Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–7, 2016

www.elsevier.com/locate/jbiosc

Evaluation of a newly developed mid-infrared sensor for real-time monitoring of yeast fermentations

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Received 9 June 2016; accepted 8 December 2016
Available online xxx

A mid-infrared (MIR) sensor using the attenuated total reflection (ATR) technique has been developed for real-time monitoring in biotechnology. The MIR-ATR sensor consists of an IR emitter as light source, a zinc selenide ATR prism as boundary to the process, and four thermopile detectors, each equipped with an optical bandpass filter. The suitability of the sensor for practical application was tested during aerobic batch-fermentations of *Saccharomyces cerevisiae* by simultaneous monitoring of glucose and ethanol. The performance of the sensor was compared to a commercial Fourier transform mid-infrared (FT-MIR) spectrometer by on-line measurements in a bypass loop. Sensor and spectrometer were calibrated by multiple linear regression (MLR) in order to link the measured absorbance in the transmission ranges of the four optical sensor channels to the analyte concentrations. For reference analysis, high-performance liquid chromatography (HPLC) was applied. Process monitoring using the sensor yielded in standard errors of prediction (SEP) of 6.15 g/L and 1.36 g/L for glucose and ethanol. In the case of the FT-MIR spectrometer the corresponding SEP values were 4.34 g/L and 0.61 g/L, respectively. The advantages of optical multi-channel mid-infrared sensors in comparison to FT-MIR spectrometer setups are the compactness, easy process implementation and lower price.

[**Key words:** Real-time monitoring; Fermentation of *Saccharomyces cerevisiae*; Monitoring of glucose and ethanol; Mid-infrared sensor; Fourier transform mid-infrared spectroscopy; Attenuated total reflection]

In recent years, there has been a strong demand for effective monitoring of biotechnological processes to increase productivity and to ensure product quality. Bioprocess monitoring is limited by its lack of well-established and reliable real-time information on educts and products (1,2). Therefore, control strategies are often based on mathematical models, which use correlations between directly measureable parameters and significant process parameters (3,4). In general, chemical and physical parameters such as dissolved oxygen concentration, pH and temperature are obtained using conventional in-/on-line probes and sensors (5,6). However, the determination of medium components like substrates (e.g., glucose or other sugars) and products is performed by time-consuming established off-line analysis, such as mass spectrometry (MS) or high-performance liquid chromatography (HPLC). For these conventional analytical methods, it is necessary to retrieve samples from the bioreactor, which increases the possibility of contamination. Furthermore, pretreatment of samples by specific labor-intensive equipment is required. In contrast, real-time process monitoring eliminates sample collection and preparation and facilitates timely intervention (5).

For a wide field of applications real-time analytical methods based on optical spectroscopy have great potential for process monitoring, especially in the chemical, pharmaceutical, and biotechnological industries. Optical measurement methods have the advantage to be fast and non-invasive. Since organic compounds have characteristic absorption bands due to vibrational excitation of the molecule, infrared spectroscopy enables simultaneous determination of multiple components within a few minutes (7,8). In the mid-infrared (MIR) region, wavenumber range from 4000 cm^{-1} to 400 cm^{-1} (wavelengths 2.5–25 μm), absorption bands mainly originate from fundamental vibration modes. In contrast, a variety of overtones and coupled modes are observed in the near-infrared (NIR) region from 12,500 cm^{-1} to 4000 cm^{-1} (0.8–2.5 μm), resulting in broad and strong overlapping absorption bands (9,10). Furthermore, the molar absorptivity in the MIR region is several orders of magnitude higher, which offers superior sensitivity against NIR spectroscopy. This results in typical detection limits for dissolved analytes of 100 ppm (0.01 wt%) for MIR and 1000 ppm (0.1 wt%) in case of NIR (11). On the other hand, the NIR region has the advantage that quartz glass can be used as optical material for cuvettes and optical fibers. Both MIR (5,7,10,12–21) and NIR (6–8,10,22–30) spectroscopy have been successfully applied for bioprocess monitoring in the liquid phase. For example, Grassi et al. (13) analyzed samples of beer fermentations from two different *Saccharomyces cerevisiae* strains by off-line FT-MIR spectroscopy. Multivariate curve resolution-alternating least squares

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.12.005

(MCR-ALS) models were developed to describe the fermentation progress of maltose conversion to ethanol (13). Doak and Phillips (15) used a diamond attenuated total reflection (ATR) probe to record in-line IR spectra of *Escherichia coli* fermentations. Partial-least-squares-based calibration and prediction models were developed for the analytes glucose and acetate using in-process samples. The resulting models had prediction errors, which were statistically equivalent to the estimated experimental errors obtained from off-line reference measurements. In a bubble column reactor, Mueller et al. (16) determined the concentration of lauric acid as well as the products mono-, di-, and trilaurin in multiphase systems by in situ mid-infrared spectroscopy. In case of near-infrared spectroscopy, Tamburini et al. (24) applied this technique for on-line and in-line monitoring of cell, substrate and product concentrations by multivariate data analysis, during aerobic and anaerobic bacterial fermentations. Alves-Rausch et al. (25) described the application of in situ NIR spectroscopy in combination with multivariate data analysis to monitor key parameters including sugar and acetoin in a *Bacillus subtilis* fermentation under industrial conditions.

A typical measurement setup for process monitoring in the liquid phase using infrared spectroscopy consists of a spectrometer (e.g., FT or grating spectrometer), a light source and a fiber optical immersion probe (transmission, reflection or ATR probe) in direct contact to the investigated process (31). The emission of the light source is transmitted by illumination fibers to the probe and into the sample, where it is partially absorbed. The non-absorbed light is transmitted by receiving fibers from the probe to the spectrometer for its detection. For many industrial applications, it is mandatory that the spectrometer and the light source are placed in an explosive and spray water protected area in distance to the process. The implementation of a complete MIR or NIR measurement system in a production plant currently exceeds US$50,000, depending on process and safety requirements. NIR spectrometer setups commonly use transmission probes with optical path lengths in the range of millimeters and centimeters due to the small molar absorptivities in this spectral region. The transmission probe is connected via quartz fibers (fused silica) to the light source and the spectrometer (32). The low self-absorbance of quartz for near-infrared light allows fiber lengths of more than 100 m (33). In contrast to NIR, MIR spectroscopy of liquid samples requires optical path lengths in the order of micrometers originating from the high molar absorptivity of the sample constituents (34). A sampling technique to realize such small path lengths is ATR (35). During total reflection, the incident light beam penetrates less than one wavelength into the sample and thereby can be partially absorbed. This measurement method is predominantly applied for the investigation of liquid samples in the MIR region (15,36–39). The ATR technique enables to monitor key analytes in a bioreactor despite the high water content, since the small realized optical path lengths lead to absorbances A of less than one ($A < 1$), where Beer–Lambert law is valid. In case of NIR spectroscopy the strong water absorption above 1800 nm (below 5500 cm^{-1}) requires optical path lengths of less than 1 mm. If transmission probes with an optical path of several millimeters are installed to monitor fermentation processes, the evaluation of NIR spectra is restricted to the spectral region from approximately 800 nm–1800 nm. Since quartz fibers offer high self-absorbance for mid-infrared light, optical fibers made of mixed silver halides or chalcogenides have to be used in the MIR region (39). However, the length of those fibers is restricted to less than five meters (12,40). Furthermore, these fragile materials become opaque over time. The lack of suitable fiber optic materials makes it difficult to implement MIR spectroscopy in a process environment (10). This is one reason why NIR spectroscopy in contrast to MIR spectroscopy has become a well-established analytical tool in chemical, pharmaceutical, and biotechnological industries (12,41).

Another approach for the implementation of MIR technology for monitoring of the liquid phase could be the use of a measurement setup avoiding optical fibers, which is directly mounted to the process. The principle design of such an instrument could be similar to IR gas analyzers. These devices are widely used in the chemical and biotechnological industries to monitor the chemical composition in exhaust lines (42). IR gas analyzers consist of a thermal IR emitter as light source, a transmission cuvette containing the gas to be analyzed, and pyroelectric or thermopile detectors equipped with optical bandpass filters. To be specific to a certain analyte, the optical bandpass filter shows high transmission in a spectral region where the analyte absorbs and blocks all wavelengths outside the defined bandwidth (43). There are standard bandpass filters available for the detection of the most important analytes such as carbon dioxide, carbon monoxide, nitric oxides, ammonia and volatile hydrocarbons like methane (42). In order to monitor the liquid phase using the basic concept of IR analyzers, the transmission cuvette has to be replaced by an ATR setup to realize the desired optical path lengths of several micrometers. This approach could lead to compact, robust and low-cost mid-infrared sensors for direct process implementation without any fibers or movable parts.

In this paper, we report on the results using a four-channel prototype MIR-ATR sensor for real-time process monitoring of an aerobic yeast fermentation. Advantages of this multi-channel ATR sensor in comparison to FT-MIR spectrometer setups are the easy process implementation and lower price (cost of all parts: US$2000–5000, depending on ATR material and optical bandpass filters). The sensor, which is described in detail in a recent publication (43), uses a thermal radiator as light source, an ATR prism made of ZnSe and four thermopile detectors, each equipped with an optical bandpass filter. In that work the esterification of ethanol and formic acid to ethyl formate and water was monitored using the developed MIR-ATR sensor. Thereby, the high concentrations of educts and products facilitated the successful application of the sensor. In contrast to chemical reactions the matrix of many biotechnological processes are dominated by water. Therefore, the concentrations of analytes such as substrates and products are comparatively low. Furthermore, the complex composition of fermentation media leads to overlapping absorption spectra of the different analytes. To overcome these circumstances a multiple linear regression (MLR) model was applied to link the measured absorbances in the four optical channels to the analyte concentrations (44–46). For the MLR calibration the analyte concentrations were obtained by high-performance liquid chromatography (HPLC) as reference method. The suitability of the MIR-ATR sensor for practical application in biotechnology was tested during aerobic batch-fermentations of *S. cerevisiae* by simultaneous real-time monitoring of carbon source glucose and product ethanol. The sensor performance was compared to a commercial FT-MIR spectrometer with diamond ATR module.

MATERIALS AND METHODS

Cultivation conditions and instrumentation of bioreactor A commercial baker's yeast (Wieninger Hefe, F.X. Wieninger GmbH, Passau, Germany) of species *S. cerevisiae* was used as inoculum for fermentation processes. The culture medium was prepared in deionized water and contained the following substances per liter: 16.21 g NH_4Cl; 1.5 g $MgSO_4 \times 7\ H_2O$; 5.57 g $NH_4H_2PO_4$; 3.0 g KCl; 0.1 g Ca-Pantothenate $\times\ H_2O$; 0.2 g m-inositol; 1.0 mg biotin; 0.8 g $CaCl_2 \times 2\ H_2O$; 0.05 g $FeCl_3 \times 6\ H_2O$; 0.03 g $ZnSO_4 \times 7\ H_2O$; 0.05 g $MnSO_4 \times H_2O$; 8.0 mg $CuSO_4 \times 5\ H_2O$; 100.0 g D(+)-glucose and 1.0 mL antifoam agent (Dow Corning RD, article number 632134D, VWR BDH Prolabo, Leuven, Belgium). Commercial baker's yeast (167.0 g, dry yeast biomass content 30 wt%) was added as inoculum to the culture medium of fermentation volume $V_F = 5$ L. Therefore, the initial dry yeast biomass concentration was 10 g/L. Bioreactor and equipment were sterilized by a solution of 70% ethanol in water. Process conditions were set to a temperature of 32°C, aeration of 1.0 vvm, and

Please cite this article in press as: Schalk, R., et al., Evaluation of a newly developed mid-infrared sensor for real-time monitoring of yeast fermentations, J. Biosci. Bioeng., (2016), http://dx.doi.org/10.1016/j.jbiosc.2016.12.005