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Application of a High Cell Density Capacitance Sensor to Different Microorganisms

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RESEARCH ARTICLE

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Abstract

In microbial cultivations there is a great need for online biomass determination, especially in such cases, where classical methods have limitations. Capacitance-based sensors are developed and widely used in stem cell and other cell cultures [1, 2].

In the present study we have tried to apply such a capacitance-based online biomass determination (Incyte, Hamilton AG) for many microorganisms of different types. Since the signal of this sensor is dependent on the polarizability of the measured cells, the applicability of the sensor should be checked for every species. Thus, we developed a method as a preliminary sensor application test which is faster and simpler than testing this sensor directly in real cultivations.

We have tested an Incyte sensor with prokaryotic species (Lactobacillus sp., Clostridium) and eucaryotic strains (Saccharomyces cerevisiae, Cryptococcus albidus, Rhizopus oryzae, Chlorella vulgaris, Nannochloropsis oculata). Furthermore, according to our best knowledge this is the first report of a capacitance sensor application for microalgae. Finally, via conductivity measurements of the same sensor, we could even follow product formation in some cases too.

Keywords

capacitance, online viable cell count, fermentation, Saccharomyces, Cryptococcus, Clostridium, Rhizopus, Lactobacillus, Chlorella, Nannochloropsis

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1 Introduction

The monitoring of microorganism concentration is one of the key tasks during fermentations and one of the biggest challenges too. According to classical methods, operators should take samples regularly then they should analyse the samples using one of the offline methods of measurement to provide information on the quantity of biomass. Because of the discrete character of the measured data, for kinetic evaluation purposes (i.e. determination of specific growth rate, production rate etc.) the fitting of continuous functions to the data points was necessary via non-linear regression [3].

These transformations can be avoided if nearly continuous measurements are possible, for which on-line sensors can play an important role. On-line sensors even have an additional benefit since they can provide information on unmanaged periods too (for example, at night, etc.).

Nowadays, Process Analytical Technology (PAT) is more often used as a tool to achieve the Quality by Design (QbD) principle [4, 5]. These also require fast online measurements, especially in terms of biomass. Therefore, control of the fermentation requires rapid and reliable determination of the viable cell count.

While most of the classical methods determine the total cell number (like the optical density, turbidity, cell dry weight, etc.) [6-8], for more important living cell number determinations generally the dilution plate (CFU-colony forming unit) [9] and Most Probable Number (MPN) [10] methods are used which are rather labor-intensive and time-consuming. For this reason, modern and rapid viable cell count determinations play key roles both in the fermentation industry and in research. Such examples are luminescence and fluorescence measurements, near-infrared (NIR) [11] and FT-IR methods [12-14] in addition to capacitance and/or impedance measurements.

Capacitance methods combine all these benefits, they are fast, online, and measure living cell numbers. Furthermore, the sensors are less sensitive against external influences with robust systems. Measurements are based on the phenomenon that ions are migrating in a high frequency magnetic field. Since the movements of ions in comparted spaces, like cells

bounded by phospholipid membranes, are inhibited by the membranes, these compartments will be polarized and after some milliseconds of induction free periods, the polarized particles relax, generating an electronic impulse, i.e. a signal. The relaxing signal corresponds to the number of comparted cells. Since the membranes of dead cells are no longer intact or at least their ion pumps are no longer functioning, ions can freely flow into and out of the dead cells, i.e. they cannot be polarized, thus they do not generate a signal. Since air bubbles cannot be polarized as well, the effect of aeration is almost completely excluded. The key parameter of this method is the polarizing frequency which should be correctly chosen for each microorganism and media. Previous capacitance sensors applied only one frequency but they were found not to be very effective, since the size and composition of the cells (and as a result of this their polarizability) changes during the cultivation. Therefore, Hamilton's innovative solution in Incyte capacitance sensor is to measure permittivity at several frequencies which can result a permittivity profile vs frequency (this function of the equipment is called F-test). These profiles can be taken every 6 seconds, allowing almost the continuous determination of the living cell number (Fig. 1).

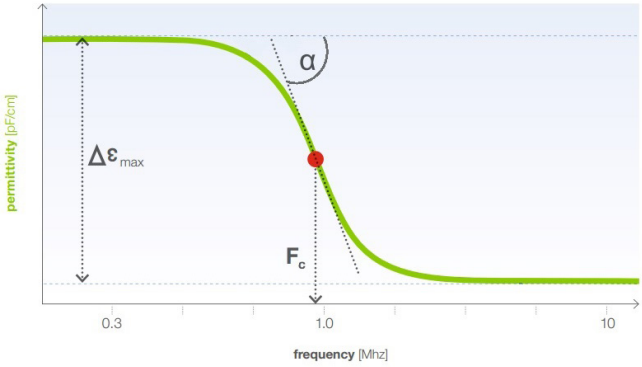

Fig. 1 Incyte sensor's permittivity profile (β-dispersion curve) in an ideal case [15]

The initial permittivity profile shows a horizontal line after the inoculation and zeroing permittivity. While the changes in permittivity at higher frequencies are negligible; thus the high frequency plateau (HFP) characterizes the media; the permittivity at lower frequency ranges increases with time and with viable cell count, resulting in a low frequency plateau (LFP). The distance between the two plateaus (Δε) is proportional to the biomass volume (the cell size x number of cells). The most innovative solution of this sensor is that for the calculation of permittivity difference (Δε) between the LFP and HFP a corresponding frequency can be selected according to the used strain: generally the HFP frequency is 10 MHz, but the LFP frequency varies depending on the microorganism (factory default is 2 MHz for yeast/fungi, 1 MHz for bacteria and cell cultures). In addition, two further parameters can also be determined: critical frequency which is the frequency of the profile's inflection point (Fc); and the slope of a tangential (α) drawn at the inflection point. [15] While the former increases with increasing cell size, the latter characterizes the cell size distribution: a smaller a reflects a more homogeneous culture.

A further advantage of this system is that the computer linked to the sensor's amplifier stores the measured permittivity for every frequency at every point in time in MS-Excel format, thus post-fermentation evaluation or re-evaluation is also possible.

Since the cells' polarizability differ among species, the application of the sensor should be tested for each process. According to the guideline of the manufacturer, the Incyte sensor is rather suitable for high cell density cultivations, especially for mammalian cell cultures, like CHO, for which the sensor has already been successfully applied [16]. While the aim of our work was to test it with different kinds of microorganisms too, we also checked its ability at low cell concentrations.

Therefore, we have developed a rapid experimental method, which can be applied without running long fermentations, and which support decision, whether the given strain in the suggested media is able to reach as high a concentration as required by the sensor for the successful determination of online viable cell counts (OVCC) or not.

In this paper we present some cases where we successfully applied the viable cell monitoring sensor both in real cultivations and in pre-fermentation tests (described detailed in Results) too.

The aim of the work was to test such cultures, which have industrial potential and use different microbial species in contrast to the mammalian cell cultures for which the sensor was originally designed.

2 Material and methods

Sensor applicability was tested for the following strains: prokaryotes: *Lactobacillus sp*. MKT878 – a lactic acid bacterium and *Clostridium butyricum* – an anaerobic bacterium; eukaryotes: *Saccharomyces cerevisiae* and *Cryptococcus albidus* – yeasts, *Rhizopus oryzae* - fungi, in addition to two isolates of *Chlorella vulgaris* and a *Nannochloropsis oculata* – a micro-algae.

A Lac 2 medium [17] for *Lactobacillus* consisted of 120 g/L glucose, 66 g/L $CaCO_3$, 30 g/L corn steep liquor (CSL), 6 g/L yeast extract, 0.5 g/L KH_2PO_4, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$ and 0.01 g/L $MnSO_4$.

A 2YT medium for *Clostridium* was prepared with 16 g/L tryptone, 10 g/L yeast extract and 5 g/L NaCl.

For the cultivation of *S. cerevisiae* we adapted the medium of Shang et al. [18] with 60 g/L glucose, 15 g/L CSL, 7 g/L $NaNO_3$, 6 g/L KH_2PO_4, 3 g/L $MgSO_4 \cdot 7H_2O$, 10 mg/L $ZnSO_4 \cdot 7H_2O$, 1 mg/L $FeSO_4 \cdot 7H_2O$ and 0.2 mg/L $CuSO_4 \cdot 5H_2O$.

For the pelleted growth of *R. oryzae* we applied a previously successfully adapted Gluc+N medium [19] of 20g/L glucose, 10 g/L $CaCO_3$, 3.3 g/L $(NH_4)_2SO_4$, 0.65 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$ and 0.088 g/L $ZnSO_4 \cdot 7H_2O$.

For the fermentation of *C. albidus* we used the medium of Hansson et al. [20] consisting of 20 g/L glucose, 3 g/L KH_2PO_4, 1 g/L yeast extract, 1 g/L tryptone, 1 g/L NH_4Cl, 1 g/L $MgSO_4 \cdot 7H_2O$, 15 mg/L $FeCl_3 \cdot 6H_2O$, 7.5 mg/L $ZnSO_4 \cdot 7H_2O$ and 0.5 mg/L $CuSO_4 \cdot 5H_2O$.

For any microalgae cultivation the BG-11 medium [21] was applied with 1.5 g/L $NaNO_3$, 75 mg/L $MgSO_4 \cdot 7H_2O$, 40 mg/L $K_2HPO_4 \cdot 7H_2O$, 36 mg/L $CaCl_2 \cdot 2H_2O$, 20 mg/L Na_2CO_3, 6 mg/L $FeNH_4SO_4$, 6 mg/L citric acid, 1 mg/L $Na_2MgEDTA$ and 1 ml/L A5 trace solution. An A5 trace element solution containing 2.86 g/L H_3BO_3, 1.81 g/L $MnCl_2 \cdot 4H_2O$, 0.391 g/L $NaMoO_4 \cdot 2H_2O$, 0.222 g/L $ZnSO_4$, 0.079 g/L $CuSO_4 \cdot 5H_2O$ and 0.05 g/L $CoCl_2 \cdot 6H_2O$. A BG-11 culture medium was prepared with deionized water (Simplicity®, EMD Millipore, Darmstadt, Germany).

The reference off-line cell concentration measurement determined the sample's optical density at 560 nm (OD_{560} for algae, after 3 times dilution) or 600 nm (OD_{600} for bacteria and yeasts, after 10 times dilution) with a spectrophotometer (Pharmacia, Ultrospec Plus, England). Every measurement was repeated three times.

For the fermentations, Biostat Q (B. Braun, Melsungen, Germany) bench-top fermenters were used with magnetic stirrers (300 rpm), as well as pH and oxygen (Mettler, Switzerland) electrodes [22]. The glass reactors had jacketed walls. The culture media and the fermenters mounted with Incyte sensor were sterilized at 121 °C for 20 minutes in autoclave (Tuttnauer 3870 ELV, Breda, Netherlands). The Hamilton Incyte on-line viable cell count (OVCC) monitoring system consisted of an Incyte sensor (Hamilton, Bonaduz, Switzerland), a Pre-Amp (Fogale Nanotech, Nimes, France) signal transmitter and an iBiomass (Fogale Nanotech, Nimes, France) controller (touchscreen computer + software). The sensor was autoclavable (max. 135 °C, max. 3 bar), 220 mm long, 12 mm diameter (DN12) with a PG13.5 process connection [15].

The off-line chemical sample analysis (glucose, lactic acid, acetic acid, 1,3-propanediol, butyric acid, ethanol) was carried out by a Waters Breeze HPLC system (Milford, CT, USA) with RI detection after separation on Bio-Rad Aminex HPX-87H (Hercules, CA, USA) at 65 °C, at flow rate of 0.5 ml/min.

We found that the conductivity signal of the Incyte sensor often correlated with the product formation (i.e. lacic and other organic acids). In such cases, on the basis of online measured conductivity Online Detected Total Products (ODTP) were calculated.

3 Results

In some real microbial fermentations the final biomass concentration we detected was too low (like poorly growing anaerobic bacteria *Propionibacterium freudenreichii*), and one could conclude that the sensor is inappropriate for those cultures. However, maybe on another more biomass producing media, that strain could also be tracked by the sensor. To resolve this contrast, we intended to test the applicability of the sensor prior to its use in real cultivations with the following process: after zeroing the sensor with cell-free media, the media was fed with a previously fermented and concentrated biomass suspension. During the addition of the biomass, Incyte tracked the capacitance and we took samples periodically to determine OD_{600} photometrically. The results give a calibration curve between on-line (calculated from permittivity) and off-line measured biomass concentration (optical densities) in addition to the limit of detection (LOD), and the measuring range. Results are presented individually for each microorganism, thus pre-fermentation tests (i.e. calibration curves) are also presented there (if available).

Clostridium butyricum

C. butyricum is a strict anaerobic microorganism, which is very sensitive to oxygen. Therefore, we developed a fermentation method, in which a batch-wise nitrogen inlet provides an anaerobic atmosphere, i.e. during sampling, a nitrogen inlet was applied. The cultivation was driven at 37 °C and at controlled pH of 7.0, while 300 rpm stirring were applied. The products of these fermentations are various extracellular enzymes of the anaerobic glycerol utilization pathway [23], of which amount correlates with the cell number. Therefore, it is very important to have on-line information about the viable cell count (OVCC). However, these cells are really small and unsegmented. In addition to this the final cell concentration used to be low, thus we expected no capacitance signal. However, in Fig. 2 we demonstrate that the permittivities calculated from 1 MHz LFP and 10 MHz HFP can correlate with off-line measured OD600, and the conductivity correlated with the total amount of different primary metabolites, i.e. on-line detected total products (ODTP), at the same time. (A temperature control failure at 17-18 h resulted a shift in online curves.)

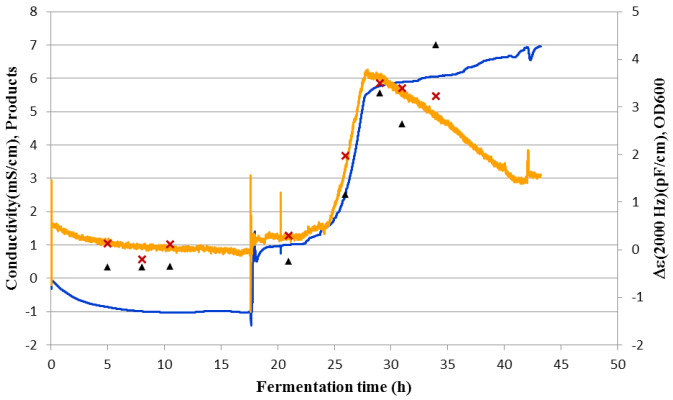

Fig. 2 Comparison of off-line determined OD600 with on-line viable cell count (OVCC = 4.5*Δε + 0.6) and comparison of off-line detected products with on-line detected total products (ODTP = 0.5*conductivity-7.5): × OD_{600}, — OVCC, — ODTP, ▲ Off-line detected total products (g / L)

Lactobacillus sp. MKT-878

During fermentation, the semi-optimized Lac-2 medium was used with $CaCO_3$ for pH regulation at 37 °C and 500 rpm. The suspended limestone made it very difficult to determine the classical off-line OD_{600} in terms of biomass concentration (the only way was to dilute the samples with 0.2 N HCl, but this can damage the cells, too). Therefore, it was advisable to apply on-line measurement for viable cell count determination. Since lactic acid bacteria are prokaryotes containing less membrane-bound segments, we expected lower polarization than in the case of yeasts, i.e. higher LOD. Since the measured and calculated 3 standard parameters (α, Fc, Δε) of the b-dispersion curves showed no significant changes during the fermentation. If the LFP values were changed from 1000 kHz (default for bacteria) to lower range (300-600 kHz) significant and evaluable changes were observed (Fig. 3B). Calibration with diluted biomass at 300 kHz gives a good correlation between Incyte sensors's signal and optical density (Fig. 3A) through OD values from 4 to 6.5. Thus in Fig. 3C the off-line measured OD_{600} values and the on-line measured OVCC calculated from $\Delta\varepsilon_{300KHz}$ are presented versus fermentation time.

During the process the conductivity increased, we assumed that this was caused by lactic acid formation, or to be more precise, the lactic acid formed was neutralized by $CaCO_3$, thus Ca-lactate was causing the increase in conductivity.

Furthermore, Fig. 3C shows the off-line measured glucose concentrations, the lactic acid concentrations (i.e. product concentrations) and calculated lactic acid concentrations on the basis of measured conductivity values too. OVCC fitted really well to OD_{600}, and conductivity based online lactic acid concentrations to offline determined one, respectively.

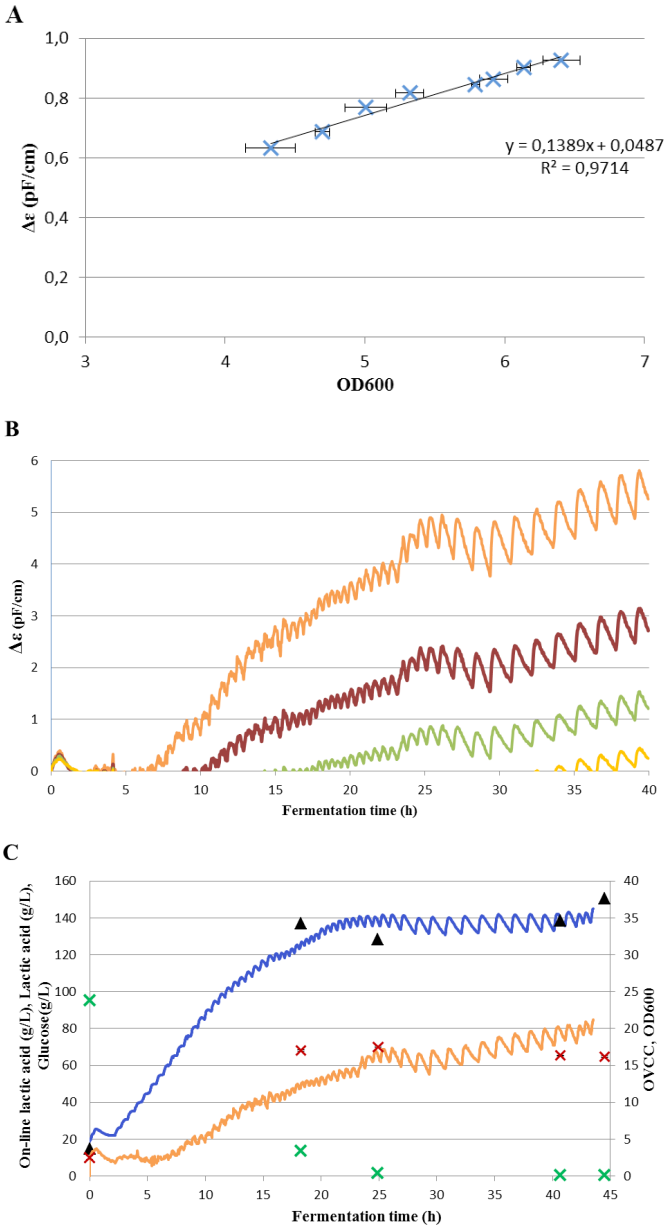

Fig. 3 *Lactobacillus sp.* lactic acid fermentation: A: Calibration curve; B: Various permittivities (Δε) calculated at different LFP frequencies, — 300 kHz — 373 kHz — 465 kHz — 578 kHz; C: Lactic acid fermentation × OD_{600}, — OVCC(=3*Permittivity$_{300\ kHz}$[pF/cm]+2.5), — Online lactic acid(=9.8*Conductivity [mS/cm]-45), ▲ Lactic acid [g/L], × Glucose [g/L]

Cryptococcus albidus

C. albidus was cultivated in whey to convert this food waste into a valuable product: ergosterol [24]. Since whey contains a lot of aggregate and exhibits high turbidity, it is really difficult to follow biomass growth off-line. While in case of lactic acid fermentation with Ca-carbonate we dissolve carbonate with the diluent 0,1N HCl before OD measurements; in case of whey a certain 10 times dilutaion resulted a less turbid and measurable suspension. Therefore, we tested an Incyte sensor in this cultivation. While Fig. 4A represents the calibration curve between the off-line measured optical density of *Cryptococcus albidus* and on-line measured permittivity differences, Fig. 4B shows the correlation between the results of the sensor and off-line optical density data during real cultivation. Interestingly, this OVCC curve is not so corrugating, than the other, but reasons could not identified until yet. The only registered difference is that while for this experiment we used reading frequency of 2 min, during the other experiments we used 0.5 min. Good applicability of the sensor was observed.

Rhizopus oryzae

For lactic acid production we also cultivated a filamentous fungus (*R. oryzae*) which is able to convert starch directly into lactic acid. This fungus is able to grow in a single cell, amorphous clump and pellet forms depending on cultivation conditions, but the latter is the most suitable for lactic acid production. However, a pellet is a really complex biomass system. Therefore, we applied the same pre-test method described above (see the beginning of the Results section) to determine whether the sensor is able to indicate the number of pellets or not. Figure 5 represents the results: the more pellets we put into the vessel, the higher the sensor signal became below permittivity of 3 pF/cm.

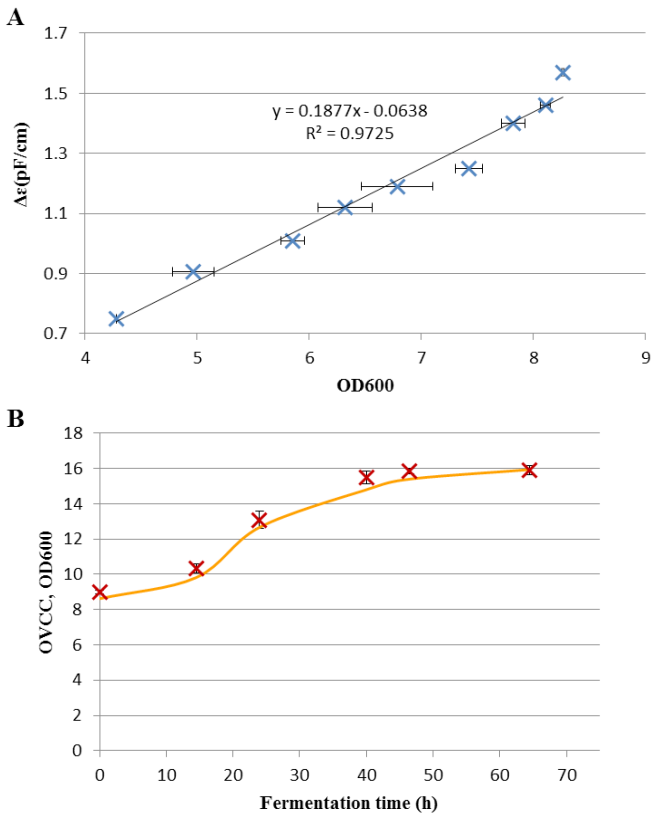

Fig. 4 Cryptococcus albidus fermentation, A: *Cryptococcus albidus* calibration; B: Time curves of off-line OD_{600}, and permittivity: × OD_{600}, ◆ OVCC(pF/cm)

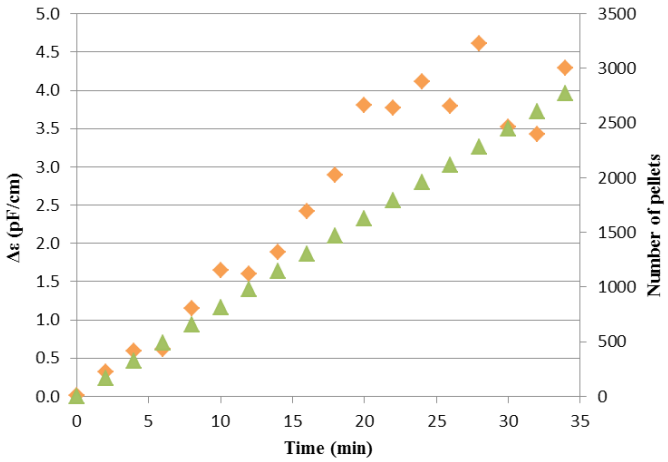

Fig. 5 OVCC sensor calibration for *Rhizopus oryzae*: ◆ $\Delta\varepsilon_{2000\ kHz}$ [pF/cm], ▲ Number of pellets (pc/L)

The sensor provided a good signal correlation with regards to the number of pellets. These new findings may open new applications of the sensor since this is the first report that follows not only single living cells but cell clusters too.

Saccharomyces cerevisiae

Before the fermentation test of *S. cerevisiae* (commercial Baker's yeast, Lesaffre, Budafok, Hungary), cells were suspended in water. An Incyte sensor was immersed in pure tap water and a high cell density suspension added slowly. By plotting Δε vs OD_{600} we obtained Fig. 6, which clearly shows the calibration curve, on which the on-line permittivity measurements start to correlate with off-line OD_{600} data at higher cell concentrations, and when OD_{600} exceeds 15 the correlation is strongly linear.

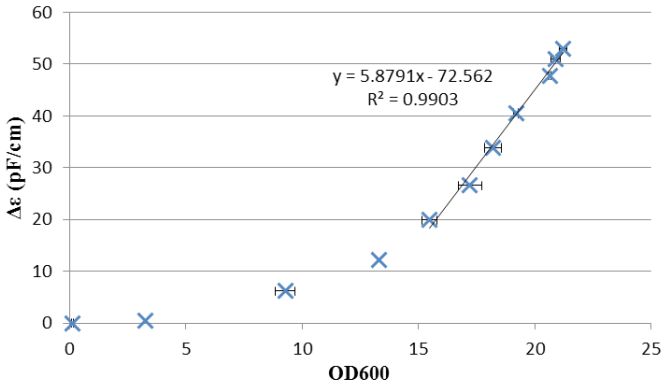

Fig. 6 OVCC sensor calibration for bakers yeast, pre-calibration curve: off-line measured OD_{600} vs. Δε [pF/cm]

For high cell density *S. cerevisiae* fermentation we ran a fed-batch cultivation of 0.8/1 L, in which an oxygen-controlled molasses feed was added to inoculated tap water to maintain as low a sugar concentration as possible. This way, Pasteur and Crabtree (or reverse Pasteur) effects [25] could be avoided. Figure 7 shows that the 1% ammonium hydroxide added to the molasses provided not only an N-source but alkaline conditions too for simultaneous pH control. Thus the pH and conductivity of the Incyte sensor stepped if the feeding pump was turned on by the increasing oxygen signal (pO_2). We applied DO = 20% as a controlled parameter for substrate feeding, which means the following: when the added sugar was consumed, DO started to increase and when it reached the desired concentration of 20%, the molasses feeding pump was turned on. This not only stopped the increase in concentration of oxygen, but even started to decrease it. When the DO reached 20% again, the feeding pump was turned off. With regards to this control method, one can see in Fig. 7 the periodic changes in DO and stepwise increase in conductivity. After the biomass formation reached the limit of detection, the sensor's signal started to rise too.

Nannochloropsis sp.

The tested strain is a seawater algae industrially used for converting the CO_2 of fuel gas into biomass [26]. In this case we also applied the previously developed pre-fermentation sensor test, and its calibration results are presented in Fig. 8.

On the basis of these calibration data, in Fig. 8 permittivity was plotted against OD_{560} resulting in a good correlation between OD_{560}=2.5 and OD_{560}=4 values.

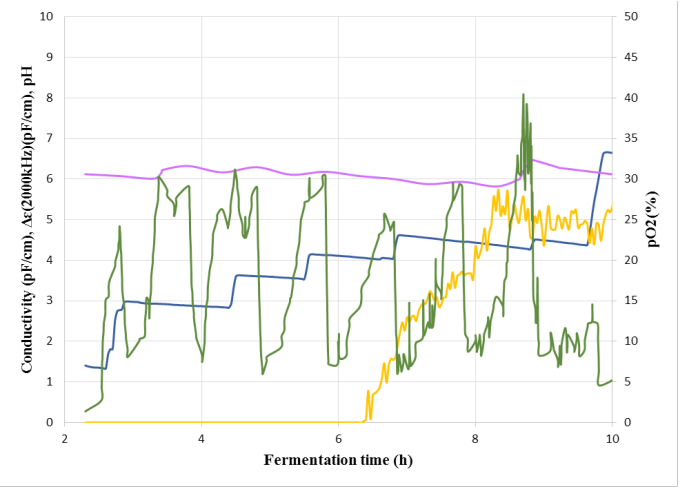

Fig. 7 *S. cerevisiae* fermentation on-line parameters: Conductivity [mS/cm], $\Delta\varepsilon_{2000\,kHz}$ [pF/cm], pH, pO_2[%]

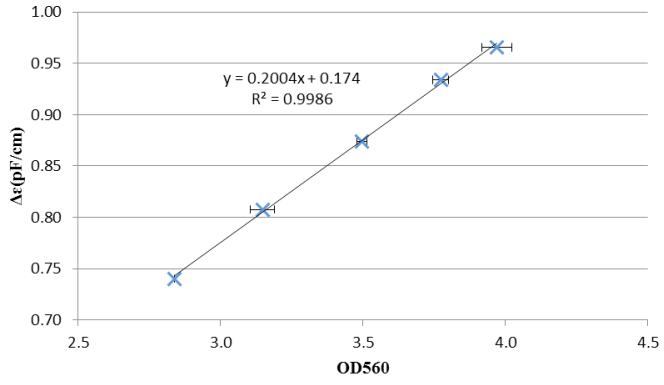

Fig. 8 OVCC sensor calibration for *Nannochloropsis sp.*: pre-calibration curve: off-line measured OD_{560} vs. Δε [pF/cm]

Chlorella vulgaris

Chlorella vulgaris was grown in a standing flask on a BG-11 medium and then concentrated by removing the supernatant. The sensor was zero calibrated with pure BG-11 media. Then the concentrated biomass was pumped at a constant flow rate into the empty media. While sensor parameters were recorded on-line, we took samples every 4 minutes for off-line OD measurements at 560 nm. Fig. 9A shows that the eukaryotic algae produces a measurable signal (Δε) already at much lower biomass concentrations (off-line OD_{560}=2) than prokaryotic cells did (Fig. 6B: LOD expressed in off-line OD_{600}=15).

With the help of the calibration above, we started a fermentation with *C. vulgaris*. According to Chisti [27], the illumination was periodically changed (in our case: 16 hours of exposure to light and 8 hours in darkness) in addition to a low level of aeration (0.2 L/min).

The obtained on-line permittivity against off-line measured OD_{560} (Fig. 9B) verified the pre-calibration curve (Fig. 9A) with almost the same gradient.

On-line and off-line measured biomass results are presented in Fig. 10. While the biomass data calculated from on-line permittivity measurements (OVCC=3.1*Δε-2.95) scattered significantly, its tendency was the same as for the off-line measured OD_{560}. The reason for this significant deviation is still under investigation in cooperation with the manufacturer (Hamilton).

These results reinforced that the preliminary calibration and the application of sensor are really useful during real cultivations.

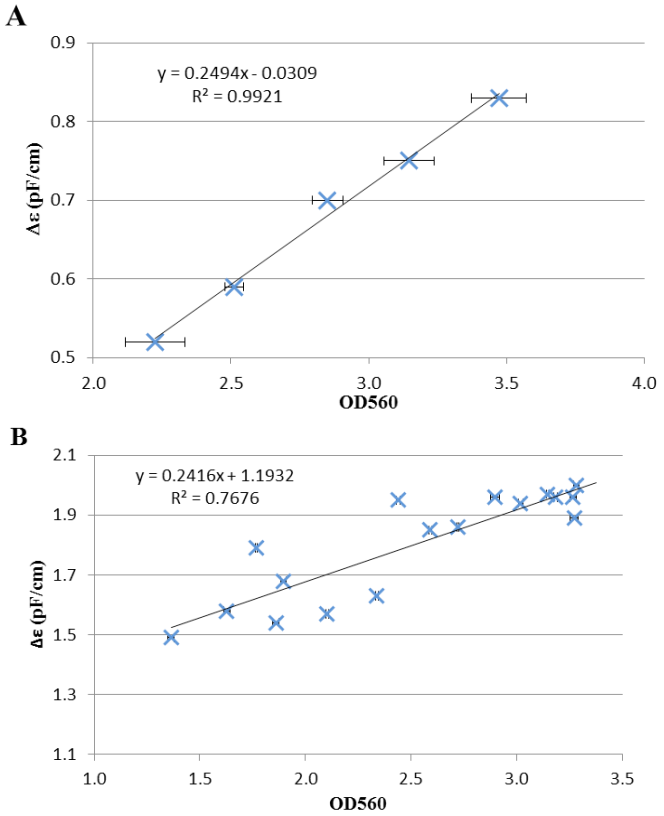

Fig. 9 Sensor calibration for *C. vulgaris:* A: pre-calibration: off-line measured OD_{560} vs. Δε [pF/cm]; B: verification of calibration with fermentation data

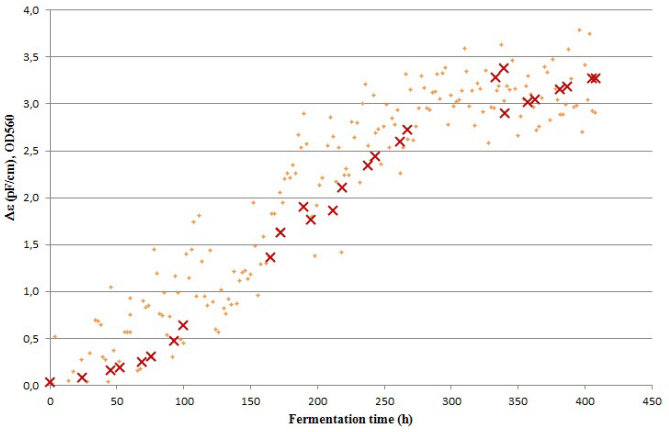

Fig. 10 Results of different biomass determinations of *Chlorella vulgaris* during fermentation: × OD_{560}, ◆ OVCC=3.1*Δε-2.95

4 Conclusions

The tested Incyte (Hamilton Bonaduz AG) sensor was found to be appropriate for viable cell count monitoring for a wide range of microorganisms (*Clostridium butyricum, Lactobacillus, Cryptococcus albidus, Saccharomyces cerevisiae, Chlorella vulgaris*) involving prokaryotic bacteria, eukaryotic yeast and

algae besides the envisaged culture of cell tissues like CHO. Furthermore, on the basis of preliminary tests, the Incyte sensor gave responses for Rhizopus pellets and Nannochloropsis cells too, but they should be further investigated in real cultivations. The elaborated calibration method provides a rapid and reliable forecast for the applicability of the sensor.

The only drawback of the tested sensor is the relatively high signal-to-noise ratio. The relative value of the noise can be affected partly by the type of measured cells and the conditions of the cultivation.

The manufacturer provided instructions for common culture conditions which should not affect the measurement of viable cell count. However, out of these parameters' range we found the sensor sensitive against external effects. Since the tendency of the growth is clearly indicated by the sensor, a potential solution could be a noise filtration algorithm to improve results for the presented fermentations.

Tests of filamentous bacteria are planned to be extended in the future.

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