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Aerobic metabolism of mixed carbon sources in sequencing batch reactor under pulse and continuous feeding

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HIGHLIGHTS

- Aerobic storage of PHB and glycogen from acetate/starch mixture was studied.
- ^{13}C NMR analysis was used to evaluate metabolic pathways of aerobic storage.
- Metabolism of glycogen from starch was not affected by the presence of acetate.
- Starch strongly affected acetate metabolism via anaplerotic and catabolic routes.
- PHB storage from acetate strongly decreased in the presence of starch.

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ABSTRACT

The aerobic metabolism of a mixture of acetate and starch was studied with main emphasis on their interaction and the effect on their storage as PHB and glycogen, respectively. Pulse feeding strongly increased the storage of both substrates; however, the presence of starch decreased PHB storage whereas the presence of acetate did not affect glycogen storage.

Indeed, ^{13}C NMR isotopomer analysis suggested an increase of acetate utilization towards TCA cycle, due to an increased request of ATP production for glycogen biosynthesis regulated by ADP-GlcPPase. This in turn influenced the partition flux for pyruvate synthesis between TCA cataplerosis and glyoxylate shunt. The corresponding reduction of PHB synthesis was in agreement with the competition for HS-CoA between KGDH activity and acetyl-CoA for PHB synthesis pathway.

As a practical consequence, bioprocesses for PHA production from volatile fatty acids could be negatively affected from other carbon sources, such as unfermented carbohydrates.

1. Introduction

The comprehension of storage phenomena under aerobic conditions is relevant for their effect of microbial population dynamics in activated sludge processes and related bulking control (Majone et al., 1996) as well as for production of biodegradable polymers by using microbial mixed cultures and volatile fatty acids as carbon source (Dionisi et al., 2004). Due to the time- or space-dependent substrate profile in the biological tanks in wastewater treatment plants (WWTPs), microorganisms frequently face with transient conditions and tend to convert the substrate into storage polymers whenever the substrate uptake rate can be higher than its direct utilization for growth and related energy needs (Majone et al., 1996). The balance between the direct growth and storage may be regulated through appropriate choice of operating conditions, e.g. organic load rate and feeding pattern (e.g. continuous vs. sequential).

The presence and relative magnitude of the storage phenomena are dependent on the type of the carbon source. Glycogen is mostly formed when the primary substrate is a compound that can be easily converted into pyruvate and reducing power, e.g. glucose, other carbohydrates, glycerol or proteins. On the other hand, polyhydroxyalkanoates (PHAs) are directly formed from the central metabolite acetyl-Coenzyme A (acetyl-CoA) (Anderson and Dawes, 1990; Doi, 1990), as in the metabolism of acetic acid and other volatile fatty acids (VFAs).

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The interaction between the metabolic pathways of different substrates has mainly been studied for pure cultures. The different carbon sources can either simultaneously or sequentially used; the latter case often occurs because microorganisms prefer the most easily accessible carbon source which allows faster growth or higher growth yield which in turn prevents the use of other, secondary, carbon sources (Gorke and Stülke, 2008). As an example, *Escherichia coli* and *Bacillus subtilis* typically utilize the carbon sources in a substrate mixture in a sequential way, with glucose being the preferred substrate (Lin, 1996). The co-utilization of a mixture of acetate and glucose has been also reported for *Corynebacterium glutamicum* (Wendisch et al., 2000). However, the consumption rates of the individual substrates were reduced during acetate–glucose co-metabolism, resulting in similar total carbon consumption rates for the three conditions (Wendisch et al., 2000). In contrast to *C. glutamicum*, *Azotobacter vinelandii* preferentially uses acetate when grown on glucose–acetate mixtures, which is due to acetate-dependent inhibition of glucose uptake and glycolysis (Tauchert et al., 1990).

Dealing with mixed microbial cultures, the interaction between carbohydrate and acetic acid metabolisms and related storage has been studied under anaerobic conditions, in the frame of enhanced phosphorus removal processes. The studies have shown the role of the glycogen metabolism in the acetic acid metabolism from Phosphorus Accumulating Organisms (PAOs), where internally stored glycogen supplies both reducing power and acetyl-CoA for PHA formation under anaerobic conditions (Yagci et al., 2007). On the other hand, glycogen is formed back during metabolism of PHA under subsequent aerobic conditions (Mino et al., 1995; Pereira et al., 1996; Serafim et al., 2002). The relative amount of PHA and glycogen formed in mixed cultures under alternating anaerobic/aerobic conditions has been found to also depend on the competitions between PAOs and Glycogen Accumulating Organisms (GAOs), the latter using glucose instead of acetic acid and forming glycogen directly with no PHA turn over.

With reference to aerobic conditions, by using a mixture of acetic, propionic, and lactic acid (Dionisi et al., 2004), a strong decrease of the removal rates of acetic and lactic acid was observed in the presence of another substrate than when using each substrate as a sole carbon source. Similarly, the individual removal of acetate and starch (and respective storage of PHA and glycogen) was observed at slightly lower rates as compared to single substrates (Karahan et al., 2008). These interactions among the different substrates suggested that they were removed by the same microorganisms that utilize same or interconnected pathways.

On the other hand, by using a mixture of glucose and acetate, Carta et al. (2001) reported that there were no differences in the uptake of acetate and glucose compared to the experiments performed with the single substrate. The rates of storage were also reported to be independent from the presence of either single or mixed substrates.

The measurement of metabolic fluxes is a useful tool for establishing which pathways are active in a given physiological state and revealing the actual rates of relevant reactions. The use of stable-isotope tracers and the analysis of the distribution of labeled carbons in various intermediates, by both mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy, allow the characterization of specific metabolic routes (Santos et al., 1994; Xavier et al., 2000). As an example, NMR spectroscopy has been used to investigate various aspects of PHA storage including the monomer compositions, cellular content, copolymer analysis and metabolic pathways (Yan et al., 2000). The use of ^{13}C NMR (13 carbon nuclear magnetic resonance) has been shown particularly suited to the in vivo study of glycogen cycling (Gaudet et al., 1992; Pereira et al., 1996).

In this context, the main aim of this study was to investigate the aerobic metabolism of mixed carbon sources in an activated sludge system, as a function of the sludge retention time (SRT) and feeding pattern (pulse vs. continuous feeding), referring primarily to the role of storage compounds under dynamic conditions. Since different storage polymers are produced from different substrates, the study was carried out with two different carbon sources: *acetate*, usually stored as PHB and *starch*, which requires hydrolysis by extracellular enzymes prior to its storage as glycogen.

The relative contribution of the direct growth and storage processes to substrate removal were investigated by traditional chemical measurements, while the metabolic pathways involved in the formation of storage polymers from these substrates were investigated by means of ^{13}carbon nuclear magnetic resonance (^{13}C NMR) analysis. Two amino acids, namely glutamate and alanine were specifically considered as they closely relate to both *glycolysis* and tricarboxylic acid (TCA) cycle, the two major catabolic pathways associated with the selected substrates. Glutamate is highly abundant in most cells and it is in rapid exchange with the TCA cycle intermediate, α-ketoglutarate (Berliner and Robitaille, 1999) whereas the labeling of alanine isotopologues reflects the labeling patterns of pyruvate derived from ^{13}C substrate metabolism such as glucose or more complex sugars.

2. Methods

2.1. SBR operation

Two identical laboratory-scale sequencing batch reactors (SBRs) were operated in parallel at six cycles a day, under pulse and continuous feeding patterns (Table 1). Each SBR had a total reactor volume, V_T, of 2.0 L, where the stationary volume, V_0, holding settled biomass and the fill volume, V_F, were 1.0 L each (V_0/V_F ratio 1.0). In each cycle, the fill volume included successive additions of nutrient and carbon source.

In both SBRs, the substrate feeding was included in the aerobic reaction phase, but it was either very quick (1 min, called "pulse") or continued during the entire reaction phase (150 min out of 180 min, called "continuous"). All other experimental conditions were the same, including the same fresh inoculum taken from the ISKI Pasakoy Advanced Biological Wastewater Treatment Plant in Istanbul, Turkey, to start the experimental SBR operation.

The mixed substrate solution was prepared according to the desired organic loading rate (OLR) by diluting sodium acetate trihydrate ($CH_3COONa.3H_2O$) and soluble starch (SolS) stock solutions to an equal carbon source concentration. The nutrient feed was obtained by diluting in distilled water a nutrient stock solution, which composition was 120 g/L NH_4Cl, 160 g/L KH_2PO_4, 320 g/L K_2HPO_4, 15 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L $FeSO_4{\cdot}7H_2O$, 2 g/L $CaCl_2{\cdot}7H_2O$, 0.5 g/L $MnSO_4{\cdot}H_2O$ and 0.5 g/L $ZnSO_4{\cdot}7H_2O$. The dilution ratio of the nutrient stock solution was adjusted so to have enough buffer capacity and to supply nitrogen and phosphorus at a non limiting concentration with respect to the carbon source.

Table 1
The phase sequences for parallel SBRs.

Phase	Time in cycle (min)	
	Pulse	Continuous
Idle (with mixing) phase (T_I)	0–10	0–10
Fill phase (T_{Fn}) (for nutrient)	10–15	10–15
Fill phase (T_{Fs}) (for substrate)	**15–16**	**15–165**
Reaction phase (T_{AX})	15–180	15–180
Excess sludge withdrawal phase (T_W)	179–180	179–180
Settling phase (T_S)	180–210	180–210
Draw phase (T_D)	210–240	210–240