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Research paper

Multivariate near infrared spectroscopy for predicting polyhydroxybutyrate biosynthesis by mixed microbial consortia cultured on crude glycerol

Jing Dai [a], Erik R. Coats [b], Armando G. McDonald [a, *]

[a] Renewable Materials Program, Department of Forest, Rangeland and Fire Sciences, University of Idaho, Moscow, ID 83844-1132, United States
[b] Department of Civil Engineering, University of Idaho, Moscow, ID 83844-1022, United States

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ABSTRACT

Polyhydroxybutyrate (PHB) is a bioplastic biosynthesized by microorganisms as an intracellular granule. In this study, PHB was produced by a mixed microbial culture fed crude glycerol in a sequencing batch bioreactor. Bioreactor operations were monitored by near infrared (NIR) spectroscopy (908–1708 nm) with a direct insertion probe to develop a model for predicting microbial PHB concentration. Multivariate statistical methods, principal component analysis (PCA) and partial least squares (PLS), were applied for modeling of the NIR spectral data. The results showed that NIR spectra can (i) be used to predict PHB content in-situ, with a calibration model and prediction model exhibiting a good fit (R^2 = 0.88 and R^2 = 0.89, respectively) and (ii) also be used to monitor substrate, glycerol, concentration.

1. Introduction

Polyhydroxyalkanoates (PHA) are a class of biologically synthesized polyesters that accumulate as intracellular carbon, energy and reducing-power storage reserves in over 300 microorganisms. PHB is the most common form of PHA and is a polymer of 3-hydroxybutyric acid monomers [1]. PHA can be biosynthesized from a variety of renewable carbon-rich resources, allowing for a potentially sustainable and closed-cycle process for the production and use of such polymers [2]. Currently, PHA synthesis at an industrial scale is based on the use of microbial isolates (pure culture) and well defined, refined substrates [30–33]. In this model, the cost of PHA produced is still too high for the polymer to compete with petrochemical plastics, and thus PHA has been relegated to niche markets. In contrast, economic evaluations have suggested that the production expense of PHA can be reduced by over half if renewable waste carbon sources together with the utilization of mixed microbial consortia rather than pure carbon feed stocks and monocultures were used [3–6]. However, recognizing the intrinsic complexities of such a process, it will be important to gain enhanced process control to accurately predict bioreactor PHA production and accumulation in real-time.

Online bioreactor measurements relating to biomass production, substrate consumption, and product concentration have not historically been routinely employed for fermentation or other similar commercial bioprocess operations [7]. Rather, traditional analytical techniques such as GC–MS and HPLC are performed off-line and require extensive time for sample preparation and analysis. As a consequence, results are obtained too late to be useful for process control [7,8]. However, a rapid and non-destructive Near infrared (NIR) spectroscopic technique has been more recently considered for low-cost on-line monitoring of bioprocesses such as food fermentation, pharmaceutical fermentation, petrochemical, and biological process [9–12]. NIR radiation covers the range from 800 to 2500 nm and the absorbance of NIR in materials is generated by combinations and overtones of molecular vibrational transitions (C–H, N–H and O–H). Due to the very low absorption coefficients, no sample pretreatment is required and multiple measurements can be obtained at the same time for monitoring several substrates or performance parameters in the bioprocess [13]. With the advancements in this technology and its application, NIR is a candidate for low-cost and real-time/on-line monitoring of PHA production, although use of this technique will also demand more sophisticated modeling to be useful and predictive.

* Corresponding author.
E-mail address: armandm@uidaho.edu (A.G. McDonald).

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In regard to modeling, multivariate calibration models are statistical tools widely used associated with spectroscopic analysis of materials [14]. Related to NIR, partial least square (PLS) regression is a popular multivariate calibration technique for quantitative analysis of spectral data. PLS regression is a dimensional reduction technique which seeks to find latent variables by maximizing the covariance of two data matrices (i.e., spectra X and concentration Y). Although this multivariate method is useful to model a calibration, the resulting model can be influenced by background and noise in the NIR spectra. Therefore, NIR spectral data is often pretreated/corrected prior to calibration. Usually the pretreatment/correction approaches include multiplicative signal correction, second derivatives, orthogonal signal correction, standard normal variation, and baseline correction. Derivatives and orthogonal signal correction (OSC) are two of the most popular methods [15–17]. Overlapping bands and systematic spectral variations may still exist in NIR spectra, which would interfere with the calibration model and influence its prediction ability [18]. In addition, a predicted model usually needs a large number of latent components to achieve desirable prediction results [15]. However, including too many components in the regression step would increase the risk of over-fitting. Therefore, removing these interferences has received increasing attention [19].

This study aimed to develop a rapid on-line, low instrument cost, NIR-based calibration model to determine PHB content in a mixed microbial consortium bioreactor fed crude glycerol containing some methanol. Calibration models were built and validated to predict PHB content together with glycerol and methanol concentrations in the bioreactor for monitoring substrate consumption and product formation. In order to avoid interferences of background, different pretreatment methods were applied to the raw NIR spectra according to each calibration model.

2. Methods

2.1. Bioreactor operation

A sequencing batch bioreactor (12 L) which produced PHB using a mixed microbial consortium (activated sludge seed from Moscow, Idaho wastewater treatment plant) was fed diluted crude glycerol (10%, amended with NH_4Cl to provide nitrogen); the stirred bioreactor was operated continuously for approximately eight months at room temperature (21 °C) [5]. The bioreactor was batch-fed every 5 d by decanting 3 L of waste liquor and replacing it with feed substrate. The solid retention time (SRT) and hydraulic retention time (HRT) both were maintained at 20 d. Aerobic condition was maintained by dispersing air through a Sanitaire membrane disc diffusers. Residual dissolved oxygen was maintained at a minimum of 2 mg/L. A biomass (200 mL) sample was collected from the bioreactor every day, centrifuged and the supernatant discarded. The pellet was further washed with water, centrifuged, and freeze dried to determine the biomass dry weight and subsequently analyzed.

2.2. Chemical analysis

2.2.1. PHB content

PHA content was determined on 113 biomass samples as methyl ester derivatives by GC–MS [2,6]. Dried biomass (20 mg) was weighed into digestion test tube to which acidified methanol (3% H_2SO_4, v/v, 2 mL) and chloroform (2 mL, containing 0.5 mg/mL benzoic acid as internal standard) were added, the tube sealed, and the mixture heated at 100 °C for 4 h. The mixture was cooled and water (1 mL) was added to the mixture, vigorously shaken, the resulting chloroform layer was removed and dried through a small column containing anhydrous sodium sulfate into a vial.

GC–MS analysis was performed on a PolarisQ instrument (ThermoQuest) in the electron impact mode. Separation was achieved on a ZB-1 capillary column (Phenomenex, 30 m × 0.25 mm) with helium carrier gas and a temperature program of 40 °C ramped to 200 °C at 5 °C/min. The Xcalibur v2.07 software was used to analyze the data. The identified compounds were confirmed by their mass spectra and retention time with known standards. A standard curve was prepared from pure PHB standard (Sigma–Aldrich) and benzoic acid after methanolysis into their methyl ester derivatives ($R^2 > 0.99$).

2.2.2. Glycerol and methanol analysis

HPLC analysis was performed on bioreactor liquor samples (4.5 mL) that were collected at defined time intervals [6]. The liquor samples were centrifuged, the supernatant was filtered (0.22 μm syringe filter, Millex-GV), diluted 5-fold and injected (20 μL) onto an HPLC system (Waters M45 HPLC pump, AS3000 autosampler (Thermo Separations), refractive index detector (HP 1047A), recording integrator (HP3393)). Separation was performed on a Rezex ROA organic acid column (7.8 mm × 300 mm, Phenomenex) at 60 °C on elution with aqueous 0.01 N sulfuric acid (0.5 mL/min). A standard concentration curve was prepared with pure methanol and glycerol standards ($R^2 > 0.98$).

2.3. NIR analysis

NIR spectra from 113 samples were obtained on a dispersive NIR spectrometer (model: NIR 128L, Control Development) ranging from 908 to 1708 nm with 6.25 nm/pixel linear dispersion. Spectral data were collected ex-situ using an optical fiber transmission Dip probe (T300-RT-VIS/NIR, 300 μm fiber diameter, 2 mm path length, Ocean Optics) with a krypton light source (SL1, StellarNet) at the same time samples were collected for chemical analysis. The number of scans was set at 32 times to obtain an average and reduce noise; the integration time was dependent on the actual setup and typically ranged from 0.08 to 0.2 s. NIR spectra were collected in the absorbance mode using air as the background (empty probe) using the CDI Spec32 software (Control Development). A total NIR spectral measurement time was between 2.6 and 6.4 s. The probe was washed with distilled water and dried between samples. PHB content and raw NIR spectral data for the 113 samples is given in Appendix A.

2.4. Data processing

Multivariate data analysis was performed using the Unscrambler® v10.3 software (CAMO). PCA was performed to determine the number of components in the model calibration; also it was used for clustering and outlier analysis. Before model calibration, the spectral region between 908 and 1708 nm was pretreated with different methods, such as 1st and 2nd derivatives applied by Savitzky–Golay algorithm, OSC, baseline, multiplicative scatter correction (MSC) and standard normal variate (SNV).

Multivariate calibration (on 90 samples) using PLS regression was performed for quantitative determination of PHB content as dry cell weight basis. The coefficient of determination (R^2), root mean square errors (RMSE) of calibration and prediction (RMSEC and RMSEP, respectively) were used for model selection and fitting. A robust full cross-validation was used to validate the calibration model, in which the model was rerun for the number of samples times and with one sample left out each time. Besides a cross-validation method (90 samples), another new spectral set (23 samples were not included in the calibration data set) was used for model prediction. Model calibrations on glycerol and methanol