Lightnovo

APPLICATION NOTE

Quantification of nutrients and metabolites during fermentation

INTRODUCTION

In industrial production of chemicals by microbial fermentation, the volumes of bioreactors and production costs are often very large, and the reproducibility and the quality of the products are crucial features that must be ensured at all times. In the pharmaceutical field, for instance, regulatory agencies have been encouraging manufacturers to innovate in the field of process control and monitoring to ensure a sufficient product quality, as demonstrated by the introduction of the Process Analytical Technology (PAT) framework by the U.S. Food and Drug Administration in 2004[1]. The PAT framework outlined a strategy for a continuous control of manufacturing processes through the monitoring of critical process parameters and critical quality attributes to ensure that the product is safe and effective. The concept of continuous process monitoring is also part of the Quality by Design (QbD) paradigm, which approaches the process validation through a continuous improvement of the manufacturing process[2]. Since a continuous control of the fermentation process is demonstrated to lead to a better product quality, monitoring and controlling tools are being continuously explored and improved. These tools include probes for temperature, pH, dissolved gases and organic carbon, and spectroscopic probes, also including Raman probes[3,4].

Besides commercial devices recently developed for monitoring of nutrients and metabolites through Raman[5,6], several examples of on-line and off-line Raman process monitoring of industrial fermentation of bacteria, fungi and mammalian cells[7] have been reported in literature over the last years. Nutrients, metabolites and by-products have been monitored to gain better knowledge of the fermentation process and to control the feeding strategy[8-10]. The yield of metabolites of interest has been predicted with the aid of complex modeling systems, as in the case of glycoproteins produced by Chinese hamster ovary[11], or, in fewer cases, with direct detection of a strongly Raman active compound[12].

TECHNOLOGY

Raman spectroscopy provides a unique opportunity to study the chemical composition of materials at the microscale.

Such capabilities come at the cost of extremely high requirements for instrumentation: lasers with stabilization of wavelength and power, low noise spectroscopic sensors, and a large clear aperture of spectrometer's optics. Therefore, demanding Raman spectroscopy and microscopy applications usually require high-end, bulky, and costly Raman instrumentation.

Lightnovo ApS found possible solutions to the most critical Raman miniaturization challenges: need for laser temperature and power stabilization, reduction of sensor dark noise, compensation on pixel-to-

pixel quantum efficiency (QE) variation, laser optical isolation and achieving high spectral resolution. As result a novel optics miniaturization strategy allows us to create **compact Raman spectrometers and microscopes** based on non-stabilized laser diodes, densely-packed optics, and non-cooled small pixel size sensors.

Details for miniRaman MRs patent

Lightnovo ApS proposed miniaturization concept based on real-time calibration of Raman shift and Raman intensity using an in-built reference channel that collects the Raman spectrum of polystyrene located in the spectrometer. We have demonstrated the miniaturization of the whole device dimensions down to several centimeters and achieved excellent sensitivity, low power consumption, perfect wavenumber and intensity calibration combined with high spectral resolution of around 7 cm^{-1} within the spectral range of 400-4000 cm^{-1}.

MATERIALS, SAMPLE PREPARATION AND MEASUREMENTS

Stock solutions of 100 mM pHCA and 100 mM CA were freshly prepared in EtOH 99%. For Raman calibration standards, pHCA was diluted in control supernatant (obtained from a non-pHCA producing E. coli strain (CBJ786)). In addition, for Raman and HPLC experiments the analytes were diluted in M9 medium. HCl 32% was used for acidification of samples and DCM as the organic phase for LLE. Stock solutions of 50 mM Phe and 50 mM Tyr were prepared in water and NaOH at pH ~14 respectively.

E. coli strains (CBJ800, CBJ786, CBJ951, CBJ789), expressing TAL and PAL-encoding genes from IPTG-inducible promoters, were grown in M9 medium with 1% glucose, 2 mM Tyr and/or 2 mM Phe, 1 mM IPTG and antibiotics for maintenance of plasmids for 22 h as described in our previous work[15].

For quantification of pHCA produced by E. coli, bacterial supernatant samples were obtained from each strain by centrifugation (10 min at 10 000g, 4 °C), and filtration through 0.2 µm filters. The concentration of pHCA in samples was measured with reversed-phase HPLC by separation on a HS-F5 column (Sigma-Aldrich, St Louis, MO, USA) with previously described mobile phases (ammonium formate buffer and acetonitrile), with an overall analysis time of approximately[15] min per sample[15]. The absorbance was measured at 333 nm for pHCA[16].

Aqueous solutions were prepared with ultra-pure water obtained from a Milli-Q purification system (Millipore Corporation, Billerica, MA, USA), and all the chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

For Raman determination of pHCA in bacterial supernatant, *E. coli* samples (CBJ 800), genetically modified to produce pHCA, were cultured in growth medium according to the methods described in our previous research[15, 16.] Bacterial aliquots were centrifuged and filtered at 0, 1.5, 4.5, 6, 7.5, 9, 11.25, 23 and 26.5 h, with the purpose of monitoring the pHCA production and the nutrient consumption at several time points throughout the culture. The concentrations of pHCA and glucose were also determined through HPLC, with the methods described in our previous publication[15].

Raman measurements were carried out at fixed laser power on the sample of 100mW (from laser excitation wavelength 785nm) and 30mW (from laser excitation wavelength 675nm) A custom Raman probe with NA = 0.08 was used in this study which provides an estimated laser spot size of around 10µm on the sample. Each spectrum was averaged over 10 repetitions at an exposure time of 1 s each. A sample volume of 500 µL was poured into a glass vial with screw cap (volume 1.5ml). The Raman signal of liquid samples was collected by focusing the laser beam in the middle of the vial through a bottom window, and each sample was collected in triplicates.

RESULTS

We used miniaturized Raman spectrometer to perform off-line quantification of pHCA produced during E. coli culture, directly measuring the Raman signal of liquid samples of bacterial supernatant (Figure 1c). Calibration samples were collected for pHCA, glucose, $MgSO_4$ and Na_2HPO_4, (Figure 1a) which were found to be the main contributions to the supernatant spectra at the beginning (black curve) and the end (red curve) of fermentation process (Figure 1b). By applying the PLS calibration model to real supernatant samples, the concentration of pHCA was found to increase, whereas glucose decreased over time, as also confirmed by the close correlation with HPLC results (Figure 2a, 2b). Also $MgSO_4$ and Na_2HPO_4 were consumed over time (Figure 2c), although no additional technique was used to validate Raman quantification in this case. Presented results demonstrate extremely high sensitivity of miniaturized Raman spectrometer with LoD for pHCA around 0.01g/L and LoD for glucose around 1g/L. To the best of our knowledge, the lowest previously reported LoD for glucose that was obtained by research grade Raman spectrometer with deep cooling CCD was around 0.55-8g/L[13,14]. It is important to mention that due to real-time laser intensity calibration and pixel-to-pixel QE compensation implemented in miniaturized Raman spectrometer we managed to obtain pHCA and glucose quantification error comparable with HPLC (Figure 2a, 2b).The presented acquisition method could be applied to more complex cases, such as the quantification of products and nutrients in tank fermentation, and, together with SERDS strategy for fluorescence reduction, could represent a high performance, compact and affordable solution for real time, at-line monitoring of bacterial fermentation. As an example, multiple miniaturized Raman spectrometers could be installed at different tank locations (top, middle and bottom) to monitor fermentation processes more efficiently. Figure 2d demonstrates quantification of Serine produced during E. coli culture, directly measuring the Raman signal of liquid samples of bacterial supernatant. Here, Raman spectra contained significant fluorescence background (Figure 1c), however, we still were able to get quantification error comparable with LC-MS (Figure 2d).

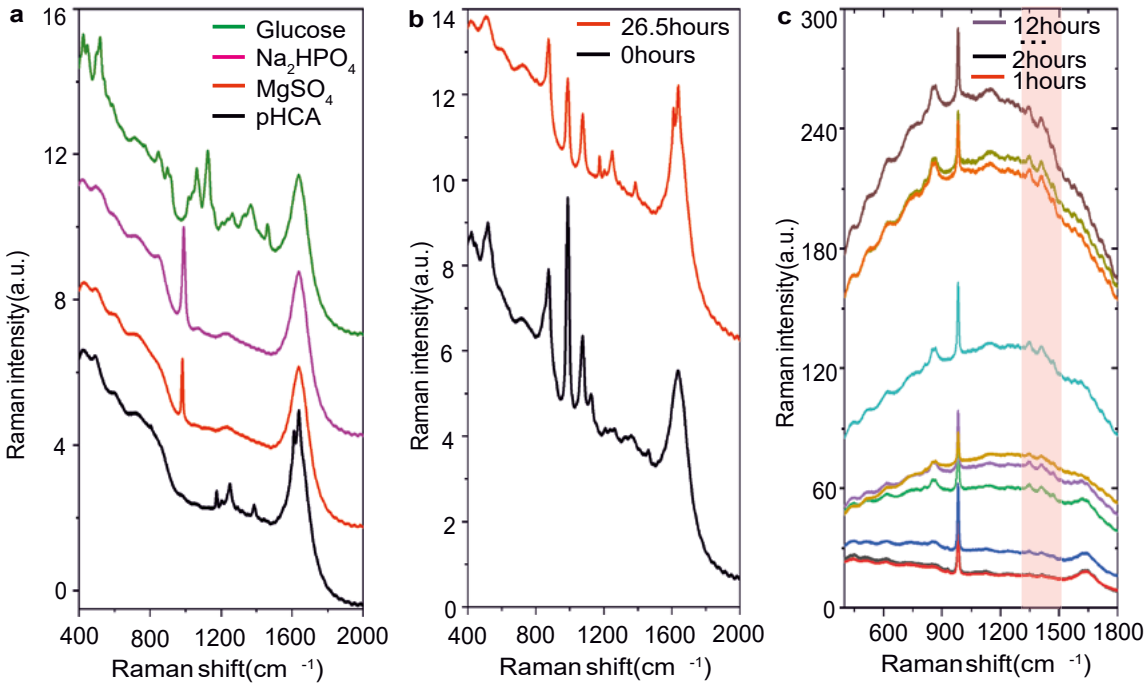

Figure 1. Miniaturized Raman spectrometer applied for fermentation monitoring. a) Raman spectra of the main components contributing to the Raman signal of bacterial supernatant, b) Raman signal of bacterial supernatant at 0 and 26.5 hours of culture, c) Raman spectra of bacterial supernatant containing Serine at 0 to 12 hours of culture collected with step of 1 hour; pink band indicates spectral region with prominent Serine peaks.

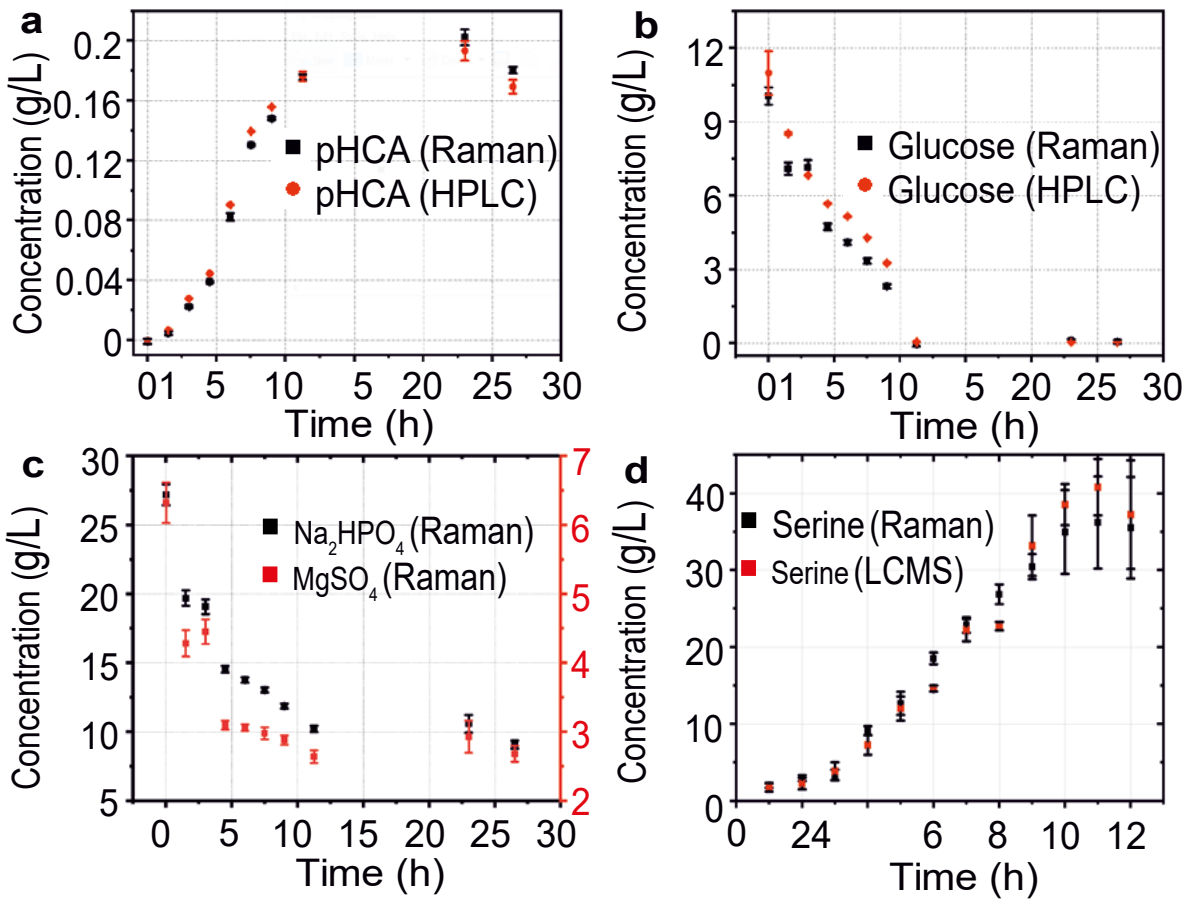

Figure 2. Miniaturized Raman spectrometer applied for fermentation monitoring. a), b), c) Raman and HPLC quantification of (a) pHCA, (b) glucose and (c) $MgSO_4$ in bacterial supernatant (each Raman point is the average of triplicate acquisitions, whereas each HPLC point is the result of duplicate injections), d) Raman and LCMS quantification of Serine in bacterial supernatant (each Raman and LCMS point is the average of triplicate acquisitions and injections, respectively).

CONCLUSION

Lightnovo ApS demonstrated technology for both miniaturizing and democratizing Raman spectrometers, making Raman spectroscopy more accessible as well as a high performance, compact and affordable solution for real time, at-line monitoring of bacterial fermentation.

Harness the power of Raman spectroscopy and make it widely accessible for the benefit of mankind.

- Lightnovo's mission

LITERATURE

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