Journal of Biotechnology 255 (2017) 28–32

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

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

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ARTICLE INFO

Keywords:
Extractable poly(hydroxyalkanoate)s (PHAs)
Sudan black stain
Novel spectrophotometric technique
Chemometrics

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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http://dx.doi.org/10.1016/j.jbiotec.2017.06.012
Received 10 April 2017; Received in revised form 30 May 2017; Accepted 12 June 2017
Available online 19 June 2017
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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

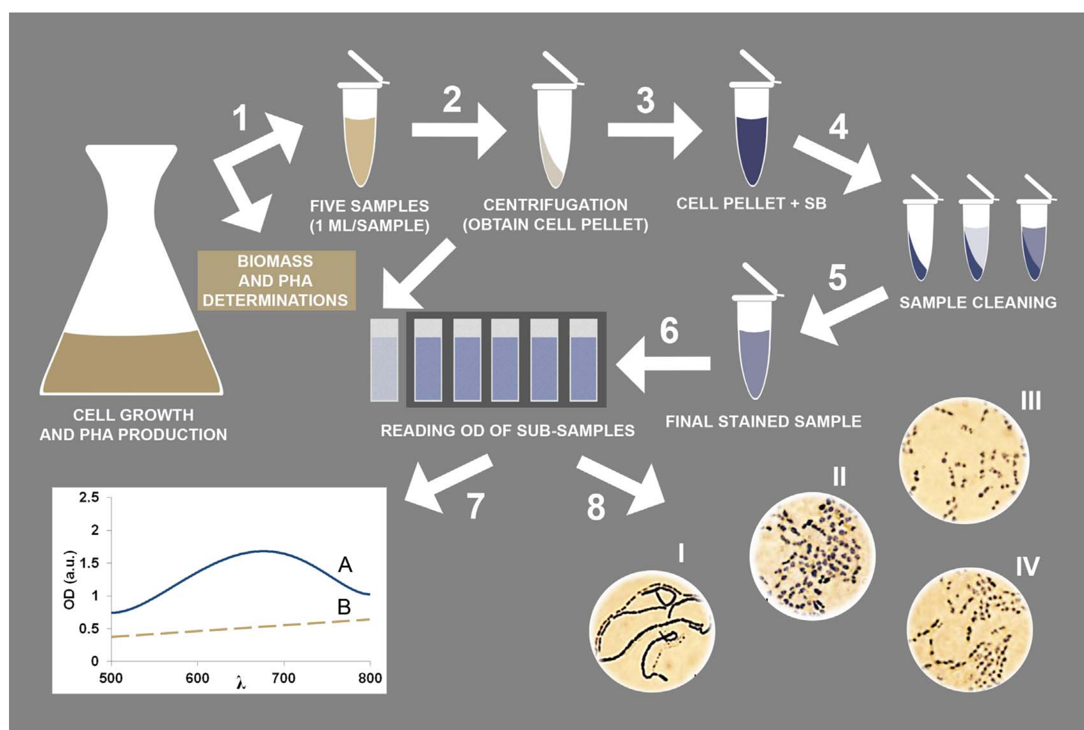

Fig. 1. Scheme of the steps of developed Sudan black quantification technique. (A) Spectrum of a stained cell sample and (B) spectrum of the same cell sample unstained. Inoculum (from solid culture) (I) and samples (from liquid culture) at 13 h (II), at 24 h (III), and at 48 h (IV).

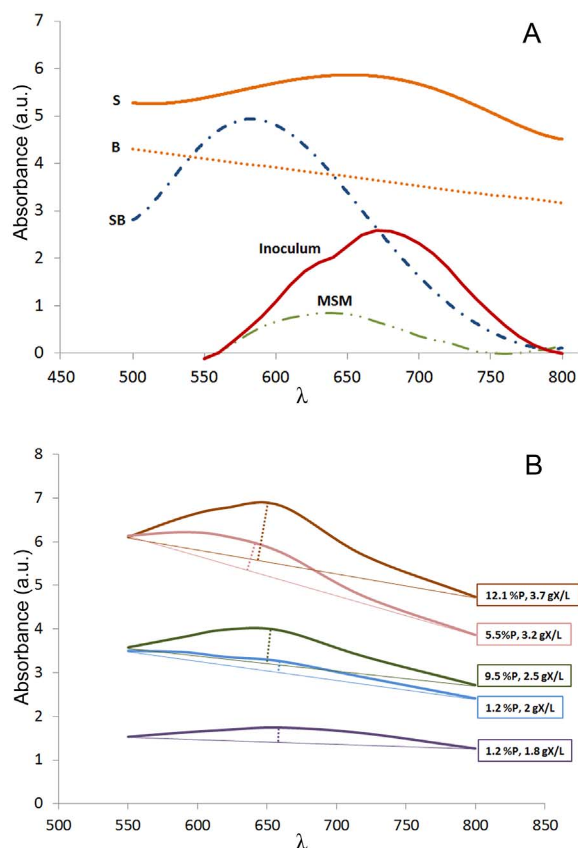

Fig. 2. Absorbance spectra for: **A.** Cell sample growth in liquid media stained with SBT (S), the same sample without dyeing (B), Sudan black spectrum in aqueous solution (SB), sample of inoculum growth in solid media stained with SBT (Inoculum) and minimal saline medium without cells stained with SBT (MSM), and **B.** Curves of spectra showed the height (vertical dotted line) and area of the spectrum curve (limited by baseline) for five cell samples cultured in liquid media and stained with SBT with different PHA (P) and biomass (X) concentrations.

spectrum of non-stained cell sample did not show any peak values. The spectrum of the dyed inoculum sample could also be observed (I in Fig. 1). This inoculum was taken directly from a plate with solid media and dissolved in sterile distilled water, so it does not contain any culture media. On the other hand, the culture medium could be observed alone (MSM), if stained following the steps of the SBT. Fig. 2B also shows the spectra of many samples stained following the SBT with the corresponding values of biomass and extracted PHA.

3.1.2. Calibration and prediction

A summary of the correlation coefficients (R^2) and errors obtained by both PLSR and LRA methods, can be observed in Table 1. Using the spectra of all calibration samples, the PLSR adjustment was better ($R^2 = 0.902$) when compared to the LRA adjustments. After performing the analysis of outliers, very similar adjustments were observed with both methodologies, although the best fit was observed with LRA using peak height ($R^2 = 0.944$). Fig. 3 showing the linear relationship between polymer concentrations and peak height of absorbance spectra

Table 1
Adjusted correlation coefficients and errors for calibration and prediction samples determined by PLSR and LRA.

Technique	Calibration				Prediction	
	R^2 Adj	E (%)	R^2 Adj[a]	E (%)[a]	E (%)	E (%)[a]
PLSR	**0.902**	**1.697(3)**[b]	**0.940**	**1.259(3)**	**1.011**	**1.040**
LRAH	0.804	2.378	0.944	1.243	3.383	0.510
LRAA	0.786	2.465	0.939	1.299	3.645	0.691

Abbreviations. PLSR: Partial Least Square Regression, **LRAH:** Sudan Black Technique based on height of peaks with LRA, **LRAA:** Sudan Black Technique based on area of peaks with LRA. **R^2 Adj:** coefficient of correlation adjusted, **E:** Methodology error (calibration and prediction for PLSR: **RMSECV** and **RMSEP**, respectively, and calibration and prediction for LRA: **RSE**).

[a] Samples without 1 outlier (PLSR) and 3 outliers (LRAH and LRAA).

[b] Values in brackets correspond to the minimum number of factors from which the minimum SEP is achieved.

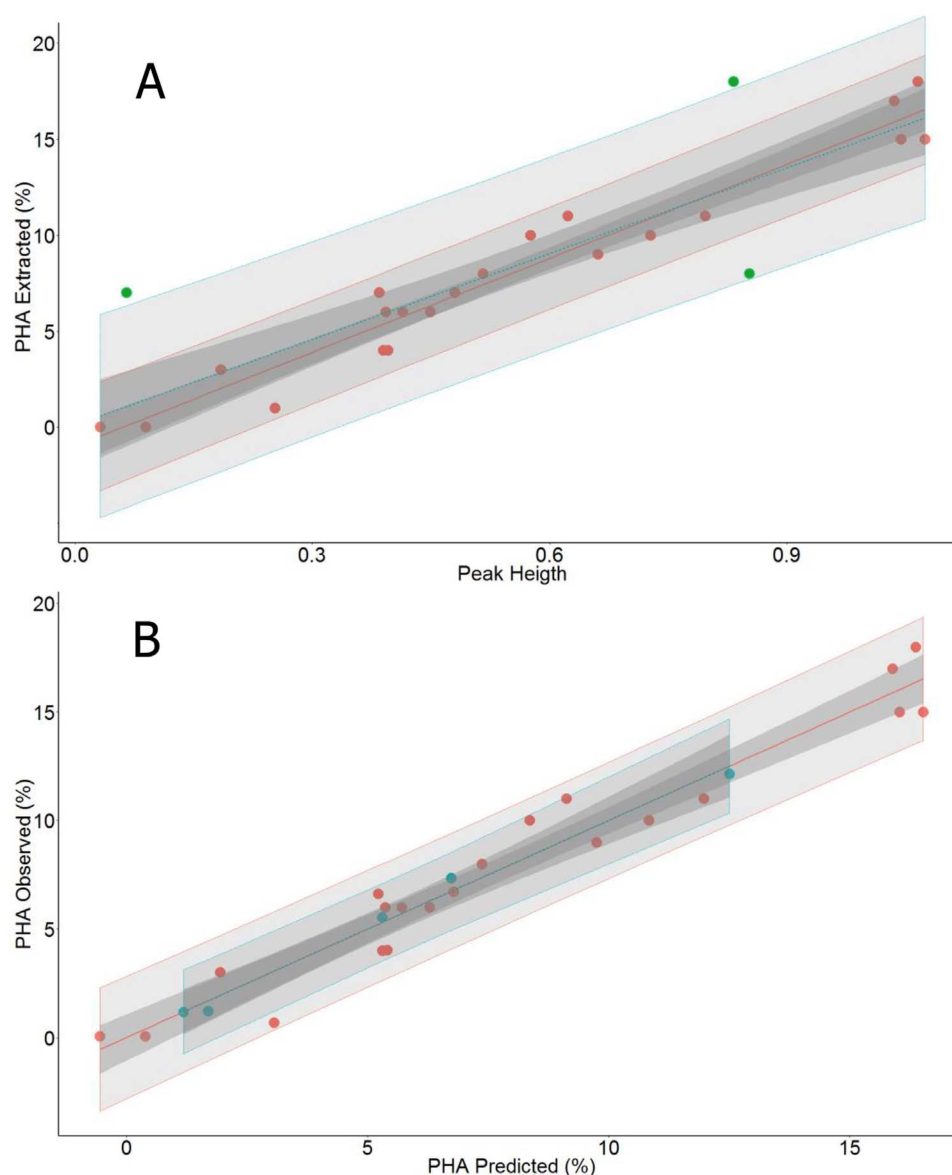

Fig. 3. Confidence and prediction regions determined by LRA. **A.** Calibration curves with (blue edges) and without (red edges) outliers, to predict the extractable PHA according to the peak height determined by SBT; in green can be seen the outlier samples. **B.** Observed and predicted curves without outliers for calibration samples (red) and prediction samples (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

obtained by LRA. For each curve can be seen the statistical errors showed as confidence and prediction regions. The following calibration equations were determined by LRA using peak height (1) and area (2) data:

$$Y_H = 16.375X_H - 1.0686 \quad (2)$$

$$Y_A = 0.959X_A - 1.0810 \quad (3)$$

In these expressions Y_i corresponds to PHA concentration for the stained sample ($Y_H = Y_A$), and X_i corresponds to the height (X_H) and the area (X_A) of the peak determined from the stained sample. LRA predictions were performed using the calibration equations showed above. While using all spectral samples for predictions, the error obtained by PLSR was about one-third of those obtained by LRA. When the analysis of outliers was performed, prediction errors obtained by LRA were approximately half of those obtained by PLSR.

4. Discussion

4.1. Sudan black technique

4.1.1. Spectral analysis

Under wet conditions the cells successfully fixed the SB dye. The spectrum of MSM stained by SBT, as well as the spectrum of SB solution (Fig. 2A), presented quite pronounced peaks. For this reason, the successive washes of the samples must be carried out until eliminating all the rest of medium and SB solution, so as to avoid its influence on the spectrum of the samples. This statement is observed in a sample of inoculum grown on solid medium and stained with SBT (see Fig. 1 sample I, and Fig. 2A), in which the cells were easily obtained without media residues. After removing all the SB dye in solution from this inoculum sample, the spectrum corresponding to the pure cell sample and its PHA content can be obtained.

In Fig. 2B can be observed the likeness between the value of extracted PHA and the height (vertical dotted line) and area of the spectrum curve (limited by baseline) obtained for the same sample. This similarity demonstrates the existence of a correlation between the spectrum curve and the intracellular PHA concentration of a sample. Therefore, the correlation was analyzed and proved to be a useful

strategy to determine the extractable content of intracellular PHA.

4.1.2. Calibration and prediction

Although the values for the calibration from the peak height data presented the best fit, the regression curve adjustment showed to be adequate using both PLSR and LRA methods. Therefore, the equations of the calibration curves were successfully used to predict the extractable PHA from the cells based on the height and area of the peak. The best prediction fit was observed when using peak heights ($RSE = 0.51\%$), once the analysis and removal of outliers was performed. The obtained prediction errors were in agreement with previous determinations obtained using FTIR as a quantification technique (Porras et al., 2016). On the other hand, the increase of the prediction error obtained by PLSR after the elimination of one supposedly atypical value, suggests that all the data contribute with relevant information to the prediction model of PLSR.

5. Conclusions

A quantification technique based on the employ of Sudan black dye is proposed in order to provide a new non-destructive alternative for the rapid determination of the extractable intracellular PHA content. The two more widely techniques employed for PHA determination, are GC for indirect quantifications and chloroform extraction for direct ones. The main problem is that both techniques are very laborious and require several steps. Moreover, they are highly time-consuming, involving more than 20 h of processing until the quantification results are obtained. The new developed quantification technique proposed here require few and simple preparation steps, processing, and reading of the samples, and can be performed using basic laboratory spectrometry equipment, with low-cost dye, and reagents commonly found in any laboratory. In fact, once the experimental data is available, less than 1 h is needed to perform the sample processing (from sampling to reading in the spectrophotometer). The prediction adjustments thus obtained prove that a robust calibration models can be obtained from this technique. As it is based on a statistical determination of the peak obtained from the spectra of cells stained with SB, this technique can also be employed in other studies related with PHA metabolism. The simplicity and flexibility of the propose technique would make it adequate for its application in routine monitoring and rapid quantification in large scale processes. Thus, this development may open new possibilities to improve the overall time of PHA determination and provide a complement for other quantification techniques.

Acknowledgments

Authors wish to thank CONICET (National Research Council of Argentine) and UNS (Universidad Nacional del Sur) for financial support.

References

Abdi, H., 2010. Partial least squares regression and projection on latent structure regression (PLS Regression). Wiley Interdiscip. Rev. Comput. Stat. 2 (1), 97–106.

Arcos-Hernandez, M.V., Gurieff, N., Pratt, S., Magnusson, P., Werker, A., Vargas, A., Lant, P., 2010. Rapid quantification of intracellular PHA using infrared spectroscopy: an application in mixed cultures. J. Biotechnol. 150, 372–379.

Burdon, K.L., Stokes, J.C., Kimbrough, C.E., 1942. Studies of the common aerobic spore-forming bacilli: I. Staining for fat with Sudan black B-safranin. J. Bacteriol. 43 (6), 717.

Dinç, E., Baleanu, D., 2002. Spectrophotometric quantitative determination of cilazapril and hydrochlorothiazide in tablets by chemometric methods. J. Pharm. Biomed. Anal. 30, 715–723.

Geladi, P., Kowalski, B.R., 1986. Partial least-squares regression: a tutorial. Anal Chim. Acta 185, l–17.

Hakan Aktaş, A., Kitiş, F., 2014. Spectrophotometric simultaneous determination of caffeine and paracetamol in commercial pharmaceutical by principal component regression, partial least squares and artificial neural networks chemometric methods. Croat. Chem. Acta. 87, 69–74.

Hartman, T.L., 1940. The use of Sudan Black B as a bacterial fat stain. Stain Technol. 15 (1), 23–28.

Jarute, G., Kainz, A., Schroll, G., Baena, J.R., Lendl, B., 2004. On-line determination of the intracellular poly($\beta-$hydroxybutyric acid) content in transformed Escherichia coli and glucose during PHB production using stopped-flow attenuated total reflection FTIR spectrometry. Anal. Chem. 76, 6353–6358.

Kansiz, M., Billman-Jacobe, H., McNaughton, D., 2000. Quantitative determination of the biodegradable polymer poly(β-hydroxybutyrate) in a recombinant Escherichia coli strain by use of mid-infrared spectroscopy and multivariative statistics. Appl. Environ. Microbiol. 66, 3415–3420.

Kulkarni, S.O., Kanekar, P.P., Nilegaonkar, S.S., Sarnaik, S.S., Jog, J.P., 2010. Production and characterization of a biodegradable poly (hydroxybutyrate-co-hydroxyvalerate) (PHB-co-PHV) copolymer by moderately haloalkalitolerant Halomonas campisalis MCM B-1027 isolated from Lonar Lake, India. Bioresour. Technol. 101, 9765–9771.

López, J.A., Naranjo, J.M., Higuita, J.C., Cubitto, M.A., Cardona, C.A., Villar, M.A., 2012. Biosynthesis of PHB from a new isolated Bacillus megaterium strain: outlook on future developments with endospore forming bacteria. Biotechnol. Bioprocess Eng. 17 (2), 250–258.

Mark, H., Workman Jr., J., 2003. Statistics in Spectroscopy, 2nd edn. Elsevier, USA.

Martens, H., 2001. Reliable and relevant modelling of real world data: a personal account of the development of PLS Regression. Chemom. Intel. Lab. Syst. 58, 85–95.

Porras, M.A., Cubitto, M.A., Villar, M.A., 2016. A new way of quantifying the production of poly(hydroxyalkanoate)s using FTIR. J. Chem. Technol. Biotechnol. 91, 1240–1249.

Porras, M.A., Vitale, C., Villar, M.A., Cubitto, M.A., 2017. Bioconversion of glycerol to poly (HB-co-HV) copolymer in an inexpensive medium by a Bacillus megaterium strain isolated from marine sediments. J. Environ. Chem. Eng. 5 (1), 1–9.

R Core Team, 2016. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.r-project.org/.

Sæbø, S., Almøy, T., Flatberg, A., Aastveit, A.H., Martens, H., 2008. LPLS-regression: a method for prediction and classification under the influence of background information on predictor variables. Chemom. Intell. Lab. Syst. 91, 121–132.

Tan, G.Y.A., Chen, C.L., Li, L., Ge, L., Wang, L., Razaad, I.M.N., N Wang, J.Y., 2014. Start a research on biopolymer polyhydroxyalkanoate (PHA): a review. Polymers 6 (3), 706–754.

Wei, X.-X., Zheng, W.-T., Hou, X., Liang, J., Li, Z.-J., 2015. Metabolic engineering of Escherichia coli for poly(3-hydroxybutyrate) production under microaerobic condition. Biomed. Res. Int. 789315.

Wold, S., 2001. Personal memories of the early PLS development. Chemom. Intell. Lab. Syst. 58, 83–84.

Xu, X.X., Gao, X.H., Pan, R., Lu, D., Dai, Y., 2010. A simple adhesion assay for studying interactions between platelets and endothelial cells in vitro. Cytotechnology 62 (1), 17–22.