogen, which appears marked in carbon 1, 2, 5 and 6. Other minor peaks at 55.29 and at 27.54 ppm could indicate the presence of glutamic acid.

Ongoing research involves the optimization of operational conditions for NMR experiments, namely concerning pH control and biofilm formation inside NMR tube.

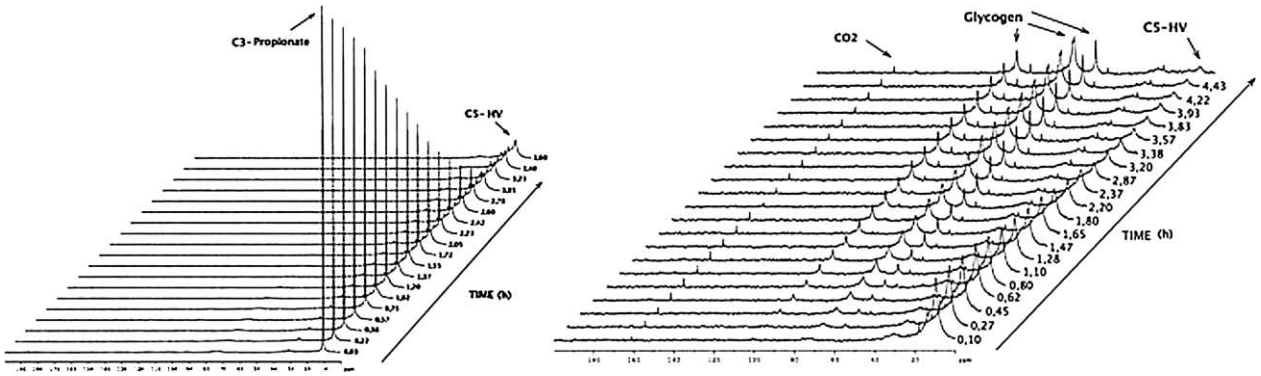

Figure 4 | NMR spectra for *Defluviicoccus-vanus* related GAO enriched culture fed with 3^{13}CPropionate. left—anaerobic phase; right—aerobic phase.

CONCLUSION

This study used a highly enriched *D. vanus* related GAO mixed culture to assess affinities for five different carbon sources. Results showed a high affinity of this population for acetate and propionate, revealing that in a full-scale EBPR, where these two carbon sources would be available, they could become important competitors. This study also showed that other VFA sources, namely butyrate and valerate, were used, but probably require a metabolic adaptation and are not so energetically favourable. However, these results also indicated that these GAOs, if used for PHA production, would use these carbon sources and convert them to PHA. Preliminary *in vivo* NMR studies showed the conversion of propionate to polyhydroxyalkanoates during the anaerobic phase, and its mobilization for glycogen production under aerobiosis.

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