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ULTRASONIC PARTICLE MANIPULATION APPROACHES IN ON-LINE VIBRATIONAL SPECTROSCOPY

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Radel, Stefan[1]; Schnöller, Johannes[2]; Dominguez-Vidal, Ana[3]; Lendl, Bernhard[4]; Gröschl, Martin[5]; Benes, Ewald[6]
[1]Institute of General Physics, Vienna University of Technology, Wiedner Hauptstraße 8-10/134, A-1040 Vienna, Austria; radel@iap.tuwien.ac.at
[2]Institute of Chemical Technology and Analytics, Vienna University of Technology, Getreidemarkt 9/164, Vienna, A-1060 Austria; jschnoel@mail.zserv.tuwien.ac.at
[3]Institute of Chemical Technology and Analytics, Vienna University of Technology, Getreidemarkt 9/164, Vienna, A-1060 Austria; adovidal@ujaen.es
[4]Institute of Chemical Technology and Analytics, Vienna University of Technology, Getreidemarkt 9/164, Vienna, A-1060 Austria; blendl@mail.zserv.tuwien.ac.at
[5]Institute of General Physics, Vienna University of Technology, Wiedner Hauptstraße 8-10/134, A-1040 Vienna, Austria; groeschl@iap.tuwien.ac.at
[6]Institute of General Physics, Vienna University of Technology, Wiedner Hauptstraße 8-10/134, A-1040 Vienna, Austria; benes@iap.tuwien.ac.at

ABSTRACT
In this work an ultrasonic standing wave (USW) field of 2 MHz was used to manipulate particles in different measurement cells for vibrational spectroscopy purposes.
These optical measurement techniques are increasingly popular in process analytical chemistry because of their ability to directly provide molecular specific information about a given sample. Especially in the regime of bioprocess control robust and reliable sensors with fast response times are needed. A target is to discriminate between biological cells and the supernatant, i.e. the suspending liquid during the measurement.
Employing the principles of ultrasonic particle manipulation we have developed a novel flow cell for a horizontal attenuated total reflection (ATR) unit connected to a portable MIR-spectrometer. Results show, that a decrease of the undesired formation of bio-films on the ATR surface (which is unavoidable when using suspensions like fermentation broth) was achieved. In addition the time resolution was increased.
Secondly an USW was used to manipulate yeast cells for assessment within the light path of a confocal Raman microscope. Due to the increased spatial concentration of matter in the focus of the laser beam an increase of the signal-to-noise ratio of the Raman spectra recorded from the yeasts was established.

INTRODUCTION
Within an ultrasonic standing wave (USW), forces are exerted on suspended particles directing them towards so-called pressure nodes, i.e. regions of vanishing sound pressure of the ultrasonic field. This technique is referred to as ultrasonic particle separation.

Ultrasonically Enhanced Settling (UES) devices based on this principle are exploited industrially as cell filters in biotechnology [1]. This is possible due to its non-destructiveness for biological cells - in contrast to the common ultrasonic cleaning devices which utilize the effect of cavitation at frequencies of a few ten kHz [2]. In fact, micro-organisms such as yeast cells, are handled very gently by a megahertz standing wave field [3].

The growing use of bioprocesses as a manufacturing route for e.g. antibiotics and other medical compounds enforces the development of reliable, automated in- or on-line sensors for bioprocess monitoring and control. These sensors are the key for optimal system performance. In order to keep control of the monitored bioprocess continued analysis is needed with fast

response times at least one order of magnitude faster in comparison to the generation time of the observed microorganism [4].

In this context advances in vibrational spectroscopy are of interest as great part of the desired (bio)chemical information can be extracted from near infrared, mid-infrared or Raman spectra. Using these techniques gaseous, liquid and solid matter can be analyzed and the concentration of practically any molecule determined if present at sufficiently high concentrations. Therefore there is great promise that also information on the physiological status of microorganism can be obtained on-line if sensor systems can be designed for robust and reliable microorganism measurement also during prolonged fermentation times.

Fourier transform infrared spectroscopy with (FTIR) in combination with attenuated total reflection (ATR) sensing elements is a currently developing, very promising means for (bio)process monitoring. ATR FTIR spectroscopy is a surface sensitive technique. It is based on the evanescent field, which decays exponentially from the interface of the ATR element – sample into the optically thinner sample layer. Therefore the closer the sample is located to the ATR surface the higher its contribution to the recorded spectrum will be. Placement of the ATR sensor horizontally and on-line, that is in bypass which is connected to the bioprocess under study, opens the possibility also to measure cells spectra in an automated fashion [5].

In Raman spectroscopy, a sample is irradiated with a focused laser beam and the inelastically scattered light measured. The recorded intensities at different frequency shifts are termed Raman spectrum which provides information on characteristic vibrational transitions in the sample under study. In case of monitoring reacting suspensions like fermentation broth Raman spectroscopy holds great promise due to possible non-invasive measurement strategies. Furthermore, based on the Raman spectrum, the physiological status of micro-organisms may be assessed [6]. This application turn out to be difficult for standard on-line Raman spectroscopy because Raman photons from the micro-organism need to be distinguished from Raman signals originating from the pure liquid phase. This problem is of special relevance in case of low concentration of suspended particles.

Thus, combination of ultrasound technology for particle manipulation and vibrational spectroscopy is an interesting avenue to increase selectivity and sensitivity of on-line measurements.

MATERIALS & METHODS

ATR FTIR flow cell: experimental set-up and procedure

An automated flow system [7] was connected via a fast loop to the fermenter (see Figure 1 left). From the fast loop small volumes of fermentation broth were taken via a T-piece by a computer controlled flow system at a flow rates of 5 ml/min and pumped via a selection valve through the homemade flow cell, which incorporates the horizontal ATR unit which was taken from a commercial three bounce diamond ATR element (Durasample II, SensIR). The ATR cell was connected to a MIR version of the portable Matrix FTIR spectrometer from Bruker (Ettlingen, Germany). To the ports of the selection valve cleaning solutions, and distilled water were connected. In doing so sequences of the solutions could be pumped over the ATR element in a computer controlled way.

The used flow cell (see Figure 1 right) comprised a cylindrical cavity (4.3 Ø x 5.2 mm) between the horizontal ATR element at the bottom and an ultrasonic transducer at the top. The PZT[1]-glass compound transducer (PZT cylindrical, 1 x 20 mm, Hoechst Tempax glass 2.77 x 25 mm) on top emitted an ultrasonic wave of around 2 MHz (Frequency Power Synthesizer FPS 4025, Psi, Austria) into the liquid. This wave was reflected by the ATR and due the superimposition of the incident wave and the reflection an USW was build up.

A suspension containing 7.5 g/L ordinary baker's yeast (Mautner Markhof Austria Hefe AG) suspended in NaAc/HAc buffer (50 mM, pH 5) was used to model fermentation broth.

[1] PZT: lead zirconate titanate

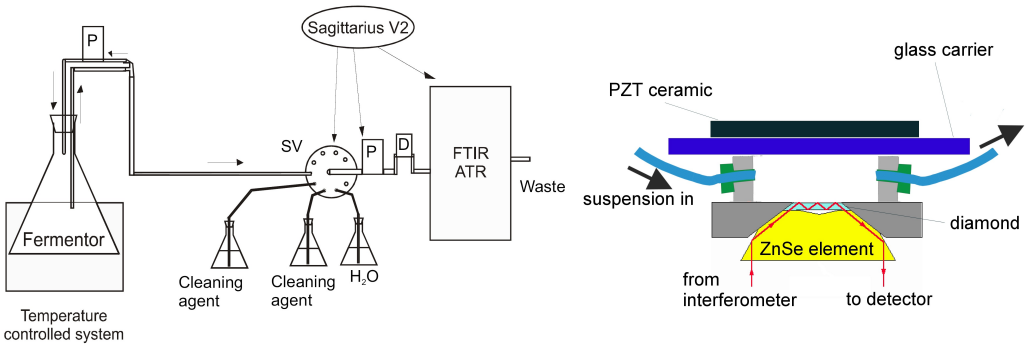

Figure 1: left: Experimental set-up comprising a peristaltic pump (P), a selection valve (V), a degassing unit (D) and the ATR FTIR; right: sketch of the ATR FTIR flow cell

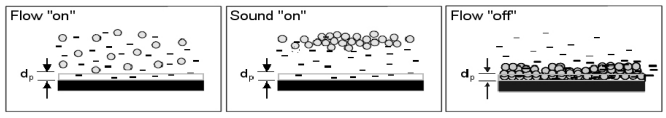

Figure 2: Illustration of the ATR measurement principle for dissolved contents (left), dissolved contents utilizing an USW (middle) and cells (right).

The system containing buffer was filled with suspension at 5 ml/min (Figure 2 left). Throughput was then switched off to let the cells sediment on the ATR (Figure 2 right). Subsequently the IR spectrum was recorded and afterwards the system was rinsed with distilled water. To test the ability of the ultrasound field to lift deposited material the USW was switched on and off for 10 s, respectively during the rinsing (Figure 2 middle). This procedure was repeated every half hour.

Spectra (intensity versus wavenumber) were recorded at a spectral resolution of 8 cm^{-1} by co-adding 128 scans recorded with at a mirror velocity of 40 kHz HeNe frequency and using a Blackman Harris 3-term apodization function. For the study of the settling speed only 10 scans were averaged for the sample spectra in order to achieve a better time resolution. Spectra acquisition and operation of the flow system was controlled by home written visual basic software program (Sagittarius II). IR spectra are displayed in absorbance units [AU], the logarithm of the ratio[2] between the absorbencies of the flow cell filled with the suspension and the background, i.e. the preceding rinse resulting in a filling with distilled water, respectively.

For the evaluation of ultrasonic enhanced settling the system was sonicated during the inflow of the suspension and the sound field was switched off together with the pump. The determination of sedimentation speed was achieved by taking spectra every 5 s and therefore detecting the increase of signal due to the settling yeast cells (Figure 2 middle and right).

Raman spectroscopy: US separator and procedure

The ultrasonic separator consisted of an aluminium spacer between two glass sheets (microscopy slides). The lid was equipped with in- and outlet thus building up a flow cell (see Figure 3 left). To one sidewall of the spacer a PZT ceramics (1x25x7 mm^3) was glued for the excitement of the USW. The other sidewall acted as a sound reflector. The device was placed under the Raman microscope with the glass sheets perpendicular to the light path.

The acoustic nodal planes where oriented parallel to the incident light beam (see Figure 3 right, allowing to control their locations relative to the light path by changing the excitation frequency (~2 MHz). The PZT was connected to an FPS 4025 frequency power synthesizer (PSI Systems, Austria). A few tens of one Watt were used as true electrical power input.

The yeast-in-water suspension was prepared with dried yeast (traditional dried active yeast, Allinson, UK) in distilled water at a concentration of a few 10^7 cells/mL

[2] Lambert Beer's law: $A[AU]=\log(I_0/I)$

The Raman spectra were recorded on a LabRAM HR 800 confocal Raman microscope from Jobin Yvon (Bensheim, Germany) using a 632.8 nm HeNe laserline (14.5 mW), a 600 grid, and a 20x magnification objective with a working distance of 20.5 mm. The confocal hole was set to 500 µm and the slit to 100 µm, recording time was 30*18 s.

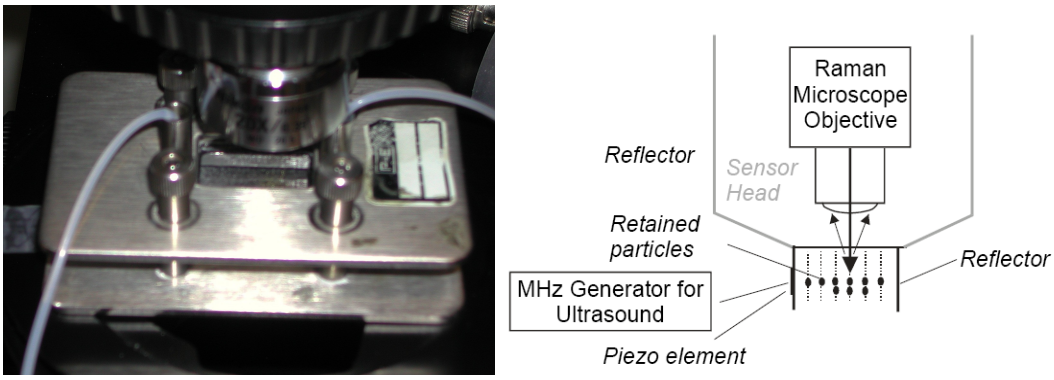

Figure 3: left: flowcell equipped with ultrasound transducer; right: sketch of yeast cells enriched by the USW in the light path of the confocal Raman microscope

RESULTS

Ultrasonically suppressed bio-film on the ATR FTIR

These observation of bio-film adherence to the ATR surface could be reproduced when working with the inactive model yeast suspension. Figure 4 shows four infrared spectra of the same yeast suspension one recorded 30 minutes after the other. A decrease of signal strength and resolution was obvious during the experiment (the black line refers to the first measurement, lines become lighter as time passed by). This is explained by the fact that a biofilm built up during the ongoing experiment was incorporated with the background spectra recorded over time. As a result the evanescent field effectively contributing to the result was decreased.

The result when the USW was switched on and off during the rinse indicated a rather clean surface suggested by very small alterations over the duration of the experiment in absorbance spectra of the yeast suspension in Figure 5. A mild coating for the amide I and II features at 1635 cm^{-1} and 1545 cm^{-1} was found, but almost no deposition of carbohydrates (1200-900 cm^{-1}).

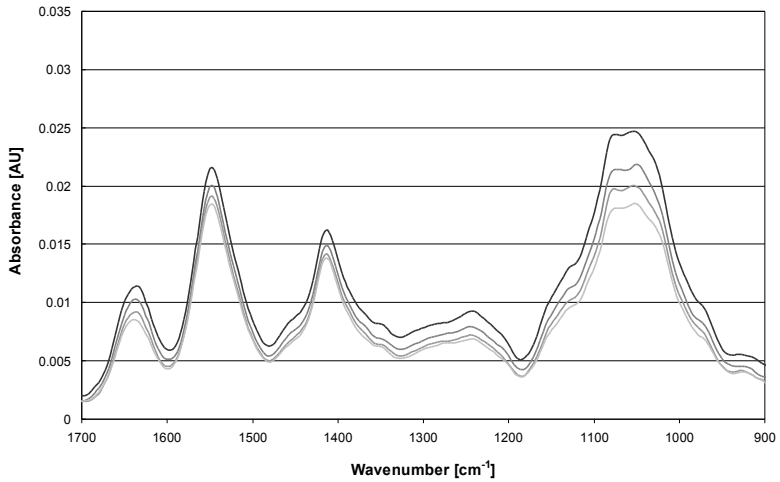

Figure 4: Consecutively recorded FTIR spectra of a yeast suspension (Δt~30 min).

Ultrasonically enhanced settling in the ATR FTIR flow-cell

Additionally to the removal of bio-film the employed flow-cell was investigated regarding the

ability to speed up the sedimentation of the yeast cells. When the yeast suspension was fed into the flow cell and the flow was stopped, the suspended yeast slowly settled on the ATR surface due to gravity. The resulting increase of absorbance due to the ATR element covered with more and more cells was recorded every 5 s. The increase of settling velocity due to ultrasonic agglomeration of the yeast cells prior to the sedimentation phase was assessed by having an USW present when the flow cell was filled with suspension. Again the absorbance was measured every 5 s.

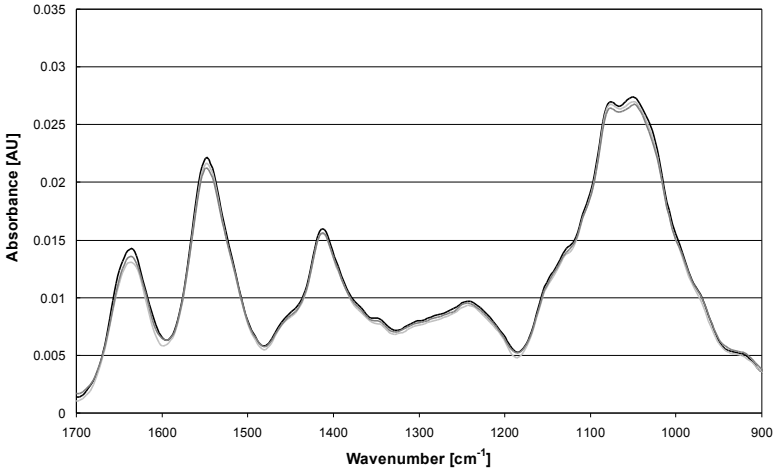

Figure 5: Absorbance spectra of yeast suspension with ultrasonic standing wave applied during the rinse.

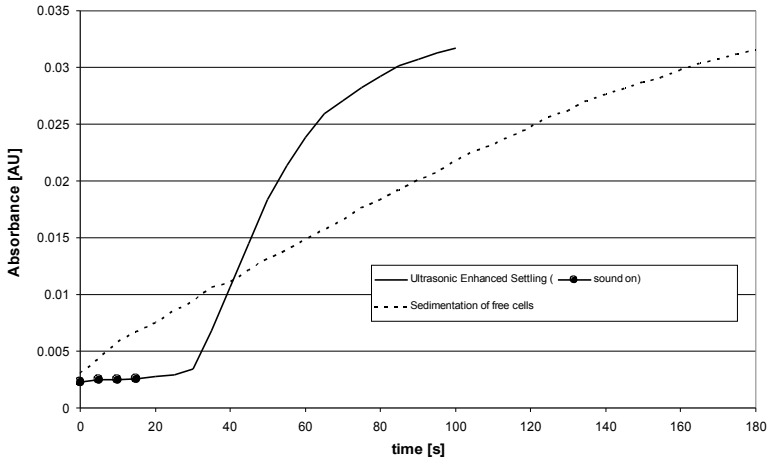

Figure 6: Sedimentation rates of freely and ultrasonically enhanced settling yeast cells. Dots represent times when an ultrasonic standing wave was applied.

This data (not shown) were further processed. Three absorbance values in the carbohydrate region were averaged and used as measure for the settle rate vs. time in Figure 6. The freely dispersed yeast cells sedimented gradually over the observed period of 180 s. The application of an USW changed the picture completely. During the first 15 s the wave field prevented the cells – dots indicate that sound was “on” – to settle at all. It took further 15 s for the settling agglomerates to be picked up be by the ATR followed by a strong increase lasting for 70 s.

Ultrasonically enhanced Raman spectroscopy

The flow cell was filled with suspensions of yeast cells in water. The black dashed line in Figure 7 shows the resulting Raman spectrum, when the optical focus was somewhere in the liquid layer. However, when an USW was applied and the Raman measurement was taken at a location within an agglomerate of yeast cells in a nodal plane the black continuous line was the

result. Significant features of yeast could be identified around wave numbers 2850 cm^{-1}, 1660 cm^{-1}, and 1437 cm^{-1}, which arise from the symmetric CH_2 stretching vibration, the amide I band and the amide III band, respectively. For reasons of comparison, Figure 7 includes the Raman spectrum of dried yeast on a quartz glass plate (grey continuous), where the scatter intensity was found to be almost twice as high as for the measurement of the agglomerate, however, the feature structure was conclusively similar.

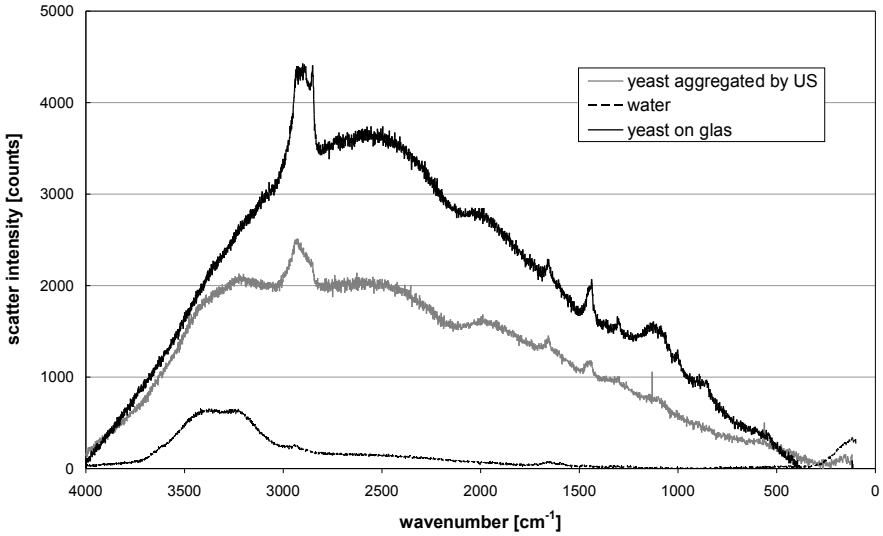

Figure 7: Raman spectra of yeast in water (black dashed), yeast cells agglomerated in the nodal plane of the ultrasonic field (grey) and for comparison dried yeast cells on quartz (black).

CONCLUSION

The presented results are promising regarding the positive influence of ultrasonics radiation forces on bio-film creation suggesting prolonged cleanness of an ATR sensor surface. Additionally an increase of time resolution was shown due to ultrasound induced aggregation which increased settling speed.. Hence the combination of the ATR FTIR sensor with the technique of ultrasonic particle manipulation has some potential, however the development of a sensor for the industrial environment more research will be necessary.

Raman spectroscopy results showed the ability of ultrasonic particle manipulation to concentrate cells in the sensitive regions. The level of scatter intensity of cell agglomerates in suspension was only slightly lower when compared to dried material. The measurement suggested, that the USW did not significantly influence the structure of the Raman spectra.

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