



Impact of Famine Length on the Structure and Performance of a PHA-Accumulating Community Under Uncoupled Feeding

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Abstract. Polyhydroxyalkanoates (PHAs) are a group of polymers that are naturally synthesized by various microorganisms and have proven to be potential alternatives to conventional plastics due to their biodegradability and versatile composition. Current industrial PHA production relies on specific bacterial strains, and therefore requires highly purified substrates and sterile environments resulting in high production costs. To improve economic viability, several strategies are being explored, including the use of mixed microbial communities (MMCs). The widely accepted method of Aerobic Dynamic Feeding (ADF) involves the ecological selection of PHA-accumulating MMCs by shifting between excess (feast) and limited (famine) availability of external substrates. To improve PHA productivity, a parallel approach, the uncoupled feeding, has been introduced within the traditional feast and famine regime, but has not yet been fully explored. This study highlights the influence of different famine periods on the selection of PHA-accumulating MMCs under uncoupled feeding by monitoring PHA accumulation during the selection step and relating to microbial community evolution. Results indicated that, lengthening the famine phase, PHA accumulation improved its performance in terms of PHA content in the biomass at the end of the feast (from 14 to 24%). However, the microbial community dynamics did not show consistent changes in the dominant PHA-accumulating bacteria (namely *Paracoccus* and *Thaurea*). This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 872053.

Keyword: Polyhydroxyalkanoates (PHAs) · Mixed Microbial Communities (MMCs) · Uncoupled feeding · Famine length

1 Introduction

Polyhydroxyalkanoates (PHAs) represent an emerging class of biodegradable aliphatic polyesters that have shown to be potential substitutes of conventional packaging materials like polyethylene and polypropylene (Rosenboom et al., 2022). The surge in PHA research is primarily driven by their biodegradability and versatile composition, rendering them highly promising for various applications, particularly in the agricultural and medical sectors (Zhou et al., 2023). These polymers are naturally synthesised intracellularly by diverse microorganisms and serve as energy and carbon reservoirs. At present, the industrial production of PHAs primarily relies on specific bacterial strains growing as pure cultures. This method needs high purity substrates and sterile environments to prevent contamination by non-PHA accumulating microorganisms. Consequently, the production cost is considerably high, rendering PHAs less competitive compared to petroleum-based polymers (Estévez-Alonso et al., 2021). To enhance the economic viability of PHA production, several strategies have been explored. These strategies encompass the use of Mixed Microbial Communities (MMCs) as biocatalysts for PHA generation under non-sterile conditions (De Donno Novelli et al., 2021). The most widely used approach to select microbial populations with an augmented capacity for PHA storage is the Aerobic Dynamic Feeding (ADF), commonly known as the feast and famine regime. In this strategy, MMCs undergo an ecological selection pressure due to shifts between excess (feast) and limited (famine) availability of an external substrate (Chen et al., 2015). However, despite this approach, the overall PHA productivity remains low. To address this limitation, more recently, a new strategy was introduced within the traditional feast and famine regime, known as uncoupled feeding. In this strategy external carbon is administered at the beginning of the feast phase and nutrients at the beginning of the famine phase, further promoting accumulation for growth and limiting the coexistence of nutrients and external organic substances. After uncoupled feeding was introduced by Oliveira et al., 2017 and Silva et al., 2017, the possibility of operating at high organic loads (OLR) has been mainly studied, but other fundamental concepts need to be further investigated to understand how to optimise this powerful selection process.

As internal limitation (enzymatic apparatus for growth) is deemed one of the fundamental parameters and a driving force for PHA storage (Dionisi et al., 2007), in this study the extension of the famine phase (almost doubled compared to control reactor) was investigated in a long term experiment in terms of PHA accumulation performance and related to microbial composition.

2 Materials and Methods

2.1 SBRs Set Up and Operating Conditions

The selection of a diverse microbial community enriched in PHA-accumulating bacteria was undertaken using a Sequencing Batch Reactor (SBR), R1, with a maximum working volume of 1.4 L. The reactor was initially inoculated with activated sludge collected from a tannery wastewater treatment plant (Tuscany, Italy). R1 was operated under a conventional feast and famine regime, modified with an uncoupled feeding strategy, where an

intermediate settling and subsequent supernatant discharge were introduced. Additionally, a specific Volumetric Exchange Ratio (VER) was set (Cruz et al., 2022) to maintain a constant Sludge Retention Time (SRT). The reactor was supplied with two different synthetic solutions: one containing 80% acetate and 20% propionate, and the other consisting of nutrients and micronutrients in restricted concentrations. Continuous aeration and stirring of the activated sludge were ensured by an air supply diffuser and a stirrer, except during settling and supernatant removal phases. Temperature was maintained at 26 °C, while pH and dissolved oxygen (DO) were not actively controlled, but their measurements were recorded and stored every minute. The reactor was operated under nitrogen-limiting conditions for approximately nine months (period I, 1–314 experimental days, data not shown). Subsequently, half of the selected biomass was utilized to inoculate an identical reactor (R2), and both reactors were subjected to phosphorous-limiting conditions (period II, 315–338 experimental days). Later, the feast-to-famine (F/F) ratio was set nearly to half in R2 (period III, 339–409 experimental days) compared to R1, only lengthening the famine phase. The operating parameters for the experimental periods II and III are reported in the Table 1.

2.2 Analytical Methods

The reactor was monitored for soluble chemical oxygen demand (sCOD), N-NH_4^+ , P-PO_4^{3-} along the working cycle analysing it through specific cuvette kits (Hach Lange, Germany). For PHA analysis, sludge samples were collected from the reactor and centrifuged, then the pellet was stored at -20 °C and later analyzed through high performance liquid chromatography (HPLC), in terms of the Polyhydroxybutyrate (PHB) content. Mixed liquor samples from the bioreactor were taken to determine total and volatile suspended solids (TSS and VSS) accordingly to the Standard Methods (APHA, 1998).

The PHB accumulation capacity was calculated both in terms of content of PHB in the biomass (as VSS) and daily productivity (assuming to purge at the end of feast).

The microbial community was monitored over time. DNA was extracted from the samples through the use of DNeasy PowerSoil Pro kit Qiagen (Germany). Subsequently, a PCR analysis on the conserved V3-V4 regions of 16S ribosomal RNA (rRNA) was carried out. The following primers were used Pro341FB (CCTACGGGNBGCWSCAG) and 805R (GACTACNVGGGTWTCTAATCC). The amplicons were sequenced using Illumina MiSeq technology at BMR Genomics Srl (Padua, Italy). The demultiplexed sequences were processed using QIIME2-2022.2. Briefly, the primers and the reads without primer were discarded using Cutadapt while DADA2 was used to cluster the reads in ASVs (Amplicon Sequence Variants). Then, the taxonomic identification was performed using SILVA 138. Finally, the data was analysed using R and package as ggplot2, vegan and DESeq2.

Table 1. Main operating parameters throughout the experiment (period II and III)

	II (R1)	II (R2)	III (R1)	III (R2)
Experimental days	315–338	315–338	339–409	339–409
F/F ratio	0.36	0.36	0.36	0.15
SRT (d)	4.4	4.4	4.4	8.8
Hydraulic Retention Time (HRT) (d)	1.3	1.3	1.3	2.5
VER (%)	39	39	39	41
OLR ($\text{mgL}^{-1}\text{d}^{-1}$)	753 ± 38	753 ± 38	780 ± 38	390 ± 19
COD:N (mass basis)	23 ± 2	23 ± 2	22 ± 1	22 ± 1
COD:P (mass basis)	238 ± 66	238 ± 66	207 ± 16	207 ± 23

3 Results and Discussion

3.1 Famine Extension: Impact on the PHA Accumulation

The performance of the two reactors was compared once the steady state was reached.

The biomass concentration was about half under more extreme starving conditions, due to the halved organic load ($\text{VSS-R1} = 575 \text{ mgCODL}^{-1}$; $\text{VSS-R2} = 346 \text{ mgCODL}^{-1}$). The uptake rates of organic matter during the feast phase are similar in R1 and R2 ($\text{OUR-R1} = 697 \text{ mgCODL}^{-1}\text{h}^{-1}$; $\text{OUR-R2} = 633 \text{ mgCODL}^{-1}\text{h}^{-1}$), as well as the concentrations of PHB at the end of the feast ($\text{PHB-R1} = 82 \text{ mgCODL}^{-1}$; $\text{PHB-R2} = 83 \text{ mgCODL}^{-1}$).

However, since the biomass concentration in R2 is less, the PHA content in the biomass appears higher when the famine length was increased (24% instead of 14%).

Therefore, if on the one hand the accumulated percentage of organic substance is higher, in terms of daily productivity, the performance of R2 is halved.

Extending the length of the famine phase seems to disadvantage growth in favour of accumulation, an assumption also supported by the rate of ammonium consumption in the feast phase: $5 \text{ mgNL}^{-1}\text{h}^{-1}$ (R1) vs $3.2 \text{ mgNL}^{-1}\text{h}^{-1}$ (R2). Nevertheless, this setup resulted in less time efficiency and higher energy costs due to extended cycle length and aeration.

Observations indicated that cycle length had a significant impact on PHA accumulation performance. Variations in other parameters may have influenced the result, such as lower OLR or higher SRT and HRT, favouring PHA accumulation. Lower OLRs did not correlate with higher accumulation capacity under uncoupled feeding selection, nor did higher HRT, a parameter that has been very little studied in this topic.

With regard to SRT, as reported by Matos et al., 2021, a higher SRT (4 instead of 2 days) leads to higher storage capacity (40% higher). However, Jiang et al., 2011, reported that an increase in cycle length (but maintaining constant SRT) led to a higher PHB content at the end of the feast phase.

3.2 Famine Extension: Impact on the Microbial Community Structure

Firstly, there is a gradual decreasing over time of *Neomegalonema* relative abundance (filamentous PHA-accumulating organisms), which was selected and then enriched by previous conditions. Once both communities reach a certain stability, they predominantly consist of *Paracoccus* and *Thauera* in both short and extended famine conditions (Fig. 1). The relative abundances of PHA-accumulating organisms seem to be lower in R2, favouring greater biodiversity (Fig. 2). Thus, the extension of the famine phase does not seem to significantly influence the most abundant genera of the microbial community.

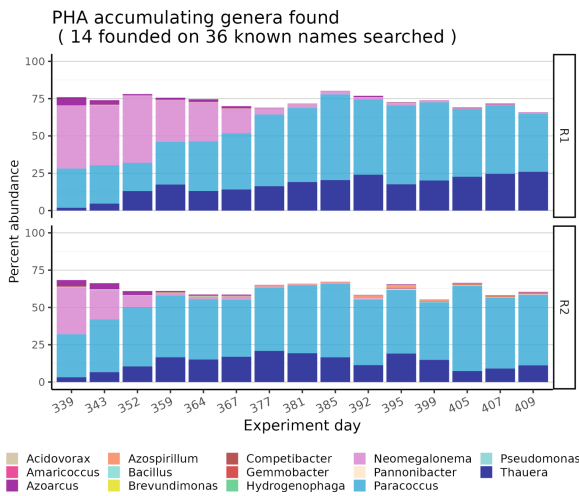


Fig. 1. Comparison between PHA accumulating genera relative abundance found in R1 and R2 after famine extension in R2

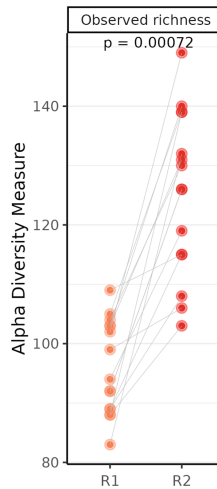


Fig. 2. Representation of the alpha diversity between R1 and R2. In particular, the observed richness in R1 (orange dots) is compared to the richness in R2 (red dots).

4 Conclusions

An improved percentage of PHA content in the biomass was observed with a longer starving period, demonstrating that extending the famine phase under uncoupled feeding promotes accumulation rather than growth. However, it is necessary to emphasize that while, from the perspective of the percentage of accumulated PHA at the end of the feast, extending the famine may be advantageous, it is not in terms of daily productivity, as it results in being halved. To conclude, the duration of the starving period appears to significantly affect accumulation performance, although not affecting the dominant genera of the community.

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