Pioneer experiences on PAT implementation of pharmaceutical biotech process development

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Introduction

By 2010, six biologics made the top twelve and eight biologics made the top twenty grades as the best selling global drug brands. Besides innovator products, biosimilar sales outside the USA exceeded 1 billion dollars.[1] These facts show the importance and magnitude of the pharmaceutical biotech industry. The process analytical technology (PAT) initiative was first proposed by the United States Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER) with the objective of achieving significant health and economic benefits by application of modern process control and tests in pharmaceutical manufacturing. The objectives for PAT implementation include better process understanding, reduction in the production cycle time by using on-line, at-line or in-line measurements for control, and cost reduction because of reduced waste and reduced energy consumption.[2] Near infrared (NIR) spectroscopy constitutes one of the major methods in PAT but a feasibility study should always be undertaken to show that NIR monitoring is possible for any particular application.[3]

Escherichia coli bacteria and Chinese hamster ovary (CHO) cell line are the two most significant expression systems applied in the biopharma field.[4] The utility of NIR monitoring was examined both for CHO[5,6] and *E. coli*[7,8] cultivations in the past. However, comparison of two different NIR techniques for monitoring a single expression system has not been published. Therefore the aim of our work was to execute a comparative feasibility study on whether dispersive NIR or FT-NIR technology is more applicable for monitoring a fermentation process executed with *E. coli*.

Both qualitative and quantitative analysis should be considered when monitoring fermentation by NIR. Qualitative analysis is only based on NIR spectra with or without pre-treatment. Using principal component analysis (PCA) on collected spectra the pathway of the bioreactor and a so-called process trajectory or process fingerprint can be derived. In this case, the overall behaviour is assessed, including changing physical and chemical properties. This method can be used for detection of disturbances and for comparison of different batches. Quantitative analysis involves developing partial least squares (PLS) models based on NIR spectra and the concentration values of compounds of the fermentation broth determined by a reference method.[9]

Materials and Methods

Samples and reference methods

To assess proper positioning of the NIR probe (pre-examinations) a simplified medium was used containing glycerol and water. The concentrations and ratio of these materials were the same as in the real fermentation broth. For real fermentation batches, *E. coli* cells were cultivated in BIOSTAT B plus Twin bioreactors (Sartorius Stedim Biotech, Aubagne, France) with 1 l working volume. Reference samples were taken every hour to provide wet chemistry values.

Concentrations of acetate (Ace), glycerol (Gly) and ammonium (NH_4^+) were measured using ion-selective membrane electrodes by BioProfile 300 equipment (Nova Biomedical, Waltham, MA, USA). Optical density (OD) was measured using an Ultrospec 500 pro (Amersham Biosciences, Uppsala, Sweden) visible spectrophotometer at $\lambda = 600$ nm.

Near infrared spectroscopy

Dispersive NIR spectra were collected every 5 min throughout the 21 h cultivation by an XDS Process Analytic MicroBundle Multiplexer (Foss NIRSystems Inc., Silver Spring, MD, USA) using 4 channels. Samples were scanned (32 scans co-added) from 800 to 2199.5 nm using an extended InGaAs detector. Data were collected every 0.5 nm (2800 data points per spectrum). FT-NIR spectra were collected every 3 min by a Bruker Matrix-F using 6 channels. Samples were scanned from12000 cm^{-1} (833.3 nm) to 4300 cm^{-1} (2325.6 nm) at intervals of 8 cm^{-1}. Transflectance immersion probes (Ingold) with 2 mm optical path length were used in both cases. Spectral data were processed using Vision 3.20 (Foss NIRSystems Inc., Silver

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Spring, MD, USA), Opus 6.5.92 (Bruker Optik GmbH, Germany), Statistica 9.1 (Statsoft Inc., Tulsa, OK, USA) and The Unscrambler 10.0 (Camo Software AS, Oslo, Norway) software.

Results and Discussion

Pre-examinations in simplified media

During fermentation monitoring by NIR the most evident spectral changes are the baseline shifts that are related to changes in physical properties (particulate matter, air bubbles). Aeration, stirring speed, temperature, pH and feeding flows also cause smaller alterations in the spectra.[9] As the process examined is carried out at constant aeration, temperature and pH, we decided to explore the effect of stirring speed (agitation) on the light scattering (signal to noise ratio) in a simplified medium without *E. coli* cells.

Aeration was kept at a constant level of 0.5 $l.min^{-1}$ but the agitation rate (rpm) was increased continuously. The effect of probe positioning (0°, 90°, 180° and 270°) and the level of agitation (400 rpm, 600 rpm, 800 rpm, 1000 rpm, 1200 rpm, 1400 rpm, 1600 rpm and 2000 rpm) were assessed. By increasing the rpm of agitation serious baseline shifts occurred in all cases; these shifts were caused by the air bubbles behaving as optical elements (Figure 1). At higher rpm values, higher absorbance levels were detected as a consequence of the lower number of photons reaching the detector, this was due to dispersion at the contact surfaces of medium and air bubbles.

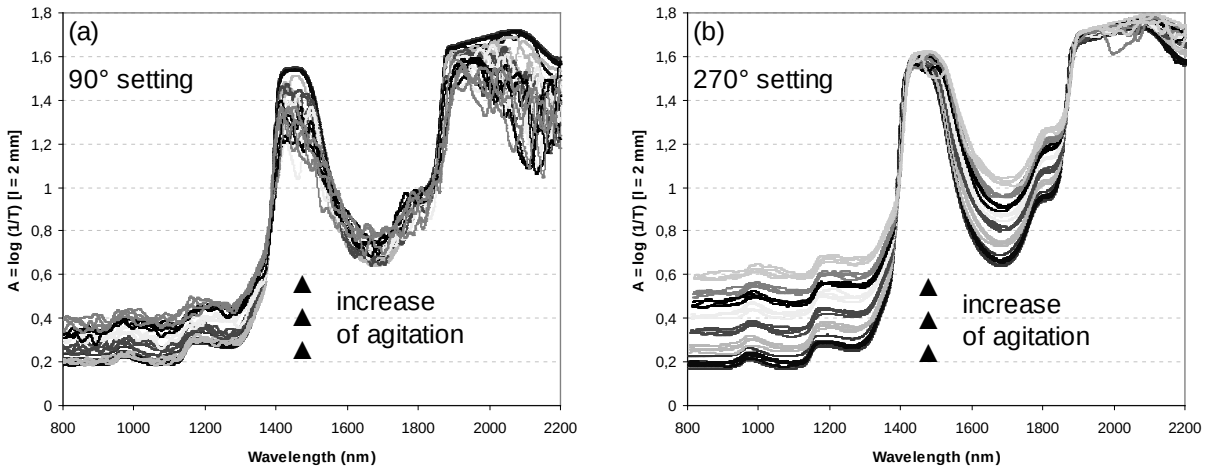

Figure 1. NIR spectra of glycerol-water mixture using 400 600, 800, 1000, 1200, 1400, 1600 and 2000 rpm agitation in the case of 90° (Figure 1a) and 270° (Figure 1b) settings.

The 270º probe position proved to be optimal when compared to the other settings (Figure 1). The probability of air bubbles entering the optical gap is the lowest in the 270º probe position, which results in a lower amount of light scattering. In further experiments some special protector elements were tested to eliminate the air bubble effect (data not shown), and the most effective of them was chosen for further work with real fermentation batches.

Qualitative analysis of fermentation batches

Four batches were monitored by dispersive NIR equipment. These batches were totally identical in terms of fermentation conditions, but two had a protector element surrounding the immersion probe (F_2 and F_4), while the other two had no protector element only 270º positioning of the probe (F_1 and F_3). Four other batches were monitored with FT-NIR. These four identical batches (F_5, F_6, F_7 and F_8) had a bigger variation in working volume compared to F_1, F_2, F_3 and F_4 runs but the process itself was the same at all the eight runs.

As the fermentation is a water-based system, we attempted to eliminate the effect of strong water absorptions in the NIR region. We decided to split the NIR region into four sub-regions and handle the spectra from the four sub-regions separately. These four sub-regions were the 3rd overtones (700–1100 nm), the 2nd overtones (1100–1550 nm), the 1st overtones (1550–2000 nm) and the combinations (2000–2500 nm). PCA was used for analysing all the eight runs with the four different sub-regions (32 analyses in total). We found that the spectra from the 3rd overtone (700–1100 nm) region had the most fermentation monitoring potential when using either dispersive NIR or FT-NIR (data not shown). This is probably a consequence of low water absorption in the 3rd overtones region compared the other three sub-regions.

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The batches with a protector element (F_2 and F_4) and the batches without a protector element (F_1 and F_3) can be easily distinguished when analysing the process fingerprints derived from dispersive NIR spectra (Figure 2a). It was also observed that the effect of the protector element disappears in the later phase of the run. The process trajectories derived from FT-NIR spectra (Figure 2b) exhibited differences between the four batches in the later phase of the run, which can be explained by the different working volumes of these runs.

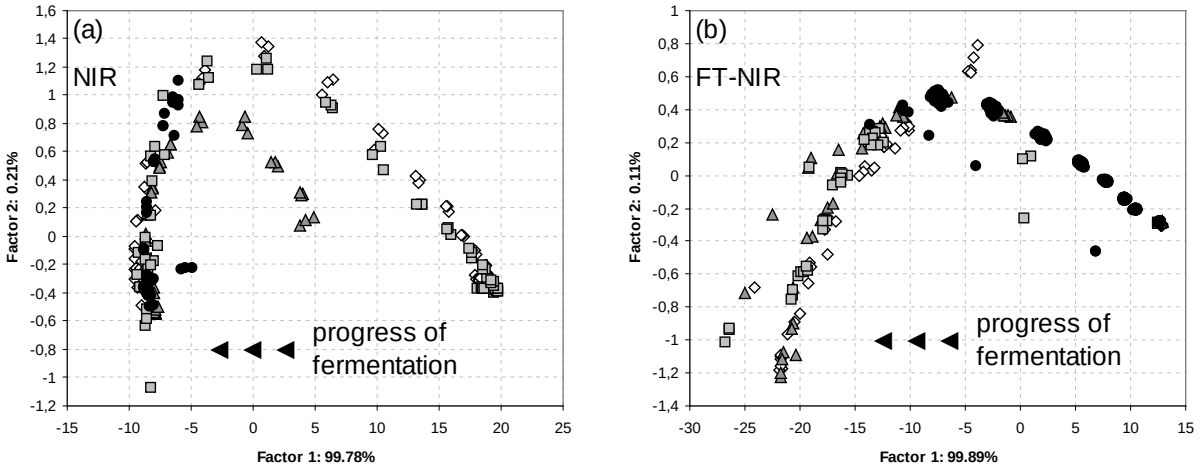

Figure 2. PCA score plots of 3rd overtone (700–1100 nm) spectra from fermentation batches F_1 (◇), F_2 (▲), F_3 (□), F_4 (●) (Figure 2a) and F_5 (◇), F_6 (▲), F_7 (□) and F_8 (●) (Figure 2b). Each point represents a spectrum from a time point of the run.

Quantitative analysis of fermentation batches

PLS calibrations were developed to predict concentrations of Ace, Gly, NH_4^+ and for OD, after checking the cross-correlations of reference data. For dispersive NIR datasets, different calibrations were processed in the case of Gly and Ace depending on whether the whole dataset was used or samples with Gly values under 2 $g.l^{-1}$ were ignored, or with Ace values equal to 0 $mmol.l^{-1}$ were left out, assuming some uncertainty of the reference method around these values. In the case of OD, fairly successful calibration and prediction could be achieved using either dispersive NIR or FT-NIR (data not shown), which is not remarkable as the reference method was also based on light absorption. The predicted concentrations of Ace, Gly and NH_4^+ throughout the fermentation process are shown in Figures 3 to 5.

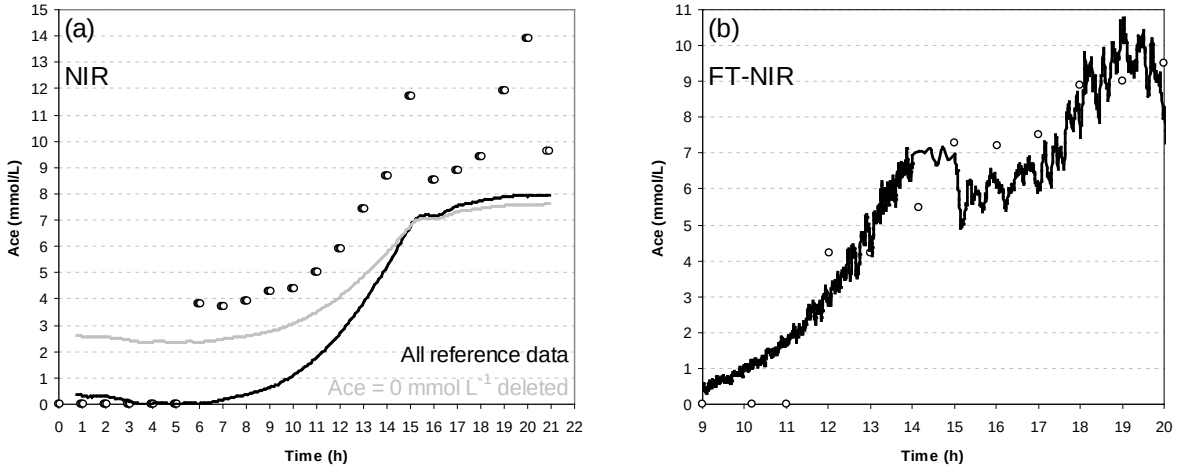

Figure 3. *E. coli* fermentation process accumulation profile for Ace ($mmol.l^{-1}$). NIR predicted values are lines smoothed by ten-point moving averages and reference chemical assays are circles.

The results in the case of Ace were acceptable in both cases. Ace prediction from dispersive NIR spectra (Figure 3a) is slightly under-predicted while PLS models based on FT-NIR spectra performed quite precisely (Figure 3b). This slight difference could be also caused by the uncertainty of the reference method.

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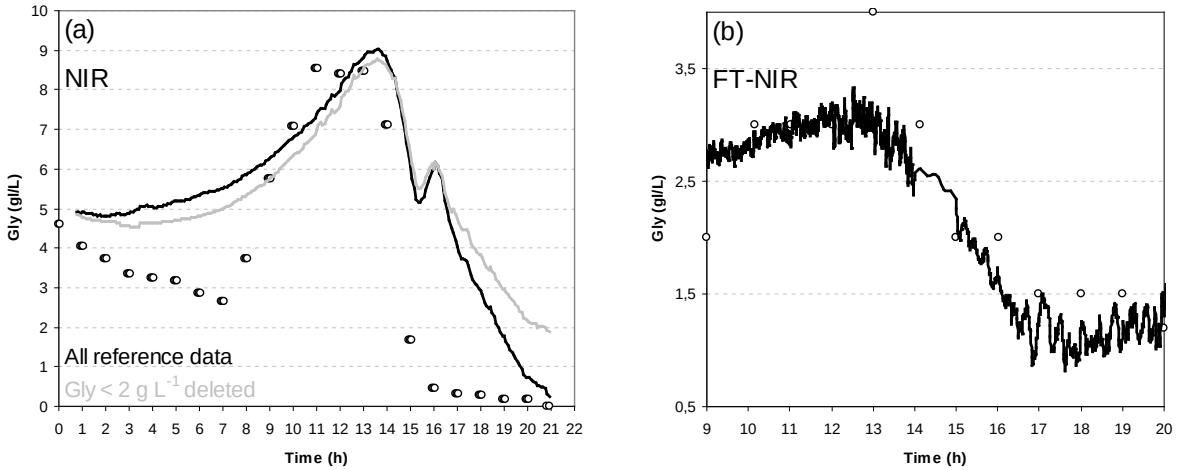

Figure 4. *E. coli* fermentation process accumulation profile for Gly ($g.l^{-1}$). NIR predicted values are lines smoothed by ten-point moving averages and reference chemical assays are circles.

As glycerol is fed during the process into the cultivation broth, glycerol is not only depleted in the system but some accumulation can also occur, so changes in Gly concentration are independent of cell proliferation. Therefore, Gly prediction is promising in both cases. The predicted values are closer to the reference values in the case of FT-NIR (Figure 4b) as compared to dispersive NIR spectra (Figure 4a).

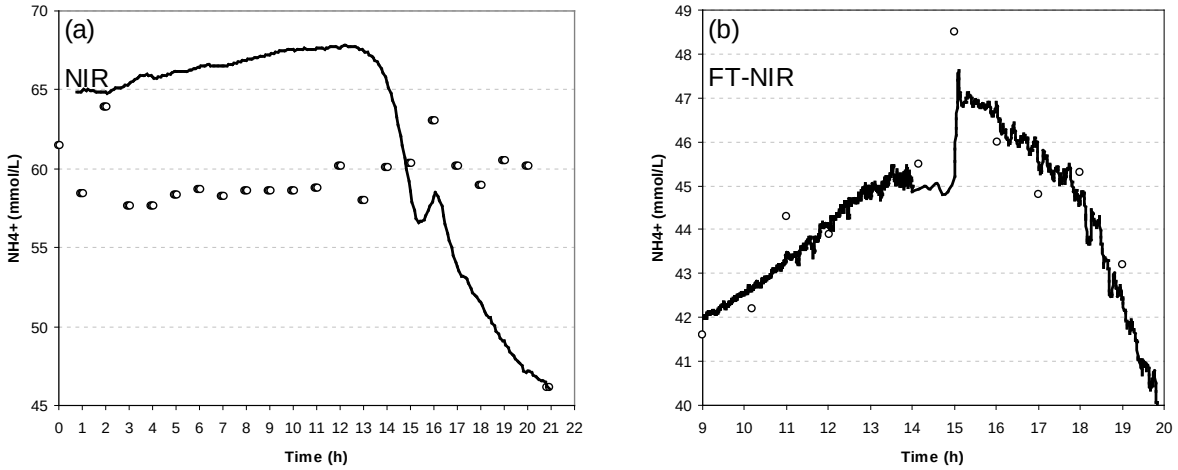

Figure 5. *E. coli* fermentation process accumulation profile for NH_4^+ (mmol L^{-1}). NIR predicted values are lines smoothed by ten-point moving averages and reference chemical assays are circles.

The models derived from dispersive NIR spectra for NH_4^+ showed a poor prediction capacity (Figure 5a) while FT-NIR based predictions are quite close to the concentrations of the reference assays (Figure 5b).

Conclusion

Based on our pre-examinations, we found the position of the NIR probe in the 1 l working volume system least affected by light scattering. Additionally we tested some protector elements to further decrease light scattering. By applying protector elements, the bubble effect was limited but it is important to mention that this influenced the process trajectory derived from NIR spectra by PCA. Qualitative fermentation could be monitored using either dispersive NIR or FT-NIR. Artificial process disturbances can be easily distinguished. In the system examined, spectra generated by the FT-NIR technique were slightly more accurate for quantitative prediction of substrate and metabolite concentrations than dispersive NIR spectra. In the case of NH_4^+ concentration there were significant differences between the two techniques. Improved models could be calculated by improving the accuracy of reference methods, increasing the number of parallel experiments and supported by further improvements in experimental design.

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