Determination of intracellular polyhydroxyalkanoates (PHA) in aerobic pulse feeding batch tests using chromatography and microscopy techniques

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Abstract: Polyhydroxyalkanoates (PHA) are biodegradable polymers that could be synthesized intracellularly by some microorganisms as a carbon and energy reservoirs. This valuable bioplastic could be produced using mixed microbial cultures and treating organic wastes in a three-stage process (Reis *et al.*, 2011; Conca *et al.*, 2020). The first stage (acidogenic fermentation) consists of the production of volatile fatty acids (VFAs) that would be subsequently fed to the following stages. The second stage of the process (selection of PHA storing organisms) is devoted to produce a biomass enriched in microorganisms able to accumulate PHA using the alternation of periods of high availability of VFAs (feast phase) and periods of absence of extracellular easily biodegradable carbon source (famine phase). This strategy favours the proliferation of those microorganisms able to store PHA and to grow using their PHA reservoirs under famine conditions. The third stage of the process (accumulation of PHA) consists of the increase of the PHA content of the purged biomass from the selection reactor by maintaining feast conditions, due to the feeding of the liquid VFA-rich stream produced in the first fermentation stage. The aim of this study is to monitor and quantify, by means of analytical and microscopy techniques, the accumulation of PHA in a biomass purged from a selection reactor where feast/famine conditions were alternated. The quantification was performed using chromatography analysis and alternative microscopy techniques with the help of Sudan Black staining and Nile Blue staining.

Two lab-scale glass reactors were operated: a selection Sequencing Batch Reactor (sSBR) to produce a biomass purge enriched in PHA-accumulating organisms and an accumulation batch reactor (aBR) where the PHA content of the purged biomass was increased due to the pulse feeding of a VFA-rich synthetic wastewater under aerobic conditions. The sSBR consisted of a jacketed reactor (3.75 L working volume) equipped with a mechanical stirrer, a dissolved oxygen probe and a pH probe. Temperature was controlled at 35 °C using a thermostatic bath, oxygen supply was carried out using air pumps and 3 peristaltic pumps performed the feeding of synthetic substrates, the biomass purge and the withdrawal of the treated effluent. The sSBR was fed (without air supply) at the start of each operating cycle (6 h) with a VFA-enriched synthetic stream (3.5 g COD_{VFA}/L) simulating a fermentation liquid from biowaste (being the proportion of acetic, propionic and butyric acids, 62.50, 18.75 and 18.75% on COD basis, respectively) but without nitrogen content. To promote the growth of biomass during the operating cycle, an ammonium nitrogen solution (94 mg NH_4Cl/L) was supplied after the biomass purge, at the end of the feast phase. The hydraulic and solid retention times were set at 1.1 and 4.2 days, respectively. The aBR consisted of a jacketed glass lab-scale reactor (1.1 L maximum working volume) equipped with a mechanical stirrer, DO and pH probes. The operating temperature was controlled at 35 °C and aeration was performed using a net-air system connected to a porous stone diffuser. In this reactor, 450 mL of purged biomass from the sSBR were initially added to the reactor and 5-6 pulses of 80 mL of a VFA- rich synthetic stream (3.5 g COD_{VFA}/L) were performed. Each pulse was performed when the previous VFAs dosed were completely depleted and a rise of the DO concentration in the mixed liquor was observed. All the analyses were performed according to the standard methods for the examination of water and wastewater (APHA, 2005). PHA was analysed by chromatography following the methodology described in Lanham et al. (2013). From mixed liquor samples, thin smears on microscope slides were prepared, and then stained with Sudan Black and Nile Blue. During microscopy observation, 100 photographs per sample were performed and analysed using ImageJ program. The images were segmented into three components, background, cells, and PHA, and to analyse automatically, a Macro for ImageJ was developed.

The monitoring of the sSBR under pseudo steady-state operation (data not shown) revealed that VFAs were completely depleted within the first 100 min of aerobic react (feast phase) of the operating cycle. The biomass tended to form a biofilm in the porous stone where oxygen was supplied, thus affecting the dissolved oxygen gas-transfer coefficient (K_La) and, consequently, the length of the feast stage during the operational cycle (Perez-Esteban et al., 2021). Although ammonium nitrogen was always supplied when the VFAs were completely depleted, 10-30 mg NH_4^+-N/L were available in the mixed liquor at the start of the feast phase of the following sSBR cycle. Regardless of this fact, the PHA content of the biomass increased during the feast stage up to 17-34% (on SS basis) and decreased during the subsequent famine period. Figure 1 shows three accumulation tests (a, b

and c) performed using 450 mL of sludge purged from the sSBR. As it can be observed, in all these cases pH remained within the range of 7.0-9.1 and the DO profile clearly showed the time when each VFA pulse was consumed (VFAs were analysed to confirm its consumption). In these tests, it could be observed that the time required for VFA consumption increased when the PHA content in the biomass also increased. The percentage of PHB in the biopolymer produced remained in the range of 87-91% for all the tests, which is in accordance with the VFA distribution of the synthetic wastewater used (the proportion of even carbon number VFAs concerning the total VFA on COD basis was 81.25%). In the third accumulation assay (test c), where the second and fifth pulse feedings were delayed with respect to the rise in the DO profile, the recorded final PHA content was lower than that obtained in tests 'a' and 'b' where all the pulse feedings were performed as soon as the DO was risen. However, in all the cases the final PHA content reached values in the range of 42-52% PHA on SS basis after the fifth pulse, which is a higher PHA content than the rough threshold of 40% that is believed to make the recovery of bioplastics commercially viable (Morgan-Sagastume et al., 2013).

When alternative microscopy techniques were applied with the help of Sudan Black and Nile Blue staining (see Figure 2), a similar tendency in the PHA content of biomass was observed and the time spent using these alternative techniques was lower. The percentage of PHA presence in biomass using the applied microscopy techniques did not provide exactly the same values, although they were well-correlated (see Figure 3a). When the PHA percentage analysed using chromatography was compared to those values obtained using microscopy techniques (see Figure 3b), it could be also observed that quite similar results were obtained, although further work needs to be performed to gain experience, reduce the dispersion of values and better clarify the correlation between results obtained using chromatography and microscopy techniques.

In conclusion, this study demonstrates that a PHA content in the range of 42-52% PHA on SS basis could be obtained in a sludge purge previously enriched in PHA-storing biomass using a VFA pulse-feeding strategy under aerobic conditions. The monitoring of the PHA content could be performed using chromatography, but also using microscopic detection of PHA with Sudan Black and/or Nile Blue staining.

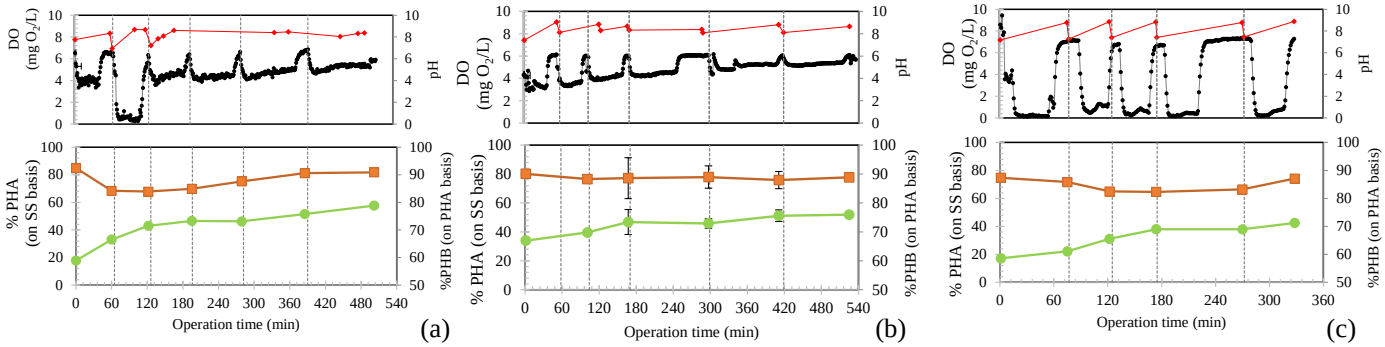

Figure 1 – DO (♦), pH (●), PHA content on SS basis (●) and PHB percentage on PHA basis (■) in three PHA accumulation tests (a,b,c) at 35 ºC (PHA analysed using chromatography).

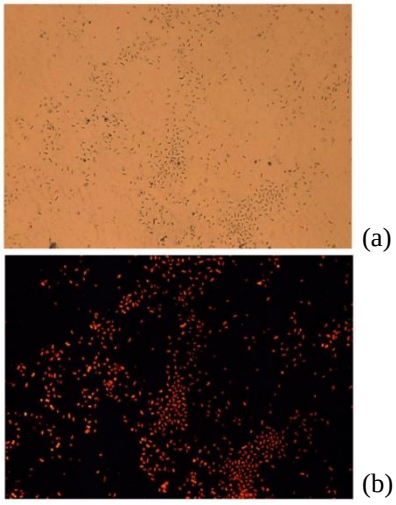

Figure 2 – Analysed sample using Nile Blue staining without (a) and with (b) fluorescence

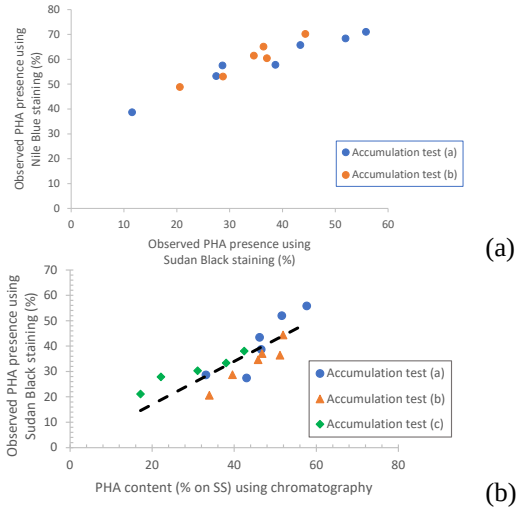

Figure 3 – Correlation between Sudan black and Nile Blue staining results for two accumulation tests (a) and comparison of the percentage of PHA analysed using chromatography and Sudan Black staining for all the accumulation tests (b)

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