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Combining the enrichment and accumulation step in non-axenic PHA production: Cultivation of *Plasticicumulans acidivorans* at high volume exchange ratios

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Highlights

- A one-stage process for simultaneous culture enrichment and PHB production.
- Production of up to 75 wt% PHB from acetate in a non-axenic, single-step process.
- Cultivation at very high volume exchange ratios compromises PHB production rate.
- Increasing the volume exchange ratio forces bacteria to grow faster (at same SRT).

Abstract

The process for non-axenic polyhydroxyalkanoate (PHA) production from organic waste generally comprises three steps: acidogenic fermentation of the waste stream, enrichment of a PHA-producing culture, and production of the PHA. This study assesses the feasibility of combining the enrichment and production step. Harvesting PHA-rich biomass directly from the sequencing batch reactor (SBR) used for enrichment of the microbial culture reduces capital cost, but may increase downstream-processing cost if the PHA content is significantly lowered. Operating an acetate-fed SBR at a volume exchange ratio of 0.75 (18 h cycles, 1 d SRT) allowed the production of biomass with 70 wt% poly(3-hydroxybutyrate) (PHB) in a single-step process. By increasing the exchange ratio to 0.83 (20 h cycles) the PHB content of the harvested biomass increased to 75 wt%, but the operational stability decreased. SBR operation at these high exchange ratios makes that bacteria have to increase their growth rate and external substrate is available for relatively long periods. This allows the establishment of larger flanking populations and negatively affected the kinetic properties of *Plasticicumulans acidivorans*, the predominant organism. Maximizing the volume exchange ratio is, therefore, a suitable strategy to produce large amounts of PHA in the SBR, but does not ensure the enrichment of a culture with superior PHA productivity.

Abbreviations: CL, cycle length; F/M ratio, food-to-microorganism ratio; HRT, hydraulic retention time; PHA, polyhydroxyalkanoate; PHB, poly(3-hydroxybutyrate); SBR, sequencing batch reactor; SRT, solids retention time; TSS, total suspended solids.

Keywords: Cycle length; microbial enrichment culture; *Plasticicumulans acidivorans*; polyhydroxybutyrate (PHB); sequencing batch reactor (SBR); volume exchange ratio

Nomenclature

k	rate constant for PHB degradation	$(Cmol/Cmol)^{1/3}/h$
μ^{max}	maximum biomass-specific growth rate	Cmol/Cmol/h
$\mu^{average}$	average biomass-specific growth rate	Cmol/Cmol/h
m_{ATP}	biomass-specific ATP requirement for maintenance	mol/Cmol/h
q_{Ac}^{max}	maximum biomass-specific acetate uptake rate	Cmol/Cmol/h
$Y_{i,j}$	modeled actual yield of compound *i* on *j*	Cmol/Cmol

1. Introduction

Polyhydroxyalkanoates (PHAs) are microbial storage polymers accumulated by many different prokaryotes as an intracellular carbon and energy reserve (Steinbüchel, 1991; Tan et al., 2014). The chemical properties of the polymer make it an interesting bioplastic that is fully biodegradable (Chen, 2009). Moreover, the PHA monomers could serve as chiral building blocks for the production of various biochemicals, and the hydroxy fatty acid methyl esters could be used as a biofuel (Chen, 2009).

The commercially available PHA is generally produced using pure cultures of *Cupriavidus necator* – formerly *Ralstonia eutropha* (Vandamme and Coenye, 2004) – or recombinant *Escherichia coli*, and glucose and propionic acid as substrate (Chen, 2009). To reduce the production cost of PHA and allow broad application, various researchers investigated the use of open microbial communities and waste organic carbon as substrate (Albuquerque et al., 2010; Bengtsson et al., 2008; Coats et al., 2007; Dionisi et al., 2005; Jiang et al., 2012). The process for non-axenic PHA production from organic waste comprises three steps: (1) acidogenic fermentation of the waste stream, (2) enrichment of a PHA-producing culture, and (3) production of the PHA (Dionisi et al., 2004; Serafim et al., 2008). The first or acidogenic fermentation step (Temudo et al., 2007) aims to convert the waste organic carbon, primarily carbohydrates, to a mixture of volatile fatty acids. These acids are a more suitable substrate for PHA production (Reis et al., 2003) and will be used as such in the following two steps. In the second step, an open microbial community is enriched in bacteria with a high PHA storage capacity. This enrichment step is performed in a sequencing batch reactor (SBR) operated under a feast-famine regime, as intermittent substrate availability creates a competitive advantage for bacteria that store substrate inside their cell as a reserve (Reis et al., 2003). Once a stable culture is obtained, the SBR will be operated as a biomass production step and the excess sludge used in step 3: maximization of the PHA content of the biomass in

a (nitrogen-limited) fed-batch reactor (Johnson et al., 2009a). Biomass with PHA contents of 70-80 wt% has thus been produced from fermented waste streams like molasses and paper or food industry effluents (Albuquerque et al., 2010; Jiang et al., 2012; Tamis et al., 2014). The highest PHA contents reported for microbial enrichment cultures are around 90 wt% and were obtained using sole acetate or lactate as substrate (Jiang et al., 2011b; Johnson et al., 2009a).

PHA-rich biomass could also be harvested directly from the enrichment reactor (Dionisi et al., 2007; Reis et al., 2003). During the feast phase of each SBR cycle, external substrate is taken up and stored as PHA before it is used for growth. At the end of the feast phase, part of the PHA-rich biomass could be harvested. Eliminating the separate accumulation step (step 3) reduces the capital cost, but may lead to increased downstream-processing cost as the maximum PHA content obtained in the SBR is generally much lower (≤ 50 wt%) than that obtained after the fed-batch accumulation step. Jiang et al. (2011a) demonstrated that the PHA content at the end of the feast phase increases if the number of cycles per solids retention time (SRT) is reduced. When they operated the SBR at a volume exchange ratio of 0.5 (12 h cycles and 1 d SRT), 53 wt% PHA was accumulated during the SBR cycle. At a volume exchange ratio of 0.75 (18 h cycles), up to 71 wt% PHA was accumulated (Jiang et al., 2011a). Operating the SBR at a maximized volume exchange ratio might, therefore, lead to sufficiently high PHA contents and allow a reduction in capital cost that outweighs increased downstream-processing costs.

The aim of this study was to assess the feasibility of combining the enrichment and accumulation step in non-axenic PHA production. To that end, an aerobic SBR inoculated with activated sludge was operated at a volume exchange ratio of 0.75, and later 0.83. The reactor was fed with acetate, and broth removal took place at the end of the feast phase in order to harvest biomass rich in poly(3-hydroxybutyrate) (PHB). To promote growth on stored PHB, the dosage of carbon and nutrients was uncoupled: growth nutrients, including ammonium, were supplied after the effluent phase. The effect of these operational conditions on the enrichment and kinetic performance of *Plasticicumulans acidivorans* (Jiang et al., 2011c) was studied.

2. Materials and Methods

2.1. Sequencing Batch Reactor for Culture Enrichment and PHB Production

The enrichment and maintenance of a PHB-producing culture and the production of PHB were performed in a single double-jacket glass bioreactor with a working volume of 2 L

(Applikon, Netherlands). The reactor was operated as a non-sterile SBR with, initially, 18-hour cycles. The exchange volume was 1.5 L, resulting in a volume exchange ratio of 0.75 and hydraulic retention time (HRT) of 1 day. As there was no settling phase, the SRT equaled the HRT. To allow the harvest of PHB-rich biomass, biomass was withdrawn from the reactor at the end of the feast phase. Each SBR cycle consisted of a carbon feed phase (15 min), reaction phase (125 min), effluent phase (20 min), nutrient feed phase (15 min), and second reaction phase (905 min). During the carbon feed phase 150 mL concentrated sodium acetate solution (0.83 M) was dosed into the reactor. After the effluent phase, the reactor was refilled with 1.35 L fresh medium comprising 5.82 mM NH_4Cl, 2.77 mM KH_2PO_4, 0.62 mM $MgSO_4 \cdot 7H_2O$, 0.80 mM KCl, 1.67 mL/L trace elements solution according to Vishniac and Santer (1957), and 5.5 mg/L allylthiourea (to prevent nitrification).

The reactor was equipped with a stirrer with three standard geometry six-blade turbines, operated at 900 rpm. The air flow rate to the reactor was set to 0.5 L_N/min using a mass flow controller (Brooks Instrument, USA). The total gas flow rate through the reactor was increased to 3.0 L_N/min by partial recirculation of the off-gas. The temperature in the reactor was controlled at 30±1°C using a thermostat bath (Lauda, Germany), and the pH was maintained at 7.0±0.1 by the addition of 1 M HCl and 1 M NaOH. Controlling of the pumps, stirrer, airflow, temperature, and pH was done by a biocontroller (Biostat Bplus, Sartorius Stedim Biotech, Germany).

Aerobic activated sludge from the municipal wastewater treatment plant Kralingseveer (Rotterdam, Netherlands) was used to inoculate the SBR. The reactor was operated for almost 4 years and cleaned once or twice a week to remove biofilm from the walls, electrodes, and other submerged reactor parts. The performance was monitored online by the length of the feast phase, which can be derived from the dissolved oxygen profile (Jiang et al., 2011b). Periodically, samples were collected to determine the biomass and PHB concentration at the end of the feast phase, and cycle experiments were conducted to characterize the enrichment culture in more detail. Moreover, biomass was collected from the SBR for accumulation experiments and analysis of the microbial community structure.

After 3 years and 4 months of continuous operation, the cycle was prolonged to 20 h and the volume exchange ratio increased to 0.83 – maintaining an HRT and SRT of 1 day. The new SBR cycle consisted of a carbon feed phase of 17 min, reaction phase of 223 min, effluent phase of 18 min, nutrient feed phase of 17 min, and second reaction phase of 925 min. The carbon feed consisted of 167 mL concentrated sodium acetate solution (1.25 M), and after the

effluent phase 1.50 L fresh medium (unchanged composition) was added. The reactor was operated at 20-hour cycles for 6.5 months and monitored as before.

2.2 Fed-Batch Reactor for Accumulation Experiments

To evaluate the maximum PHB storage capacity of the enrichment culture, accumulation experiments were conducted in a similar double-jacket glass bioreactor (Applikon, Netherlands) operated as a non-sterile fed-batch reactor. At the beginning of each experiment the reactor was filled with 1.5 L effluent from the SBR, and 0.5 L carbon- and ammonium-free medium (otherwise the same composition). If the effluent did not contain residual acetate, the production of PHB was initiated by feeding a pulse of 60 mmol sodium acetate. Further carbon source was continuously supplied to the reactor via pH control, using a 1.5 M acetic acid solution instead of the 1 M HCl solution used in the SBR (Johnson et al., 2009a). As the ammonium concentration in the effluent from the SBR was zero, microbial growth was prevented throughout the experiment. When necessary a few drops of antifoam B (Sigma-Aldrich) were added and after 10 h the experiments were stopped.

2.3. Analytical Methods

During cycle and accumulation experiments the reactor was monitored closely by both online (dissolved oxygen, temperature, pH, acid and base dosage, off-gas O_2 and CO_2) and offline (acetate, ammonium, TSS, PHB) measurements.

Samples taken to determine the acetate and ammonium concentration in the reactor were immediately filtered with a 0.45 μm pore size filter (PVDF membrane, Millipore, Ireland) to remove the biomass. The ammonium concentration in the supernatant was determined spectrophotometrically using a commercial cuvette test kit (Hach Lange, Germany). The acetate concentration was analyzed using a high-performance liquid chromatograph with a BioRad Aminex HPX-87H column and a UV detector (Waters 484, 210 nm). The mobile phase – 1.5 mM H_3PO_4 in Milli-Q water – had a flow rate of 0.6 mL/min and a temperature of 59°C.

Samples taken to determine the total suspended solids (TSS) and PHB concentration in the reactor were collected in 15 mL tubes with five drops of formaldehyde (37%) to stop all biological activity. The samples were centrifuged for 10 min at 4500 rpm (3850*g*) before removing the supernatant and freezing the samples (-20°C). The samples were subsequently freeze-dried for 24 h (-40°C, 10^{-4} atm) to yield the TSS. The PHB content of the freeze-dried cells was determined using a gas chromatograph (Agilent 6890N, USA) equipped with a flame ionization detector (FID) and a HP-INNOWax column. A detailed description of the

procedure can be found elsewhere (Johnson et al., 2009a). The PHB content, expressed as the weight percentage PHB of TSS, was calculated using pure PHB (Sigma-Aldrich, CAS 26063-00-3) as standard, and benzoic acid as internal standard. The determined PHB content was subtracted from the TSS to obtain the concentration of active biomass in the reactor. The biomass composition (excl. PHB) was assumed to be $CH_{1.8}O_{0.5}N_{0.2}$ and its molecular weight 25.1 g/Cmol (including ash).

2.4. Data Treatment and Modeling

The data collected during cycle and accumulation experiments was evaluated according to the approach proposed by Johnson et al. (2009b). The gathered online and offline measurements were corrected for the air pressure and effects of sampling, the addition of liquids, and inorganic carbon dissolution. After correction, carbon and electron balances were setup to assess the accuracy of the measurements. On average 96% (±5) of the carbon and 99% (±6) of the electrons present in the substrate could be traced back in the active biomass, PHB, and carbon dioxide production or oxygen consumption. Finally, the corrected data was evaluated using a metabolic model to determine the biomass-specific reaction rates and other kinetic parameters. The metabolic reactions, stoichiometry, and kinetic equations can be found in Johnson et al. (2009b). The efficiency of the oxidative phosphorylation (P/O ratio) was assumed to be 2.0 mol ATP/mol NADH. The half-saturation constants for acetate (K_{Ac}) and ammonium (K_N) – both acting merely as a switch function – were set to 0.2 Cmmol/L and 0.0001 mmol/L, respectively.

The initial data treatment was performed in Microsoft Excel. The metabolic model was written in Matlab™, where the built-in function *ode113* was used to solve the differential equations and *fmincon* was used to minimize the sum of the squared relative error (SSqRE) between the measured and modeled data. For each compound (acetate, biomass, PHB, NH_4^+, CO_2, and O_2) the SSqRE was calculated according to Eq. 1. These squared relative errors were subsequently summated to yield the overall error.

$$SSqRE_i = \sum_0^t \left(c_i^{measure}(t) - c_i^{model}(t) \Big/ c_i^{model}(t) \right)^2 \quad (1)$$

2.5. Microbial Community Analysis

In order to analyze the microbial community structure by PCR-DGGE, biomass samples were collected from the SBR and washed with TE buffer. The genomic DNA was extracted using the Ultra Clean Soil DNA extraction kit (MoBio Laboratories, USA) and subsequently used as template DNA. 16S rRNA gene fragments of the different community constituents were

obtained using a touchdown PCR program with the primers 341F-GC and 907R (Table 1) (Schäfer and Muyzer, 2001). The 16S rRNA gene amplicons were loaded onto 8% polyacrylamide gels with a denaturing gradient from 20 to 70% DNA denaturants (100% denaturants is a mixture of 5.6 M urea and 32% formamide (Schäfer and Muyzer, 2001)). The DNA was visualized by UV illumination after staining with SYBR® Safe, and photographed with a digital camera. Individual bands were excised from the gel with a sterile razor blade and incubated overnight in 50 μL water at 4°C. Re-amplification was performed using the same primer pair (Table 1) and the PCR products were sequenced by a commercial company (BaseClear, Netherlands). The sequences have been stored in GenBank under accession numbers: **KT634309-KT634310**.

In addition to the PCR-DGGE analysis, FISH was performed to confirm the predominance of *P. acidivorans* on a more regular basis. A detailed description of the procedure can be found in Johnson et al. (2009a). The general probe mixture EUB338I-III was used to visualize all bacteria in the sample and the specific probe UCB823 to indicate the presence of *P. acidivorans*. The probes (Table 1) were commercially synthesized and 5' labeled with respectively FLUOS and the sulfoindocyanine dye Cy5 (Thermo Hybaid interactive, Germany).

3. Results

3.1. Culture Enrichment and Microbial Characterization

The SBR operated in this study – serving the simultaneous enrichment of a PHB-producing culture and production of PHB – was fed with acetate as model substrate. It was, initially, operated at a volume exchange ratio of 0.75 (i.e. 18 h cycles and 1 d SRT). The typical feast-famine response established immediately after inoculation with activated sludge, but the feast phase length remained relatively long and variable (5.5±0.9 h) throughout the first months of operation (not shown). After eight months the feast phase length had decreased to around 180 min. It took almost two years (23 months), though, to reach a stable and final feast phase length of 128 (±12) min. During the 16-month operational period thereafter, the reactor performance remained stable. Fig. 1 shows the feast phase length during the last six months of operation.

Analysis of the microbial community structure by FISH after 9 and 20 months of operation showed that the enrichment culture was dominated by *P. acidivorans* (Fig. 2a,b). Small but not negligible fractions of other bacteria were also present, especially in the first sample. At

the end of the stable operational period, the continued predominance of *P. acidivorans* was confirmed by PCR-DGGE analysis (Fig. 2c, band B1, 99.8% similarity).

3.2. SBR Performance at 18-hour Cycles

To evaluate the kinetic performance of the enrichment culture in detail, multiple cycle experiments – covering the feast (10) and/or famine phase (4) – were conducted throughout the final 16-month operational period. Table 2 gives an overview of the main observed and model-derived variables that were obtained, while Fig. 3 shows the results of a single, representative feast and famine experiment.

The maximum biomass-specific acetate uptake rate (q_{Ac}^{max}) of the culture was 2.15 (±0.17) Cmol/Cmol/h. The consumed acetate was primarily stored as PHB ($Y_{PHB,Ac}$ = 0.59 Cmol/Cmol) and at the end of the feast phase the PHB content reached 70±1 wt%. To promote the growth on stored PHB, growth nutrients – including ammonium – were supplied after the feast phase and nitrogen availability during the feast phase was limited. Direct growth on acetate was not prevented since the ammonium concentration reached zero only shortly before substrate depletion. The maximum biomass-specific growth rate on acetate (μ^{max}) was found to be 0.12 (±0.03) Cmol/Cmol/h (Table 2). Five percent of the consumed carbon was traced back as active biomass ($Y_{X,Ac}$ = 0.05 Cmol/Cmol) and around 30% of the total ammonium consumption occurred during the feast phase.

After 140 min, 75% of the broth liquid was removed from the reactor – to harvest the PHB – and replaced by fresh medium. In the period between substrate depletion and fresh nutrient supply (±0.5 h) already a slight reduction in the PHB content was observed (1-2 wt%, data not shown). After nutrient supply, the accumulated PHB was rapidly degraded and used for biomass synthesis. The initial biomass-specific growth rate ($\mu^{max,famine}$) was around 0.2 Cmol/Cmol/h, but this slowed down as the intracellular PHB content decreased towards 3±1 wt% at the end of the cycle. The average biomass-specific growth rate during the famine phase ($\mu^{average,\ famine}$) was 0.07 Cmol/Cmol/h.

3.3. SBR Performance at 20-hour Cycles

After 39 months of operation the volume exchange ratio of the reactor was increased to 0.83 (i.e. 20 h cycles and 1 d SRT). The reactor was not re-inoculated with activated sludge and no clear adaptation period was observed (Fig. 4). The feast phase length and its variability increased considerably: throughout reactor operation (6.5 months) the feast phase length was

354±40 min. Analysis of the microbial community structure by PCR-DGGE (after 2 months, Fig. 2c) and FISH (after 6 months, not shown) showed that *P. acidivorans* remained the predominant microbial species. The sequence derived from band B2 (Fig. 2c) showed 99.8% similarity to that of *P. acidivorans*.

As before, cycle experiments – covering the feast (6) and/or famine phase (3) – were conducted to evaluate the kinetic parameters of the enrichment culture. The results of a representative feast and famine experiment are shown in Fig. 5, and an overview of the observed and model-derived variables is given in Table 2. The acetate was taken up slower than in the first, 18 h setup: the maximum biomass-specific acetate uptake rate (q_{Ac}^{max}) was only 1.34 (±0.14) Cmol/Cmol/h. Nevertheless, the consumed acetate was largely stored as PHB ($Y_{PHB,Ac}$ = 0.56 Cmol/Cmol) and upon substrate depletion the PHB content reached 75±3 wt%. The nitrogen availability during the feast phase was limited and ammonium got depleted after roughly 3 h. Despite the absence of ammonium during a significant part of the feast phase, the role of biomass growth increased: 40% of the overall ammonium consumption occurred during the feast phase. The biomass yield ($Y_{X,Ac}$) and maximum specific growth rate on acetate (μ^{max}) increased slightly to 0.07 Cmol/Cmol and 0.14 (±0.02) Cmol/Cmol/h, respectively.

The effluent phase started 4 h after the start of the cycle. At that point, not all acetate had been consumed yet and the PHB content of the harvested biomass was 73±3 wt%. As afterwards the reactor was being refilled with ammonium-rich medium, the remaining acetate was used for PHB production as well as fast biomass growth (Fig. 5). Once the acetate had been depleted, stored PHB was degraded and used for biomass synthesis. The maximum and average biomass-specific growth rate during the famine phase were, respectively, 0.2 and 0.08 Cmol/Cmol/h. At the end of the famine phase the PHB content had decreased to 9±4 wt%. This is higher than observed in the 18 h setup (this study) or previous studies (Jiang et al., 2011a), and suggests that the growth rate on PHB has approached its maximum.

3.4. PHB Storage Capacity

In addition to characterization of the normal SBR performance at 18 and 20 h cycle length, the maximum PHB storage capacity of the enrichment culture was determined in fed-batch accumulation experiments. Part of the SBR effluent was transferred to another bioreactor, where additional carbon source (acetic acid) was supplied via pH control. As the ammonium concentration in the SBR effluent was zero, these experiments were conducted under ammonium-limited conditions. Fig. 6 shows the accumulation of PHB during duplicate feast

and accumulation experiments with biomass from the SBR operated at 18 and 20 h cycles, respectively.

Biomass harvested from the 18 h setup had a maximum PHB storage capacity of 86±1 wt% (Table 2). This is slightly lower than the PHB storage capacities previously reported for *P. acidivorans* predominated enrichment cultures (88-89 wt%) by, e.g., Johnson et al. (2009a) and Jiang et al. (2011b). The maximum PHB content reached in the culture enriched at 20 h cycles was further reduced: 83±1 wt% after 11 h or more. Aside from the lowered maximum PHB content, the PHB production rate also decreased considerably. Where the culture enriched at 18 h cycles still accumulated 80 wt% PHB in 4-5 h, the culture enriched at 20 h cycles required 6-7 h to reach this level (Fig. 6 and Table 2).

4. Discussion

4.1. Enrichment Progress

The enrichment of *P. acidivorans* in an acetate-fed SBR operated at 18 h cycles and 1 d SRT has been studied once before. Jiang et al. (2011a) reported a feast phase length similar to that observed in this study, namely 137 min. However, contrary to the long enrichment period in the present study (23 months), they reached this feast phase length within a few weeks after inoculation with activated sludge. The fast stabilization of their system may well be ascribed to the bioaugmentation with 1% *P. acidivorans* predominated biomass. The inoculated biomass originated from an enrichment reactor (SBR, 12 h cycles, 1 d SRT, 30°C) that had been running for several years and for which the gradual increase of the culture's kinetic performance has also been reported (Johnson et al., 2009a). Johnson et al. (2009a) presented three accumulation experiments – conducted after respectively 2, 7, and 16 months of operation – that show a clear increase of the biomass-specific PHB production rate and the PHB storage capacity over time. Furthermore, the feast phase was reported to last 50 min (Johnson et al., 2009a), while later a feast phase length of 38 min has been reported for the same enrichment culture (Jiang et al., 2011b).

The timescale of the current enrichment period (2 years), together with the continued predominance of *P. acidivorans* (Fig. 2) and significant reduction of the feast phase length, suggests that the enrichment process involved more than just the wash-out of flanking populations. To explain the reduction of the feast phase length from 180 to 128 min solely by the wash-out of bacteria, the flanking population should have formed at least 30% of the culture. Assuming that the flanking population is competing with *P. acidivorans* for the

acetate at around half the biomass-specific rate of *P. acidivorans*, more than 50% of the culture should have consisted of bacteria other than *P. acidivorans*. Although other bacteria were present (roughly 10%, Fig. 2a,b), the flanking population was not large enough to fully explain the long feast phase lengths. The kinetic properties of *P. acidivorans* itself – especially its maximum biomass-specific acetate uptake rate – will likely have changed as well. In the future, analysis of the genome and/or gene expression of *P. acidivorans* in biomass samples collected throughout the enrichment period may reveal what changed and provide more insight into possible cellular adaptation occurring alongside the microbial selection.

4.2. Impact of the Exchange Ratio

As the SBR was operated at higher volume exchange ratios, the relative feast phase length increased significantly. In previous acetate-fed SBRs that were dominated by *P. acidivorans* and operated at volume exchange ratios between 0.04 and 0.50 (1-12 h cycles) the feast phase occupied around 5% of the total cycle (Fig. 7) (Jiang et al., 2011a, 2011b). At an exchange ratio of 0.75 (18 h cycles) the relative feast phase length increased to 12%. This was observed in the present study as well as in the study by Jiang et al. (2011a). SBR operation at an exchange ratio of 0.83 (20 h cycles), finally, resulted in the presence of external substrate during 30% of the cycle (Fig. 7 and Table 2). The increase of the relative feast phase length along with an increase of the volume exchange ratio or cycle length is partially due to the increased substrate-to-biomass (F/M) ratio. Assuming a constant biomass-specific acetate uptake rate (q_{Ac}^{max}), the relative feast phase length will increase as shown by the dashed line in Fig. 7. However, as the biomass-specific acetate uptake rate of the enrichment culture decreased (Table 2), the actual increase was more pronounced – especially for the highest exchange ratio.

Compared to the SBR previously operated at 12 h cycles (Jiang et al., 2011b) the specific acetate uptake rate in the 18 h setup was roughly half, and that in the 20 h setup only one-third (Table 2). Just as for the speed of the enrichment process (section 4.1), the flanking population alone (roughly 10%) cannot explain these differences. There is no conclusive answer as to why the substrate uptake rate of *P. acidivorans* decreased so much, though. Cellular stress caused by elevated acetate concentrations (up to 100 mM) or the storage of large amounts of PHB (up to 75 wt%) forms one possible explanation, but no specific indications for this assumption were found. The overall acetate uptake rate was still reasonably constant throughout the feast phase and the observed small increase could well be

explained by biomass growth (Fig. 3 and 5). The repeated storage of large amounts of PHB may also have affected the size of the bacterial cells. The cell-size has previously been reported to increase significantly during the accumulation of PHB (Jiang et al., 2011b; Pedrós-Alió et al., 1985). An increased cell-size will reduce the specific surface area and may thus result in a lower biomass-specific substrate uptake rate.

Another possible explanation for *P. acidivorans*' decreased acetate uptake rate is the increased growth rate. In contrast to the growth in a continuous stirred-tank reactor (CSTR), the average growth rate (μ) in an SBR does not simply equal the dilution rate. Instead, the average growth rate of the culture increases with the applied cycle length or volume exchange ratio – at the same SRT (μ = ln[1/(1-CL/SRT)]/CL). To prevent being washed-out, the average growth rate of a culture in an SBR operated at 20 h cycles has to be 55% higher than in a similar SBR operated at 12 h cycles. While increasing the volume exchange ratio, increasing growth rates were indeed observed for the feast as well as the famine phase (Table 2). The average growth rate during the famine phase, for example, increased from 0.06 Cmol/Cmol/h in the 12 h setup to 0.07 and 0.08 Cmol/Cmol/h in the 18 and 20 h setup, respectively. The increased growth rates reduce the cellular overcapacity that can be used for PHB production and may affect the overall biomass-specific substrate uptake rate.

4.3. High Exchange Ratio as Enrichment Strategy

Jiang et al. (2011a) previously suggested that the number of cycles per SRT should be minimized to enrich bacteria with a high PHA storage capacity. Operating the SBR at higher volume exchange ratios – increasing the F/M ratio – leads to higher maximum PHA contents in the SBR itself. This was observed in the present study, as well as by Jiang et al. (2011a) and Valentino et al. (2014). However, it does not necessarily select for the highest PHA storage capacity or for a higher substrate uptake and PHA production rate. Although Jiang et al. (2011a) reported increasing rates for SBRs operated at cycle lengths increasing from 1 to 12 h, the specific substrate uptake rate in their SBR operated at 18 h cycles was considerably lower again – just as in this study (Table 2). Moreover, Valentino et al. (2014) reported decreasing uptake and storage rates for SBRs operated at increasing cycle lengths (2-8 h). For successful PHA production, both high biomass-specific rates and a high PHA storage capacity are important. Maximization of the volume exchange ratio is, therefore, a suitable strategy for the production of PHA-rich biomass in the SBR, but not necessarily the best strategy to enrich a microbial culture with a superior PHA productivity. At very high volume exchange ratios,

bacteria will have to increase their growth rate and external substrate will be available for growth for a relatively long period (section 4.2).

4.4. Feasibility of Combining the Enrichment and Accumulation Step

Operating the SBR at a high volume exchange ratio (0.75) allowed the production of biomass with 70 wt% PHB in a single-step process from acetate. The reactor was operated for more than three years and already before the final 16-month operational period biomass with a high PHB content could be harvested (66-70 wt%, not shown). The PHB content reached upon substrate depletion was lower than the theoretical maximum that can be calculated from the applied volume exchange ratio (79 wt%, Fig. 7) (Jiang et al., 2011a). The calculation is based on the assumption that growth and maintenance during the feast phase can be neglected, while in reality biomass growth did occur. As a result, the PHB yield decreased (Table 2) and lower PHB contents were obtained (Fig. 7). Still, the PHB content of the harvested biomass ranks among the higher values reported for non-axenic PHB production (Jiang et al., 2012; Serafim et al., 2008).

All in all, it does not seem feasible to produce much more than this 70 wt% PHB when omitting the accumulation step. The PHB content of the harvested biomass was increased up to 75 wt% by further increasing the volume exchange ratio, but this reduced the operational stability (Fig. 4). Moreover, fermented organic waste streams – containing also compounds that are less suitable for PHA production – will ultimately be used as substrate. This will lead to the co-enrichment of non-PHA-storing bacteria and thereby to a reduction of the culture's PHA content (Jiang et al., 2012; Tamis et al., 2014).

Depending on the intended application and required product purity, recovery of the PHA may still be economically feasible (Koller et al., 2013). However, besides the lower PHA content, combining the enrichment and accumulation step in a single reactor also showed other drawbacks: the storage rate, yield, and capacity of the enrichment culture were severely compromised (Table 2) and the existing imbalance in the oxygen transfer required during the feast and the famine phase – becoming apparent from the typical dissolved oxygen profile for feast-famine systems (Jiang et al., 2011b) – was enlarged. As biomass is withdrawn from the reactor after the feast phase, the reactor contains least biomass during the period with the lowest biomass-specific oxygen uptake: the famine phase. To guarantee an efficient and robust PHA production process and the enrichment of a culture with a high productivity, the initial SBR setup with 12 h cycles and 1 d SRT (Johnson et al., 2009a) seems more optimal.

To save on the equipment cost, the feast and accumulation step could be combined in a single reactor, while the growth on stored PHA is facilitated in a separate famine reactor.

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Figure Captions

Fig. 1 Performance of the SBR during the final 6 months of operation at 18 h cycles. Black diamonds indicate the length of the feast phase (min), blue triangles the PHB content of the harvested biomass (wt% of TSS). The grey asterisks on the x-axis indicate when cycle and/or accumulation experiments were conducted.

Fig. 2 FISH microscopic photographs and DGGE gel. (a, b) Fluorescence microscopy images of the enrichment culture after respectively 9 months (40× magnification) and 20 months (100× magnification) of reactor operation at 18 h cycles. The biomass was stained with a FLUOS-labeled probe for Eubacteria (EUB338I-III, green) and Cy5-labeled probe for *P. acidivorans* (UCB823, blue). (c) DGGE gel of PCR-amplified 16S rRNA gene fragments from the enrichment culture collected during operation at 18-hour (lane 18^{h}) and 20-hour cycles (lane 20^{h}). SmartLadder SF (Eurogentec) was loaded in lane M. The bands labeled B1-2 were excised and re-amplified for microbial identification. Previous analyses have shown that the bands just above B1-2 also belong to *P. acidivorans* (Jiang et al., 2011a).

Fig. 3 Results of a cycle experiment during normal SBR operation at 18 h cycles. The symbols represent measured data, the lines modeled data.

Fig. 4 Performance of the SBR during operation at 20 h cycles (6.5 months). Black diamonds indicate the length of the feast phase (min), blue triangles the PHB content of the harvested biomass (wt% of TSS). The grey asterisks on the x-axis indicate when cycle and/or accumulation experiments were conducted.

Fig. 5 Results of a cycle experiment during normal SBR operation at 20 h cycles. The symbols represent measured data, the lines modeled data.

Fig. 6 The production of PHB (wt% of TSS) during accumulation experiments with biomass enriched at 18 h (black diamonds) and 20 h cycles (blue triangles). The filled and open symbols present the results obtained during duplicate experiments. The horizontal grey, dashed line indicates the maximum PHB storage capacity of *P. acidivorans* (Johnson et al., 2009a).

Fig. 7 Impact of the volume exchange ratio on the PHB content obtained upon substrate depletion (blue triangles, wt% of TSS) and the relative feast phase length (black diamonds, % of the cycle length). The filled symbols are data points obtained in this study, the open symbols data points from literature (Jiang et al., 2011a, 2011b). The grey, dashed lines show the theoretical impact of the volume exchange ratio on the PHB content and the feast phase length, assuming negligible biomass growth during the feast and a constant biomass-specific acetate uptake rate (using the 12 h cycle (Jiang et al., 2011b) as reference point).

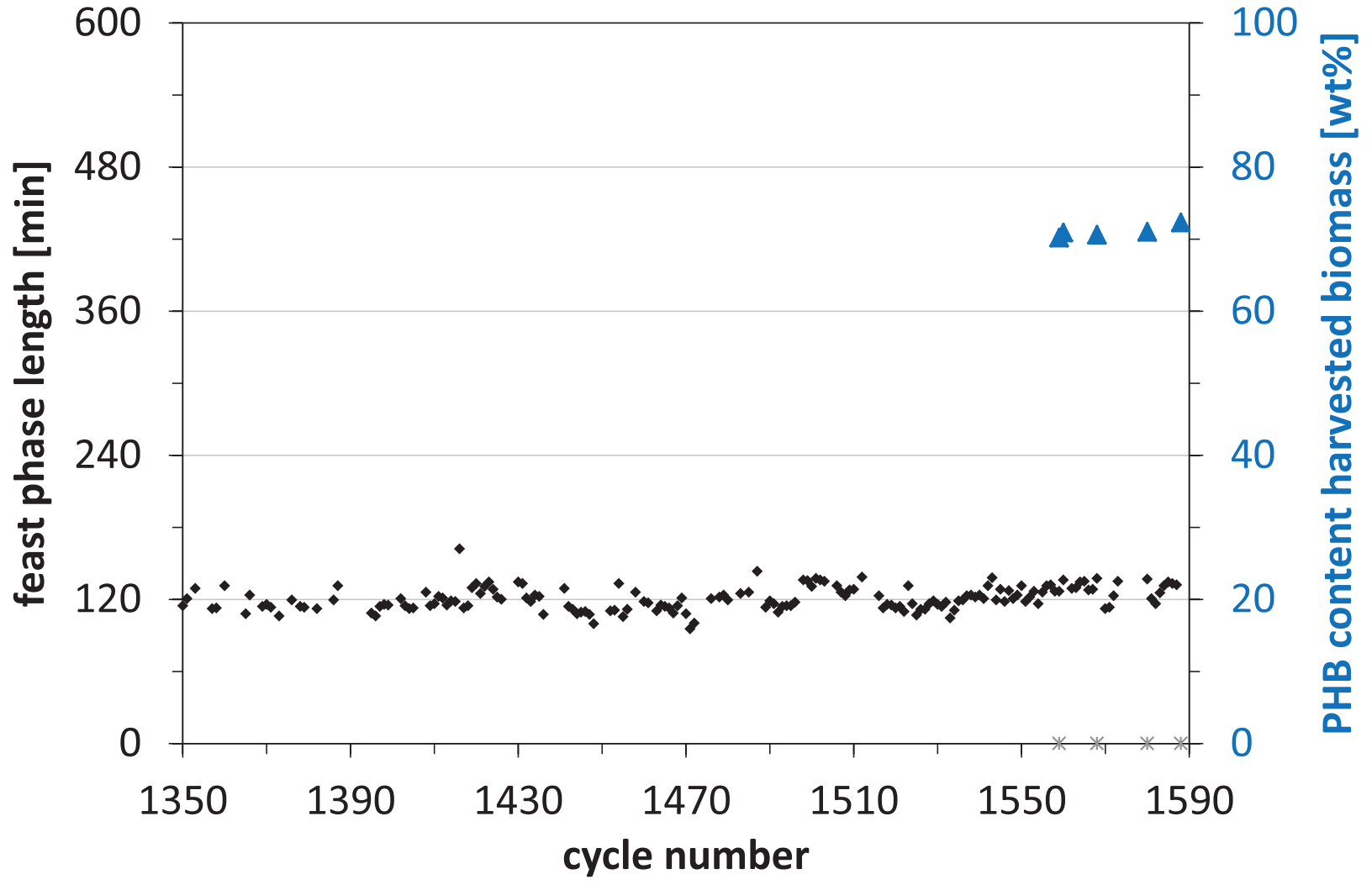

Figure 2

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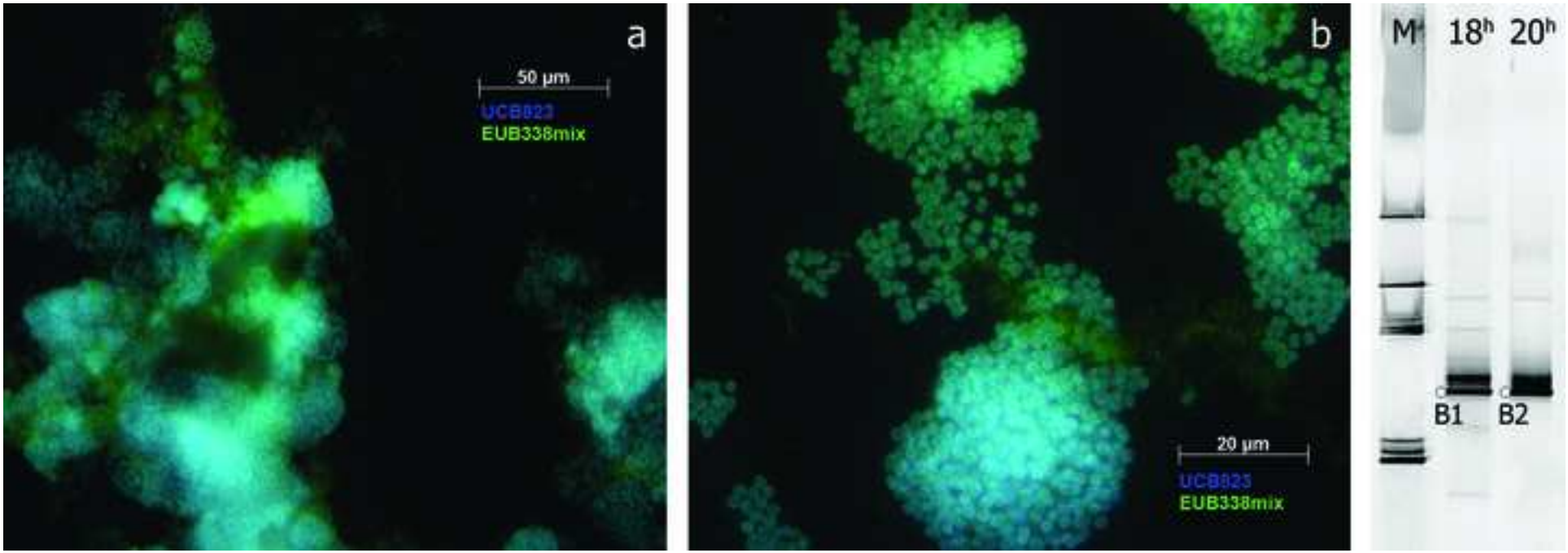

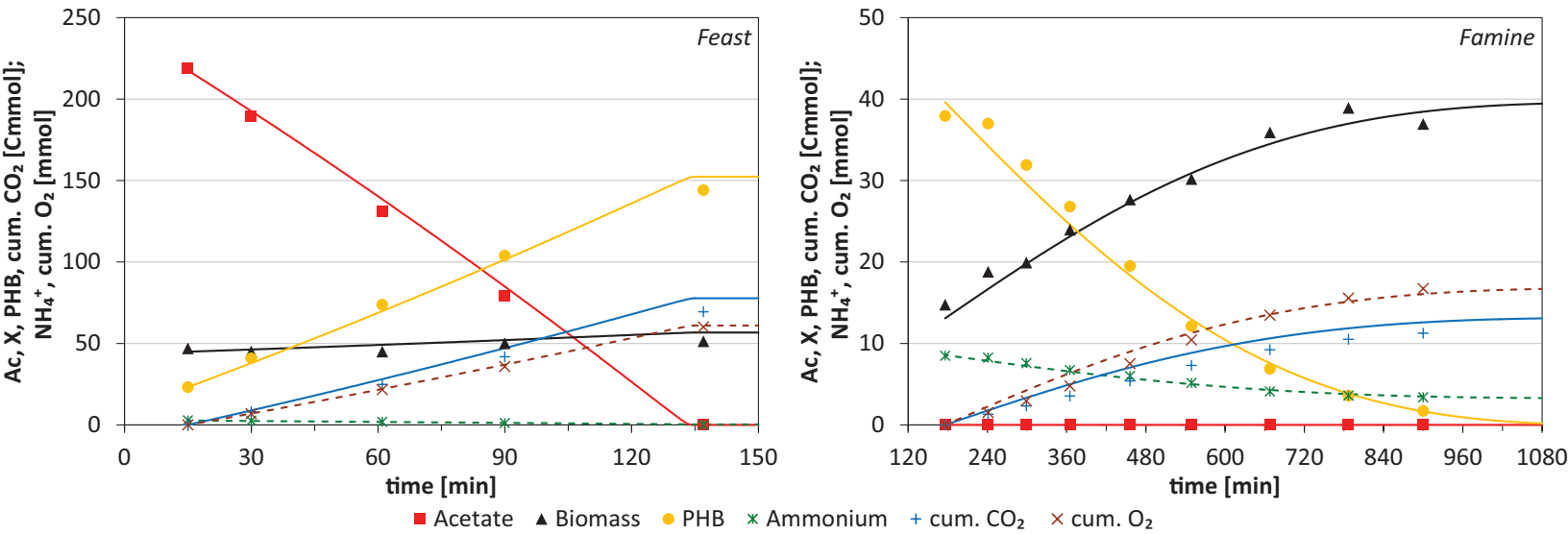

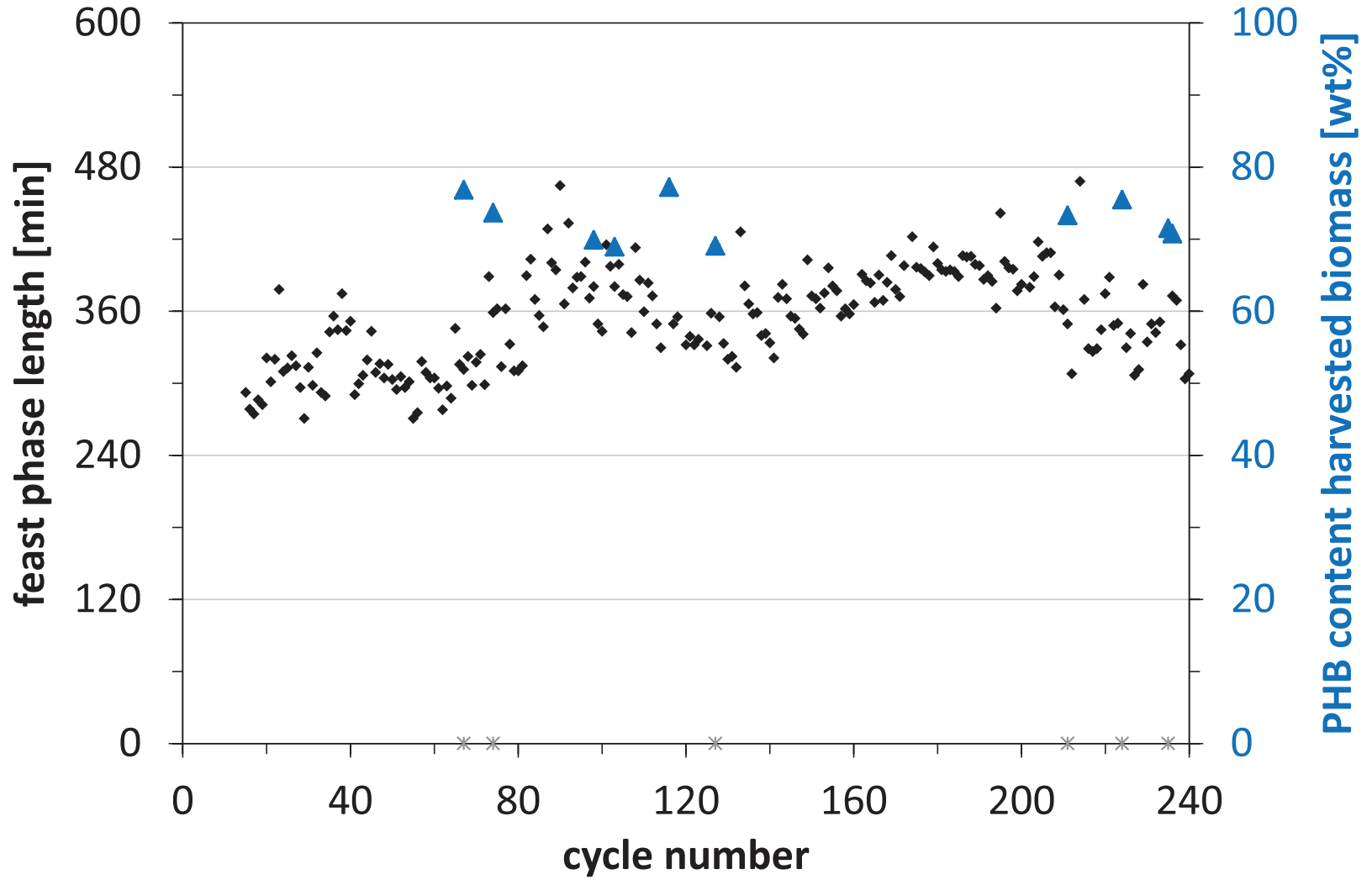

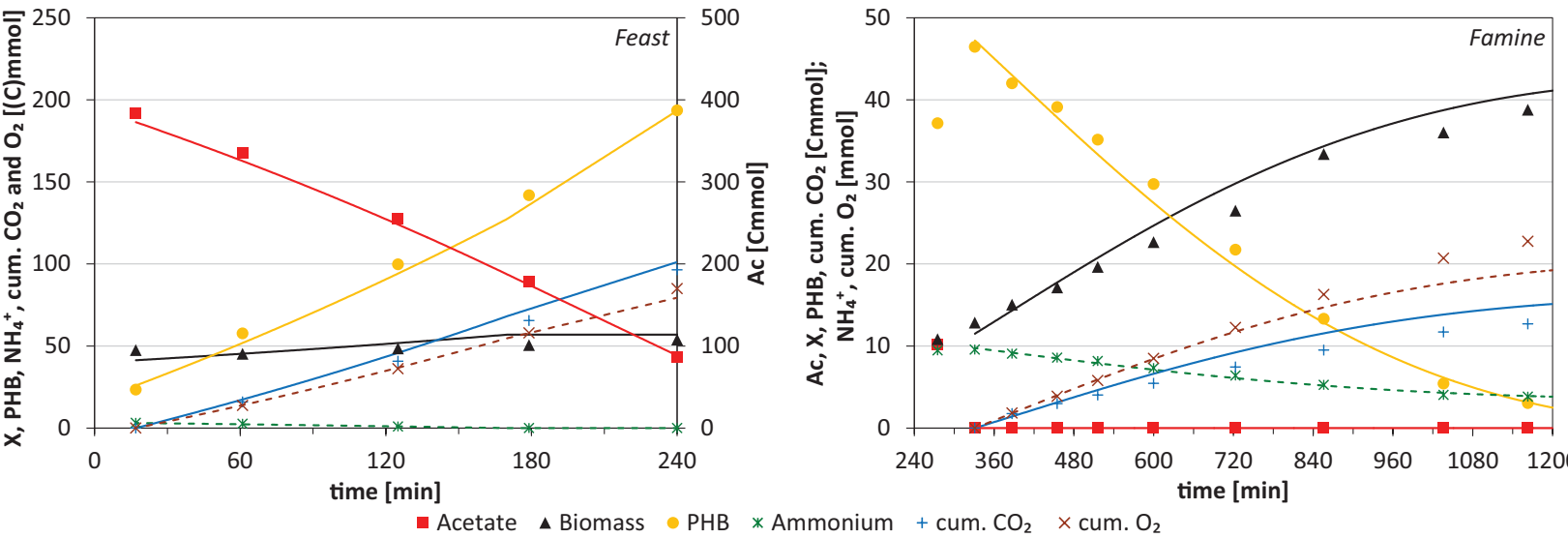

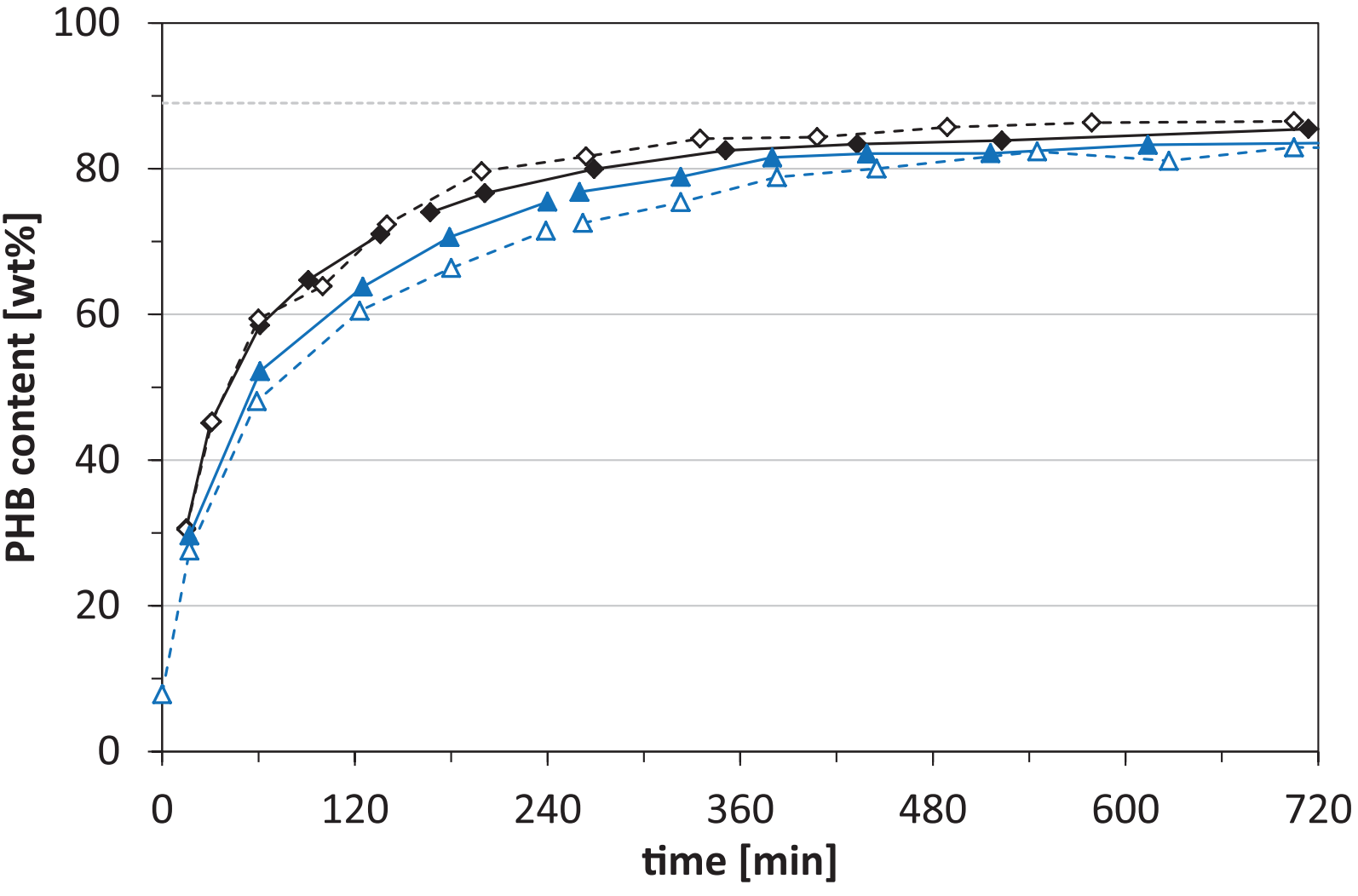

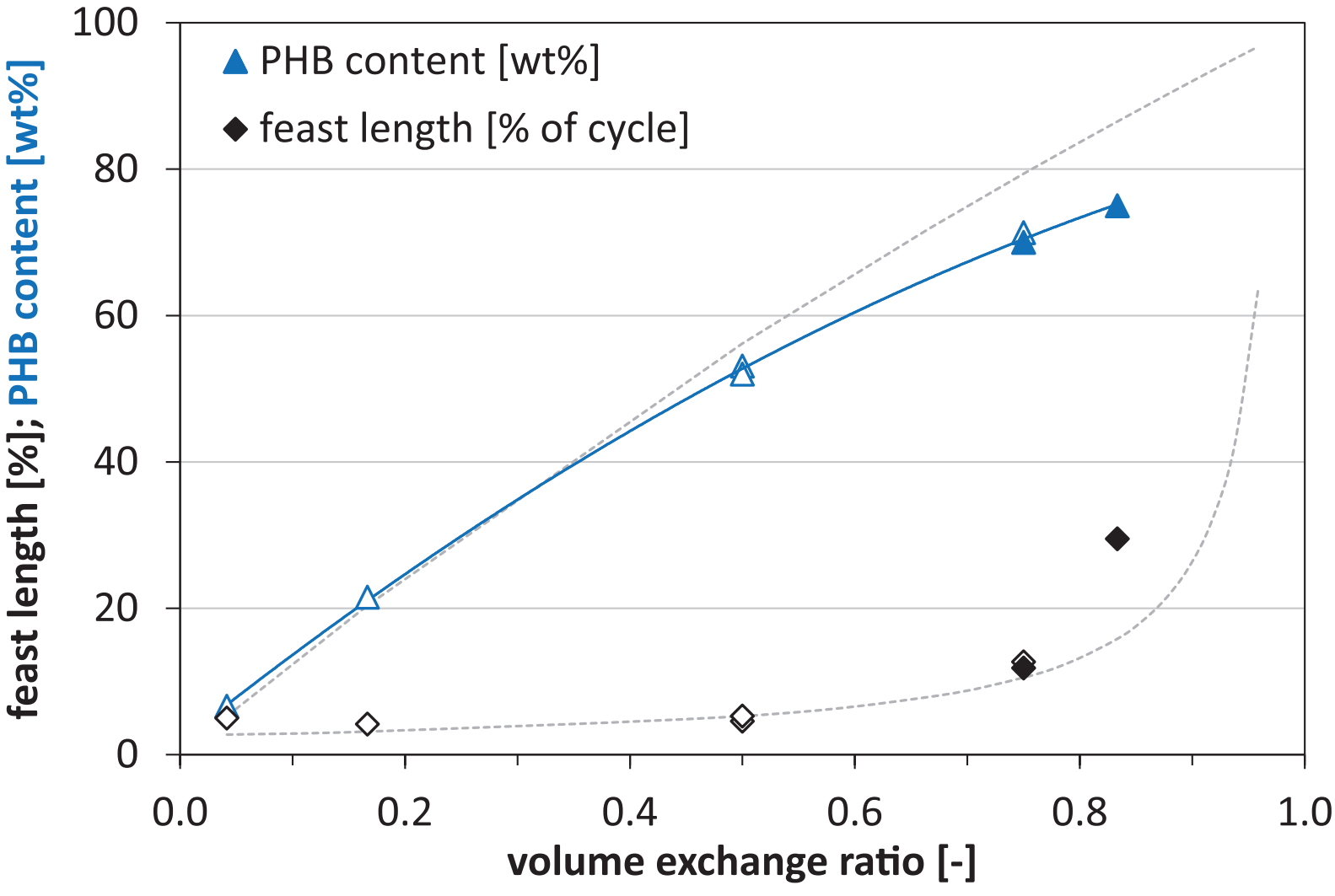

Table 1 Oligonucleotide probes for FISH analysis and primers for PCR-DGGE analysis used in this study.

Code	Function	Sequence (5'-3')	Specificity	Reference
EUB338 I	Probe	gct gcc tcc cgt agg agt	Bacteria	Amann et al. (1990)
EUB338 II	Probe	gca gcc acc cgt agg tgt	Bacteria	Daims et al. (1999)
EUB338 III	Probe	gct gcc acc cgt agg tgt	Bacteria	Daims et al. (1999)
UCB823	Probe	cct ccc cac cgt cca gtt	*P. acidivorans*	Johnson et al. (2009a)
341F-GC	Primer	cct acg gga ggc agc ag*	Bacteria	Schäfer and Muyzer (2001)
907R	Primer	ccg tca att cmt ttg agt tt	Bacteria	Schäfer and Muyzer (2001)

* Contains GC-clamp (5'-cgcccgccgcgccccgcgcccgtcccgccgcccccgcccg-3') at the 5' end of the primer.

Table 2 Overview of observed variables, and model-derived yields and biomass-specific rates during normal SBR operation and accumulation experiments. The presented average values and standard deviation for the SBR are based on the results of at least three experiments. The accumulation experiments were performed in duplicate.

		SBR 1	SBR 2	SBR 3
		12 h cycle	**18 h cycle**	**20 h cycle**
		Jiang et al. (2011b)	*This study*	*This study*
SBR operation/Cycle experiments				
Observed				
Length feast phase	[min]	38	128 ± 12	354 ± 40
Length feast phase	[% of CL]	5	12 ± 1	30 ± 3
PHB max. feast	[wt%]	52	70 ± 1	75 ± 3
Model-derived (feast)				
$Y_{PHB,Ac}^{feast}$	[Cmol/Cmol]	0.67	0.59 ± 0.01	0.56 ± 0.02
$Y_{X,Ac}^{feast}$	[Cmol/Cmol]	0.00	0.05 ± 0.01	0.07 ± 0.02
$Y_{CO2,Ac}^{feast}$	[Cmol/Cmol]	0.33	0.36 ± 0.00	0.37 ± 0.01
q_{Ac}^{max}	[Cmol/Cmol/h]	4.38	2.15 ± 0.17	1.34 ± 0.14
$\mu^{max,\ feast}$	[Cmol/Cmol/h]	0.00	0.12 ± 0.03	0.14 ± 0.02
m_{ATP}^{feast}	[mol/Cmol/h]	0.00	0.00 ± 0.01	0.00 ± 0.01
Model-derived (famine)				
k	[(Cmol/Cmol)$^{1/3}$/h]	-0.16	-0.17 ± 0.01	-0.14 ± 0.02
$\mu^{max,\ famine}$	[Cmol/Cmol/h]	0.14	0.20 ± 0.04	0.20 ± 0.06
$\mu^{average,\ famine}$	[Cmol/Cmol/h]	0.06	0.07 ± 0.01	0.08 ± 0.01
m_{ATP}^{famine}	[mol/Cmol/h]	0.00	0.00 ± 0.00	0.01 ± 0.01
Accumulation experiments				
Observed				
PHB max. acc.	[wt%]	88	86 ± 1	83 ± 1
PHB max. acc.	[Cmol/Cmol]	8.3	7.1 ± 0.4	5.9 ± 0.3
Time PHB max.	[h]	9.2	10-12	11-14
Time PHB >80 wt%	[h]	4.2	4-5	6-7