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Quantification of Exopolysaccharide, Lactic Acid, and Lactose Concentrations in Culture Broth by Near-Infrared Spectroscopy

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Near-infrared spectroscopy (NIRS) was used for the simultaneous prediction of exopolysaccharide (EPS; 0−3 g/L) and lactic acid (0−59 g/L) productions as well as lactose (0−68 g/L) concentration in supernatant samples from pH-controlled batch cultures of *Lactobacillus rhamnosus* RW-9595M in supplemented whey permeate medium. To develop calibration equations, the correlation between the second derivative of 164 NIRS transmittance spectra and concentration data obtained with reference methods was calculated at the wavelength between 1653−1770 and 2041−2353 nm, using a partial least-squares method (PLS). The lactic acid and lactose concentrations were measured by HPLC, and the EPS concentration was estimated by a new ultrafiltration method. The PLS correlation coefficient (R^2) and the standard error of cross-validation for the calibrations were 91% and 0.26 g/L for EPS, 99% and 2.54 g/L for lactic acid, and 98% and 3.32 g/L for lactose, respectively. The calibration equations were validated with 45 randomly selected culture samples from 6 cultures that were not used for calibration. A high agreement between data of the reference methods and those of NIRS was observed, with correlation coefficients and standard errors of prediction of 99% and 1.64 g/L for lactic acid, 99% and 4.5 g/L for lactose, and 91% and 0.32 g/L for EPS. The results suggest that NIRS could be a useful method for rapid monitoring and control of EPS lactic fermentations.

KEYWORDS: Exopolysaccharide quantification; lactose; lactic acid; NIR spectroscopy; culture broth; lactobacilli

INTRODUCTION

For accurate control of fermentation processes, it is very important to monitor accurately and rapidly substrate and product concentrations during incubation. However, the analysis of these compounds requires sample preparations, and in some cases several steps of purification are needed, which delays obtaining the final results from analyses. Recently, there has been an increasing interest for the production of exopolysaccharides (EPS) by lactic acid bacteria (*1*). The EPS produced in complex fermentation media could be used as bioingredients, for their ability to enhance rheological properties in lactic fermented products (*1*). However, their production is very low, in the range of 0.05−2.5 g/L, compared with other microbial polysaccharides (*2*). Additionally, EPS quantification is a long process that takes one day using a new ultrafiltration methodology (*3*) or up to two weeks using a conventional method (*4*). Quantification of lactic acid and lactose in culture medium by HPLC methodology also requires several hours for sample preparation and analysis. Thus, the development of a simple, rapid, and noninvasive method for monitoring simultaneously EPS and lactic acid production, as well as lactose consumption, in a complex medium during fermentation is desired.

Near-infrared transmittance spectroscopy (NIRS) offers the advantage of rapid sample processing with results available within 5 min. It is a nondestructive technique that requires no sample preparation and can measure several broth constituents simultaneously (*5*). Furthermore, authentic standards are not necessary after the prediction equation is developed. With the use of NIRS as a routine analysis method, opportunities for on-line control processes and fault analysis are much greater than can presently be achieved with existing measurements, such as HPLC (*5*). Nevertheless, the NIRS method also has disadvantages, such as interference caused by unexpected components in the sample, with overlapping spectral features, which can introduce errors in the analysis (*6*). However, the main advantage in using NIRS is its ability to monitor in real time the progress of fermentation. This allows the optimization of the harvesting time during batch culture, the selection of feeding times with growth-limiting substrate during feed-batch fermentation, and the detection of steady state for continuous fermentation. In all cases NIRS could save medium and operation time (*7*). NIRS has mainly been used for the evaluation of nutritional components in animal feed (*8*). Recently it has been used for determining natural products in plants (*9*), monitoring cell (*10*,

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11) and biomass concentrations (*12*), characterizing polymers (*13*), enhancing production of lactic acid in a fully automated plant-controlled process (*7*, *14*), and also for analyzing dairy foods (*15*).

Our objective was to determine the feasibility of NIRS for quantifying simultaneously the EPS and lactic acid productions, as well as the lactose concentration, during batch cultures of *Lactobacillus rhamnosus* RW-9595M in complex whey permeate medium with different supplement formulations.

MATERIALS AND METHODS

Samples. Twenty-nine pH-controlled batch cultures were carried out with the EPS producer strain (*16*), *L. rhamnosus* RW-9595M, obtained from the Lactic Acid Bacteria Research Network culture collection (Dairy Research Centre STELA, Université Laval, PQ, Canada), in whey permeate (whey permeate powder reconstituted at 7.8% w/w total solids; Foremost, Baraboo, WI) and yeast extract medium (0.5%, Difco, Detroit, MI). Yeast extract was ultrafiltered using a tangential filtration system (Minitan filter plates, Millipore, Bedford, MA) with a 10 kDa membrane to remove polysaccharides (*17*). This medium was supplemented with Tween 80 and individual or combinations of the nutrient groups of basal minimal medium (BMM) (*18*): vitamins (V), salts (S), and amino acids (AA), as previously described (*19*). Concentrated solutions of nutrients were prepared separately. The concentrated vitamin solution contained (per liter) 0.1 g of folic acid, 1 g of nicotinic acid, 1 g of pantothenic acid, 2 g of pyridoxal, and 1 g of riboflavin. It was sterilized by filtration (0.22 μm, Puradisk, Whatman, Ann Arbor, MI). The amino acid (L form) stock solution contained (per liter) 1 g of arginine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine, as well as 2 g of aspartic and glutamic acids and cysteine. The salt stock solution contained (per liter) 0.5 g of $MnSO_4 \cdot 7H_2O$, 5 g of $MgSO_4 \cdot 7H_2O$, 60 g of CH_3COONa, 10 g of $(NH_4)_2HC_6H_5O_7$, and 0.20 g of $FeSO_4 \cdot 7H_2O$. Vitamins and amino acids were obtained from Sigma Chemical Co. (St Louis, MO) and salts from BHD Inc. (Toronto, ON, Canada). Cultures were performed for 56 h at 37 °C with mixing at 100 rpm, and pH was controlled at 6.0 by addition of 5 N NH_4OH. Samples were aseptically withdrawn at different culture times and heated at 100 °C for 15 min to destroy hydrolytic enzymes that could degrade the polysaccharide and to liberate polysaccharides attached to cell walls. The culture samples were centrifuged at 13218*g* and 4 °C for 20 min (Sorval Instrument, DuPont, Newtown, CO) to recover supernatants, which were used for the analysis of EPS, lactose, and lactic acid contents by reference and NIRS methods. For calibration model development, a set of 164 samples from 23 cultures randomly selected was used. For validation, a set of 45 samples from the 6 remaining fermentations, which were not used for calibration, was tested.

Reference Analytical Methods. Residual lactose concentration and lactic acid production in supernatant were determined by HPLC (Waters, Millipore Co., Montreal, PQ, Canada) with a Phenomenex ion column (Phenomenex, Torrance, CA), and 0.0064 N H_2SO_4 as eluent at a flow rate of 0.4 mL/min. Diluted samples were previously filtered using an acrodisc LC 13 filter (0.2 μm; Acrodisc, Gelman Sciences, Ann Arbor, MI). Analysis was performed in duplicate.

EPS concentration was determined in duplicate by an ultrafiltration method (*3*), with six stirred cells having cutoff levels of 30 kDa (Omegacell; Filtron Technology Co., Northborough, MA). Cells were connected to a reservoir (RC800 minireservoir; Millipore, Bedford, MA) containing 0.1 N NaCl solution and connected to a laboratory air-regulated reservoir that provides air pressure. A volume of 2 mL was accurately measured and added to the stirred cell unit, and then the stir bar/pressure cap assembly was inserted into the cell. By applying pressurized NaCl solution, the ultrafiltration was driven as a diafiltration process. Filtration was conducted with agitation at 650 rpm. The diafiltration process was carried out until no carbohydrates could be detected in the filtrate by the phenol−sulfuric acid method (*20*). A sample of the retentate was then taken for EPS quantification according to the phenol−sulfuric method, using glucose as standard. The EPS productions were then reported as milligrams of glucose equivalents per liter.

NIR Spectroscopy. NIR transmittance spectra were collected at room temperature (21 °C) using a Bomem MB series spectrophotometer (ABB Bomem Inc., Quebec, PQ, Canada), in the 741−2500 nm region at 16 cm^{-1} resolution (128 scans) using a 0.5 mm quartz cell. Data collection was done with a water spectrum background. Only single analysis of samples was carried out, because during preliminary trials no difference was observed between a single analysis and the mean of two measurements.

Statistical Analysis. Data collection and spectral mathematical treatment using a partial least-squares method (PLS) were performed using Grams/32 software (Galactic Industries Corp., Salem, NH) supplied with the NIRS instrument. All regression analyses were performed on the second-derivative of the spectral data using the Savitzky−Golay algorithm (polynomial 2 and point 13) in the 1653−1770 and 2041−2353 nm spectral ranges. Samples presenting spectral or concentration ratios ≥ 3.0, evaluated by the Mahalanobis distance, were considered to be outliers and were removed from the calibration set. For representation NIR derivative spectra were multiplied by −1.

PLS is a multivariate statistical procedure that uses a mathematical technique known as spectral decomposition to reduce the quantity of spectral data. By using mathematical combinations of these new and reduced data, called factors, it is possible to regenerate or model the original data. Generally the first factors account for the majority of the analytical spectral variation. The individual factors calculated from the PLS spectral decomposition are useful for finding spectral regions of importance in the calibration of certain components (*21*).

The correlation coefficient (R^2) and standard error of calibration or standard error of differences between reference and NIR data (SEC) are indicators of calibration performance. R^2 indicates the percentage of total variability explained by the PLS model. The SEC gives an indication of the quality of the NIRS calibration fitting of the model. For calibration data, the number of factors was assessed by the cross-validation procedure. The standard error of cross-validation (SECV) was obtained by removing and predicting one sample at a time while all of the remaining samples were used in the calibration until every sample had been predicted once. The predicted values gave validation errors, which were combined into SECV. The standard error of prediction (SEP) were calculated with all PLS predictions. The SEP represents the standard deviation of the differences between the predicted and measured validation data. The percent errors, %SECV and %SEP, calculated by the ratios of SECV and SEP to maximum concentration range of the compound × 100, were used to compare the relative errors for the three compounds calibrated by NIRS.

RESULTS

NIR Spectra of EPS, Lactic Acid, and Lactose. To determine the specific bands and their relationships with the concentrations of EPS, lactic acid, and lactose, the NIR second-derivative spectra of standard solutions at different concentrations of the three compounds were analyzed in the particular regions between 1653−1770 nm and 2041−2353 nm (**Figure 1**). In these regions, the NIR bands that presented the strongest intensity were identified and associated with the corresponding components. For EPS, the principal bands were located at 1683, 1722, 1752, 2253, 2291, and 2328 nm. For lactic acid and lactose, these bands were located at 2236, 2278, and 2322 nm and at 2244 and 2290 nm, respectively (**Figure 1**). EPS was the only compound that showed large distinctive bands in the region between 1653 and 1770 nm. A correlation between band intensity and concentration was observed for the three compounds, except for the 2290 nm band for lactose (**Figure 1**).

NIR Spectra of Culture Samples. The changes of the second-derivative spectra of supernatant samples from a pH-controlled batch culture in whey permeate yeast extract medium (WPYE) supplemented with vitamins, amino acids, and salts, as a function of time, is shown in **Figure 2**. The relationship between band intensity and concentration is also observed. Indeed, the strongest NIR bands that increased with incubation

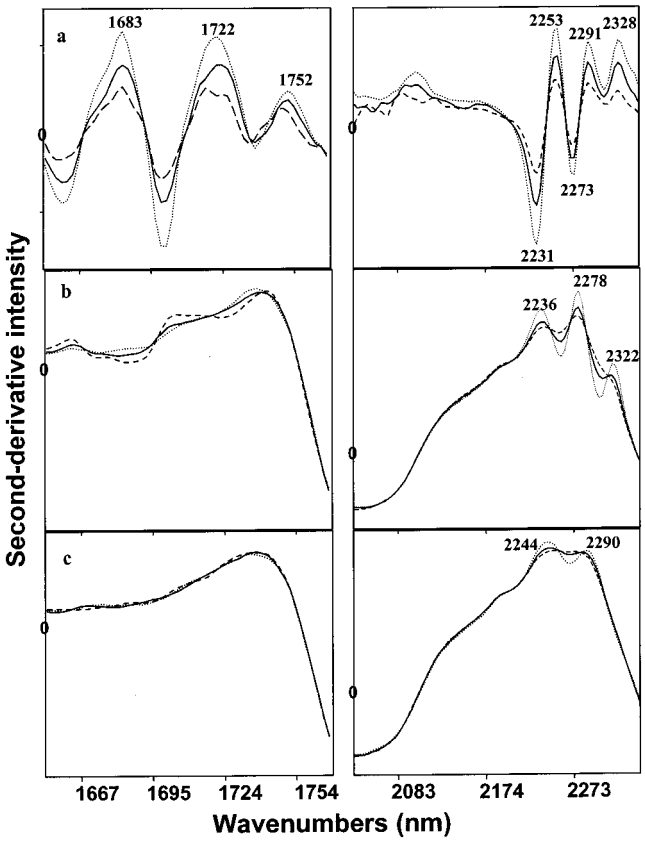

Figure 1. NIR second-derivative spectra of standard solutions of (a) EPS, (b) lactic acid, and (c) lactose at different concentrations of 3% (- - -), 5% (—), and 6% (•••).

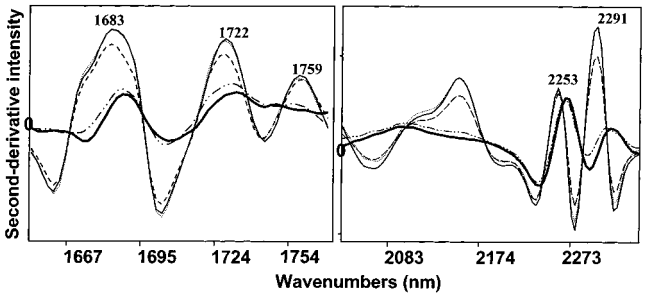

Figure 2. NIR second-derivative spectra of supernatants obtained from a pH-controlled batch culture in whey permeate based medium as a function of time: 0 h (–), 8 h (–••–), 15 h (- - -), 32 h (••••), and 56 h (—).

time are located in the same wavelength regions as for EPS, lactic acid, and lactose bands (**Figure 1**). During the first 8 h of incubation, when EPS and lactic acid production was low (0.35 and 11.4 g/L, respectively) and lactose concentration was high (43.3 g/L), a low intensity in the bands associated with EPS at 1683, 1722, and 1730 nm and a shift of bands in the region between 2247 and 2353 nm was observed. After 15 h of incubation, when all of the lactose was already consumed and EPS as well as lactic acid production became important (2.4 and 44.0 g/L, respectively), the strongest NIR bands were placed at 2253 and 2291 nm and the bands in the region of 1653−1770 nm became important. The intensity of EPS bands increased with EPS concentration in the sample.

NIR Determination of EPS, Lactic Acid, and Lactose. Calibration and validation were carried out on separate subsets of culture samples obtained from pH-controlled batch cultures with different medium compositions and which represented

Table 1. Chemical Composition of Supernatant Samples from *L. rhamnosus* RW-9595M Cultures for NIRS Calibration

component	mean (g/L)	minimum (g/L)	maximum (g/L)	SD[a] (g/L)
EPS-UF	0.92	0.013	2.9	0.05
lactic acid-HPLC	28.40	0	59.0	0.19
lactose-HPLC	28.20	0	68.0	0.30

[a] Standard deviation of reference method.

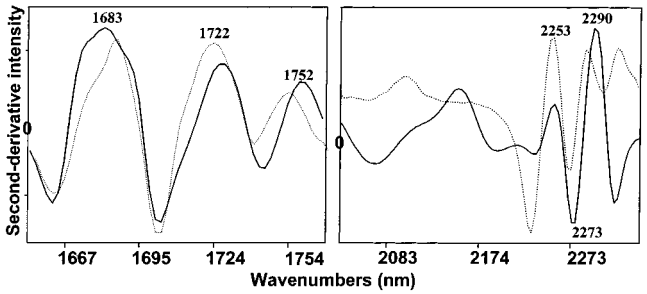

Figure 3. NIR second-derivative spectra of EPS standard solutions at 6% (—) and EPS factor (•••).

concentration ranges typically found for lactic acid fermentation (**Table 1**). The best calibration equations for individual compounds were those with the lowest SECV and the highest R^2. For all models, the spectra factors were evaluated to ensure that adequate spectral information was available for calibrations. **Figure 3** compares EPS standard solution spectrum (6%) and the first PLS calibration factor for EPS. Both spectra showed the same spectral features. Indeed, the principal absorption bands in the factor spectrum correspond to the EPS NIR bands for the regions between 1653−1770 and 2041−2353 nm.

Figure 4 shows the calibration curves and **Table 2** reports the calibration and validation statistical data for EPS, lactic acid, and lactose. The highest R^2 and the lowest SECV were obtained for nine PLS factors for EPS and for five PLS factors for lactic acid and lactose. The NIRS model accurately fitted lactic acid and lactose concentration data. The calibration data predicted by NIRS were in good agreement with those measured by the reference method (HPLC) as shown by high R^2 values of 99.1 and 98.1% and low SECV values of 2.54 and 3.32 g/L, respectively. In contrast, the EPS calibration was less accurate as indicated by an R^2 of only 90.6% and a large SECV value of 0.26 g/L (**Table 2**). The percent error (% SECV) for EPS (9.0%) was 2-fold higher than for lactic acid and lactose (4.3 and 4.9%, respectively).

To test the validity of calibration equations, compositions of samples from six culture experiments that were not used for calibration were predicted using the calibration model developed. The validation data for the three calibration compounds are summarized in **Table 2** and **Figure 5**. Lactic acid and lactose concentrations were accurately predicted by NIRS with high R^2 values of 99.4 and 98.7%, respectively, and SEP values of 1.64 and 4.50 g/L for lactic acid and lactose, respectively, but EPS prediction was less accurate with R^2 and SEP of 90.3% and 0.32 g/L, respectively. The % SEP was 2-fold higher for EPS (11.0%) than for lactic acid or lactose (5.8 and 6.6%, respectively; **Table 2**).

DISCUSSION

The spectra recorded with pure solutions of EPS, lactic acid, and lactose (**Figure 1**) allowed the precise location of NIR bands for each component and clearly showed the relationship between

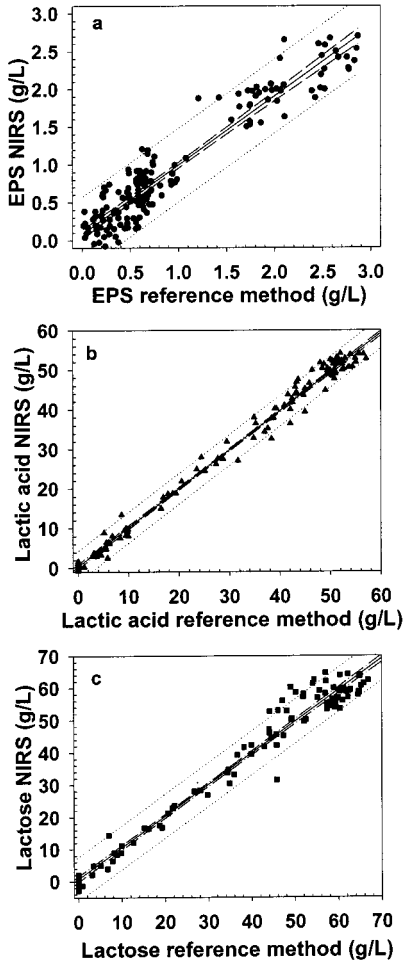

Figure 4. NIR calibrations for (a) EPS ($y = 0.913x + 0.0808$, $R^2 = 90.6$), (b) lactic acid ($y = 0.982x + 0.3314$, $R^2 = 99.1$), and (c) lactose ($y = 0.977x + 0.7172$, $R^2 = 98.1$); prediction (···) and 95% confidence (–•–) intervals.

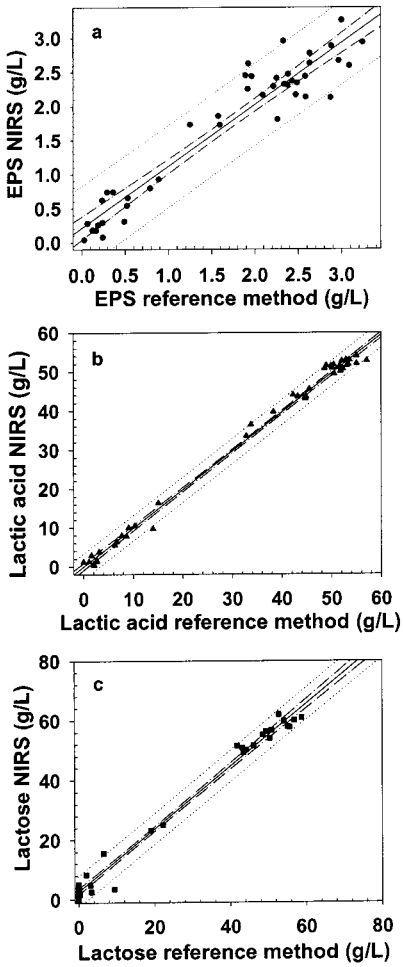

Figure 5. NIR validation curves for (a) EPS ($y = 0.907x + 0.2070$, $R^2 = 90.3$), (b) lactic acid ($y = 0.990x + 0.0295$, $R^2 = 99.4$), and (c) lactose ($y = 1.051x + 2.645$, $R^2 = 98.7$); prediction (···) and 95% confidence (–•–) intervals.

Table 2. Statistical Descriptors for NIRS Calibration and Validation for EPS, Lactic Acid, and Lactose Concentrations in Supernatant Culture Samples

	calibration				validation		
component	PLS factor[a]	R^2 [b] (%)	SECV[c] (g/L)	SECV[d] (%)	R^2 [b] (%)	SEP[e] (g/L)	SEP[d] (%)
EPS	9	90.65	0.26	9.0	90.30	0.32	11.0
lactic acid	5	99.11	2.54	4.3	99.42	1.64	5.8
lactose	5	98.14	3.32	4.9	98.68	4.50	6.6

[a] Partial least-squares mathematical method. [b] Coefficient of correlation. [c] Standard error of cross-validation. [d] Percent error, defined as SECV or SEP divided by the maximal concentration range of the component × 100. [e] Standard error of prediction.

concentration and band intensity. For the three compounds, the strongest bands were situated between 2041 and 2353 nm, where the OH-stretching and deformation regions are located (*22*). The decrease in the intensity of bands at 2273 nm when EPS concentration increased may reflect a decrease in the molar fraction of water in solution (*23*). In contrast, the region from 1653 to 1770 nm showed a strong correlation only for some NIR bands and EPS concentrations. This region enhanced the overall performance of calibration for this compound, but it did not provide information on lactic acid and lactose. Indeed, calibration of lactic acid and lactose was based only on the region from 2041 to 2353 nm, where differences in absorption bands and intensity of signal were observed. Band assignments for lactic acid have not been well documented in the literature. The NIR bands identified for lactic acid have been reported to largely depend on other compounds present in the solution. Riley et al. (*24*) observed two distinct absorbance bands for lactate (2262 and 2288 nm) in the presence of glucose. McShane et al. (*25*) observed two characteristic bands at 2250 and 2300 nm in the presence of glucose, glutamate, glutamine, and ammonia, whereas in our study lactic acid bands were observed at 2236, 2278, and 2322 nm. This band shift could be due to the presence of EPS and lactose bands in the same spectral region or to the complexity of the culture medium. Lactose and EPS bands have not been reported in the literature. The spectral region between 1818 and 2000 nm showed the high interference mainly due to water and protein absorption (*22*) and for this reason was not considered for calibration.

Bands that exhibited strong variation in the spectra, as a function of culture time (**Figure 2**), were the same EPS bands identified in **Figure 1**. However, a slight shift of bands was observed for supernatant spectra of culture samples compared with standard solutions, probably due to medium complexity. The characteristic lactic acid and lactose bands from 2041 to 2353 nm were overlapped by EPS bands (**Figure 2**), but a relationship between bands and lactic acid and lactose concentrations was found with PLS regression. These data illustrate the advantage of the PLS chemometric model, which is able to calibrate systems with several constituents and with large

concentration variations. Our data suggest that NIR spectroscopy can be used to quantify EPS and lactic acid production, as well as lactose consumption, in culture samples.

For calibration, the NIRS method requires a substantial number of samples for which chemical components (the desired and interfering compounds) have different concentrations representing the range found in future unknown samples. The concentration of individual compounds in the samples must be also independent and not correlated. The number of calibration samples required should be ~6 times the number of varying components (*11*). Additionally, it is important to obtain samples across the entire expected concentration range. This is necessary to ensure that the slope of the calibration curve is linear across the entire range (*26*). The models developed in this study for EPS, lactic acid, and lactose concentrations were robust because they were calculated with 164 randomly selected samples, which had a large concentration range corresponding to real concentrations found in lactic fermentation (**Table 1**).

Calculation of the second-derivative of the spectra could enhance spectral features and reduce baseline offsets with no obvious degradation in the analytical result (*27*). This is an important parameter to take into account for calibration of samples, such as culture samples, which contain a complex mixture of components (proteins, amino acids, vitamins, and minerals) and a high concentration of lactate and lactose. Moreover, complex nutrient sources contain many different organic species that generate highly overlapping absorption bands and increase spectra variation (*24*).

Calibrations for lactic acid and lactose were accurately performed by NIRS calibration because models showed high R^2 and low SECV (**Table 2**), and only five factors were needed for developing the model. Although SECV provides a good estimate of model accuracy, validation of the model with an independent set of samples is still necessary to determinate the real model accuracy (**Table 2**). For validation, the R^2 and SEP obtained in this study for lactic acid and lactose are in agreement with lactic acid data reported by Vaccari et al. (*7*, *28*) and for acetic and ethanol production during rice vinegar fermentation (*29*).

On the other hand, EPS calibration required nine PLS factors, which means that a substantial number of wavelength combinations were used for the calibration model. Riley et al. (*6*) found that the number of PLS factors required to generate satisfactory calibration models increased with the number of components. The first PLS factor spectrum for EPS calibration is shown in **Figure 3**. Factor spectral features were similar to EPS standard solution spectrum. This figure clearly shows that the PLS calibration was developed using PLS factor spectral information, which was related to EPS concentration. However, a shift of bands was observed for the EPS factor spectrum from culture samples, probably due to the complex medium interferences. To our knowledge, this is the first study showing a correlation between NIR specific bands and polysaccharide concentration.

On the other hand, the high dispersion of EPS data measured by NIRS compared with lactic acid and lactose could be due to the large difference of concentrations for EPS and lactic acid or lactose concentration in culture samples. Indeed, lactic acid and lactose mean concentrations were ~10−20-fold higher than the mean EPS concentration (**Table 1**), which may explain the 2-fold higher percent errors (% SECV and % SEP) for EPS than for lactic acid and lactose (**Table 2**). Riley and Crider (*24*) found that SEP is a function of the compound concentration. They quantified the concentration of five compounds produced during animal cell cultivation at different initial concentrations and found that SEP determined values for each compound as a function of its concentration. They also found that for a reduced concentration range (0−0.1 mM) the percent error was ~11% of initial concentration. In contrast, for the largest concentration range (0−30 mM), the percent error decreased to 1.6% of the concentration range.

Interference of components in the broth could explain the low performances for EPS calibration ($R^2 = 91\%$) and the high value of the *y*-axis intercept for the validation equation (0.28; **Figure 5a**). Indeed, the medium used was complex, and large composition changes during fermentation occurred. Actually, in fermentation processes it is difficult to ensure that all sources of spectral variation, whether identified or not, have been included in the calibration model (*27*). In fact, the real utility of the NIRS predictive model is its ability to quantify rapidly and simultaneously EPS, lactic acid, and lactose concentrations in supernatant samples from pH-controlled batch cultures which were not previously used for model development, even if NIRS errors are higher than reference method errors. Less accurate models for fermentation processes are still acceptable because the reproducibility of a measurement, in a process control context, is much more important that its accuracy. Thus, even if accuracy is reduced, it is preferable to get more robust models that can be applied to processes with different medium formulations (*30*). For this reason the performance of an indirect method, such as NIRS, can be seen from the perspective of NIRS prediction efficacy versus reference methods. Furthermore, the accuracy of NIR EPS prediction could be enhanced by using sophisticated mathematical treatments, such as artificial neural networks (*31*, *32*) or other kinds of algorithms (*25*, *33*, *34*). It has been demonstrated that the use of simulated spectra could provide a substantial control of the spectra noise, baseline, and absorbance band heights and widths (*11*).

Data from this study demonstrate the applicability of NIRS to predict EPS and lactic acid productions as well as lactose consumption during fermentation. Quantification of EPS by the NIRS method is much less time-consuming (<5 min) compared with the rapid ultrafiltration reference method (~8 h) and the conventional method, which involves many purification steps and takes ~10 days (*3*, *4*). The speed of NIRS analyses will reduce costs and could improve control and optimization of lactic acid fermentation processes. Data from this study indicate that NIRS may be a powerful alternative methodology for real-time quantification of other microbial polysaccharides, particularly those produced at high concentrations during fermentation such as xantane or alginate (*2*).

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