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Improved intracellular PHA determinations with novel spectrophotometric quantification methodologies based on Sudan black dye

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ABSTRACT

The presence of intracellular polyhydroxyalkanoates (PHAs) is usually studied using Sudan black dye solution (SB). In a previous work it was shown that the PHA could be directly quantified using the absorbance of SB fixed by PHA granules in wet cell samples. In the present paper, the optimum SB amount and the optimum conditions to be used for SB assays were determined following an experimental design by hybrid response surface methodology and desirability-function. In addition, a new methodology was developed in which it is shown that the amount of SB fixed by PHA granules can also be determined indirectly through the absorbance of the supernatant obtained from the stained cell samples. This alternative methodology allows a faster determination of the PHA content (involving 23 and 42 min for indirect and direct determinations, respectively), and can be undertaken by means of basic laboratory equipment and reagents. The correlation between PHA content in wet cell samples and the spectra of the SB stained supernatant was determined by means of multivariate and linear regression analysis. The best calibration adjustment (R^2 = 0.91, RSE: 1.56%), and the good PHA prediction obtained (RSE = 1.81%), shows that the proposed methodology constitutes a reasonably precise way for PHA content determination. Thus, this methodology could anticipate the probable results of the above mentioned direct PHA determination. Compared with the most used techniques described in the scientific literature, the combined implementation of these two methodologies seems to be one of the most economical and environmentally friendly, suitable for rapid monitoring of the intracellular PHA content.

1. Introduction

One of the most promising biomaterials to replace petrochemical-based plastics are polyhydroxyalkanoates (PHAs). These biopolyesters are naturally synthesized by bacteria and stored as cytoplasmic granules of 0.2–0.5 μm diameter, providing carbon and energy for the bacteria under certain conditions (Khanna and Srivastava, 2005). Due to its biodegradability, biocompatibility, and its synthesis from renewable resources, PHAs have attracted considerable commercial interests. Their properties and chemical diversity permitted various applications ranging from biodegradable packaging materials to medical products (Tan et al., 2014).

The presence of PHA granules is usually tested using alcoholic Sudan black B (SB) solution in both Gram positive and Gram negative bacteria (Aswathy, 2015; Dalgaard, 1995; Hartman, 1940). Hartman used wet preparations only, suspending the bacteria in a solution of Sudan black B containing 70% alcohol. Fat droplets were recognized as blue-black bodies in a colorless cytoplasm (Hartman, 1940). On the other hand, quantitative PHA determinations are performed using indirect and direct techniques. Although gas chromatography (GC) is reported as the preferred indirect method for PHA quantification (Tan et al., 2014), cellular PHA content can also be indirectly determined by different techniques, such as crotonic acid assays (Law and Slepecky, 1961; Ward and Dawes, 1973), liquid chromatography (Grubelnik et al., 2008), and Fourier transform infrared spectroscopy (FTIR) (Hong et al., 1999), among others. However, the "gold standard" for the quantification of PHAs remains the direct determination by gravimetric techniques with solvent extraction (Godbole, 2016; Valappil et al., 2007a; Yu and Chen, 2006). In any case, the main drawback associated with these techniques is the time delay required to obtain the measurement results. These aspects difficult the use of these techniques as a basis to support rapid decisions that require the performance evaluation of the PHA producing strains. The same happens to establish the operating conditions for on-line control and process optimization.

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Furthermore, quantification techniques are generally destructive and unfriendly to the environment.

Concerning the equipment employed for PHA content determinations, the spectrophotometer is undoubtedly the simplest and most economical to be used in any laboratory (Hakan Aktaş and Kitiş, 2014). Despite this, few methodologies use spectrophotometry. One of them measures the turbidity of the PHA granules released from cells after the digestion of bacterial wall with sodium hypochlorite solution (Williamson and Wilkinson, 1958). The other methods, and more widely used, employ the crotonic acid absorption band at 235 nm in the ultraviolet (UV) spectrum (Law and Slepecky, 1961; Ward and Dawes, 1973). However, it is known that the crotonic acid assay overestimates the biopolymer content and is only useful in poly(3-hydroxybutyrate) (PHB) determinations (Valappil et al., 2007b).

Spectrophotometric data can be used in quantitative analyzes that use chemometric techniques, which allow obtaining equations to summarize or describe the data relationships. The powerful chemometric methods, i.e. classical least square (CLS), inverse least square (ILS), principal component regression (PCR), multiple linear regression (MLR) and partial least squares regression (PLSR), are commonly used in spectral data analysis (Chen and Wang, 2001; Dinç and Baleanu, 2002; Hogan et al., 1970). Among all these, the most employed in biological sciences is the PLSR. It combines features of PCA and MLR (Abdi, 2010), but differs from them because data with strong collinearity (correlated), noisy, and numerous X-variables, can only be studied through PLSR analysis (Wold et al., 2001). The PLSR method can also be widely applied in analytical, physical and clinical chemistry, while the industrial process control may also benefit from the use of this methodology (Geladi and Kowalski, 1986; Martens, 2001). In addition, it is particularly useful in natural sciences, where the number of samples obtained from experiments is generally quite limited, unlike other sciences (Sæbø et al., 2008). The robustness of this method can be observed when new samples are included in the population used for calibration, where model parameters do not change significantly (Geladi and Kowalski, 1986). Sometimes, the problem of estimating equations can be solved with simple regression methods, based on a single covariate, an independent "predictor" variable x, and a response, a dependent variable y, with a linear relationship (Stauffer, 2007). Model adequacy must be evaluated looking for potential misspecification, failure in the selection of the important variables, inclusion of unnecessary variables, or unusual/inappropriate data. In case the model is inadequate, it must be corrected and its parameters estimated once more. This process may be repeated several times until an adequate model is obtained (Montgomery et al., 2012). Once the best model (that which correlates the observed and predicted variables) is found, its equations can be used to predict (estimate) the values corresponding to unknown samples.

In a previous work, a rapid, reliable and non-destructive spectrophotometric methodology for PHA quantification using SB dye and basic spectrophotometric equipment, was developed and described (Porras et al., Since the results obtained in this reference will often be used here for comparative purposes, in the rest of this paper it will be called "the previous work"). As with all new quantification techniques, the optimal conditions to carry out the assays must be studied and determined. This is necessary to improve reproducibility, as well as the robustness of the methodology and the speed in obtaining results. Working in optimal conditions also decreases the error in predictions. Response surface methodology (RSM) is generally used to achieve this end. RSM consists of a group of mathematical and statistical techniques used in the development of an adequate functional relationship between a response of interest (y) and a number of associated control (or input) variables (x_i) (Khuri and Mukhopadhyay, 2010). RSM can be complemented with the desirability-function to determine the best interactions and simultaneously optimize multiple functions (Derringer and Suich, 1980). Therefore, in the present work the optimal amount of SB, together with the optimal conditions that should be used for carrying the SB methodology (named here the Direct Sudan Black methodology, DSB), were determined following an experimental design by hybrid RSM and the desirability-function.

In addition, carrying out the DSB assays under optimal conditions gave rise to an interesting issue, which in turn gave the basis for the development of a new and alternative PHA quantification methodology. This issue was the difference that in these conditions could be observed with the naked eyes, between the initial and final color of the dye solution during the staining process. As will be described below, the amount of PHA produced could also be determined, in this case indirectly, through the difference in the absorbance of both dyeing solutions.

At the same time, it will be shown that this extra methodology can complement and add robustness and predictability to the previous DSB. The indirect methodology was developed using quantitative spectroscopic analysis, based on multivariate statistics (PLSR and Simple Linear Regression Analysis).

2. Materials and methods

2.1. Bacterial strain

The strain used was characterized as *Bacillus megaterium* (GenBank database accession number: HM119600.1) in a previous work (López et al., 2012) and named BBST4. *B. megaterium* BBST4 is capable to produce PHB (López et al., 2012) and P(HB-co-HV) (Porras et al., 2017b).

2.2. Biomass and PHA determinations

The experiments were carried out based on a technique developed in the previous work. *Bacillus megaterium* BBST4 cell growth was performed in 27 shaking flasks of 250 mL with 100 mL of minimal saline medium (MSM). Starch (Anedra, Argentine) and glucose (Anedra, Argentine) were used as carbon source for the production of different types of PHA in order to be quantified using SB methodologies (described in the next sections). The *B. megaterium* inoculum employed was a cell suspension obtained as described in a previous work (Porras et al., 2017b). To obtain different PHA concentrations, flasks were stirred in a shaker at 150 rpm and 30 °C and taken at different time intervals. These time intervals were selected in the stationary growth phase, which is reached at 18 h for this strain. From each flask, 1 mL of culture medium was taken and employed for both SB methodologies. Cell weight and PHA content were determined with the remaining culture medium. For each flask, bacterial suspension was centrifuged at 2000*g* for 15 min to collect cells. The obtained pellet was lyophilized (RIFICOR L-A-B3-C, with a vacuum pump WELCH 1402) and cell weight (g/L) was determined. Lyophilized biomass was used for PHA extraction and purification (as is described in Porras et al., 2017b). The correlation between the PHA content (%) in the lyophilized cell samples and SB assays data was determined as follows.

2.3. Sudan black assays

The steps of both methodologies are outlined in Fig. 1. First, 1 mL of sample from each culture flask was harvested in an Eppendorf tube. Culture medium traces of each sample were removed by washing twice with distilled water and centrifugation (7500*g* for 5 min). Subsequently, each cell sample pellet thus obtained was mixed with the SB solution (Sigma-Aldrich, Germany) at different dye concentrations (SB suspensions) following an experimental design involving a hybrid response surface methodology (HRSM) in conjunction with desirability-function. This experimental design was used to obtain the most economical and efficient results in the dyeing step. In this way, the optimal amount of SB solution and the optimal time and temperature for sample shaking were determined. The optimal conditions found in this step were used

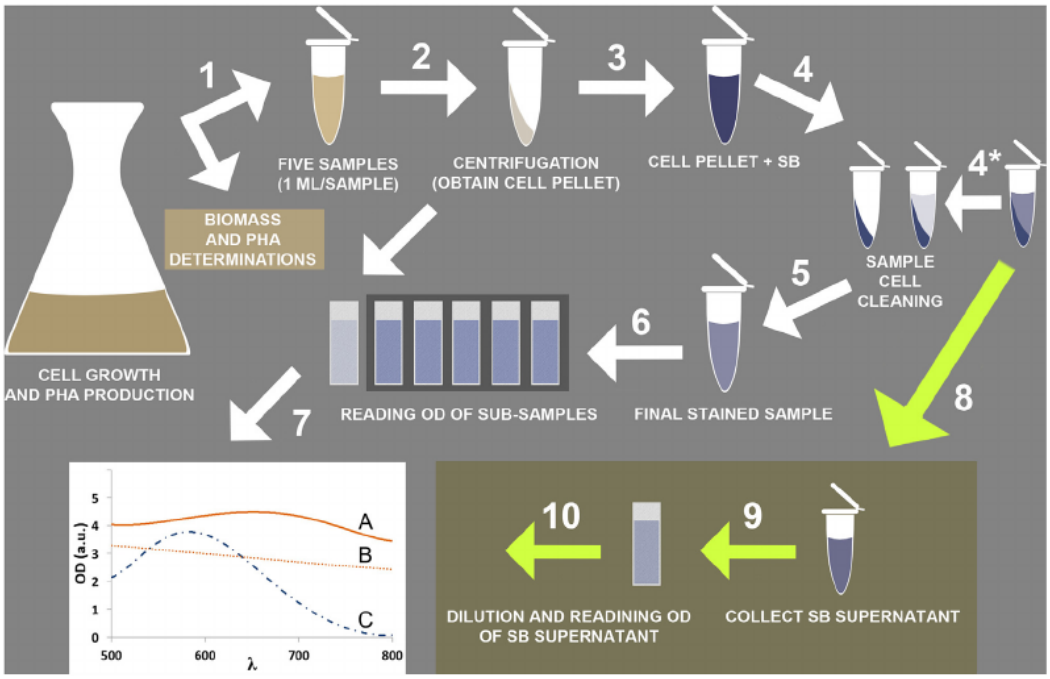

Fig. 1. Scheme of the steps developed in Sudan black quantification assays. (A) Spectrum of a stained cell sample, (B) spectrum of the same cell sample unstained, and (C) spectrum of the first SB diluted supernatant. ISB steps (8–10) in the bottom box (Modified from Porras et al., 2017a).

in the application of the SB quantification methodologies described below.

2.3.1. Indirect Sudan black methodology (ISB)

The SB solution not fixed by the cells was used to develop the ISB presented in this paper (Fig. 1, steps 8–10). In order to obtain representative spectral data, absorbance at 660 nm should be less than 0.9; otherwise, PHA values would be underestimated and the correlation could not be maintained. When absorbance of the samples exceeded the value of 0.9, dilutions were made. In these cases, the corresponding reading was corrected with the dilution factor. In all the spectra, distilled water was used as a reference (blank). A calibration curve was generated based on the correlation between PHA content in the sample (expressed as mass percentage) and the spectrum of the supernatant employing all the information of the curve (PLSR) or the height or peak area (LRA). Unknown samples were used for the predictions.

2.3.2. Direct Sudan black methodology (DSB)

Stained samples used for DSB were obtained following the steps described in the previous work. To complement and reinforce the DSB results, 21 sample data obtained in the previous work were used. Therefore, data of 42 samples were finally used in the calibration of DSB.

2.4. Statistical analysis

From the 27 shaking flasks experimented, 21 were used for calibration and 6 for prediction. 1 mL of sample was taken from each flask, and the spectral peak data was determined using DSB and ISB. The relationship (correlation) between these data and the extracted PHA content (expressed as mass percentage) was studied for each sample. The results of both methodologies were statistically compared. To achieve these tasks, the coefficient of determination (R^2) and the errors was determined. PLSR was based on 221 variables for ISB and 251 variables for DSB (values of each point of the curve, with 1 nm of data acquisition frequency), and LRA was based on data of height and area of the peak determined using second derivative filter (see Porras et al., 2015). All samples were performed in triplicate or quintuplicate and the data were employed as mean ± error (as standard deviation, not reported here). Therefore, 110 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 500 and 800 nm was read. Complementary, a correlation between direct and indirect data was determined and a model equation was obtained. For PLSR calibrations the root-mean-square error of cross-validation (RMSECV) was employed and for predictions the root-mean-square error of prediction (RMSEP) was used. Residual standard error (RSE) was employed for LRA as a measure of the calibration and prediction values. The performance of the calibration 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. Predictions of extractable PHA (X_i) using SB spectral data (Y_i) were determined with LRA through the following equation:

$$b \quad (1)$$

The estimation of the parameters a and b was made by least squares. The detection of outliers (atypical values), for PLSR was performed based on Hotelling T^2 ellipses (with 95% confidence) and residuals, based on QQ-plot, and from the confidence and prediction intervals for LRA. Statistical analysis was performed using R software (R Core Team, 2017).

3. Results

3.1. Sudan black assays optimization

3.1.1. Basis of the optimization

As was demonstrated in the previous work, under wet conditions cells successfully fixed the SB dye, and this property, as already explained in Section 2.3, was used to quantify the amount of intracellular PHA in a given sample. This methodology, called above DSB methodology, was developed and used in the previous work. As a subsequent step of that original experimentation plan, the optimal conditions for

PHA quantification through the DSB methodology are investigated and reported here. In addition (as will be seen in Section 3.2), the results obtained from this step, also permits the development of another new methodology capable to determine the amount of intracellular PHA.

The optimal conditions for the DSB methodology were found as follows. As a certain amount of SB molecules will be fixed by the PHA granules during the staining process (approximately 1.16 g of SB/g of PHA, determined under the optimal conditions), a variation in the color of the SB supernatant solution should be expected. However, this variation is not apparent to the naked eye when there is an SB excess in the staining experience. This situation gave the basis of an optimization of the amount of SB solution to be added, always caring for the complete staining of the granules of PHA present in the cell sample. Namely, the minimum amount of SB solution that guarantees complete staining.

In other words, the optimal value of SB solution for the DSB methodology will be that which verifies that: a) additional aggregates of SB solution, predict the same production in all experiences, b) smaller aggregates of SB solution, predict smaller PHA productions than those predicted through the optimum. That is, the addition of SB below the optimum would lead to underestimating the amount of PHA produced, especially in situations of high biopolymer production.

Besides the amount of SB, the main variables involved in the experimental determination of the PHA produced were optimized. The procedure implemented to achieve this goal is described below.

3.1.2. Optimization experiences

The amount of SB fixed by PHA granules, and therefore the amount of SB to be added, depends on the concentration of cells and PHA present in the analyzed sample. In turn, it will depend on the temperature and time needed to experience a complete fixation. Therefore, these three variables (amount of SB, temperature and stirring time) were selected for the optimization step, which was conducted at a constant stirring speed of 200 rpm. Table 1 shows the experimental design for optimum conditions determination. Using desirability-function, the respective values finally determined were: 440 µL of SB solution, and 34 °C and 16 min for the stirring process (desirability = 0.96). As central value for SB solution in the HRSM design, the volume needed to stain the maximum PHA produced by the cells of the studied strain (about 400 µL of SB solution to stain 20% of PHA) was selected. In this case, the optimum determined (440 µL) exceeded in about 2% that PHA production.

3.2. Development of a simple and fast technique for PHA determination

As already mentioned, experiences conducted under the optimal conditions found above, permitted an obvious but interesting observation: the supernatant of SB solution, once the staining process was concluded (after step 4, Fig. 1), presented a color which significantly differed to the original one. This circumstance gave rise to the idea that PHA production could also be determined indirectly. In fact, it must be related in inverse proportion to the amount of SB remaining in the supernatant.

All this led to a new development, already mentioned in Section 2.3.1 and called the Indirect Sudan Black (ISB) methodology. Its logic and performance are described in what follows, in permanent comparison with the DSB methodology developed previously. The above determined optimal conditions were used to analyze the results in both SB methodologies. Based on the best peak definition of each spectra, data between 500 and 720 nm were selected for ISB methodologies, and between 550 and 800 nm for DSB methodologies.

Table 1
Optimal conditions determinations for SB assays based on hybrid response surface methodology.

Run	T (°C)	Time (min)	SB (µL)	Peak Height
1	49	30	260	0.229
2	21	30	260	0.183
3	35	30	680	0.554
4	35	2	260	0.220
5	25	10	540	0.320
6	35	58	260	0.208
7	35	30	120	0.102
8	45	10	540	0.460
9	45	50	540	0.549
10	35	30	400	0.550
11	25	50	540	0.500

3.2.1. Indirect Sudan black methodology

Stationary growth phase is an indispensable condition for the ISB methodology. This is because the amount of fixed dye is directly related with the concentrations of both PHA and cells in the sample, which in turn determine the concentration of free dye in the supernatant. In this growth phase, supernatants from colored samples obtained through the DSB methodology (Fig. 1) were recovered to be employed in the ISB methodology. In this case, the first step consisted in finding an inverse correlation between the amount of PHA produced and the area or height of the peak corresponding to the residual SB in the sample. This inverse correlation is illustrated in Fig. 2.

Based on the scheme showed in Fig. 3 A, the residual amount of dye in the supernatant of the DSB sample (SB_s) used in the ISB methodology, must be equal to the difference between the existing dye in the initial SB solution (SB_o) and the amount of dye retained in the cell wall (SB_c) and in the PHA granules (SB_g).

3.2.2. Direct Sudan black methodology

Fig. 3 B shows the possible principle that governs the DSB methodology, wherein the absorbance (A) of stained cells without PHA determines a lineal spectrum (OD_o, curve 1), while the absorbance of stained cells with PHA determines a curved spectrum (OD_{DSB}, curve 2).

3.2.3. Calibration and prediction

Correlation coefficients (R^2) and errors obtained by PLSR and LRA methods for both SB methodologies, can be observed in Table 2. Good fits and low errors were obtained for all analysis. However, adjustments obtained for the DSB methodology seem to be better than those obtained for the ISB methodology for both calibration and prediction. For all calibration samples spectra, the PLSR adjustment obtained for the DSB methodology was better than the adjustments determined by LRA.

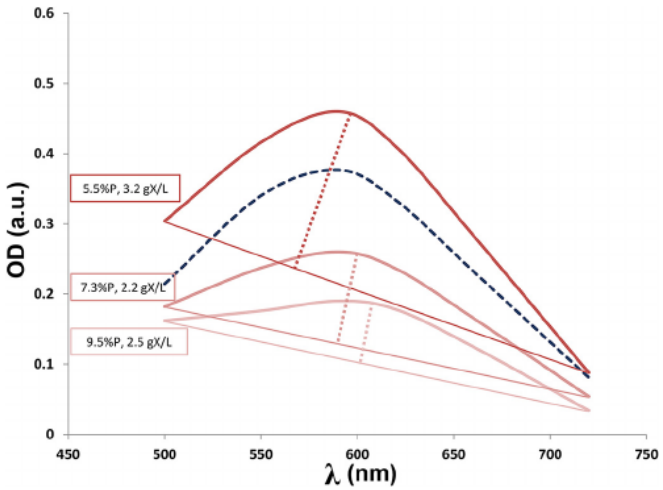

Fig. 2. Comparison between supernatant spectra (solid lines) of DSB assay samples used for ISB assay, based on different concentrations of biomass (X) and PHA (P). Spectrum of Sudan black solution (broken line). The PHA and biomass was determined as is described in Section 2.2. %P: PHA percentage, gX/L: grams of biomass per liter of sample, a.u.: arbitrary units.

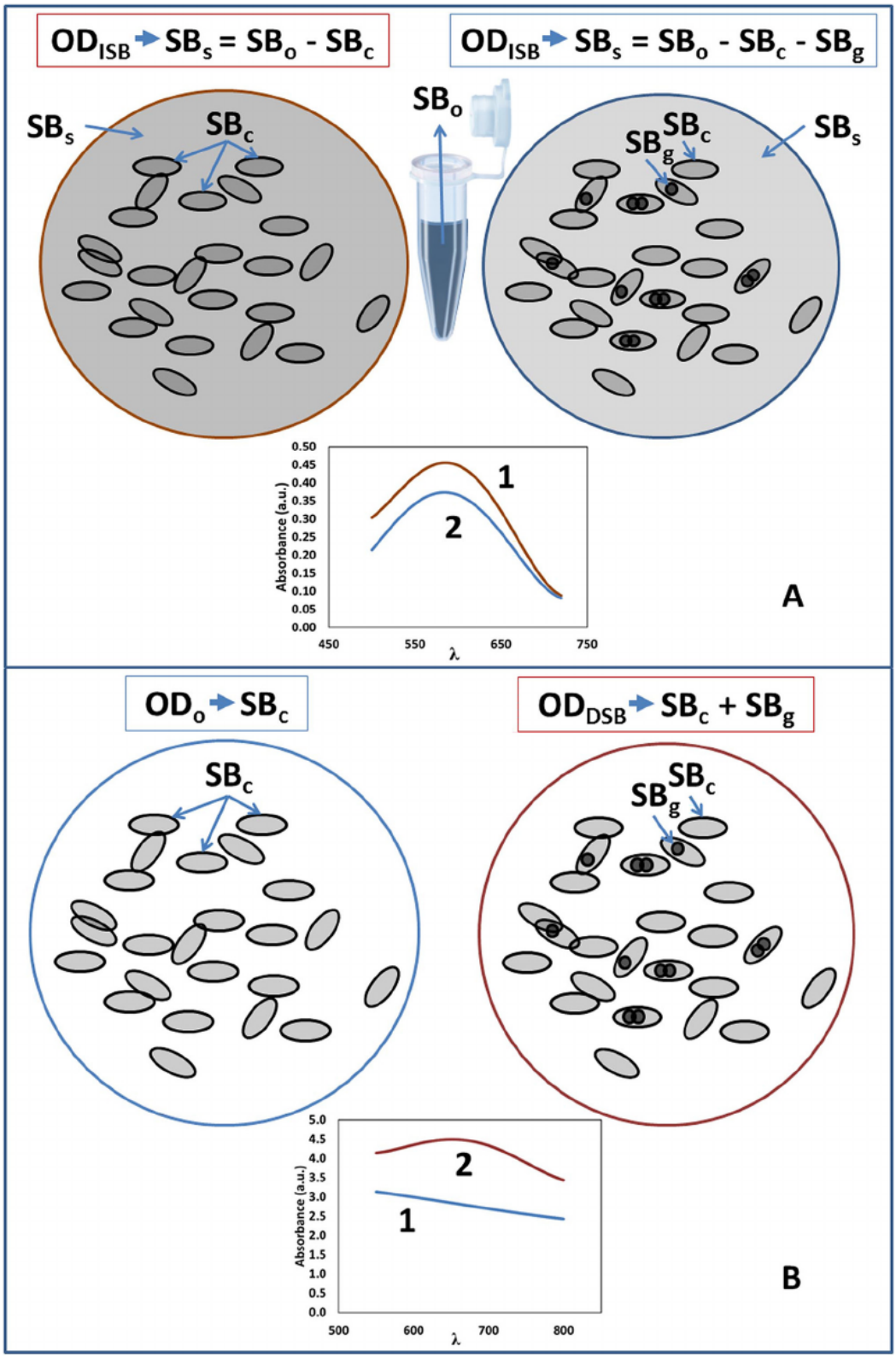

Fig. 3. Simplified Sudan black quantification assays principles. A. Principle for ISB assay, and B. Principle for DSB assay. OD_o: original optical density, OD_{DSB}: DSB optical density, OD_{ISB}: ISB optical density, SB_0: initial dye in the SB solution, SB_c: SB of stained cells, SB_s: SB in solution, and SB_g: SB of stained granules. 1. Stained sample without PHA and 2. Stained sample with PHA. a.u.: arbitrary units.

Similar results were obtained in the previous work. Before performing the analysis of outliers, better adjustments were observed for calibrations of the DSB methodology, although the best adjustment was observed using PLSR (R^2 = 0.915). Compared with results obtained in the previous work, PLSR calibration using DSB data shows a fit improvement, with an increase of the correlation coefficient (from 0.940

Table 2
Adjusted correlation coefficients and errors for calibration and prediction samples for SB assays determined by PLSR and LRA.

Technique[a]	Calibration				Prediction		Reference
	R^2 *Adj*	*E (%)*	R^2 *Adj*[a]	*E (%)*[a]	*E (%)*	*E (%)*[a]	
$DPLSR_0$	**0.902**	**1.697(3)**[b]	**0.940**	**1.259(3)**	**1.011**	**1.040**	Porras et al. (2017a)
$DLRAH_0$	0.804	2.378	0.944	1.243	3.383	0.510	
$DLRAA_0$	0.786	2.465	0.939	1.299	3.645	0.691	
DPLSR	**0.915**	**1.542(2)**[b]	**0.972**	**1.128(3)**	**1.203**	**0.985**	Present work
DLRAH	0.877	1.977	0.943	1.363	3.884	0.809	
DLRAA	0.861	2.103	0.931	1.504	4.228	0.961	
IPLSR	**0.795**	**1.927(2)**	**0.911**	**1.564(3)**	**2.159**	**1.814**	
ILRAH	0.678	3.336	0.886	1.953	4.249	1.944	
ILRAA	0.669	3.379	0.876	2.032	4.429	2.201	

Abbreviations. PLSR: Partial Least Square Regression for DSB (DPLSR) and ISB (IPLSR) assays, LRAH: Sudan Black Technique based on height of peaks with LRA for DSB (DLRAH) and ISB (ILRAH) assays, LRAA: Sudan Black Technique based on area of peaks with LRA for DSB (DLRAA) and ISB (ILRAA) assays. 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). Bold text corresponds to multivariate analysis.

[a] Samples without 2 outlier (PLSR), 2 outliers (DLRA) and 3 outliers (ILRA).

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

to 0.972) and a decrease of RMSECV (from 1.26 to 1.13%). Otherwise, adjustments and errors of the LRA calibration obtained for the DSB methodology were similar to those obtained in the previous work. Predictions also showed similar results: for the DSB methodology better fits and lower errors were obtained when compared to those corresponding to the ISB methodology. The prediction errors obtained for the DSB methodology showed lower values than those obtained in the previous work using PLSR, while opposite results were obtained by means of LRA.

Regression curves plotted with the adjustments of the calibration curves using LRA for the ISB methodology, and based on the best fit obtained (with peak height data, and with and without outliers), can be observed in Fig. 4. The linear relationship between PHA concentration and peak height of absorbance spectra shows a significant variation in slope, demonstrating the outlier influence. The confidence and prediction regions are shown as statistical errors for each curve.

The following calibration equations for the DSB methodology (Eq. (2)) and the ISB methodology (Eq. (3)) were determined based on LRA, using peak height data:

$$0.726 \quad (2)$$

$$23.313 \quad (3)$$

In these expressions, Y refers to PHA content of the stained sample ($Y_D = Y_I$), while X indicates the height value of the peak in the spectrum of stained DSB (X_D) and ISB (X_I) samples. Subsequently, calibration equations were used to predict the PHA content in unknown samples. Prediction errors obtained for the DSB and the ISB methodologies by PLSR were approximately one-third and a half, respectively, of those obtained by means of LRA while using full spectra of the samples. These results were similar to those obtained in the previous work. On the other hand, when the analysis of outliers was performed, prediction errors obtained by LRA were similar of those obtained by PLSR, whilst prediction errors obtained by LRA in the previous work were significantly smaller, about the half of those obtained by PLSR.

Considering the peak height data of all calibration samples spectra, the correlation between DSB and ISB methodologies can be shown in Fig. 5. The correlation coefficient was 0.792 (RSE = 0.11%).

When the third outlier detected in the ISB methodology was removed, R^2 raised to 0.901 (RSE = 0.053%). As expected, when values of DSB data increase, values of ISB data exhibit an inversely proportional variation, following the formula:

$$(4)$$

4. Discussion

4.1. Sudan black technique

4.1.1. Spectral analysis

The correspondence between the height (dotted line) and the area (limited by the baseline) of each spectral curve, with the PHA content of the sample under analysis (%P), can be seen in Fig. 2. The observed relationship indicates a strong correlation between the spectrum of the supernatant, obtained from the stained sample, and its PHA concentration. Consequently, the lower the height and the area of the observed peak, the higher the PHA content present in the sample. Therefore, the extractable content of intracellular PHA could, in principle, be determined by means of a univariate (using either peak height or area values), or a multivariate (using all spectral data) correlation analysis.

4.1.2. Calibration and prediction

The best fit for the calibration (either through PLSR or LRA), was obtained from the values of peak height data. In both cases, the adjustment of each regression curve was adequate. Similar results were observed in the previous work. Therefore, the calibration equations demonstrate to be a useful tool for the prediction of extractable PHA content. Using peak heights without outliers, the best prediction fit was observed with DSB when applying LRA (RSE = 0.809%). However, no significant differences are found when applying the other statistical analysis for DSB.

The use of 42 sample data in DSB served to improve the analysis and robustness of PLSR results obtained in the previous work. Comparing with those of the previous work, a decrease in PLSR prediction errors, together with an increase in LRA prediction errors of DSB, was observed. As already said, these results demonstrate a higher robustness of PLSR analysis with respect to LRA. In fact, the addition of new information to the PLSR analysis produced better predictions, while such improvements were not necessarily observed using LRA.

Strengths and weaknesses of the techniques generally used for PHA quantification, together with the costs of equipment and reagents commonly used, are shown in Table 3. The times involved and the sample sizes required to perform each methodology are also included. Even with the smaller sample quantities required when applying the methodologies developed in the present and in the previous work (Table 3 (cont.)), it can be seen that the values of the obtained adjustments have a good accuracy (R^2 = 0.972 and 0.911 using PLSR, for DSB and ISB respectively.

More than 43,000 samples can be processed with only 25 mg of SB

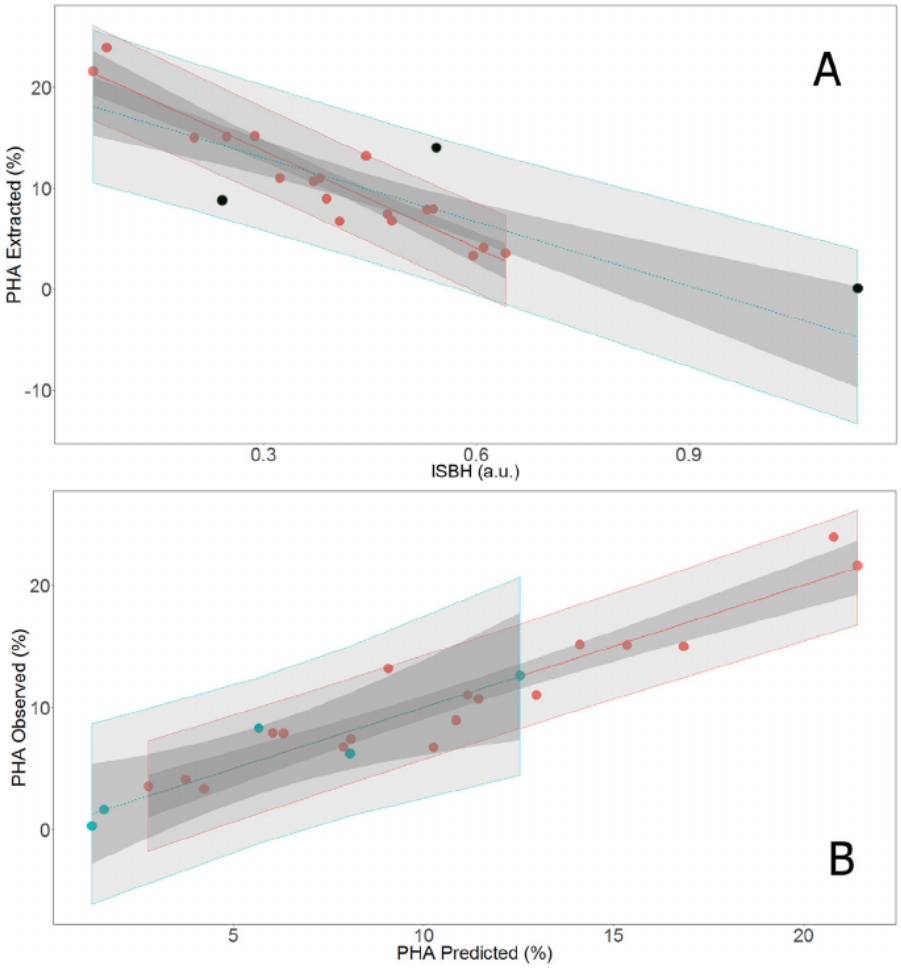

Fig. 4. Confidence and prediction regions determined by LRA for ISBH. A. Calibration curves with black (edges) and without (red edges) outliers, to predict the extractable PHA; black dots are the outlier samples. B. Observed and predicted curves without outliers for calibration samples (red dots) and prediction samples (blue dots). a.u.: arbitrary units. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

powder, which represents an added expense of about US$ 0.005/sample. This value, practically negligible, permits to assume that the costs of the application of both SB methodologies, will be mainly determined by the price of the equipment to be used. Now, in terms of the equipment required, quantification assays by means of SB methodologies turns to be the most economical option, together with the well-known crotonic acid assay. However, that alternative demands a greater variety of chemical compounds, and the use of environmental

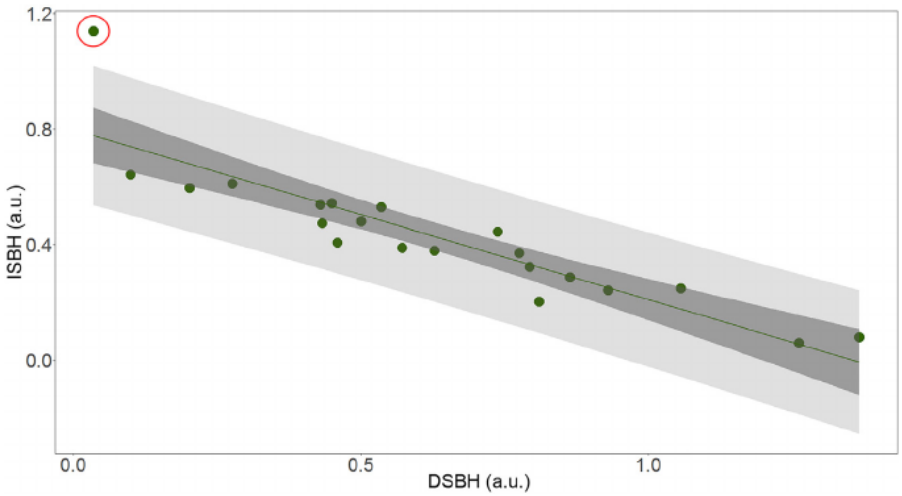

Fig. 5. Confidence and prediction regions determined by LRA for the correlation between peak height data of DSB and ISB. ISB outlier: dot surrounded by red circle. a.u.: arbitrary units. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Methods for detection and indirect quantification of PHA in biomass.
Modified from Tan et al., 2014.

Technique	Strengths	Weaknesses	References
Sudan Black assays	Sample size small and not lyophylized. Short analysis time. Non-solvent usage. Provide quantitative information. Easy operation. Inexpensive. Detect intracellular PHAs.	Cannot discriminate between different PHAs. ISB assay is only useful in stationary phase.	Present work
Flow cytometry and Spectrofluorometry (Nile Red assays)	Sample size relatively small and not lyophylized. Provide quantitative information. Inexpensive. Detect intracellular PHAs.	Cannot discriminate between different PHAs.	Gorenflo et al. (1999), Karmann et al. (2016), Marose et al. (1998)
Crotonic acid assay	Small sample size and not lyophylized. Easy operation. Inexpensive.	Result can be interfered by other endogenous components and can result in overestimation of PHB content. Quantitative determination is limited to P3HB.	Karr et al. (1983), Law and Slepecky (1961), Sulo et al. (1996)
Fourier transform infrared spectroscopy (FTIR)	Small sample size. Short analysis time. Solvent usage is optional. Provide quantitative information. Relatively inexpensive. Enables online and real-time PHA analysis.	Cannot discriminate between different PHAs. Generally, requires cell lyophilization.	Arcos-Hernandez et al. (2010), Hong et al. (1999), Porras et al. (2015)
Liquid chromatography (HPLC)	Sample size small. Short analysis time. Provides quantitative and qualitative information. Coupling with mass spectrometer (MS) detector enables identification of novel PHA monomers.	Requires cell lyophilization. Low separation power that is currently limited to analysis of scl-PHA monomers unless coupled to MS detector. Unable to distinguish between PHA homopolymers and copolymers. Expensive.	Grubelnik et al. (2008)
Nuclear Magnetic Resonance (NMR)	Small sample size. Provides quantitative and qualitative information.	Requires cell lyophilization. Requires PHA extraction. Expensive.	Doi et al. (1986), Jan et al. (1996), Linton et al. (2012)
Gas chromatography (GC)	High separation power. High sensitivity. Provides quantitative and qualitative information. Can be applied for tentative identification of novel PHA monomers when coupled to MS detector.	Requires cell lyophilization. Long sample preparation time. Requiring the use of hazardous and volatile solvents. Unable to distinguish between PHA homopolymers and copolymers. Difficult in the reproducibility because it undergoes an incomplete conversion of PHB to the esters and their extraction. Expensive.	Furrer et al. (2007), Hahn et al. (1995), Smolders et al. (1994)

Technique (cont.)	Equipment (prices)[a]	Average Equipment Price	Reagents (prices)[b]	CL/PD[c]	Time process	Sample size[d]	References
Sudan Black assays	Spectrophotometer (VIS: US$ 1000–2000[e]; UV–VIS: US$ 3000–5000)	US$ 1500/4000 (VIS/UV–VIS)	SB (25 g/US$210)	NO/NO	minutes	1000 μL drectly from culture (1.5 mg of dry cells[f])	Present work

(*continued on next page*)

Table 3 (*continued*)

Technique (cont.)	Equipment (prices)[a]	Average Equipment Price	Reagents (prices)[b]	CL/PD[c]	Time process	Sample size[d]	References
Flow cytometry and Spectrofluorometry (Nile Red assays)	Flow cytometer (US$ 10,000–30,000); Spectrofluorometer (US$ 5000–20,000)	US$ 20,000/12500 (FC/ Spectrofluorometer)	Nile Red (100 mg/US$282)	NO/NO	minutes	50 µL of diluted, stained sample	Karmann et al. (2016)
Crotonic acid assay	Spectrophotometer (UV–VIS: US$ 3000–5000)	US$ 4000 (UV–VIS)	Sulfuric acid (100 mL/US$339); acetone (2.5 L/117US$); ethanol (1 L/US$357); chloroform (1 L/US$232)	YES/YES	minutes	ND	Law and Slepecky (1961), Sulo et al. (1996)
Fourier transform infrared spectroscopy (FTIR)	FTIR Spectrometer (US$ 10,000–30,000); Lyophilizer (US$ 2000–10,000)	US$ 20,000/6000 (FTIR/ Lyophilizer)	Potassium bromide (25 g/ US$352)	NO/NO	hours-days	10 mg of dry cells	Porras et al. (2015)
Liquid chromatography (HPLC)	HPLC (US$ 10,000–50,000); Mass Spectrometer (US$ 10,000–30,000); Lyophilizer (US$ 2000–10,000)	US$ 30,000/20000/6000 (HPLC/MS/Lyophilizer)	Sulfuric acid (100 mL/US$339); Hexane (1 L/US$ 331); Toluene (100 mL/US$ 120); Formic Acid (2.5 L/US$ 0)	YES/YES	hours-days	1000 mg DWC/ 1–5 mg DWC/2 g of extracted PHA	Karr et al. (1983)/Del Don et al. (1994)/ Grubelnik et al. (2008)
Nuclear Magnetic Resonance (NMR)	NMR imaging analyzer (US$ 40,000–80,000); Lyophilizer (US$ 2000–10,000)	US$ 60,000/6000 (NMR/ Lyophilizer)	Sulfuric acid (100 mL/US$339); Deuterochloroform (50 mL/ US$611)	YES/NO	hours-days	15 mg of dry cells	Linton et al. (2012)
Gas chromatography (GC)	GC analyzer (US$ 10,000–40,000); Mass Spectrometer (US$ 10,000–30,000); Lyophilizer (US$ 2000–10,000)	US$ 25,000/20000/6000 (GC/ MS/Lyophilizer)	HCl (500 mL/ US$380); 1-Propanol (1 L/ US $272); Dichloromethane (1 L/ US$88)	YES/YES	hours-days	50 mg of dry cells	Furrer et al. (2007), Riis (1988), Elhottová (2000)

ND: Not Defined in the bibliography.

[a] Laboratory equipment prices.

[b] Usually employed, based on Sigma-Aldrich prices and buying from Argentina.

[c] CL: Cell Lysis: the solvents used for cell lysis are pollutants for the environment. PD: Polymer Destructive.

[d] Approximate values.

[e] Minimum six data points for LRA determinations.

[f] Approximate value, using starch as the sole carbon source, and in a stationary phase for *B. megaterium* BBST4 strain.

polluting solvents in the cell lysis process. Besides their convenience from the economic and environmental standpoints, the SB methodologies require small amounts of sample, which can be directly obtained during the course of experimentation. Finally, yet importantly, the whole process to obtain the final measurement results takes only few minutes (about 23 min for ISB and 42 min for DSB, in optimized conditions).

All the previous reasoning permits to assert that SB methodologies can be considered as a promising alternative to quantify the amount of PHA produced.

Now, comparing both SB methodologies among themselves, the ISB presented in this paper reveals to be faster than the DSB, and simpler to perform. In turn, DSB seems to produce determinations that are more precise. Most interesting, these two contradictory facts can be advantageously exploited, just by implementing both methodologies together. In fact, the ISB only requires: little extra work when the DSB assays is being performed, and no extra equipment or reagents. Due to this, ISB can be very useful as a complementary methodology to DSB in industrial practice. It can serve as an anticipatory warning of a possible malfunctioning of the PHA production process. Also, it may be useful as a validation test that could avoid any malpractice during the additional washing and dilution steps required by the DSB.

5. Conclusions

To complete the previous work, where a methodology called DSB to quantify the amount of PHA produced had been developed, the first part of this paper is devoted to finding the optimal conditions to carry out assays of this methodology. Conducting experiences under optimal conditions, a significant color variation was observed in the supernatant of the staining solution. This issue permitted the development of a new methodology for PHA determination. It was based on the amount of the initial SB consumed during the staining process, and therefore was called the Indirect Sudan Black methodology (ISB). Compared with the commonly used techniques, this new quantification assay provides a fast, simple, cheap and environmentally friendly alternative for the determination of extractable intracellular PHA content. Furthermore, in situations where the DSB is currently being employed, the ISB can be easily instrumented in parallel and at a very low cost. Although it will provide a slightly lower accuracy, due to its particular features, it can be very useful in the process industry as a faster and contrasting assay.

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