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Monitoring a bioprocess for ethanol production using FT-MIR and FT-Raman spectroscopy

S Sivakesava, J Irudayaraj and A Demirci

Department of Agricultural and Biological Engineering, Pennsylvania State University, 249, Agricultural Engineering Building, University Park, PA 16802

The application of Fourier transform mid-infrared (FT-MIR) spectroscopy and Fourier transform Raman (FT-Raman) spectroscopy for process and quality control of fermentative production of ethanol was investigated. FT-MIR and FT-Raman spectroscopy along with multivariate techniques were used to determine simultaneously glucose, ethanol, and optical cell density of *Saccharomyces cerevisiae* during ethanol fermentation. Spectroscopic measurement of glucose and ethanol were compared and validated with the high-performance liquid chromatography (HPLC) method. Spectral wave number regions were selected for partial least-squares (PLS) regression and principal component regression (PCR) and calibration models for glucose, ethanol, and optical cell density were developed for culture samples. Correlation coefficient (R^2) value for the prediction for glucose and ethanol was more than 0.9 using various calibration methods. The standard error of prediction for the PLS first-derivative calibration models for glucose, ethanol, and optical cell density were 1.938 g/l, 1.150 g/l, and 0.507, respectively. Prediction errors were high with FT-Raman because the Raman scattering of the cultures was weak. Results indicated that FT-MIR spectroscopy could be used for rapid detection of glucose, ethanol, and optical cell density in *S. cerevisiae* culture during ethanol fermentation. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 185–190.

Keywords: Fourier transform midinfrared spectroscopy; Fourier transform Raman spectroscopy; multivariate analysis; principal component regression; partial least-squares regression

Introduction

The ability to control fermentation is of paramount importance in the optimization of biomass and product synthesis. Parameters such as biomass, nutrient, by-product and product concentrations play a major role in modulating the kinetics during fermentation. Several labor-intensive analytical techniques are required to measure biomass, nutrients, and product. An increase in the efficiency of measurement and monitoring procedures has a high potential to result in an increase in the efficiency of these bioprocesses.

Generally bioprocesses give rise to materials that progress from translucent to increasingly opaque matrices as the microbial cells multiply and become highly light-scattering and give rise to intense molecular vibrational signals. Monitoring specific molecular vibrations using an appropriate spectroscopic technique will allow specific fingerprinting of singular or multicomponents and hence their determination. Spectroscopic techniques are rapid (assay time ~2 min), nondestructive, require no sample preparation and provide the opportunity to measure several broth constituents simultaneously. Present spectroscopic instrumentations are robust, precise, and can be easily operated by unskilled personnel. Other techniques, such as flow injection analysis or HPLC, generally involve more sample preparation, longer analysis times, skilled staff, and require specific protocol for measurement of individual chemical species, in any given sample.

There has been an increasing interest in the use of near-infrared (NIR) [2–5,13,15,16,22,29] spectroscopy in conjunction with chemometric techniques to monitor biological processes. Although a number of diverse process systems have been investigated, most of the results reported to date have been on NIR. No work has been done with FT-Raman spectroscopy and only a limited amount has been done with mid-infrared (MIR) [1,7,9,20,28] spectroscopy to monitor biological processes and no published information on the applicability of MIR spectroscopy to monitor ethanol fermentation is available. A computer-based technique for classifying and identifying bacterial samples using FT-MIR spectroscopy was described [18]. Classification schemes were tested for selected series of bacterial strains and species from a variety of different genera. The concentrations of ethanol [3], fructose, glycerol, glucose, ethanol [10], and the yeast cell density [12] have been quantified by spectroscopic methods.

MIR spectroscopy measures the fundamental vibrational modes of molecules. As a consequence, it has a unique advantage over NIR spectroscopy: not only known species can be quantified, but also unknown species that frequently arise in complex systems can be detected and identified. This method is unconstrained by dynamic range and within the performance limits of the instrument components and has excellent sensitivity [6]. The attenuated total reflection (ATR) technique using an ATR accessory in the FTIR spectrometer has evolved as a suitable method for quantitative characterization of components during a bioprocess. A comparative study was conducted using FTIR–ATR and a cylindrical internal reflection (CIR) accessory for the determination of sugars in soft drinks and fruit juices [11]. Analytical sensitivity obtained using the ATR cell was three times higher than that obtained using the CIR unit.

Quantitative FTIR spectroscopy has been used in a wide range of applications. The determination of glucose in whole blood [17], human plasma protein mixtures [25] and the spectral analysis of

Correspondence: J Irudayaraj, Department of Agricultural and Biological Engineering, Pennsylvania State University, 249, Agricultural Engineering Building, University Park, PA 16802, USA
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human blood plasma [21] have been carried out. FTIR spectroscopy was used to study ethanol production from whey [7] and on-line analysis of lactose and lactic acid [8]. Most spectroscopic quantitative analysis procedures require the use of multivariate statistics or chemometrics.

Partial least-squares (PLS) and principal component regression (PCR) methods are some of the most relevant chemometric methods used for spectral data analysis. They are useful for solving the collinearity problem as well as for providing additional graphic information about the relationship between spectrum and chemical composition. In this analysis the X variables are wave numbers and Y the corresponding intensity. These methods compress X variables (wave numbers) to a small number of so-called components or scores. These components are linear combinations of the original spectral values and describe the variation of data. In PCR, the components are simply the classical principal components of the **X**-matrix (PC's). In PLS, they are obtained by maximizing the covariance between linear combinations of X and Y using information about the X and Y variables. In both methods, the components are then used as independent variables in a regression equation for prediction.

PLS and PCR methods have been used to develop calibration equations for determining sugars in aqueous mixtures [27]. The best results were obtained with PLS first-derivative calibration method. The standard errors of prediction obtained by the PLS first-derivative regression are: 0.334 g/l for glucose; 0.286 g/l for fructose; and 0.365 g/l for sucrose. The results predicted by this method were used to predict sugar profile in synthetic mixtures and real beverage samples. The NIR spectroscopic method was developed to noninvasively measure the concentration of alanine, glucose, glutamine, and leucine in samples removed from an Sf-9 insect cell culture bioreactor [26]. Calibration models developed from spiked spent medium produced better measurements than its synthetic counterpart.

In the present study, FT-MIR and FT-Raman methods for quantitative determination of glucose, ethanol, and optical cell density of *Saccharomyces cerevisiae* were investigated. The spectral data were correlated with HPLC data using multivariate statistical analysis. The main objective is to develop a rapid and simple FT-MIR and FT-Raman procedure and a suitable calibration model to detect glucose, ethanol, and optical cell density during ethanol fermentation.

Materials and methods

Microorganism and media

S. cerevisiae (ATCC 24859) was grown in a medium containing (in grams per liter of water) 20 glucose, 6 yeast extract (Becton Dickinson, Sparks, MD, USA), 0.23 $CaCl_2 \cdot 2H_2O$, 4 $(NH_4)_2SO_4$, 1 $MgSO_4 \cdot 7H_2O$, and 1.5 KH_2PO_4, and stored at 4°C. A 24-h static culture grown at 30°C was used as inoculum (1%) in all fermentations. Flask fermentations contained varying concentrations of glucose (30–70 g/l) as carbon source. Samples were taken for analysis from the flasks at different times during fermentation, to span the entire analytes concentration range desired. Spectral data were acquired using FT-MIR and FT-Raman spectroscopic techniques directly without any sample preparation.

FT-MIR measurements

FT-MIR spectra were recorded on a Nicolet model 870 (Madison, WI, USA) spectrometer equipped with a deuterated triglycine sulphate (DTGS) detector. The sampling station was equipped with an overhead ATR accessory, comprised of transfer optics within the chamber through which infrared radiation was directed to a detachable ATR zinc selenide crystal mounted in a shallow trough for sample containment. Distilled water was used for background spectra, and 256 coadded scans were taken for each sample from 4000 to 400 cm^{-1} at a resolution of 16 cm^{-1}. Single-beam spectra of the samples were obtained, and corrected against the background spectrum of water, to present the spectra in absorbance units. The ATR crystal was carefully cleaned with water between successive analyses and dried using nitrogen gas. Spectra were collected in duplicate and used for multivariate analysis.

FT-Raman measurements

FT-Raman spectra were obtained using a Nicolet 870 spectrometer with the Nicolet Raman module 32B (Madison, WI, USA) and HeNe laser operating at 1064 nm with a maximum power of 2 W. The system was equipped with an InGaAs (indium–gallium arsenide) detector, XT-KBr beamsplitter with 180° reflective optics, and a fully motorized sample position adjustment feature. A laser output power of 1.2 W was used, which was low enough to prevent possible laser-induced sample damage and a high signal-to-noise ratio. Data were collected at 32 cm^{-1} resolution with 256 scans. Spectra were used in the Raman shift range between 200 and 3600 cm^{-1} at 2-cm^{-1} data intervals. The system was operated using the OMNIC 5.1 software and spectra were acquired in triplicate.

Reference methods

Bacterial growth was followed by measuring optical cell density at 620 nm using a Bausch & Lomb Spectronic 20 spectrophotometer. Ethanol and glucose concentrations were measured by using Waters high performance liquid chromatography (HPLC) equipped with column heater, auto sampler, computer controller, and Waters model 2410 refractive index detector. Ethanol and glucose were separated on a Bio-Rad Aminex HPX-87H column (300×7.8 mm) at 65°C, using 0.012 N sulfuric acid as the mobile phase at a flow rate of 0.8 ml/min with a 20-μl injection volume.

Multivariate analysis

The sample set from *S. cerevisiae* cultures consisted of 37 samples for calibration and 13 for validation. The spectral data from the selected spectral regions were analyzed with GRAMS 32 (Galactic Industries, Salem, NH) software using PLS and PCR methods. PLS [14] and PCR [23] regressions were employed to determine the concentrations of glucose, ethanol, and optical cell density of *S. cerevisiae* cultures. In the set of binary or multicomponent mixtures, these procedures have the potential to detect the various components simultaneously.

The HPLC and spectral data are used for calibration and the models developed were used to predict the concentrations of glucose and ethanol, and the optical cell density in the samples of the validation set. Calibration models were developed with spectra in absorbance units using PLS and PCR analysis with original and first-derivative transformed spectra. Both these methods use cross-validation to assess the average predictive ability. The cross validation method is a resampling technique based on "leaving one sample out" procedure. This means that the calibration is

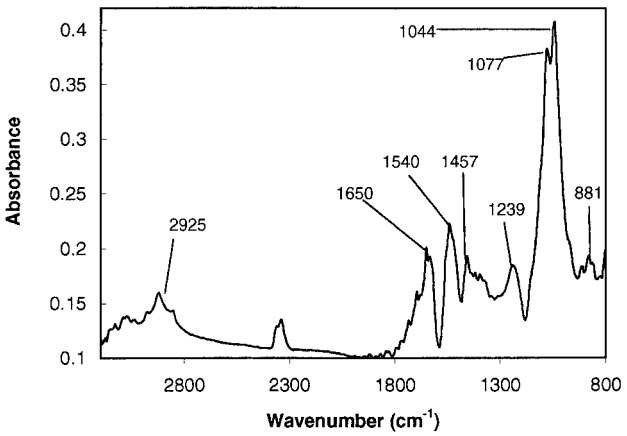

Figure 1 FT-MIR spectrum of *S. cerevisiae* culture with the key band assignments.

performed N times (N is the number of samples in the calibration data set), each time leaving one sample out and testing the calibration equation on this single sample. The PLS and PCR methods must be computed for each number of components and optimum number of factors for calibration was selected based on R^2 and the predicted residual sum of squares (PRESS), which should be minimized. The model with the best prediction ability is usually selected by computing the standard error of prediction (SEP) and standard error of calibration (SEC).

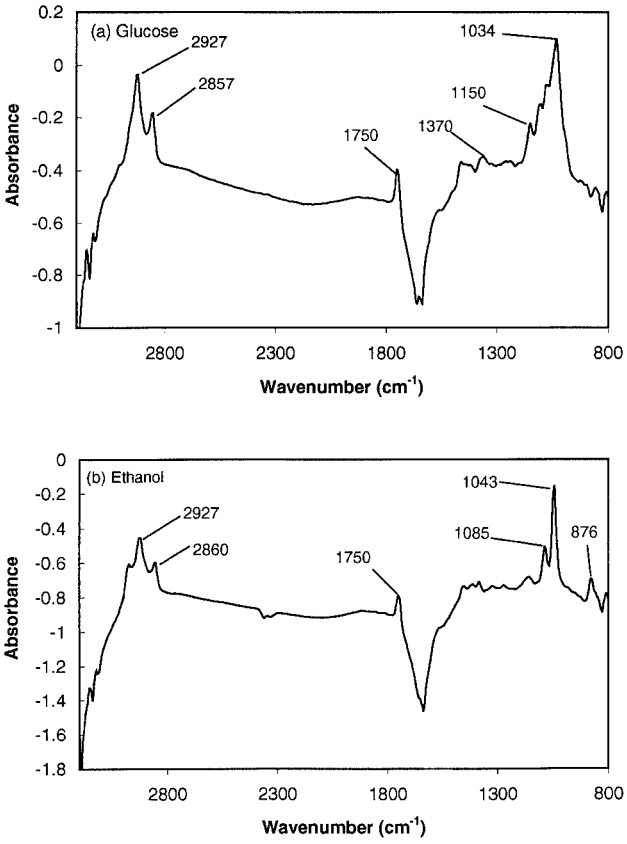

Figure 2 FT-MIR spectra of pure (a) glucose and (b) ethanol with the key band assignments.

Table 1 Functional groups, and their vibration modes obtained from the FT-MIR spectra of *S. cerevisiae* culture medium

Wave number	Functional group	Mode of vibration
800–1200	C–O (glucose and ethanol)	stretching
	C–C (glucose and ethanol)	stretching
1239	P=O phosphodiester groups (of cells)	stretching
1457	CH_3 (methyl groups of proteins)	asymmetric stretching
1540	C=O, C–N (amide II)	bending
	N–H (cell)	stretching
1650	C=O, C–N (amide I)	bending
	N–H (cell)	stretching
1612	O–H (water)	bending
2925	C–H (fatty acids of the membrane)	stretching

$$\mathrm{SEP} = \sqrt{\sum_{i=1}^{N} \left(y_{\mathrm{actual}} - y_{\mathrm{predicted}}\right)^2 \Big/ N - 1}$$

$$\mathrm{SEC} = \sqrt{\sum_{i=1}^{N} \left(y_{\mathrm{actual}} - y_{\mathrm{predicted}}\right)^2 \Big/ N - f - 1}$$

Here N, is the number of independent samples in the calibration set and f is the number of factors. In general, models with fewer factors are less likely to exhibit over fitting, and tend to be more stable and have better generalization ability. The optimal calibration method will be selected based on the highest R^2 and lowest SEC in calibration sets.

Results and discussion

FT-MIR spectroscopy

A typical FTIR spectrum of a *S. cerevisiae* culture is shown in Figure 1. The spectrum provides composite information of all the components in the medium including microbial components (cell

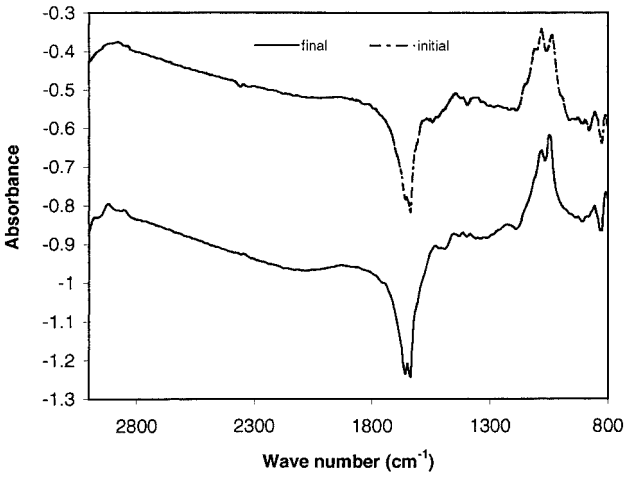

Figure 3 FT-MIR spectra of *S. cerevisiae* culture at the initial and final stages of fermentation.

Table 2 Calibration and validation results for estimation of glucose, ethanol, and optical cell density of *S. cerevisiae* using FT-MIR spectroscopy

Calibration method	Calibration			Validation	
	Factors	R^2	SEC	R^2	SEP
Glucose, g/l					
PLS	8	0.985	2.59	0.955	2.837
PLS first derivative	6	0.992	1.938	0.979	1.819
PCR	10	0.992	2.460	0.979	1.922
PLS first derivative	12	0.996	2.610	0.980	1.745
Ethanol, g/l					
PLS	8	0.996	1.089	0.983	1.805
PLS first derivative	4	0.995	1.150	0.991	1.243
PCR	8	0.996	1.104	0.985	1.680
PLS first derivative	9	0.996	1.008	0.992	1.284
Cell $OD_{620\ nm}$					
PLS	10	0.916	0.572	0.764	0.634
PLS first derivative	6	0.895	0.507	0.744	0.590
PCR	11	0.918	0.565	0.757	0.627
PLS first derivative	12	0.925	1.008	0.732	0.614

R^2, correlation coefficient; SEP, standard error of prediction, grams per liter; SEC, standard error of calibration, grams per liter; PLS, partial least squares; PCR, principal component regression.

walls, membranes, proteins, nucleic acids, etc.), glucose, ethanol, and salts.

The spectra of cultures show absorbance bands at 881, 1044, 1077, 1239, 1457, 1540, 1650, and 2925 cm^{-1}. These bands represent the chemical groups of components present in the culture. Assignment of functional groups corresponding to the vibration modes was based on identification of the spectrum peaks and matching the frequency with the corresponding chemical groups that absorb in the MIR region. The fingerprint region between 800 and 1500 cm^{-1} mainly contains absorptions from the C–O bending modes of the polysaccharide component. Structure in the region 800–1200 cm^{-1} reflects composition of glucose and

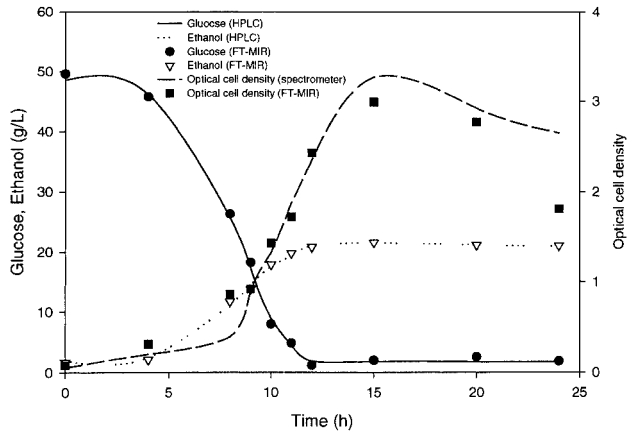

Figure 4 Comparison of HPLC and FT-MIR analysis of ethanol fermentation with *S. cerevisiae*.

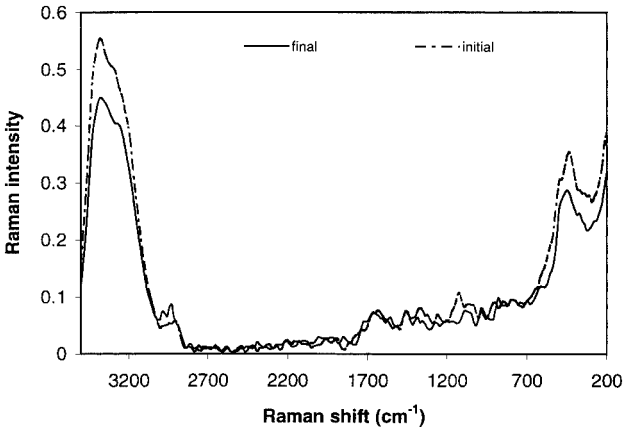

Figure 5 FT-Raman spectra of culture at the initial and final stages of fermentation.

ethanol as shown in the FT-MIR spectra of pure glucose and ethanol (Figure 2a and b). The region 1500–1580 cm^{-1} shows strong absorbance of amide II functional groups (NH deformation bands). The peak 1650 cm^{-1} contains amide I carbonyl absorptions, which indicate the presence of protein. Both amide I and II peaks are the most intense bands in nearly all bacterial species examined [24]. This region provides evidence of relevant quantitative and qualitative relationships between the various

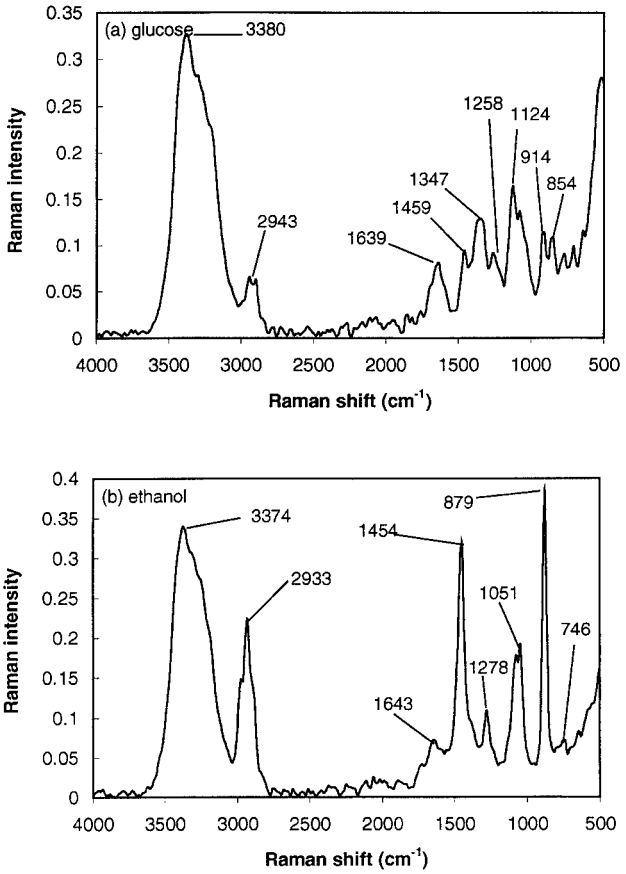

Figure 6 FT-Raman spectra of pure (a) glucose and (b) ethanol with the key band assignments.

Table 3 Calibration results for estimation of glucose, ethanol, and optical cell density of *S. cerevisiae* using FT-Raman spectroscopy

Calibration method	Factors	R^2	SEC
Glucose			
PLS	2	0.958	6.810
PLS first derivative	2	0.724	12.070
PCR	1	0.839	10.520
PLS first derivative	1	0.801	10.243
Ethanol			
PLS	2	0.912	3.340
PLS first derivative	2	0.872	4.110
PCR	2	0.907	3.505
PLS first derivative	2	0.853	4.392
Cell $OD_{620\ nm}$			
PLS	2	0.814	0.640
PLS first derivative	2	0.777	0.717
PCR	2	0.808	0.676
PLS first derivative	3	0.755	0.765

R^2, correlation coefficient; SEC, standard error of calibration, grams per liter; PLS, partial least squares; PCR, principal component regression.

secondary substructures of proteins. The peak at 2925 cm^{-1} denotes CH stretching absorptions, which indicate the presence of lipids. The assignments of functional groups and their vibration mode corresponding to the structure of major components for *S. cerevisiae* culture are listed in Table 1.

Quantitative analysis

Figure 3 shows FT-MIR spectra of culture at the initial and final stages of fermentation. Visual inspection of these spectra indicates that there are some qualitative differences in selected regions, although at least some complex quantitative differences between them were observed. Such spectra, essentially uninterpretable by the naked eye, readily illustrate the need to employ multivariate statistical techniques for their analyses. The most sensitive and selective regions for multivariate analysis were those between 2800 and 3200 and 800 and 1500 cm^{-1} (Figure 3). These regions are dominated by characteristic absorptions due to glucose, ethanol, and proteins (cells) and hence were selected for PLS and PCR calibration methods.

Table 2 summarizes the performance of the different calibration models using PLS, PLS first-derivative, PCR, and PCR first-derivative for predicting the concentration of glucose, ethanol, and optical cell density of *S. cerevisiae* cultures. All calibration methods gave satisfactory results for estimation of glucose and ethanol concentrations. Correlation coefficient for the estimation of optical cell density could be improved by including larger data sets. Results show that PLS first-derivative was slightly better than other methods. The optimum number of factors was the least (6, 4, and 6 factors for glucose, ethanol, and optical cell density, respectively) for PLS first-derivative compared to other calibration methods (Table 2). The highest correlation (R^2) between the predicted and actual values was 0.992, 0.995, and 0.895 for glucose, ethanol, and optical cell density, respectively (Table 2), using the PLS first-derivative method. The SEC values were 1.938 g/l (for glucose), 0.15 g/l (for ethanol), and 0.507 (for optical cell density) using PLS first-derivative calibration method.

The calibrations were then applied to the corresponding validation data sets for the computation of glucose, ethanol, and optical cell density of culture samples. Concentration profiles of glucose and ethanol, and optical cell density measured by both HPLC and FT-MIR methods during selected ethanol fermentation are shown in Figure 4. Validation results show that the FTIR spectroscopic technique could accurately predict the concentrations of glucose and ethanol though the R^2 for optical cell density are low (Table 2). The precision of this method could be improved by studying larger multicomponent data sets. The statistical characteristics using PLS first-derivative calibration method used a smaller number of factors and hence can be recommended for quantitative analysis of glucose and ethanol.

FT-Raman spectroscopy

Figure 5 shows the FT-Raman spectra of culture at the beginning and end of fermentation. The functional groups of glucose, ethanol, and proteins generally show much weaker bands in Raman than in FT-MIR spectra. In general, Raman scattering for the culture sample is weak, which suggests fairly high concentration of the target analytes are required to be measured by the Raman technique. This was confirmed by taking FT-Raman spectra of high concentrations of glucose and ethanol (Figure 6a and b), which show several clear characteristic Raman bands. The regions between 250 and 600, 950 and 1200, and 2900 and 3300 cm^{-1} are important in quantitative analysis (Figure 5) and were used for quantitative analysis using PLS and PCR methods.

The calibration parameters for glucose, ethanol, and optical cell density using different statistical procedures are given in Table 3. In general, SEC values are high compared to results using FT-MIR, though the number of optimum factors is less. Quantitative analysis using FT-Raman spectra of aqueous solutions with low concentrations of the components are not accurate because of attenuation of the spectra due to water [19]. Lower analyte concentrations can be studied using the technique of resonance Raman spectroscopy, where the exciting laser wavelength can be adjusted to the absorption range of particular chromophores within the sample [4]. Additionally Raman spectroscopy involves the use of powerful lasers and their optical alignment to produce an intense focused path of radiation on the sample, hence requiring moderate operator skill in the alignment and operation of the equipment compared with FT-MIR spectroscopy. Raman scattering of a culture can also be improved by using a more sensitive germanium detector, which needs liquid nitrogen for cooling. Further research on various fermentations is required for any conclusive decision on the use of FT-Raman as an alternative to FT-MIR for quantitative analysis.

Conclusions

The concentrations of glucose and ethanol, and the optical cell density of *S. cerevisiae* during ethanol fermentation were measured simultaneously and accurately by FT-MIR spectroscopy without any sample preparation. FT-Raman spectroscopy is suitable for analysis of major constituents in bioprocesses when they are present at high concentrations. The developed method can be used to measure other chemical components in the culture medium such

as salts by noninvasive methods. The method provided in this paper could be developed for rapid analysis of the overall metabolic rate of the cells, because the FT-MIR method has a potential to determine theoretically most of the chemical components relating to the metabolism as well as sugars.

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