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The link of feast-phase dissolved oxygen (DO) with substrate competition and microbial selection in PHA production

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are biobased and biodegradable polyesters with the potential to replace conventional plastics. Aeration requires large amounts of energy in PHA production by mixed microbial cultures (MMCs), particularly during the feast phase due to substrate uptake. The objective of this study was to investigate the impact of DO concentrations on microbial selection, substrate competition and PHA production performance by MMCs. This represents the first study investigating DO impact on PHA production while feeding the multiple volatile fatty acids (VFAs) typically encountered in real fermented feedstocks, as well as the substrate preferences at different DO levels. Efficient microbial cultures were enriched under both high (3.47 ± 1.12 mg/L) and low (0.86 ± 0.50 mg/L) DO conditions in the feast phase containing mostly the same populations but with different relative abundance. The most abundant microorganisms in the two MMCs were *Plasticicumulans, Zoogloea, Paracoccus,* and *Flavobacterium.* Butyrate and valerate were found to be the preferred substrates as compared to acetate and propionate regardless of DO concentrations. In the accumulation step, the PHA storage capacity and yield were less affected by the change of DO levels when applying the culture selected under low DO in the feast phase (PHA storage capacity >60% and yield > 0.9 Cmol PHA/Cmol VFA). A high DO level is required for maximal PHA accumulation rates with the four VFAs (acetate, propionate, butyrate and valerate) present, due to the lower specific uptake rates of acetate and propionate under low DO conditions. However, butyrate and valerate specific uptake rates were less impacted by DO levels and hence low DO for PHA accumulation may be effective when feed is composed of these substrates only.

1. Introduction

Polyhydroxyalkanoates (PHAs) are biologically synthesized and biodegradable polyesters with a wide range of thermal and mechanical properties. As environmental friendly bioplastics, PHAs are considered to have the potential to compete with conventional plastics in the near future (Reis et al., 2011). High PHA production up to 80%–90% of cell dry weight has been achieved by pure cultures (Venkateswar Reddy et al., 2012). However, the high costs due to specific substrates, operation under sterilized conditions and polymer extraction have limited a wider industrial application. Hence, mixed microbial cultures (MMCs) was proposed for PHA production by using open systems that potentially require lower operational costs (M G E Albuquerque et al., 2010a; Johnson et al., 2009).

In order to reduce the costs of PHA production by MMCs, industrial wastes/by-products were used as feedstocks, such as olive oil mill effluent (D. Dionisi et al., 2005a,b), sugar cane molasses (Albuquerque et al., 2007) and paper mill wastewater (Bengtsson et al., 2008), through a 3 stage PHA production process. Stage 1: carbon is converted to volatile fatty acids (VFAs) by acidogenic fermentation; stage 2: VFAs that produced in stage 1 are fed to the culture selection reactor for the enrichment of PHA accumulating organisms; and stage 3: the VFAs produced in the stage 1 and the culture enriched in stage 2 are used for enhanced PHA accumulation (Albuquerque et al., 2010a). The feast and famine regime, also known as aerobic dynamic feeding (ADF), is the most commonly used strategy for MMCs selection in stage 2. In the short feast period, microorganisms use external substrate to store PHAs, and in

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the long famine period, when external carbon is unavailable, bacteria use intracellular PHAs as carbon and energy source for cell growth and maintenance (van Loosdrecht et al., 1997).

The enrichment of PHA accumulators depends on the operational conditions applied to the selection reactor. Many studies were conducted in recent years to investigate the impact of operational conditions on culture selection and PHA accumulation, such as pH (Villano et al., 2010), organic loading rates (OLRs) (Dionisi et al., 2006), and feeding frequency (Valentino et al., 2014; Jiang et al., 2011). Compared to these operational conditions, oxygen input is more directly related to process cost, since a large amount of energy is required through the aerobic process of PHA production. However, only a few studies can be found linking the impact of dissolved oxygen (DO) with the PHA production process by mixed microbial cultures. Third et al. (2003) observed that in the feast phase of sequencing batch reactor (SBR) operation fed with acetate, biomass growth was enhanced by excessive aeration rate, yet the PHB conversion yield was improved at low DO level, since more ATP is required for cell growth than polymer synthesis. Moralejo-Gárate et al. (2013), using glycerol as substrate, fostered the culture with higher production of polyglucose over PHB under limited oxygen supply. Polyglucose can be produced from glycerol at a lower energy expense as compared to PHB. Both studies demonstrated that with less oxygen supply, MMCs consumed substrates through the pathways with lower energy requirement. However, these works only focused on individual substrates. With the wide use of fermented industrial wastewater which contains multiple VFAs (acetate, propionate, butyrate and valerate), substrate composition becomes a crucial factor determining microbial communities enrichment and PHA production performance (Albuquerque et al., 2013). A recent study of Coats et al. (2016) investigated the impact of aeration on PHA accumulating culture selection by applying fermented dairy manure as carbon sources. They found that different aeration strategies (K_La 4, 8, 12 and 20 hr^{-1}) had no statistically significant impact on PHA synthesis rates. Thus far, no studies have investigated the link between the DO level supplied and the substrate uptake preferences by MMCs.

The amount of energy (ATP) for the conversion of each VFA into PHA is different. The metabolism of PHA production is dependent on the substrate composition, as pointed out in Table 1. Both acetate and butyrate are converted to acetyl-CoA, then to PHB. Propionate is converted to propionyl-CoA, then to PHV by binding with acetyl-CoA produced either from other VFAs or through decarboxylation from propionyl-CoA, or to PH2MV by coupling 2 propionyl-CoA. Valerate is converted to both acetyl-CoA and propionyl-CoA, then to PHV (Dias et al., 2008; Pardelha et al., 2012). Each mole of VFAs needs 2 mol ATP for PHA synthesis: 1 mol ATP for active transport, and another mole of ATP for intracellular conversion (Dias et al., 2008). Therefore, VFAs with longer carbon chains, like butyrate and valerate, need less ATP to produce one C-mol of PHAs as compared to acetate and propionate.

In this work the impact of DO levels on MMC selection, and on substrate preference was studied. The rationale for the latter was based on the hypothesis that with less oxygen supplied (less ATP generation), valerate and butyrate will be preferred as substrates than acetate and propionate due to the lower ATP requirement. Thus, the selection reactor was operated at different DO levels during the feast phase, when substrate uptake competition takes place. PHA production kinetics in the PHA accumulation stage was determined for the different selected cultures to assess substrate competition at different DO levels.

2. Materials and methods

2.1. Experimental setup

The experimental setup consisted of two bench-scale reactor systems. The selection of PHA-accumulating cultures was carried out in two sequencing batch reactors (SBRs), each with 2 L working volume, subjected to the feast and famine regime. SBR1 was operated under higher DO conditions in the feast phase (3.47 ± 1.12 mg/L) while SBR2 was operated at a lower DO level in the feast phase (0.86 ± 0.50 mg/L). The cultures selected were then inoculated to batch reactors for PHA accumulation testing the impacts of different DO concentrations on substrate competition and process performance of each selected culture.

2.1.1. Culture selection

The two SBRs were inoculated with activated sludge from the Chelas WWTP in Portugal. The 12 h working cycle of both SBRs consisted of four periods, feeding (10 min), aeration (11 h), settling (40 min) and withdrawal (10 min). In the feeding periods, 50 ml of mixed VFAs (25% C-mol based acetic acid, propionic acid, butyric acid and valeric acid) were fed as carbon sources, together with 0.9 L mineral nutrients medium composed of (per liter of deionised water): $MgSO_4 \cdot 7H_2O$ (670 mg), EDTA (110 mg), $CaCl_2 \cdot 2H_2O$ (80 mg), K_2HPO_4 (48 mg), KH_2PO_4 (38 mg) and 1 ml of trace elements solution ($FeCl_3 \cdot 6H_2O$: 1.5 g/L, H_3BO_3: 0.15 g/L, $CuSO_4 \cdot 5H_2O$: 0.03 g/L, KI: 0.03 g/L, $MnCl_2 \cdot 4H_2O$: 0.12 g/L, $Ma_2MoO \cdot 2H_2O$: 0.06 g/L, $ZnSO_4 \cdot 7H_2O$: 0.12 g/L and $CoCl_2 \cdot 6H_2O$: 0.15 g/L) (Serafim et al., 2008). Thiourea (10 mg/L) was also added in the medium to inhibit nitrification. 50 mL of an NH_4Cl solution (4.28 g/L) was added 2 h after the start of the cycle to increase the selective pressure for PHA accumulators (Oliveira et al., 2016). The C/N/P ratio was kept at 100:8:1. The pH of each feed was adjusted to 6.5 before being added to the SBRs. The hydraulic retention time (HRT) of each SBR was kept at one day.

Air was supplied to both SBRs by air pumps through ceramic diffusers. Stirring was kept at 200 rpm by overhead stirrers (VELP-BS). pH was controlled <8 by dosing HCl (0.5 M), where the actual pH varied from 7 to 8. Both the reactors were kept at 22 °C by a water bath. The cycle operations included feeding and withdrawal by peristaltic pumps, aeration and stirring, which were automatically controlled by a software developed in our research group that was also used in other studies (Carvalheira et al., 2014). The software acquired pH and DO data at the same time in order to manipulate the acid dosing pump and air input valves. On/off valves were connected between air pumps and reactors, which were controlled by the software to keep the DO in the SBRs at the designated values according to the DO data acquired. The average

Table 1
VFAs uptake stoichiometry for the production of PHA precursors, on a C-mol basis.

Reaction	Stoichiometry
Acetate → Acetyl-CoA	$CH_2O + ATP \rightarrow CHO_{0.5} + 0.5 \cdot H_2O$
Propionate → Propionyl-CoA	$CH_2O_{2/3} + 0.67 \cdot ATP \rightarrow CH_{4/3}O_{1/3} + 0.33 \cdot H_2O$
Butyrate → Acetyl-CoA	$CH_2O_{0.5} + 0.5ATP \rightarrow CHO_{0.5} + 0.5 \cdot NADH_2$
Valerate → Acetyl-CoA + Propionyl-CoA	$CH_2O_{2/5} + 0.4 \cdot ATP \rightarrow 0.4CHO_{0.5} + 0.6CH_{4/3}O_{1/3} + 0.4 \cdot NADH_2$