Image Analysis for Automatic Characterization of Polyhydroxyalcanoates Granules

Daniela P. Mesquita[1], Gianluca Selvaggio[1], Jorge R. Cunha[1], Cristiano S. Leal[2], A. Luís Amaral[1,2], and Eugénio C. Ferreira[1,*]

[1] IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal
{daniela,ecferreira}@deb.uminho.pt,
{gianluca.selvaggio,apolicunha}@gmail.com
[2] Instituto Superior de Engenharia de Coimbra, Instituto Politécnico de Coimbra, Rua Pedro Nunes, Quinta da Nora, 3030-199 Coimbra, Portugal
{lpamaral,cristiano.leal}@isec.pt

Abstract. A new monitoring approach for polyhydroxyalcanoates (PHA) granules identification and characterization based on image analysis procedures is proposed. PHA granules were analyzed by Sudan Black B (SBB) staining in an enhanced biological phosphorus removal (EBPR) system. Color images captured on an optical microscope were analyzed through quantitative image analysis. The distribution of PHA granules was estimated by determination of the proportion of blue-black pixels. A relationship was found between image analysis parameters and PHA concentration. In conclusion, it may be inferred that the present image analysis procedure is suitable to quantify PHA granules in SBB staining images and a promising alternative to standard analysis.

Keywords: Image analysis, Enhanced biological phosphorus removal (EBPR), Polyhydroxyalcanoate granules (PHA), Sudan black B (SBB).

1 Introduction

Polyhydroxyalcanoates (PHA) are polyesters and a promising alternative to petrochemical plastics with good biodegradable and biocompatible properties. PHA have a potentially broad range of commercial applications with thermoplastic characteristics of polypropylene and polyethylene. These polymers, usually from lipid nature, are accumulated intracellularly by microorganisms in the form of storage granules for carbon and energy, allowing microbial survival under stress conditions [1]. Conventional PHA production based on pure culture is a well know process, however, this can lead to high costs. Nowadays, it was found a substitute process to produce PHA using mixed cultures from activated sludge systems. Enhanced biological phosphorus removal (EBPR) is performed by operating the system through sequentially alternating feast and famine conditions. This strategy was developed to favor a specific group

* Corresponding author.

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of microorganisms versus other heterotrophic organisms competing for organic substrates. In the anaerobic stage, these organisms store them as intracellular storage polymers, namely PHA. Chemically, they consist mostly of poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV) or copolymers (poly-β-hydroxybutyrate-*co*-3-hydroxyvalerate).

Microscopic examination of activated sludge is a very useful way for determining the physical nature of the flocs and the type and abundance of filamentous organisms [2]. Based on microscopic examination, quantitative image analysis has been already used to characterize activated sludge and relate those characteristics with operational parameters [3-8].

Gram and Neisser staining procedures are routinely used for flocs and filaments characterization. However, additional procedures with more specialized uses include staining tests to detect intracellular storage products such as PHA. Nile Red, Nile Blue A and Sudan Black B (SBB) are the most used dyes for selective staining of PHA granules [9-12]. Despite the useful information provided by these staining protocols, quantitative image analysis has never been applied with SBB staining for specifically quantify PHA in activated sludge flocs. On the other hand, PHA off-line analyses are labor intensive, time consuming, and not adequate to be performed in full-scale plants. Thus, in the present work, a novel monitoring approach based on quantitative image analysis was tested using SBB to quantify PHA production from mixed cultures in an EBPR system.

2 Materials and Methods

2.1 Experimental Setup and Synthetic Medium

A 4 L sequential batch reactor was operated at 30 °C, under 6-h cycle that consisted of anaerobic (120 min), aerobic (180 min), settling (55 min), and withdrawing periods (5 min). The synthetic medium (described below) was added at the beginning of the anaerobic stage. Nitrogen and compressed air were used to ensure anaerobic and aerobic conditions, respectively, using two on/off control valves. The hydraulic retention time (HRT) was 12 h and the sludge retention time (SRT) was kept at approximately 8 days by wasting mixed liquor at the end of the aerobic stage. Synthetic medium containing acetate as the main carbon source and a phosphate solution containing K_2HPO_4 and KH_2PO_4 were fed to the system in the first 5 min of the anaerobic step. The concentration of other nutrients added in the synthetic medium are listed below (g L^{-1}) [13]: 0.59 NH_4Cl, 0.95 $MgSO_4 \cdot 7H_2O$, 0.44 $CaCl_2 \cdot 2H_2O$, 0.0116 allyl-N thiourea, 0.03 EDTA, and 3.16 mL of a trace metals solution. The trace metals solution [13] are listed below (g L^{-1}): 1.5 $FeCl_3 \cdot 6H_2O$, 0.15 H_3BO_3, 0.03 $CuSO_4 \cdot 5H_2O$, 0.18 KI, 0.12 $MnCl_2 \cdot 4H_2O$, 0.06 $Na_2MoO_4 \cdot 2H_2O$, 0.12 $ZnSO_4 \cdot 7H_2O$, 0.15 $CoCl_2 \cdot 6H_2O$.

2.2 Analytical Procedures

Mixed liquor samples were taken at the end of anaerobic and aerobic stages and centrifuged during a monitoring period of 60 days. The supernatant was discharged and

the pellet was lyophilized during at least 48 h. PHB and PHV content was measured by gas chromatography (GC) using the adapted method developed by Smolders et al. (1994) [13]. Pre-weighed samples of lyophilized sludge were transferred to glass tubes. The polymers were esterified with HCl:1-Propanol and extracted with dichloromethane including an internal standard (benzoic acid). The mixture was digested at 100 °C for 3.5 h. Quantification was made in a GC system (Varian 3800 instrument, Varian Inc., USA) equipped with a flame ionization detector. PHB and PHV were separated using a TRWAX capillary column (Teknokroma, Spain), with helium as the carrier gas. Temperatures of the split injection and detector were 220 °C and 250 °C, respectively. The initial oven temperature was 50 °C for 2 min, with a 10 °C min^{-1} ramp to 225 °C and then maintained for 10 min. Data were analyzed using the acquisition and integration software Star Chromatography Workstation v. 6.30 (Varian Inc., USA) and calibration curves obtained for PHB and PHV. In this work, PHA was considered the sum of PHB and PHV.

2.3 Staining Procedure and Bright-Field Image Acquisition

Biomass samples taken at the end of anaerobic and aerobic stages were disrupted using a tissue grinder. A smear of biomass suspension (200 μL) was carefully and uniformly deposited on a glass slide and thoroughly air dried. It was stained with SBB (0.3 % w/v in 60 % v/v ethanol) for 10 min and counterstained with safranin O (0.5 % w/v in deionised water) for 10 s [2]. Images were acquired in the upper, middle and bottom of two slides resulting in a total of 60 images (2 × 30 images per slide). Samples were examined by means of a microscope Olympus BX51 (Olympus, Tokyo, Japan) at 1000× total magnification under oil immersion. Images were acquired at 2040 × 1536 pixels, and 24-bit RGB format (8 bit red, 8 bit green and 8 bit blue channels) through the commercial software Cell^B (Olympus, Tokyo, Japan).

2.4 Image Analysis Procedure

The image processing and analysis was based on the identification and quantification of PHA granules using Matlab 7.8.0 (The Mathworks, Natick, MA). A more detailed description of the image processing methods is presented below.

The first step of the image processing program splits the RGB image into the three composing channels (red, green and blue). Only the red channel was subsequently used for the remaining treatment, given the fact that SBB stained PHA granules in blue-black color. To enhance the image contrast and to increase the definition of the PHA granules contours, a logarithmic transformation (natural logarithm followed by normalization) and a contrast enhancement filter (3-by-3 pixels unsharp filter) was performed, followed by a 50 pixels kernel size (sigma = 20) Gaussian filter. This procedure created a blob of uniform color in the image containing all the granules and easily segmented by a simple threshold. After a first rough segmentation, an adaptive thresholding algorithm which separates the information required from the background was used. In mixed cultures, the biomass structure can be considerably different from sample to sample, regarding the extensive fraction of flocs, and the amount of PHA granules which generally are combined as clusters. Thus, to surpass the complexity of image segmentation, color segmentation was performed, where all the pixels related

to the red and green color were removed. The color segmentation was based on determining the blue/red and green/blue ratios. It was considered that the pixels related to the red color presented a blue/red ratio below 0.9, whereas the pixels related to the green color presented a green/blue ratio above 1.1. Afterwards, a 2-by-2 pixels disk erosion guided by the brightness was applied for the separation of each PHA granule independently. However, even considering this processing step, some clusters are still present, mostly due to insufficient color gradient. Therefore, an algorithm for the separation based on distance transform was implemented where the solidity (below 0.8) in conjunction with an area above 100 pixels were selected as the criteria to decide where to apply the separation. Identified granules were post-treated in terms of debris elimination by a 3-by-3 pixels square erosion, dilation and filling. In the last refining step, a brightness guided erosion was performed since some of the granules were not perfectly segmented, so to eliminate the outer part that is usually very bright a technique from signal processing that eliminate the less important part of the information was applied. With this operation the brightest pixels (3%) that form the granules were eliminated since they are probably part of the background.

The recognized PHA granules from the collected images were then characterized into the most relevant morphological parameters described below. The total area, real area (without border objects), roundness, diameter, perimeter, length, width, form factor and color intensity were quantified for each PHA granule. Furthermore, PHA granules were also classified according to size: small granules (S) (less than 100 pixels), intermediate (I) (between 100 and 400 pixels), and large (L) (more than 400 pixels). Images accounting for the main image analysis procedure steps are presented in Figure 1.

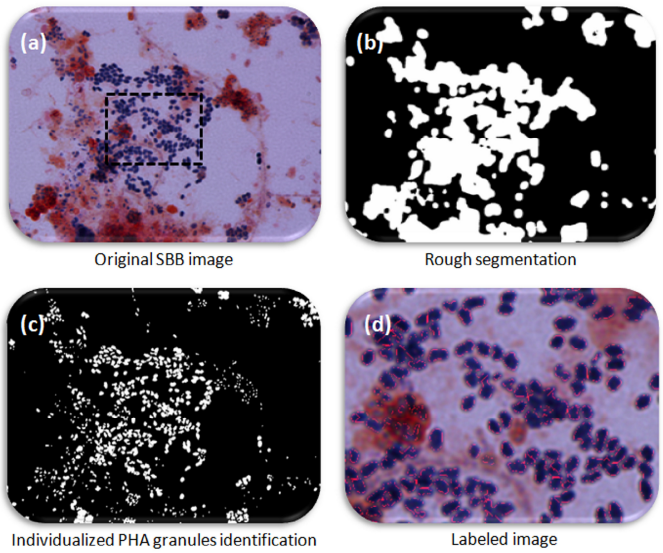

Fig. 1. (a) SBB original image, (b) rough segmented image, (c) individualized PHA granules identification, and (d) partial labeled image

2.5 Multivariate Statistical Analysis

Partial least squares (PLS) was applied to predict PHA concentrations using the data from quantitative image analysis provided by the routine specifically design for SBB staining. The model was performed with a total of 30 samples (15 aerobic and 15 anaerobic), divided randomly into a training set (67% of the observations to calibrate the model) and a validation set (33% of the observations to validate the final model).

3 Results and Discussion

The possibility of using quantitative image analysis data for monitoring PHA intracellular storage polymers based on SBB staining from an EBPR system was sought in this work. First, a gas chromatography analysis was performed to quantify those intracellular storage polymers. Figure 2 shows the behavior of PHA concentration during the monitoring period at the end of anaerobic and aerobic stages.

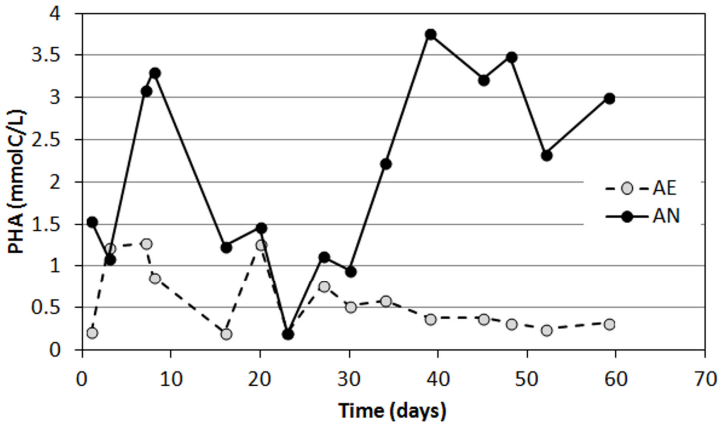

Fig. 2. Intracellular PHA concentrations at the end of anaerobic (AN) and aerobic (AE) stages

Regarding the results obtained at the end of the anaerobic phase, higher PHA concentrations were achieved. These results could be explained by the microbial communities' metabolism. In anaerobiosis, PHA are stored, differing to the lower concentrations obtained at the end of the aerobic stage, when these polymers are oxidized.

Regarding image analysis parameters, Figure 3 illustrates the behavior of total area obtained for small, intermediate, and large PHA granules for anaerobic and aerobic samples.

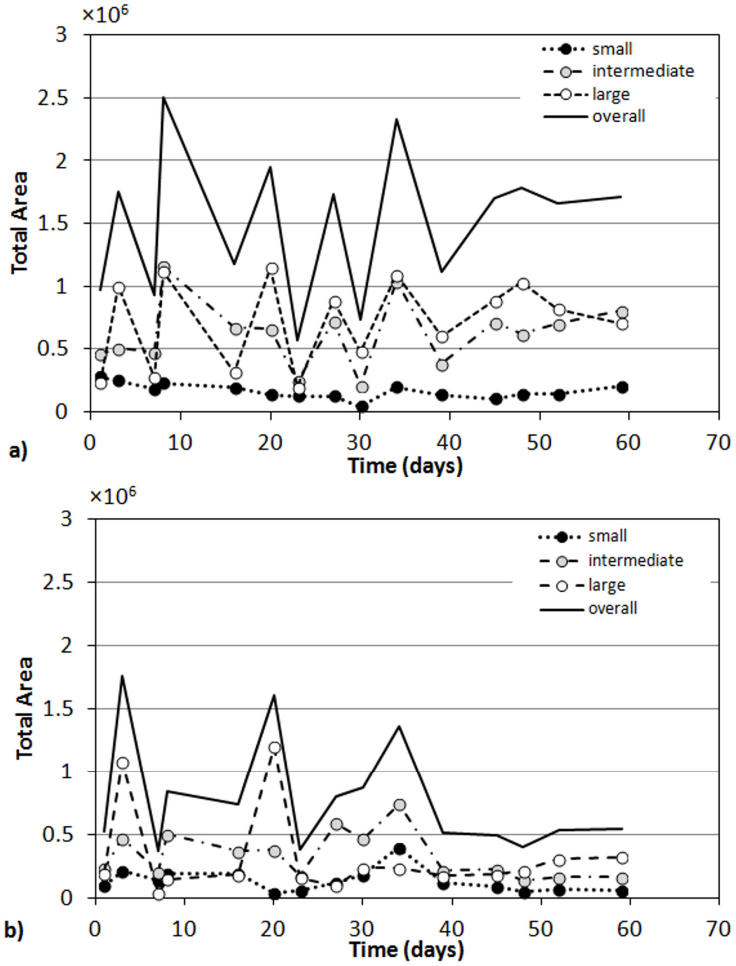

Fig. 3. (a) Total Area of PHA granules obtained at the end of AN stage, (b) Total Area of PHA granules obtained at the of AE stage

With respect to the area distribution of the granules, in anaerobic stage (Fig. 3a) there is a greater difference between the small granules total area, on one hand, and the intermediate and large granules, on the other, with respect to the aerobic stage. This can be explained from the fact that during this stage the microorganisms involved in this biological process store organic substrates as intracellular storage polymers, i.e. PHA in the form of granules. Regarding the results obtained for the aerobic stage (Fig 3b) it was found, until the end of the monitoring period, a decrease of intermediate and large granules, indicating that, under the presence of aerobic conditions, those microorganisms oxidize the internal stored PHA for growth. Consequently, image analysis results seem to corroborate the standard analytical results.

The possibility of predicting the intracellular PHA concentration was sought using a multivariate partial least squares analysis. This analysis was performed combining image analysis from SBB staining and standard analytical parameters provided by GC analysis. The obtained linear regression is presented in Figure 4.

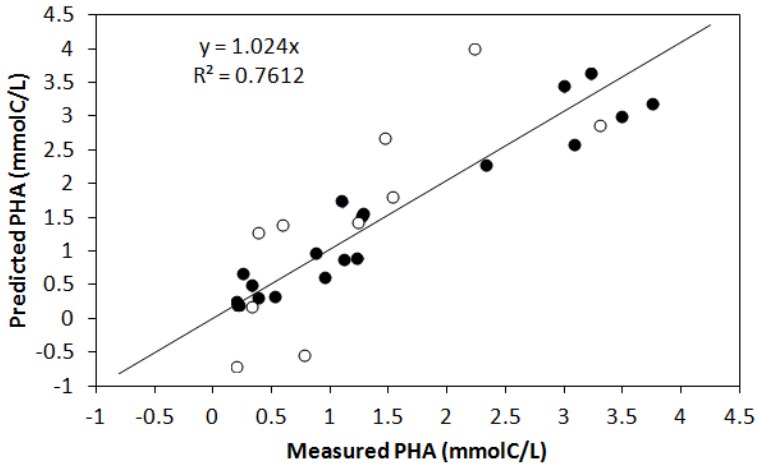

Fig. 4. Measured *vs* predicted intracellular PHA concentrations

Regarding the predicted and measured intracellular PHA concentrations (Fig. 4), a satisfactory correlation was obtained (R^2 of 0.76) by SBB staining, although for a reduced number of samples. Although the obtained correlation factor is still somewhat distant from 1, these results can be seen as promising to effectively quantify intracellular accumulation of PHA granules in EBPR systems.

4 Conclusions

A specific image analysis routine for PHA granules characterization using SBB staining was developed in this work. A good recognition of objects contours was found, however, since this is a user-friendly procedure it is possible to improve the sensitivity and specificity of the procedure by changing the segmentation parameters depending on the images to process. The most important value that can be chosen by the user is the window size for the adaptive threshold (larger window size for images with larger granules and/or smoother backgrounds, and smaller window size for images with smaller granules and/or uneven backgrounds). The cluster separation can also be changed by the user, increasing or decreasing the radius for a granule in order to divide, respectively less or more, the cluster. It is known that in bright-field microscopy, and especially in color acquisition, images can brighten or darken depending on the light intensity. Thus, this procedure is able to increase the background control value comprising a better division of the real granules from the misrecognized background. Regarding the results obtained from quantitative image analysis, a suitable prediction ability was found between the measured and predicted PHA concentration.

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