On-line measurement of the glycerol concentration in a *Pichia pastoris* fermentation using FT-IR/ATR

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Abstract
The glycerol concentration was measured on-line in a *Pichia pastoris* fermentation process using Fourier Transform Infrared (FT-IR) spectroscopy, utilizing an Attenuated Total Reflection (ATR) tunnel cell. The calibration models for this quantitative measurement were calculated with the Partial Least Squares (PLS) method. The accuracy achieved, when High Performance Liquid Chromatography (HPLC) was used as the reference method, was a Standard Error of Prediction (SEP) of 1.6 g/L.

Introduction and purpose
In the strive towards better understanding of fermentation processes, the availability of on-line measurement systems, that monitors the chemical composition of the fermentation broth, is considered a key factor. FT-IR spectroscopy, in particular when combined with the ATR-technique, is a promising alternative for this purpose. Since FT-IR spectroscopy measures the fundamental vibrations in molecular bonds, the technique enables both qualitative and quantitative measurements, i.e. a compound can both be identified and quantified. Furthermore, using mathematical algorithms, unknown components that appear in the fermentation broth can also be detected and identified. In this study, the usability of FT-IR/ATR spectroscopy for on-line measurement of the glycerol concentration in a C-5 epimerase producing *Pichia pastoris* fermentation was investigated.

Methods
The fermentations were carried out in a 3.5 L Chemap CF3000 bioreactor, with a working volume of 2 L. The studied organism was a recombinant Mut’ (methanol utilization positive) strain of *Pichia pastoris*. The part of the process that was monitored with the FT-IR was the glycerol batch phase. The FT-IR was a WorkIR 100 from Bomem Inc., equipped with a cylindrical ZnSe ATR tunnel cell, TNL-131 C, from Axiom Analytical. Calibration models were calculated with the PLS-method, using the software PLSplus/IQ ver. 3.0. The reference measurements of glycerol were carried out with a HPLC equipped with a Fam-pak column (Waters, Millipore Corporation). The calibration models were based on pure glycerol spectra and spectra collected from off-line samples of the fermentation broth during five previous fermentations. In the collection of these absorbance spectra water was used as the reference substance. Five spectra were collected for every sample, and each spectrum consisted of 16 co-added cosine-apodized scans at 4 cm⁻¹ resolution. In the on-line setup the fermentation broth was continuously pumped to the ATR in a re-circulation loop configuration. The sampling interval was one minute. The initial reactor content was used as reference in the on-line application. The on-line measurement was tested in two fermentations.

Results
When water was used as reference, the spectral features of the fermentation broth were dominated by components other than glycerol. In figure 1, a spectrum of the fermentation broth is plotted together with a pure glycerol spectrum with roughly the same glycerol concentration as the fermentation broth spectrum. As can be seen, the glycerol features are hardly visible in the fermentation broth spectrum. However, if this spectrum is subtracted with a spectrum from a fermentation broth sample in which the glycerol concentration was zero, i.e. from the end of the batch phase, the constant parts of the background matrix are removed. In this case it could be shown that the changes in the mid-infrared spectra during the glycerol batch phase are dominated by the changes in the glycerol concentration (figure 1).

In the first fermentation there was a lag phase of about 30 hours, which led to that only one reference measurement could be carried out during the period of exponential growth. However, as can be seen from figure 2, the trend displayed by the on-line measurement seemed reasonable and the experiment was therefore repeated in order to get enough reference measurements for the computation of the SEP.

In the second fermentation, 13 reference measurements were carried out and the SEP was calculated to 1.6 g/L. Figure 3 shows the on-line results. Based on these results the measurement application was successful. However, as figure 4 shows, a considerable drift in the baseline occurred during this measurement. Based on the appearance of the crystal, it was concluded that this had been irreversibly harmed by some compound in the fermentation broth. This compound was probably either amino acids or proteins.

Conclusions
FT-IR spectroscopy can be used for on-line measurement of the glycerol concentration in this *Pichia pastoris* fermentation process. The SEP of 1.6 g/L achieved in this study can be described as satisfactory. However, ZnSe is an unsuitable crystal material for this measurement application. On the other hand, since the changes in the mid-infrared spectrum during the glycerol batch phase are dominated by the changes in the glycerol concentration, it is suggested that this measurement application is a fairly easy to implement and that it would also be possible to use diamond-composite probes and possibly also Germanium crystals.