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Novel spectrophotometric technique for rapid determination of extractable PHA using Sudan black dye

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ABSTRACT

Classical techniques employed to determine the amount of extractable poly(hydroxyalkanoate)s (PHAs) from cells, are laborious and destructive. Sudan black staining is commonly used in the laboratory to investigate the presence of intracellular PHA. The aim of the present study was to develop a low-cost alternative technique to achieve a quick determination of extractable intracellular PHA. This methodology employs a basic laboratory spectroscopy equipment and Sudan black dye for spectra determination. The correlation between the content of PHA in cell samples taken directly from the culture flask and its spectra was determined using partial least square regression analysis and simple linear regression analysis. The best fit obtained for calibration correlation analysis (R^2 = 0.944, *RSE*: 1.24%), together with the good extractable PHA predictions (*RSE* = 0.51%) demonstrate that the proposed methodology constitutes a fast way with high potential for the determination of extractable PHA. Based on its simplicity and flexibility, its application would be suitable in routine monitoring and rapid quantification in large-scale processes involving PHA metabolism.

1. Introduction

Poly(hydroxyalkanoate)s (PHAs) are a family of natural biopolyesters synthesized by various microorganisms. These biopolymers have generated significant commercial and research interest due to its biodegradability, biocompatibility, chemical diversity, and their possibility of being produced from renewable carbon sources (Wei et al., 2015). In addition, they can be employed for packaging and coating materials, as carriers for slow delivery of drugs and agrochemicals and for preparation of medical devices in the biomedical field (Kulkarni et al., 2010).

Currently, to assist in the development of more efficient processes of fermentation and to control the biopolymer production, a rapid feedback about PHA content in the cells is required (Kansiz et al., 2000). Usually, cellular PHA content is directly determined by solvent extraction or indirectly by crotonic acid assay, gas chromatography (GC), liquid chromatography (generally high-performance liquid chromatography) and Fourier transform infrared spectroscopy (FTIR) (Tan et al., 2014). The time delay to get the determination results through these methods constitutes the main difficulty to achieve on-line control and optimization, making them inadequate to take fast decisions for the evaluation of producing strains as well as different operating conditions. Except FTIR method, the above mentioned quantification techniques are destructive. Furthermore, crotonic acid assay is only useful in determining poly(3-hydroxybutyrate) (PHB). On the other hand, PHA production is qualitatively tested using dyes, typically with alcoholic Sudan black B (SB) solution in gram positive and gram negative PHA producers (Hartman, 1940; Burdon et al., 1942). Sudan black B is also commonly used in histological works. Related to this dye, Xu et al. (2010) established a spectrophotometric method to label adherent platelets with a linear correlation between the absorbance of SB and the number of platelets. Spectrophotometry is by far the instrumental technique of choice of industrial laboratories, owing mainly to simplicity, often demanding low cost equipment (Hakan Aktaş and Kitiş, 2014). However, quantitative analysis based on spectrophotometric data commonly requires the use of chemometric techniques (Dinç and Baleanu, 2002). Partial least squares regression (PLSR) is a recent multivariate technique that combines features and generalizes principal component analysis and multiple linear regression. It is used to predict a set of variables dependent on a set of independent variables or predictors (Abdi, 2010). Unlike multiple linear regression and principal

Abbreviations: PLSR, partial least square regression; LRA, linear regression analysis; MSM, minimal saline medium; PHA, poly(hydroxyalkanoate); RMSECV, root mean square error of cross-validation; RMSEP, root mean square error of prediction; RSE, residual standard error; SB, Sudan black dye; SBT, Sudan black technique

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component regression methods, PLSR can analyze data with strongly collinear (correlated), noisy, and numerous X-variables (Wold, 2001). PLSR is originated in the social sciences but became popular first in chemometrics (i.e., computational chemistry) (Abdi, 2010). In analytical, physical and clinical chemistry, PLSR gained importance, while industrial process control may also benefit from the use of this methodology (Geladi and Kowalski, 1986; Martens, 2001). This method can be widely applied in natural sciences, where the number of samples run in experiments is usually rather limited by comparison (Sæbø et al., 2008). The model parameters do not change significantly when new calibration samples are included in the total population, which is a robustness indication of the method (Geladi and Kowalski, 1986). Through Linear Regression Analysis (LRA), it is possible to find the best straight line that fits through the percentage of extracted versus predicted PHA, by minimizing the residuals. The line obtained is known as calibration curve, and its equation can be used to predict the concentration of unknown samples (Mark and Workman, 2003).

In this context, the aim of the present work was to develop a simple, fast and non-destructive technique to determine the intracellular content of extractable PHA using samples of cells taken directly from culture flasks and stained with SB. *Bacillus megaterium* BBST4, a typical gram positive strain that produces both poly(3-hydroxybutyrate) homopolymer (PHB) as well as copolymers, was selected to develop the new quantification technique. The novel technique is based on spectroscopic quantitative analysis using multivariate statistical, Partial Least Squares Regression (PLSR), and Simple Linear Regression Analysis (LRA).

2. Materials and methods

2.1. Bacterial strain

The strain using in the present work was isolated from sediments of Bahía Blanca Estuary in a previous work (López et al., 2012). It was characterized as *Bacillus megaterium* (GenBank database accession number: HM119600.1) and named BBST4. This strain is capable to produce PHB (López et al., 2012) and PHA copolymers (Porras et al., 2017).

2.2. Biomass and PHA determinations

Bacillus megaterium BBST4 cell growth, was conducted in 31 flasks of 250 mL with 100 mL of minimal saline medium (MSM) and different carbon sources (glucose, glycerol and starch) using a cell suspension inoculum of the strain, obtained as described in a previous work (Porras et al., 2017). Different carbon sources were used to generate different type of PHA to be quantified using the Sudan black technique (SBT). Flasks were stirred in a shaker at 150 rpm and 30 °C, and taken at different time intervals to obtain different concentrations of PHA. One mL of culturing medium was taken from each flask and employed in the SBT, described in Section 2.3. The remaining culture medium was used to determine the cell weight and PHA content. The bacteria suspension from each flask was centrifuged at 2000*g* for 15 min to collect cells. The pellet was washed twice with distilled water and lyophilized (RIFICOR L-A-B3-C, with a vacuum pump WELCH 1402). Subsequently, the lyophilized cell weight (g/L) was determined in an analytical balance (Mettler AE 163, Mettler-Toledo Ltd., Leicester, UK). The resulting lyophilized biomass was used for PHA extraction and purification as is described by Porras et al. (2017). The correlation between the content of PHA (%) in the sample of lyophilized cells and the SBT data was determined.

2.3. Sudan black technique

The schematic steps of the technique are showed in Fig. 1. First, 1 mL sample was withdrawn from each culture flask and stored in an EP tube. Then, each sample was centrifuged at 7500*g* and washed with distilled water to eliminate all traces of culture medium. The next step involved the addition of 400 µL of SB staining solution to the wet cells sample. SB solution was prepared and optimized using different concentrations of solid SB in ethanol. In a fourth step, cellular suspension was continuously stirred for 20 min at 35 °C in an oven. The stained sample thus obtained was centrifuged at 7500*g* and washed thrice with distilled water to remove the SB not fixed by the cells. In a fifth step, the resulting stained cell pellet was suspended with 1 mL distilled water, homogenized with the use of a vortex and the final sample was divided into five sub-samples of 200 µL. Therefore, 155 sub-samples were used for spectrophotometric determinations and PHA quantification.

Each sub-sample was diluted to 1 mL with distilled water and its spectrum between 550 and 800 nm was read. The stained samples shown to be and could be stored for up to 48 h in the refrigerator without appreciable changes in color. The stained cell samples could be directly observed at an optical microscope with no need of contrast dye (Step 8). For comparative purposes, the inoculum and the media used in the experiments were also stained with SBT.

2.4. Statistical analysis

One mL sample was taken from each of the 31 flasks of the experiment, 24 were employed for calibration and the remaining 7 for prediction. For each sample, the relationship between the data obtained from each spectral peak determined using SBT and the extracted PHA content (expressed as mass percentage) was studied. To accomplish this task, the coefficient of determination (R^2) was used, based on 251 variables (values of each point of the curve between 550 and 800 nm with 1 nm of data acquisition frequency) for PLSR, and on height and area of the peak for LRA. Data are presented as mean ± error. For PLSR calibrations the root-mean-square error of cross-validation (*RMSECV*) was used to indicate the predictive ability of the model within the calibration set and the optimal number of factors, and the root-mean-square error of prediction (*RMSEP*) was employed to evaluate the response of the established calibration model versus an independent test sample set used (Jarute et al., 2004). Residual standard error (*RSE*) was used for LRA as a measure of the calibration and prediction adjustment. The performance of the calibration models was evaluated using the full cross-validation method for PLSR and the analysis of variance (ANOVA) for LRA, in which the results were considered significant when *p*-values were less than 0.05. The predictions of extractable PHA (X_i) using SBT spectral data (Y_i) were determined with LRA through the following equation:

$$Y_i = aX_i + b \quad (1)$$

The detection of atypical values (outliers), was performed based on the ellipses (Hotelling T^2 with 95% confidence) and residuals for PLSR evaluating the distances of the samples in the model space and in the residual space, respectively (Arcos-Hernandez et al., 2010). For LRA, outlier detection was performed based on QQ-plot and confidence and prediction intervals. The statistical analysis was performed using R software (R Core Team, 2016).

3. Results

3.1. Sudan black technique

3.1.1. Spectral analysis

The optimal concentration of SB that completely stain the PHA of the *B. megaterium* BBST4 strain cells, was 30% solid SB in 70% ethanol. Higher concentrations of SB did not increase the staining of the cells. Fig. 2A shows the difference between the spectra of the stained and the non-stained cell sample cultured in liquid media. After applying the baseline correction to the spectra samples, stained cell sample spectra showed a maximum absorbance value at around 660–680 nm, while the