

On-line infrared spectroscopy for bioprocess monitoring

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Abstract One of the major aims of bioprocess engineering is the real-time monitoring of important process variables. This is the basis of precise process control and is essential for high productivity as well as the exact documentation of the overall production process. Infrared spectroscopy is a powerful analytical technique to analyze a wide variety of organic compounds. Thus, infrared sensors are ideal instruments for bioprocess monitoring. The sensors are non-invasive, have no time delay due to sensor response times, and have no influence on the bioprocess itself. No sampling is necessary, and several components can be analyzed simultaneously. In general, the direct monitoring of substrates, products, metabolites, as well as the biomass itself is possible. In this review article, insights are provided into the different applications of infrared spectroscopy for bioprocess monitoring and the complex data interpretation. Different analytical techniques are presented as well as example applications in different areas.

Keywords Infrared spectroscopy · Bioprocess · On-line measurement · On-line monitoring · Sensor

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Introduction

Real-time monitoring and control of bioprocesses is necessary for increased productivity, efficiency, and reproducibility. The International Conference on Harmonisation (ICH Q8 “Pharmaceutical Development”, 2006) suggests that all critical process parameters (CPPs) that have an influence on the critical process attributes should be controlled. Two years earlier, the Process Analytical Technology initiative of the American Food and Drug Administration articulated the same intention.

Established sensor technologies for some of the CPPs, including temperature, pH, and dissolved oxygen, are already in common use for process control (Scheper et al. 1984, 1996, 1999; Marose et al. 1999; Hantelmann et al. 2005; Knuettel et al. 2006). However, research is still underway on sensor systems for other CPPs such as medium composition, biomass concentration, product concentration, and the metabolic state of the cells (Table 1). Many of these methods require that a sample be withdrawn from the bioreactor in order to obtain the desired information. Using this approach means that there is a time delay in the process data. Furthermore, these techniques often lack robustness, simple procedures, and handling, and often cause sterility problems.

Optical sensors in general offer substantial advantages for bioprocess monitoring. They are non-invasive, and no removal of a sample from the process is necessary. The sensors can readily be miniaturized and multiplexed, and they deliver information instantaneously. Figure 1 is an overview of optical spectroscopic sensors. Among all of the options, sensors in the near-infrared spectrum (NIRS) and the mid-infrared spectrum (MIRS) have gained importance in bioprocess analysis research during the last few years. These spectroscopic sensors are interesting

Table 1 Current works

Microorganism/cell line	Analyte	Method	Source
<i>B. subtilis</i> 168	Biomass	NIR	Kiviharju et al. (2007)
<i>B. longum</i> ATCC 15707 and DSM 14583	Biomass	NIR	Kiviharju et al. (2007)
<i>B. pertussis</i>	Biomass	NIR	Soons et al. (2008)
CHO	Glucose Lactate Glutamine Ammonia	NIR	Arnold et al. (2003)
CHO	Glucose Lactate	MIR	Rhiel et al. (2001)
CHO	Glucose Lactate	NIR	Roychoudhury et al. (2007a, b)
CHO	Antibody	MIR	Sellick et al. (2010)
<i>E. coli</i>	Biomass	NIR	Arnold et al. (2002)
<i>E. coli</i>	pH	MIR	Schenk et al. (2008)
<i>E. coli</i> XL-1 blue strains QMX-8b and TXC-1a	Biomass	NIR	Kiviharju et al. (2007)
<i>G. xylinus</i>	Fructose Acetic acid Ethanol Gluconacetan	MIR	Kommann et al. (2003)
<i>G. xylinus</i>	Fructose Ethanol Acetate Gluconaceton Phosphate	MIR	Kommann et al. (2004)
Combination of yeast and <i>Acetobacter</i> (so-called kombucha)	Glucose Gluconic acid	MIR	Franco et al. (2006)
<i>L. casei</i>	Glucose Lactate Cell density	MIR NIR	Sivakesava et al. (2001)
<i>L. plantarum</i> BPT 197 and BPT 186	Biomass	NIR	Kiviharju et al. (2007)
<i>L. fermentum</i> ES15	Biomass Glucose Lactic acid Acetic acid	NIR	Tosi et al. (2003)
<i>L. lactis</i> GRS71, NZ9800, NZ9010 and mutant (ptrfut5)	Biomass	NIR	Kiviharju et al. (2007)
PC3 (human prostate cancer)	Glucose Lactate Glutamine Ammonia	NIR	Rhiel et al. (2002)
PC3 (human prostate cancer)	Glucose Lactate Glutamine	NIR	Rhiel et al. (2004)
<i>Phaffia rhodozyma</i>	Cell density Astaxanthine Glucose	NIR	Yamane et al. (1996)
<i>P. pastoris</i>	Biomass Glycerol Methanol	NIR	Crowley et al. (2005)
<i>P. pastoris</i>	Methanol	MIR	Schenk et al. (2006)
Probat 505	Biomass	NIR	Kiviharju et al. (2007)

Table 1 (continued)

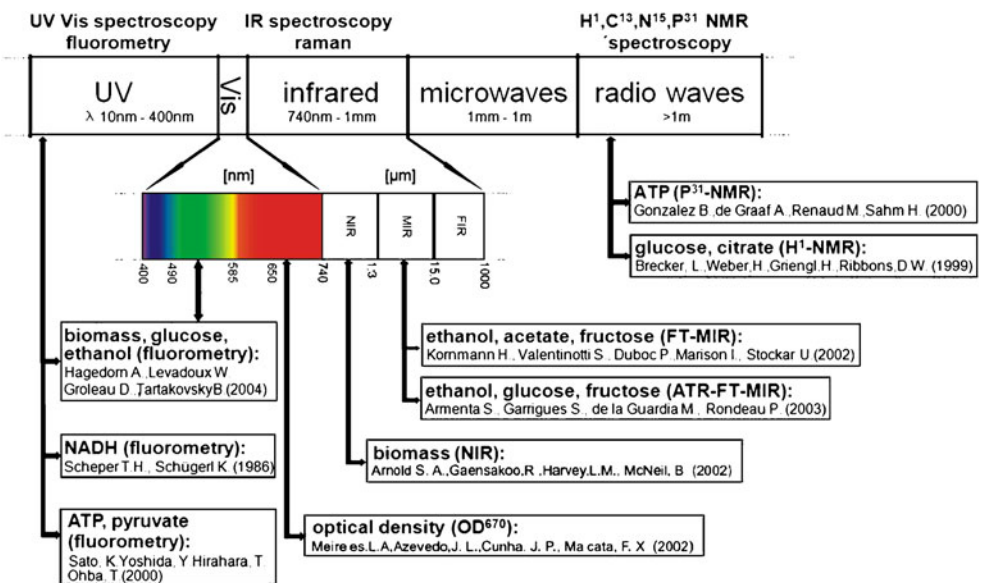
Microorganism/cell line	Analyte	Method	Source
<i>S. cerevisiae</i>	Cell density	NIR	Ge et al. (1994)
<i>S. cerevisiae</i>	Biomass	NIR	Kiviharju et al. (2007)
<i>S. cerevisiae</i>	Glucose Ethanol	MIR	Mazarevica et al. (2004)
<i>S. cerevisiae</i>	1-Phenyl-1,2-propandion ®-1-Hydroxy-1-phenyl-2-propanon (S)-2-Hydroxy-1-phenyl-1-propanon (1R,2S)-1-Phenyl-1,2-propandiol	MIR	Trevisan and Poppi (2008)
SF-9 (insect cell line)	Glucose Glutamine	NIR	Riley et al. (1997)
<i>Sphingomonas paucimobilis</i>	Biomass Gellan gum	NIR	Giavasis et al. (2003)
<i>S. xylosus</i> ES13	Biomass Glucose Acetic acid Lactic acid	NIR	Tosi et al. (2003)
<i>Streptomyces spec.</i>	Biomass	NIR	Nordon et al. (2008)
<i>S. clavuligerus</i>	(Metabolic and morphologic changes during cultivation process)	NIR	Ferreira and Menezes (2006)
<i>S. clavuligerus</i>	Clavulanic acid Nitrogen sources Carbon sources	NIR	Rodrigues et al. (2008)
<i>S. clavuligerus</i>	Biomass Ammonia Glucose Methyloleat	MIR	Roychoudhury et al. (2006)
<i>S. clavuligerus</i>	Clavulanic acid	MIR	Roychoudhury et al. (2007a, b)
<i>S. coelicolor</i>	Glucose Ammonia	NIR	Petersen et al. (2009)
<i>S. fradiae</i>	Biomass	NIR	Vaidyanathan et al. (2003)
<i>S. peucetius</i>	Biomass	NIR	Kiviharju et al. (2007)
<i>S. thermophylus</i> ES17	Biomass Glucose Lactic acid Acetic acid	NIR	Tosi et al. (2003)
<i>Trichoderma reesei</i>	Biomass Cellulase Humidity	NIR	Hongqiang and Hongzhang (2008)
<i>V. cholerae</i>	Biomass Glucose Lactate	NIR	Navrátil et al. (2005)

because excellent sensor systems exist that can easily be adapted for use in bioprocess monitoring. Additionally, NIRS and MIRS sensors can simultaneously detect several compounds. Because of the nature of the spectral signals from these techniques, multivariate analytical techniques such as principal component analysis (PCA) and partial least squares analysis (PLA) are necessary to obtain the

desired information. Thus, the spectroscopic sensors must be supported by appropriate chemometric algorithms for successful bioprocess monitoring.

Since IR, and especially near-infrared (NIR), analysis is used for pharmaceutical analyses, more and more specific guideline for the application exists. These guidelines also must be followed for biotechnological applications (ASTM 1997).

Fig. 1 Spectroscopic methods



The goal of this review is to summarize the actual research work in the area of NIR and mid-infrared (MIR) spectroscopy techniques for bioprocess monitoring. The principles and the potential of these spectroscopic techniques are shortly explained; major emphasis is given to the various practical applications and especially to data analysis.

Overview of infrared spectroscopy

Molecular vibrations from organic compounds have spectral signatures in the infrared region. They are subdivided into three categories of spectroscopy: far infrared (wave number range, 10–200 cm^{-1}), MIR (wave number range, 200–4,000 cm^{-1}), and NIR (wave number range, 4,000–13,000 cm^{-1}). Within this paper, we review the application of NIRS and MIRS for bioprocess monitoring in different areas.

Most excitations of fundamental molecular vibrations are in the MIR region, whereas the overtones and vibration combinations are excited in the NIR region in most cases. A quantum theoretical explanation is given by the anharmonic oscillator demonstrated in Fig. 2.

Due to the minor energetic distances of the energy levels in NIRS, the resulting spectra are less defined, whereas the MIR spectra exhibit high absorption capacity and well-defined peaks. Figure 3 shows spectra of aqueous solutions of different important components [concentration 100 mM each in phosphate buffer (pH 7.4)].

Data analysis for IR spectroscopy

The evaluation of IR spectra is rather complex because the absorption bands of different molecules overlap, especially

in NIR spectra. The fingerprint region of 1,500–500 cm^{-1} is used to identify compounds because they produce different and mostly specific patterns in this part of the spectrum. The identification procedure is carried out by a comparison with spectra from known compounds. However, if the spectrometer is used as a process analyzer, the results of the evaluation must be available in real time. The main focus here is a quantitative analysis of the spectra to obtain concentration values to monitor and control the process. The culture broth is a multicomponent solution (and typically multi-phase, as well) so that, for many applications, it is not possible to evaluate the spectra using a linear combination of the spectra obtained with each individual component. Therefore, multivariate evaluation procedures are required (Miller 2000; Lavine 2000) that can deal with the co-linear data sets. These evaluation procedures are able to bypass the missing selectivity and sensitivity of individual measurement points in an IR-spectrum and can extract the relevant information from the spectra, i.e., the pure concentration or value of important process variables. Unfortunately, no single routine method exists that will provide the optimal evaluation results for all applications. Frequently, combinations of different methods are used.

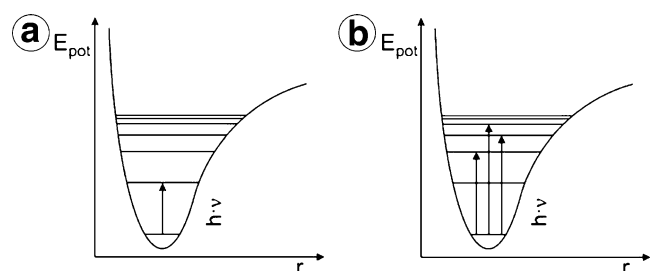


Fig. 2 Schematic illustration of the fundamental transitions by MIR (a) and the excitations of the overtones by NIR (b)

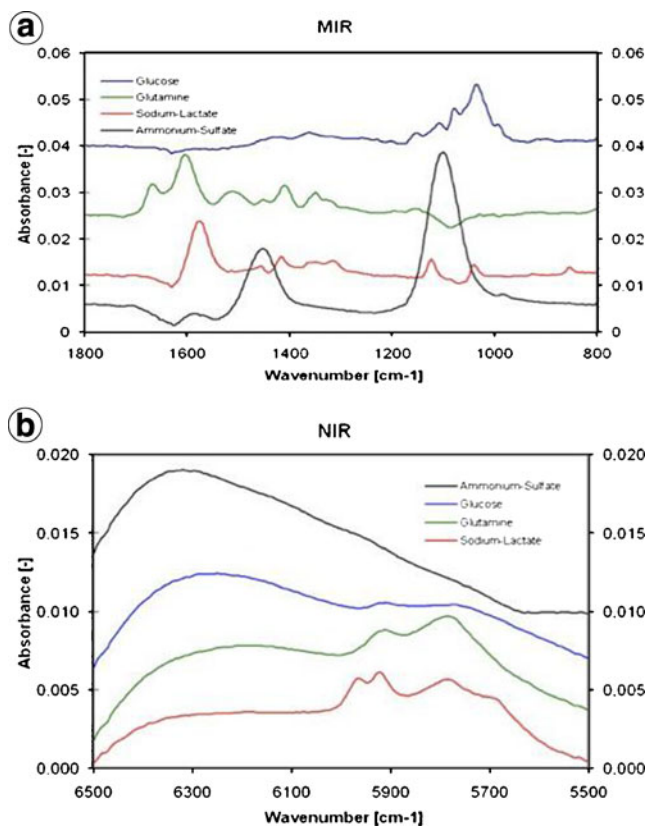


Fig. 3 Typical spectra obtained by MIRS (a) and NIRS (b)

For almost all evaluation procedures, a pretreatment of the data is required to extract useful information from the spectra. For this purpose, subtraction of background spectra (actual spectra minus the spectra of the sample matrix without the analytes), baseline (offset) correction, centering of data, and sometimes scaling can be used to improve the prediction capability of the evaluation method under consideration. Furthermore, if the data are noisy, smoothing is required. One method by which this can be carried out by is the Savitzky–Golay filter, which is based on a least squares polynomial regression in a fixed window. Sometimes, the information is hidden in the derivative of the signal, and this can also be obtained using the Savitzky–Golay method (Gorry 1990). An additional pretreatment is the detection and removal of outliers from the data set (Leardi 1994).

Before a regression method is applied to obtain quantifiable information, a variable selection method, which uses selected specific absorbance values out of the whole spectra, is performed so that meaningless parts of the spectra will not be used for the evaluation. Brown (1992) presented a simple method for wavelength selection for multicomponent NIRS evaluation. He chose those wavelengths that are specific to each pure component as compared with the other components (including the medium) for the varying compositions. However, this

approach can only be applied if the number of components giving significant signals in the spectra is small. The variable selection can be done by a priori knowledge about the process or by using an optimization procedure such as a genetic algorithm (Leardi, 1994). Goicoechea and Olivieri (2003) showed that a procedure based on a genetic algorithm complemented with PLA is able to alleviate over-fitting without the need of preparing an independent monitoring sample set. Bras et al. (2008) used a bootstrap-based strategy to select spectral intervals for partial least squares (PLS) regression. They demonstrated that interval partial least squares bootstrap enables an improvement to the model predictive ability, with a greater impact on external validation. The improvement of the root mean square error of prediction with respect to the full spectra was 16%.

Shamsipur et al. (2006) used an ant colony optimization procedure to select wavelengths for a regression model (based on classical least squares, inverse least squares, and PLS) resulting in an improved quality of the calibration model compared with a full-spectrum procedure. They used four different NIR and UV/Vis data sets as well as various ant colony optimization approaches to demonstrate how to improve the predictive ability of the evaluation method.

Heise et al. (2001) developed an expert system in which different chemometric tools were implemented. Their system enables the evaluation of the spectra with a minimum of user interaction. The authors selected the optimal wavelength ranges based on a mathematical criterion, applied spectral background strategies, and used high-quality reference spectra in a procedure that allowed the adaptation of non-linearity effects. Coen et al. (2006) proposed a methodology to initialize and optimize tuning parameters for least squares support vector machine regression with radial basis function kernel to evaluate NIR spectra. They used complex spectra of manure samples to demonstrate that this procedure simplifies the least squares support vector machine regression technique for new users.

Udelhoven and Schütt (2000) used artificial neural networks (ANN) for the evaluation of diffuse reflectance spectra with various learning algorithms. They demonstrated that all learning algorithms performed well using principal component scores of the first derivative spectra as input for the supervised training. They verified that the performance of the combination of PCA and artificial neural networks was better than that of standard multivariate statistical tools such as stepwise multiple linear regression and PCA.

Acha et al. (2000) used the attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) technique to monitor chlorinated aliphatic hydrocarbons in a fixed-bed bioreactor. The Fourier-transform technique

uses a Michelson interferometer with one fixed and one moving mirror. Due to the movement of the one mirror, different interference pattern (intensities) are obtained with a so-called center burst if both mirrors have an equal distance to the beam splitter, and all frequencies will constructively interfere. The obtained interferogram has to be Fourier-transformed to get the spectrum. Acha et al. used the ATR technique to connect the sensor with the bioreactor. They showed that the spectra could be evaluated using PLS calibration models. The standard error of prediction ranged between 0.6 and 1 ppm, with a relative standard error of prediction of 3–6%. They concluded that the sensor in combination with the evaluation procedure could be used directly to perform routine continuous on-line monitoring of the bioprocess.

A review describing several chemometric methods for the evaluation of NIR spectra is presented by Roggo et al. (2007) discussing several applications in pharmaceutical technologies.

Theory and application of MIR spectroscopy in bioprocess monitoring

MIR theory

The greater resolution of MIRS enables the detection and quantification of components in aqueous solutions at significantly lower concentrations than is achievable with NIRS. In particular, MIRS has advantages for the detection of organic compounds in complex culture media owing to the possibility of identifying them on the basis of their typical fingerprint.

Practical on-line applications have been made possible by the development of suitable fiber optic probes using ATR spectroscopy. ATR spectroscopy utilizes the total reflection of an evanescent wave, which penetrates the sample touching the crystal. The absorbing path length (0.5–5 μm) can be enhanced by multiple reflections as shown in Fig. 4. A primary requirement for an ATR measurement is that the refractive index of the crystal be significantly larger than that of the sample. Typical ATR crystals have refractive indices at $2,000\text{ cm}^{-1}$ of about 2.4. The most appropriate material is diamond, whose cost disadvantage is compensated by its very long shelf life and high stability. The most commonly used materials are zinc selenide and germanium. ATR is the fastest IR technology and also has the highest level of reproducibility.

First attempts of real-time MIR measurements have been performed using ATR flow cells, whereby a sample stream is piped through the measuring chamber of the FTIR spectrometer in a bypass (Fig. 5a).

In the past, in-line MIR measurements were hindered by the lack of suitable fiber optic materials and the poor

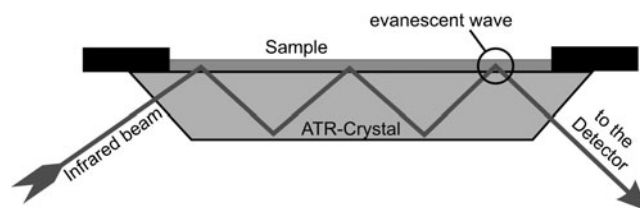


Fig. 4 Principle of ATR illustrating a threefold reflection for increased interaction with the sample

stability of the available ATR crystals. By reason of the high attenuation of the beam by the fiber optic materials, mirror combinations like the ReactIR[®] MP (Fig. 5b) have been utilized for the transport of the infrared beam towards the reactor and back to the spectrometer. The development of chalcogenide and silver halide fibers of high quality as well as the substitution of conventional ATR materials with diamond ATR crystals has led to the launch of several commercially available probes such as ReactIR[®] (Mettler Toledo Intl. Inc., Greidensee, Switzerland) or Reaction-View[®] (Remspec Corporation, Charlton, MA, USA). These probes are autoclavable and can be inserted in a reactor leading to the setup shown in Fig. 5c.

Applications of MIR via ATR probes for bioprocess monitoring

The bacterium *Gluconacetobacter xylinus* produces the food-grade exopolysaccharide gluconacetan. Kormmann et al. (2004) utilized attenuated total reflection-mid-infrared (ATR-MIR) for an on-line measurement of the substrate concentrations. This allowed the monitoring of the production and accumulation of the biomass by adding ethanol in a fed-batch process. The calibration was performed with synthetic solutions of the major medium components like fructose, acetate, ethanol, phosphate, and ammonium.

Roychoudhury et al. (2006) showed that ATR-MIR could be used to measure critical parameters like the biomass of a filamentous organism, despite effects such as an increase in the medium viscosity. Cultivations of the bacterium *Streptomyces clavuligerus* were used to create PLS models that predicted the concentrations of biomass, glucose, and ammonia with a maximum variation of 5%. The same group also demonstrated that the ATR-MIR approach with mathematical models could predict the concentrations of the product clavulanic acid in *S. clavuligerus* cultivations with a variation of approximately 2% (Roychoudhury et al. 2007a, b).

Schenk et al. (2006) used Fourier-transform-mid-infrared (FT-MIR) and ATR for the investigation of fed-batch cultivations of the methylotrophic yeast *Pichia pastoris*. The observed concentrations of methanol were validated via high-performance liquid chromatography

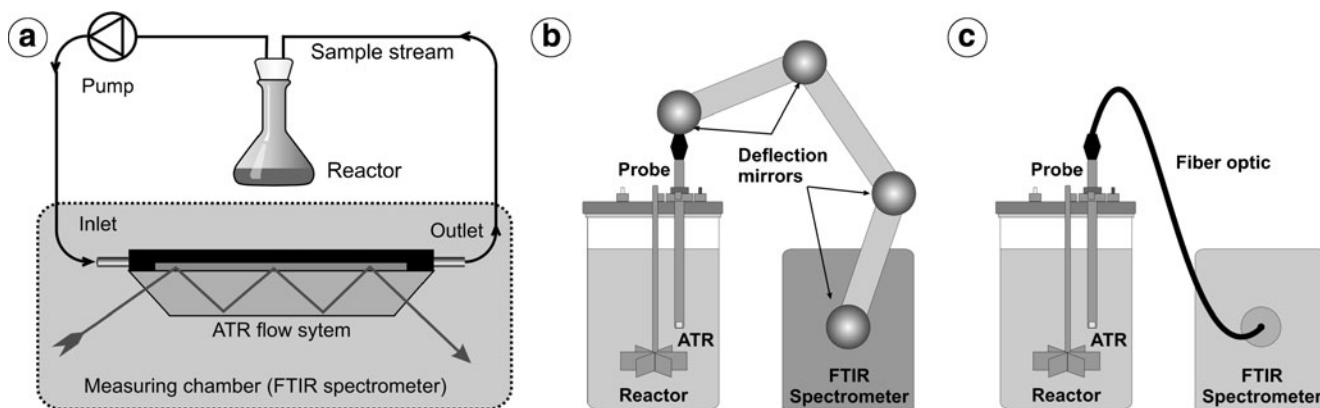


Fig. 5 Different setups for real-time measurements: **a** ATR flow system, **b** FTIR-arm based on mirror technology, **c** experimental setup using fiber optics

(HPLC). This work shows that time-consuming calibrations of the spectrometer can be avoided by analyzing only one component of the cultivation medium. It was assumed that only the substrate concentration changed significantly during the cultivation, and the absorption is proportional to the concentration. Only two spectra were used for a linear calibration. The on-line measurements were in good agreement with the off-line HPLC analysis (variations of 1–10%).

Mazarevica et al. (2004) used the common combination of FT-MIR and ATR. They present mathematical models for the prediction of yeast cultivations with a variation of <1% for the concentrations of glucose and ethanol. The models were created with data from only three cultivations. Common problems during the measurements caused by gas bubbles and biofilms on the ATR surface could be avoided by using an automated flow system.

Trevisan and Poppi (2008) also used this combination of FT-MIR and ATR for the investigation of a biotransformation process. They observed different biotransformation reactions in *Saccharomyces cerevisiae* for the creation of two intermediates and the final product. The creation of the mathematical models was based on PLS regression. The concentrations of the substances could be predicted with a variation of approximately 5%.

Sellick et al. (2010) uses FT-MIR spectroscopy to predict antibody concentrations in mammalian cell cultivation. The used spectroscope was equipped with a deuterated triglycine sulfate detector. Measurements were performed in samples of CHO and NS0 (murine myeloma) cell cultivations. The spectra were normalized by PCA. Antibody concentrations could be predicted with a low error (8% and 9%, respectively) compared with the ELISA data.

Other applications of MIR for bioprocess monitoring

Franco et al. (2006) investigated the cultivation of the kombucha mushroom, which contains several different

microorganisms like *Acetobacter* and yeast species, with a barium fluoride cell for the FT-MIR interface. They predict the concentrations of the substrate (glucose) and the product (gluconic acid) with an accuracy of 2%. The data were analyzed with several multivariate techniques, including PLS and ANN.

Theory and application of NIR spectroscopy in bioprocess monitoring

NIR theory

Most of the bands of the near-infrared result from overtones (in the region below $5,500\text{ cm}^{-1}$) and from vibrations of the central IR region (in the region between 400 and $4,000\text{ cm}^{-1}$). The stretch vibrations of the hydrogen bonds in the region between $3,600$ and $2,400\text{ cm}^{-1}$ hold great potential for the identification of compounds that contain hydrogen bound to a heteroatom. In addition to aliphatic or aromatic carbon compounds with their C–H bonds, the N–H bonds of proteins and the O–H bonds of alcohols are also important targets. Since IR radiation is low in energy, it is simply absorbed by molecules with the relevant covalent bonds with the possibility that the dipole moment of rotations or vibrations will change. According to the definition of the American Society for Testing and Materials, the NIR region lies between the wave numbers of $4,000$ and $13,000\text{ cm}^{-1}$. As a result of the overtones and the low energy, the absorption characteristics of NIR spectra are poorly developed, and spectrometers with high signal to noise ratios are required. For interfacing the spectrometer with the bioprocess, there are the possibilities of an in situ (or in-line) and an ex situ measurement approach. While for in situ measurements a fiber optic probe, which is directly immersed into the fermentation broth, is utilized, the ex situ approach can be realized by either using a flow-through cell

or loop or by a reflectance probe on the glass wall of the reactor. The influence of the probes to the spectra is described in a detailed review by Cervera et al. (2009). It is also shown what effects the fermenter conditions and the organism have on the generated spectra.

Applications of NIR to bioprocess monitoring

The usage of Fourier-transform near infrared spectroscopy (FT-NIR) spectroscopy for the simultaneous determination of multiple compounds of drug fermentation process and the development of a precise NIR calibration that is independent of the reactor scale was described by Rodrigues et al. (2008). Clavulanic acid, a β -lactamase inhibitor, is produced in a fed-batch process with *S. clavuligerus*, a filamentous microorganism. The measurements were performed in situ, utilizing transfection probes that were in direct contact with the medium in both pilot-scale and industrial-scale bioreactors. The spectra that were obtained from the in situ measurements were used as inputs to PLS-based models. The RMS errors were 0.03% (w/w) for the clavulanic acid concentration, 150 cp for the viscosity, 0.01% (w/w) for the nitrogen sources, and 0.4% (w/w) for the carbon sources.

Petersen et al. (2009) used semisynthetic samples for the calibration of an NIR spectroscope. These samples contain fermentation broth of five different fermentations, different amounts of glucose, ammonium and biomass of *Streptomyces coelicolor*. The prediction of the glucose concentration shows an error respectively of approximately 5% and 16% for ammonium.

Holm-Nielsen et al. (2008) used NIR spectroscopy for the process monitoring of a thermophilic anaerobic digestion of a mixture of manure and organic industrial food waste boosted by glycerol. Representative sampling within the very heterogeneous fermentation mixture was made possible through the using of a transflexive embedded near-infrared sensor integrated into an external recurrent loop on the fermentation reactor. A PLS regression was used to determine the glycerol concentration from the spectral data. The same approach was applied for the concentrations of the volatile fatty acids (acetic, propionic, iso-butanoic, butanoic, iso-valeric, and valeric acid). Good correlations were obtained between the on-line NIR measurements of the glycerol and volatile fatty acid concentrations and analytical laboratory results.

In-line NIR for the monitoring and control of the fermentation process of *Staphylococcus xylosus* ES13 was used by Tosi et al. (2003). This approach was used to measure the concentrations of biomass, glucose, lactic acid, and acetic acid through the utilization of an intertance fiber optic probe, which was immersed into the culture broth and connected to a NIR instrument. For the prediction

of the concentrations, PLS regression models of the second derivative of the NIR spectra in the 700–1,800 nm region were used. The fermentations were performed as batch, repeated batch, and continuous process and thereby successfully automatically controlled through the interfacing of the NIR to the bioreactor control unit.

A comparison of the NIR spectra collected in-line during the fermentations of *S. xylosus* ES13, *Lactobacillus fermentum* ES15, and *Streptococcus thermophilus* ES17 shows the successful transfer of a calibration model originally developed for *S. xylosus* ES13 to other strains, which have different growth patterns but were cultivated in the same medium under the same fermentation conditions. The NIR approach was found to be general enough that several biochemical parameters of the different strains could be determined via only slight changes in the model without a separate calibration for each individual strain.

Crowley et al. (2005) used NIR spectroscopy for high cell density industrial *P. pastoris* fed-batch cultivation for the production of a therapeutic mammalian protein. A complex medium was used in this cultivation, resulting in chemical and physical changes in the liquid phase due to cell growth. Furthermore, both glycerol and methanol were used as carbon sources, added at different rates at different times, and thus, this application presented a considerable challenge for the use of NIR spectroscopy for modeling the key analytes. Models for the biomass and the concentrations of glycerol, methanol, and the protein product were developed, and different methods of spectral collection and mathematical procedures were used depending on the analyte. The models for the biomass, glycerol, and methanol emerged as good external validation methods, while the model for the product concentration delivered poor results.

A comparison of two different on-line probes for biomass measurements in bioreactor cultivations, one based on NIR light absorption spectroscopy and one based on dielectric spectroscopy, was performed by Kiviharju et al. (2007). These two probes were used to estimate the biomass concentration during cultivations of *Bacillus subtilis* 168; *Bifidobacterium longum* ATCC 15707, and DSM 14583; *Escherichia coli* XL-1 blue strains QMX-8b und TXC-1a; *Lactobacillus plantarum* BPT 197 and BPT 186; *Lactococcus lactis* GRS71, NZ9800, NZ9010, and the mutant (ptrfut5) Probat 505; *S. cerevisiae*; and *Streptomyces peucetius* var. *caesius* N47. Differences between the results obtained off-line and from the NIR probes revealed that the factor with the highest impact on the results was the cell shape. Overall, the authors concluded that NIR spectroscopy is further developed and more dependable for on-line biomass analysis than is dielectric spectroscopy; however, the usefulness of NIR is limited when the cultivation medium is very turbid or when adsorbents or solid matrixes are used to support microbial growth.

Nordon et al. (2008) used NIR spectroscopy for in situ monitoring of the seed stage of a *Streptomyces* fermentation process. They found that the main spectral change occurred in the first derivative spectrum at a wave number of $7,263\text{ cm}^{-1}$. By comparison with off-line measurements of the spectra of components of the fermentation broth, it was possible to relate this wave number with the biomass concentration. The biomass signal stayed constant for the first 20 h of the seed stage, and then decreased briefly before it finally increased. At the same time as this minimum of the biomass concentration, a maximum in the carbon dioxide evolution rate could be measured. By relating these changes in the spectrum to the growth and the fragmentation of mycelia, a change in the metabolic activity was indicated, which made it possible to determine the optimal transfer time for the seed stage of a fermentation via in situ NIR spectroscopy.

Soons et al. (2008) applied a controlled random search procedure for the selection of an optimal window of wavelengths giving a good calibration model for the biomass concentrations during the cultivation of *Bordetella pertussis*. Furthermore, it was possible to overcome difficulties of interpreting IR spectra that result from the large number of measured wavelengths and the overlapping of absorption peaks. IR-based predictions of the biomass concentration were compared with assessments obtained with a software sensor for biomass and the specific growth rate, derived from standard measurements of oxygen consumption. This comparison revealed that the IR-based predictions depend on the quality of the training dataset, which needs to compass all possible sources of temporary disturbances like pH and dissolved oxygen. If these phenomena are not included in the training data, then the software sensor was the better choice for the feedback control of the biomass concentration and the specific growth rate.

At-line as well as in situ NIR spectroscopy was used by Arnold et al. (2002) for the monitoring of the biomass concentration in a complex industrial *E. coli* fed-batch process. Factors including a range of temperature changes and vigorous stirring and aeration represented serious challenges to the in situ measurement. Good results were obtained using both modes of NIR measurements. The authors noted that signal optimization were necessary for in situ measurements because of the loss of wavelength regions and an increased background signal. Although the modeling process based on at-line measurements was a valuable guide for the formulation of models for in situ data, the structure of the two models may differ significantly.

The possibility of monitoring state transitions arising from metabolic and morphologic changes of the microorganisms during fermentation via the analysis of on-line NIR spectra by sample-sample two-dimensional correlation

spectroscopy (SS-2DCoS) was investigated by Ferreira and Menezes (2006). SS-2DCoS has been proven to be helpful for extracting process information directly from on-line measurements to establish the relationship between two different samples. In this report, the authors monitored the process of the industrial fermentation of *S. clavuligerus* in complex medium for the production of an active pharmaceutical ingredient. The complexity of this process and the great number of metabolic reactions involved prohibited the direct determination of the concentration profiles of all of the relevant metabolites. However, it was possible to maintain relevant information concerning the fermentation state transitions. SS-2DCoS revealed the moments at which metabolic or morphologic changes occurred. For process monitoring, this might be as important as the knowledge about the concentrations of particular metabolites.

Vaidyanathan et al. (2003) investigated the influence of cell morphology on the NIR-based determination of mycelial biomass in cultivations of *Streptomyces fradiae*. The use of optical techniques for the measurement of the concentration of mycelia-forming microorganisms is challenged by the morphological heterogeneity of the culture. In this study, a variant of *S. fradiae* was used that had been modified to form multiple different morphological shapes. For the characterisation of these morphologies, a computer-based picture analysis was used. PCA was applied for the determination of the variation of the single spectral measurements and for the correlation with the different morphologies. Although the NIR transmission spectrum was dependent on the morphology, the influence was lower than in the visible region. Furthermore, the spectral information at longer wavelengths (1,600–2,350 nm) was more robust with respect to morphological differences. Hence, the information in the long-wave NIR region was the most useful for determination of the biomass concentration during fermentations of mycelia-forming microorganisms.

NIR spectroscopy also finds applications in mammalian cell culture. Henriques et al. (2009) describe the usage of NIR for these processes. Spectra of five 10-L batch cultivations were used for an adequate prediction of the concentrations of glucose, lactate, cell density, and ammonia. The publication describes the chemometric validation very detailed. An included case study pictures the development of the models for quantitative validation.

Arnold et al. (2003) applied NIR spectroscopy for the in situ analysis of the four key analytes glucose, lactate, glutamine, and ammonia in a CHO-K1 mammalian cell culture in serum-free medium for the production of a human protein. A steam-sterilized fiber optic probe was used. Models for the key analytes were developed that yielded estimates standard errors of 0.399 mM for glucose, 0.162 mM for lactate, 0.308 mM for glutamine, and

0.036 mM for ammonia. This was the first demonstration of the use of an in situ fiber optic probe with a model to measure multiple analytes at very low concentration ranges in a fed-batch process with temperature changes.

A novel calibration strategy for the measurement of the two key analytes in a CHO cultivation, glucose and lactate, was developed by Roychoudhury et al. (2007a, b). The use of NIR for bioprocess monitoring has been restricted by its dependence on a calibration derived from a single bioreactor. In this work, a multiplexed calibration technique was applied using several bioreactors operating in parallel. The model predictions based on this approach were shown to have only slightly inferior values than those from the common calibration and measurement method with one bioreactor: the r^2 values for glucose were 89.4% with the single bioreactor vs. 88% for the multi-reactor set and 92% vs. 91.8% for lactate. In addition, the number of factors increased because the model incorporated the inter-probe variability. Since on-line models could be successfully set up and confirmed by external measurements, this first realization of multiplex-NIR spectroscopy for the monitoring of multiple bioreactors is a good approach for decrease in time required and improvement of process control.

Applications of combined IR measurements

While individual MIR- or NIR-based measurement approaches are clearly useful, additional value may be obtained by combining different types of IR monitoring or through combinations of IR and other advanced measurement methods.

Sivakesava et al. (2001) performed a comparative investigation of FT-MIR, NIR, and FT-Raman spectroscopy. Data from these three techniques were used for mathematical modeling of a *Lactobacillus casei* cultivation process with the goal of predicting the concentrations of glucose, lactate, and biomass. The respective spectra were analyzed with PLS and principal component regression. The applications of NIR and FT-Raman spectroscopy yielded predictions with accuracy of 4–14%. The predictions based on the FT-MIR spectroscopy had variations of only 2%.

NIR spectroscopy and electronic nose mapping (ENM) were used by Navrátil et al. (2005) for monitoring and control of a cholera toxin-producing *Vibrio cholerae* fed-batch cultivation, carried out at laboratory and production scales. Models for the prediction of the biomass and the concentrations of glucose and acetate from the NIR spectra were developed using PLS regression. The standard errors of the predictions from these models were 0.20 g/L for the biomass, 0.26 g/L for the glucose, and 0.28 g/L for the

acetate. To account for disturbances caused by aeration, a compensation algorithm was incorporated into the model calculation, improving the predictions, especially for the biomass concentration. This compensation was obtained by combining in-line ENM data (identified using PCA) with the NIR data to generate a trajectory representing the fed-batch cultivation. A correlation between the ENM results and the cultivation progress could also be determined and was helpful during the monitoring of the cultivation quality. It was possible to easily detect variations from the normal cultivation behavior and the trajectory improved the detection of bacterial contaminations. The concentration of the cholera toxin product could be predicted with an accuracy of 0.020 g/L using the NIR data. The on-line biomass predictions from the NIR data were used for the control of the acetate production of the *V. cholera* culture. The controller compared the actual specific growth rate with the prediction of the growth rate for the critical acetate concentration and calculated the glucose feed rate from the difference, so that the critical acetate concentration was always avoided. This way, the limitation effect of this by-product could be minimized.

General conclusions

NIR and MIR spectroscopy offers the possibility of gaining a direct non-invasive insight into the medium composition without a time delay. Modern chemometric methods are necessary to acquire valid data of the complex spectra; however, the large numbers of application show the potential of this spectroscopic monitoring technique. In the future, better interface components will improve the broad applicability and even the possibility to apply the method to single-use bioreactors.

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