Simultaneous Determination of Multiple Components in Nisin Fermentation Using FTIR Spectroscopy

Sivakesava Sakhamuri, Jamie Bober, Joseph Irudayaraj, and Ali Demirci

Department of Agricultural and Biological Engineering, The Pennsylvania State University, University Park, PA 16802, USA.

Correspondence:
Dr. Joseph Irudayaraj
Department of Agricultural and Biological Engineering
The Pennsylvania State University
249, Agricultural Engineering Building
University Park, PA 16802
Phone: 814-865-2807
Fax: 814-863-1031
Email: josephi@psu.edu

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Abstract

The potential of noninvasive determination of glucose, lactic acid, and nisin in *Lactococcus lactis* subsp. *lactis* biofilm fermentation was investigated through fourier transform mid-infrared (FTIR) spectroscopy. Samples obtained from a biofilm bioreactor were analyzed with traditional methods and FTIR spectroscopy. The FTIR spectra were interpreted by using suitable spectra wavenumber regions through multivariate statistical techniques such as partial least square (PLS) and principal component regression (PCR). The standard error of calibration for the PLS-1st derivative calibration models for glucose, lactic acid, and nisin were 3.87 g/l, 2.62 g/l, and 189.6 IU/ml, respectively. Prediction errors were low for glucose and lactic acid, whereas nisin could be reliably quantified when its concentration is higher than 800 IU/ml. Results indicated that FTIR spectroscopy could be used for rapid detection of glucose and lactic acid concentrations, and nisin activity in nisin fermentation.

Key words: Fourier transform mid infrared spectroscopy; partial least squares, principal component regression, chemometrics, lactic acid; bacteriocin.

1. Introduction

Nisin, a proteinaceous compound produced by *Lactococcus lactis* subsp. *lactis*, inhibit the growth of gram-positive bacteria including food spoilage organisms and pathogens. Nisin was approved for use in the United States in 1988 (Jay, 2000) and is, to this day, the only approved bacteriocin for use in foods in the U.S. Nisin is primarily effective against gram-positive microorganisms, such as the pathogenic *Listeria*, *Bacillus* and *Clostridium* species, and inhibits the germination of bacterial spores (Delves-Broughton, 1990). Nisin is commercially made predominantly by the fermentation of various strains of *Lactococcus lactis* subsp. *lactis* (Qiao et al., 1997).

In view of the growing consumer demand for natural food additives, much applied research is currently focused on the economic production of nisin. In bioprocesses, the control task is often difficult, due to lack of information relating to the concentration of various key components. Generally analyses of biological compounds are performed by off-line analytical techniques such as wet chemical analysis or high-

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performance liquid chromatography (HPLC). These methods are highly sensitive but are not generally suitable for continuous on-line measurements. The most common method available at present for analysis of nisin is the diffused agar method (Wolf and Gibbons, 1996). This involves the incubation of a nisin-sensitive microorganism (indicator microorganism) into the bioassay medium agar plates in presence of nisin. Since the gram-positive microorganism in the agar is inhibited by nisin, there will be an absence of microbial growth forming a circular "zone of inhibition" after a period of incubation, which is typically 24 hours. The diameter of these zones is measured and compared against a standard curve relating the diameter of the zone to nisin concentrations. Such a procedure is time consuming and laborious.

To aid in the development of a more efficient nisin fermentation processes and to monitor production, rapid feedback on the state of fermentation in terms of the nisin content is very crucial, because nisin activity starts to decrease after a certain time during fermentation, due to the release of non-specific proteolytic enzymes (De Vuyst and Vandamme, 1991). Therefore, fermentation needs to be stopped before nisin activity decreases, which is possible by a rapid/on-line analysis of nisin. Thus, there is a need for on/at–line methods for the characterization of bioprocesses that will enhance process analysis and control. The ideal method would be rapid, noninvasive, precise, and economical. Monitoring of bioprocesses requires sensors providing real-time measurement of several variables to analyze, model, and control optimally the time course of these processes in bioreactors (Phillips, 1990). FTIR spectroscopy is a promising tool for the rapid, noninvasive, and multi-parameter analysis of aqueous biological systems. This is a well-established analytical method for process monitoring and identifying chemical species (Doyle, 1992 and Mao et al., 1995).

FTIR is an analytical chemical technique used to study molecular structure, however when it is applied to fermentation culture medium, the resulting spectra reflect the total biochemical composition of the culture medium. FTIR spectroscopy can thus provide a total simultaneous chemical analysis. The use of FTIR spectroscopy for the analysis of aqueous process systems has been limited primarily by the broad intense absorption bands of water, which obscure the infrared (IR) spectra of analytes. The attenuated total reflectance (ATR) technique gives a fairly general solution to the problem of strong water absorption. ATR-based FTIR spectroscopic sensors appear promising for the noninvasive quantification of reactants and products in complex

systems such as fermentation broths (Doak and Phillips, 1999). For quantification, the spectra had to be calibrated with known reference values for the analyte, as determined by an established method such as HPLC. This allows the development of mathematical models with the spectra as input to predict the analyte concentration. The most common multivariate methods used for this purpose are the partial least squares (PLS) and principal component regression (PCR) methods.

In this study, FTIR spectroscopy was applied for simultaneous measurement of multiple components such as glucose, lactic acid concentrations, and nisin activity in a nisin fermentation process. The FTIR spectral data were correlated with data from standard assay methods using multivariate statistical analysis. The main objectives of this research were to (1) characterize the FTIR spectra and identify appropriate spectral regions and (2) to develop, validate, and compare the prediction models for glucose, lactic acid, and nisin activity.

2. Materials and Methods

2.1. *Microorganism and media*

Lactococcus lactis subsp. *lactis* (NIZO 22186) was grown in three different media as shown in Table 1. Biofilm fermentations in repeated batches with plastic composite supports were carried out using a 1.5 l Cellogen bench top bioreactor (New Brunswick Scientific, Edison, NJ) at 30^{o}C and 75 rpm with 800 ml of working volume. The pH was controlled at 6.8 by adding 4N NaOH. The inoculum (1%) was grown in the same medium in a flask in a shaker incubator at 30^{o}C and 75 rpm for the first batch. At the end of each batch spent media was drained and new sterile media was added. Samples were drawn from the bioreactor at regular intervals to span a wide range of analyte concentration and analyzed for glucose, lactic acid concentrations, and nisin activity. In all, 89 samples from the six different fermentation batches were collected and analyzed by FTIR spectroscopy and other reference methods.

Table 1. *L. lactis* fermentation medium composition

1X	0.5X	0.25X
Glucose 100 g/l Yeast extract 10 g/l Soy peptone 10 g/l KH_2PO_4 10 g/l NaCl 2g/l $MgSO_4.7H_2O$ 0.2 g/l	Glucose 100 g/l Yeast extract 5 g/l Soy peptone 5 g/l KH_2PO_4 10 g/l NaCl 2g/l $MgSO_4.7H_2O$ 0.2 g/l	Glucose 100 g/l Yeast extract 2.5 g/l Soy peptone 2.5 g/l KH_2PO_4 10 g/l NaCl 2g/l $MgSO_4.7H_2O$ 0.2 g/l

2.2. *FT-MIR measurements*

FT-MIR spectra were recorded using a Nicolet model 870 (Madison, WI, USA) spectrometer equipped with deuterated triglycine sulphate (DTGS) detector. The sampling station was equipped with an overhead ATR accessory, comprising 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 the 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 between successive analysis with water and dried using nitrogen gas. Spectra were collected in triplicate and used for multivariate analysis.

2.3. *Reference methods*

Lactic acid and glucose concentrations were measured by using Waters high performance liquid chromatography (Milford, MA) equipped with column heater, auto sampler, computer controller, and Waters model 2410 refractive index detector. Lactic acid and glucose were separated using a Bio-Rad Aminex HPX-87H column (300 x 7.8

mm) (Cambridge, MA) at 65°C, with 0.012 N sulfuric acid as the mobile phase at a flow rate of 0.8 ml/min with a 20-µl injection volume.

Samples were analyzed for nisin production by the modified agar diffusion method (Wolf and Gibbons, 1996) using *Micrococcus luteus* (ATCC 10240), which will be maintained and cultured under the same conditions as described previously by Tramer and Fowler (1964). A 100 µl of sample was dispensed into the individual well, and incubated at 30°C for 48 h until growth of the test organism could be easily observed. The zones of inhibition are measured by a digital caliper, which was then converted into nisin activity using a standard curve.

2.4. *Chemometrics*

The FTIR spectra set from *L. lactis* subsp. *lactis* cultures were divided into calibration (67 samples) and validation (22 samples) data sets. The spectral data from the selected spectral regions were analyzed with GRAMS 32 (Galactic Industries Corporation, Salem, NH) software using PLS and PCR methods. Partial least square (Haaland and Thomas, 1988) and PCR (Martens and Naes, 1988) regressions were employed to determine the concentrations of glucose, lactic acid, and nisin activity in *L. lactis* culture. PLS and PCR calibration methods with original and 1st derivative transformed spectra were used to build calibration models using the data from calibration data set. The standard error of calibration (SEC) and standard error of prediction (SEP), repeatability (S_r) were computed to assess the degree of fit and predictive ability of the model. The SEC (equation 1) is calculated using the calibration data set, while the SEP (equation 2) provides a measure of the predictive ability of the model for independent data (for validation data set).

$$SEC = \sqrt{\frac{\sum_{i=1}^{n}(actual - predicted)^2}{n - f - 1}} \quad \cdots\cdots\cdots\cdots(1)$$

$$SEP = \sqrt{\frac{\sum_{i=1}^{n}(actual - predicted)^2}{n}} \quad \cdots\cdots\cdots\cdots(2)$$

Here 'n' is the number of samples in the data set and 'f' is the number of factors used in the calibration model. Cross-validation was used in all cases to minimize the risk of over-fitting the calibrations when evaluating calibration accuracy. Repeatability is expressed as a mean standard deviation (S_r) of multiple determinations performed under identical conditions and is calculated as

$$S_r = \sqrt{\frac{\sum_{j=1}^{n}\sum_{i=1}^{r}(xj,i-\bar{x}_j)^2}{n(r-1)}} \quad \cdots\cdots\cdots\cdots (3)$$

where r is the number of replicates, $x_{j,i}$ is the result of the i^{th} replicate of the j^{th} sample and ξ_j is the average result of the j^{th} sample.

3. Results and Discussion

FTIR analysis of aqueous systems is difficult because of the interference from broad vibrational bands of water that overlap the spectral information of analytes, which usually cannot be deconvoluted into their constituents. However the effect of water can be minimized/removed by using water as background. Figures 1a and b show the characteristic absorption spectra of the culture medium at the beginning and end of fermentation using water as background spectrum. The spectrum provides composite information of all the components in the medium (glucose, lactic acid, nisin, and other salts) including microbial components such as cell walls, membranes, proteins, nucleic acids. The distinctiveness of an individual spectrum, which is determined by the chemical structure of each component and the degree to which each component contributes to the spectrum could be directly related to the concentration of the specific component in the sample.

Figures 1a and 1b show the changes in the FTIR spectrum of culture medium during the course of fermentation. The decrease in the absorbance in the 1000-1200 cm^{-1} range results from the consumption of both glucose and the phosphorylated

carbohydrates formed during the sterilization. The increase in absorbance between 1500 and 1600 cm^{-1} is due to the accumulation of lactic acid. The spectral region between 3000

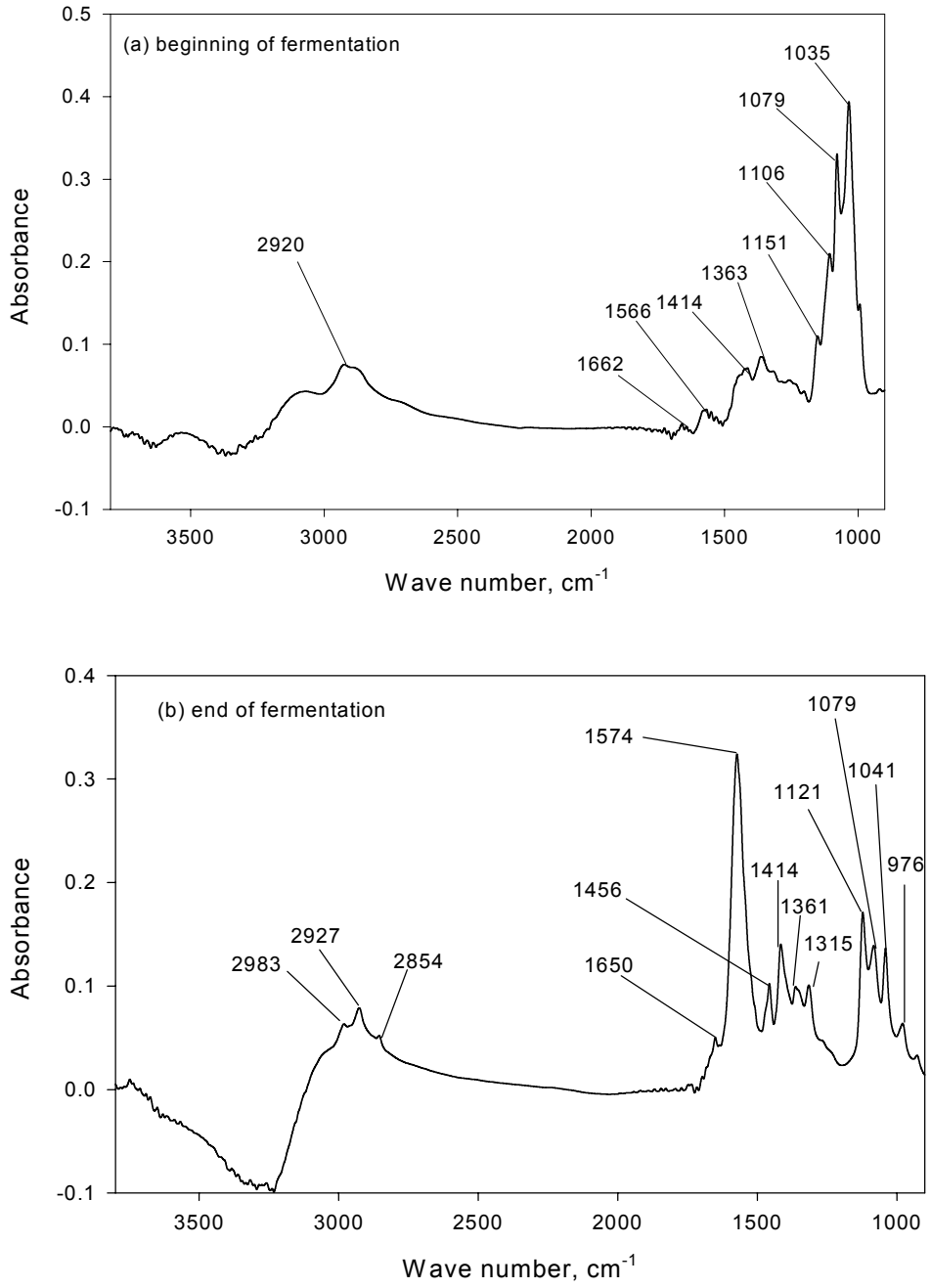

Figure 1. FTIR spectrum of *L. lactis* subsp. *lactis* culture at the (a) initial and (b) final stages of fermentation with the key band assignments

and 2800 cm^{-1} is dominated by the $–CH_3$, $>CH_2$, and >CH-stretching vibrations of groups usually present in fatty acid components. The region between 1700 and 1500 cm^{-1} is dominated by protein bands while the range 1450 to 1200 cm^{-1} denote carboxylic groups of proteins. The vibrational characteristics of mono- and polysaccharides generally dominate the region between 1200 and 900 cm^{-1} because of the C-O stretch bonds related to sugar. The assignments of functional groups with their corresponding vibration mode observed are listed in Table 2.

Table 2. Chemical assignments of bands obtained from FT-MIR spectrum of *L. lactis* fermentation medium.

Wave number	Functional group	Mode of vibration	Comments
976	P=O		pyrophosphate
1035	C-O	Stretching	glucose
1079	P=O (phosphodiester)	stretching	phosphate, Backbone of nucleic acids (DNA and RNA) and phosphorylated protein and polyphosphate storage products
1121,1151	C-O, C-C	stretching	
1315	N-H	bending	secondary amide
1361	C-H	bending	$-CH_3$
1414	C-O	symmetric stretching	$-COO^-$, carboxylate
1456	C-H(CH_3), C-H (CH_2)	asymmetric deformation symmetric deformation	Proteins Proteins
1574	C=O	strething	Lactic acid (lactate ion)
1650,1662	C=O C-N	stretching bending	Amide I band; may also contain contributions from C=C stretches of olefinic and aromatic compounds.
2854	C-H (CH_2)	symmetric stretching	Fatty acids
2920,2927	C-H (CH_2)	asymmetric stretching	Fatty acids
2983	C-H (CH_3)	asymmetric stretching	Fatty acids

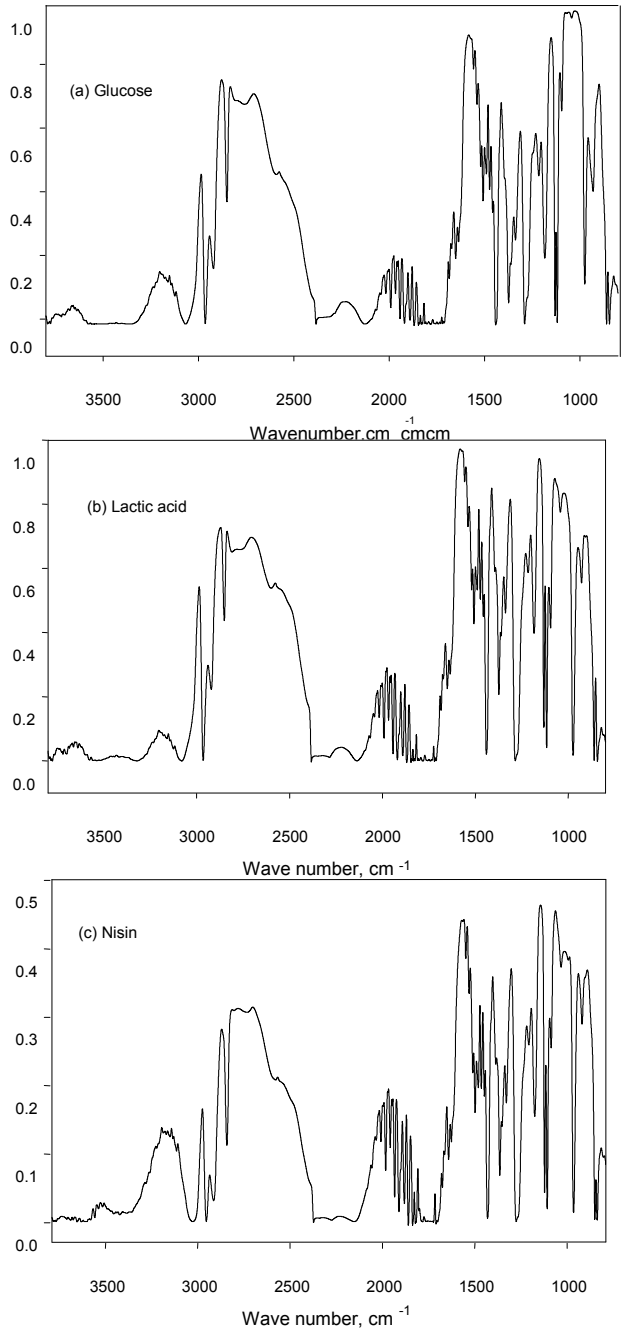

Figure 2: Correlation (R^2) spectrum for (a) glucose, (b) lactic acid, and (c) nisin activity with respect to wavenumber.

3.1. *Quantitative analysis*

The FTIR absorbance at specific wavenumbers corresponding to various generic functional groups given in Table 2 could be correlated to related fermentation products. The interpretation of lower concentrations of nisin in this experiment was difficult because of the high concentration of overlapping absorbance bands. Multivariate calibration techniques such as PLS or PCR were used to extract relevant information from the spectra and to reduce the dimension of the dataset.

To build calibration models, the spectral range dictates which spectral points are used in the computation of the unknown variables. The spectral range should include information describing the concentration variation of the analyte and other matrix constituents while excluding regions dominated by noise or other artifacts that might distort the model. Suitable spectral ranges can be identified by computing the correlation coefficient spectrum for each constituent of interest (Figures 2a-c). This is done by calculating the correlation of the absorbance at every wavelength in the training data set to the concentrations of glucose, lactic acid, and nisin activity. Figures 2a-c show high correlation bands at 1000-1165 cm^{-1} for glucose, 1470-1700 cm^{-1} for lactic acid, and 1200-1800 cm^{-1} for nisin. These regions are dominated by characteristic absorptions due to glucose, lactic acid, and nisin and hence selected for multivariate model development.

Table 3 summarizes the performance of the different calibration models using PLS, PLS-1st derivative, PCR, and PCR-1st derivative for predicting the concentrations of glucose and lactic acid concentrations, and nisin activity in *L. lactis* subsp *lactis* culture using FTIR spectroscopy. Results show that PLS-1st derivative was slightly better than other methods. The optimum numbers of factors were the least (3, 3, and16 factors for glucose and lactic acid concentrations, and nisin activity, respectively) for PLS-1st derivative compared to other calibration methods (Table 3). The correlation (R^2) between the predicted and actual values were 0.995, 0.992, and 0.842 for glucose and lactic acid concentrations, and nisin activity, respectively, using PLS-1st derivative method (Table 3). The SEC values were 3.87 g/l, 2.62 g/l, and 189.6 IU/ml for glucose, lactic acid, and nisin activity, respectively, using PLS-1st derivative calibration method. The repeatability values are considerable lower except in the case of nisin. On-line methods using mid-

infrared spectroscopy have been used to monitor chlorinated aliphatic hydrocarbons (Acha et al., 2000) and fermentations (Fayolle et al., 2000; Sivakesava et al., 2001). The work reported in this research is the first attempt in monitoring nisin fermentation.

Table 3. Calibration and validation results for estimation of glucose, lactic acid, and nisin activity in *L. lactis* culture using FTIR spectroscopy (*selected spectral region)

Analyte/Calibration method	Calibration				Validation		
	Factors	R^2	SEC (g/l)	S_r (g/l)	R^2	SEP (g/l)	S_r (g/l)
Glucose (g/l) (1000-1165 cm^{-1})*							
PLS	4	0.994	4.7	0.81	0.997	6.3	0.75
PLS-1st derivative	3	0.995	3.8	0.63	0.998	2.9	0.61
PCR	4	0.993	5.1	0.89	0.987	10.9	0.91
PCR-1st derivative	3	0.995	4.5	0.72	0.996	4.5	0.83
Lactic acid (g/l) (1470-1700 cm^{-1})*							
PLS	4	0.992	2.7	1.23	0.975	4.3	1.69
PLS-1st derivative	3	0.992	2.6	0.42	0.986	2.2	0.46
PCR	6	0.993	2.9	1.43	0.978	2.7	1.56
PCR-1st derivative	5	0.993	2.8	0.51	0.987	2.3	0.57
Nisin activity (IU/ml) (1200-1800 cm-1)*							
PLS	22	0.853	195	31	0.813	505	37
PLS-1st derivative	16	0.842	189	28	0.678	484	33
PCR	24	0.782	243	43	0.713	555	45
PCR-1st derivative	22	0.801	226	40	0.753	513	48

R^2: Correlation coefficient; SEC: standard error of calibration, g/l; SEP: standard error of prediction, g/l; PLS: partial least square; PCR: principal component regression.

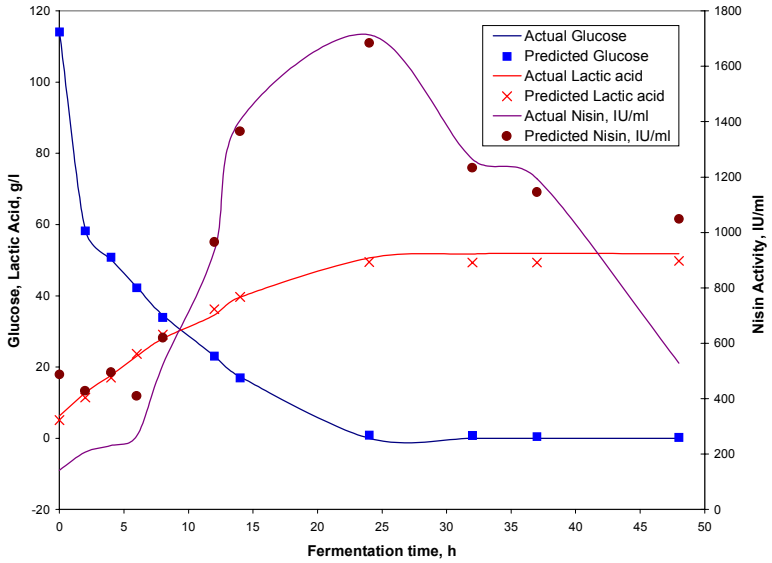

Figure 3. Comparison of standard methods and FTIR analysis of nisin production with *L. lactis* subsp. *lactis*

The calibrations were then applied to the corresponding validation data sets for the computation of glucose, lactic acid, and nisin activity in culture samples (Table 3). The profiles of glucose and lactic acid concentrations, and nisin activity measured by both reference and FTIR methods during a validated experiment are shown in Figure 3. Validation results show that the FTIR spectroscopic technique could accurately predict the concentrations of glucose and lactic acid (Table 3). Results showed that error in predicting the nisin concentration increased with decrease in actual nisin activity. A critical factor in the success of FTIR spectroscopy for nisin detection is the activity of nisin produced in the culture medium. The higher the nisin concentration (>800 IU/ml), the richer the spectral information and lower the error of prediction. Indeed, the nisin activity above 800 IU/ml has more importance for a successful fermentation.

The statistical characteristics using PLS-1st derivative calibration method gave lower errors of prediction, lower values of repeatability, and used less number of factors, and hence can be recommended for quantitative analysis. The accuracy of the above model was considered sufficient for glucose and lactic acid prediction, since testing was done with samples that cover a broad range of concentrations and the errors of prediction were considerably low. The use of initial media as background instead of water might facilitate quantitative measurement of nisin activity with lower error of predictions. Other

suggestions for improved predictions are to eliminate the noise due to carbondioxide and water interference and to increase the calibration data set and replications. The ATR crystal surface should also be examined for fouling periodically.

4. Conclusions

The present work has demonstrated that FTIR spectroscopy can be an ideal tool for monitoring chemical changes during nisin fermentation in a nondestructive and noninvasive manner. The use of FTIR spectral information and multivariate techniques showed potential for the simultaneous detection of multiple components in nisin fermentation system using *L. lactis* subsp *lactis*. The methods adopted do not require any sample preparation and are simple. The developed method can also be used to measure other chemical components in the culture medium such as salts by noninvasive methods.

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