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RESEARCH ARTICLE

MONITORING OF FERMENTATIONS WITH ONLINE CAPACITANCE SENSOR

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ABSTRACT

In biotechnological experiments the development and kinetics of the microbial culture are crucial information. Therefore researchers made lot of efforts to develop different cell measuring systems. Classically offline methods were widely used but they do not give real-time and reliable results enough. Among them some are able to follow even the living cell-number, which has probably the highest importance. A new, innovative online living-cell measurement sensor (InCyte (Hamilton) were received from Hamilton Bonaduz AG to test it with different art of microorganism. In the present investigation we elaborated a method for preliminary sensor application test, which is more fast and simple than testing this sensor directly in real cultivations. We already tested InCyte sensor with a prokaryotic lactobacillus (*Lactobacillus sp.*) and with eucaryotic yeast (*Saccharomyces cerevisiae*) furthermore with a microalgae strain (*Chlorella vulgaris*), which latter is also eukaryotic as well. We could confirm, that in some cases only high cell density could result high signal enough for the sensor, but according to our findings, InCyte sensor is applicable even for microalgae. Furthermore via conductivity measurements it can give information on product formation, too

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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 have to analyse the samples with one of the offline measurements to provide information on the quantity of biomass. Because of the discrete character of the measured data, for kinetic evaluation (i.e. determination of specific growth rate, production rate etc.) the fitting of continuous functions to the data points were necessary via non-linear regression (Mitchell *et al*., 2004). These transformations can be avoided if almost continuous measurements are possible, for which in-line sensors can play an important role. Nowadays, Process Analytical Technics (PAT) is more often used as a tool to achieve Quality by Design (QbD) principle (Rathore 2009, Konold *et al*., 2009). These also require fast online measurements, especially in terms of biomass. Therefore, control of the fermentation is requiring rapid and reliable determination of viable cell count. While most of the classical methods determine the total cell number (like microscopic Buerker chamber, the optical density, the turbidity, cell dry weight, etc.) (Havlik *et al*., 2013, Leduy *et al*., 1977, Meireles *et al*., 2002), for the more important living cell number determinations only dilution plate method (Thomas *et al*., 2015) and Most Probable Number (MPN) (Woomer *et al*., 1994) technique are available, which are rather labour-intensive and time-consuming methods. For this reason, modern and rapid viable cell count determinations play key-roles both in fermentation industry and research. Such examples are luminescent, fluorescent measurements, near infrared (NIR) (Sivakesava *et al*., 2001) and FT-IR methods (Dean *et al*., 2010, Horton *et al*., 2011, Menga *et al*., 2014) furthermore capacitance and/or impedance measurements.

The capacitance methods combine all these benefits (fast, online, measuring of living cell numbers), furthermore they are less sensitive sensor against external influences with robust systems. The measurement is based on the phenomenon that ions are migrating in high frequency magnetic field. Since the movements of ions in comparted spaces (like cells bounded with phospholipid membranes) are inhibited by the membranes, these compartments will be polarized, and after some milliseconds of magnet free periods, the polarized particles relax, which generate an electronic impulse, i.e. a signal. The relaxing signal corresponded to the amount of (comparted) cells. Since the membranes of dead cells are no more intact or at least the ion pumps of them are no more functioning, ions can freely flow into and from the dead cells, i.e. they cannot be polarized, thus they do not generate signal. Since air bubbles can not be polarized as well, the effect of aeration is also excluded. The key-parameter of this method is the polarizing frequency, which should be correctly chose for each

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microorganism and media. Previous capacitance sensors applied only one frequency, but it was found not really effective, since the size and compositions of the cells (and through this their polarizability) is changing during the cultivation. Therefore Hamilton's innovative solution in InCyte capacitance sensor is to measure permittivity at several frequencies resulting a permittivity profile vs frequency (this function of the equipment is called F-test). These profiles can be taken in every 7seconds allowing almost continuous determination of living cell number (Fig. 1).

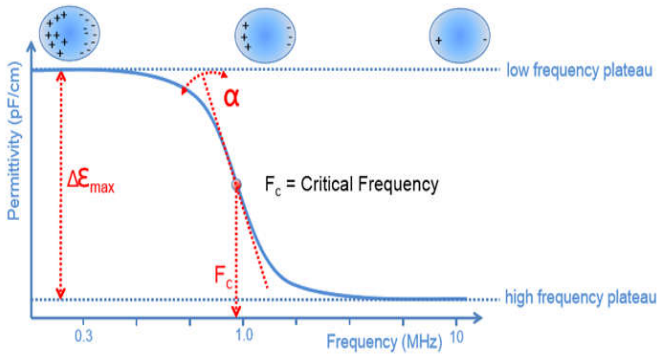

Figure 1. InCyte sensor's permittivity profile (β–dispersion curve) in ideal case

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

A further advantage of the sensor is, that it stores the measured permittivities for every frequencies at every time-point in MS-Excell format, thus post-fermentation evaluation or re-evaluation is also an opportunity. Since the cells polarizability differ among species, the application of the sensor should be tested for each processes. Therefore we developed a rapid experimental method, which can be applied without running long fermentations, and support decision, whether this sensor will be suitable for online viable cell count (OVCC) determination for the given strain and cultivation or not. According to the guideline of the manufacturer InCyte sensor is rather suitable for high cell density cultivations and especially for mammalian cell culture, for which the sensor was already successfully applied (Zhang *et al*. 2015). However, the aim of our work was to test it with different kind of microorganism, too. If the sensor would be tested only in real microbial fermentations, in some cases the final biomass concentration could be too low, and conclusion would be made false.

These were our observation for some weakly growing anaerobic bacteria, like *Clostridium butyricum* or *Propionibacterium freudenreichii*, too (data not shown). To overcome this problem, we intended to test the applicability of the sensor prior to use in real cultivations with the following process: after zeroing the sensor with cell-free media, fed the media with previously fermented and concentrated biomass suspension. During the biomass addition, InCyte followed the capacitance and we took sample periodically to determine photometrically OD_{600}. The results will be a calibration curve between on-line (calculated from permittivity) and off-line optical density, furthermore the limit of detection (LOD), and the measuring range. In this research we present some cases where we successfully applied both the sensor test method before real cultivation and the sensor in real fermentation, too. The aim of the work was to test such cultures, which have industrial potential and use different art of microorganism out of mammalian cell cultures for which the sensor was originally designed.

MATERIALS AND METHODS

The sensor applicability were tested for the following strains: prokaryotic: *Lactobacillus sp*. MKT878 –lactic acid bacterium; eukaryotes*: Saccharomyces cerevisiae* - yeast and *Chlorella vulgaris* - micro algae. The used culture media compositions are listed in Table 1.

Table 1. Media Compositions; * for petri dishes

Component	*Lactobacillus inoc* (MRS)	*S.cerevisiae inoc.*	*C.vulgaris* (BG-11)
Glucose	20 g/L	20 g/L	-
Yeast extract	5 g/L	15 g/l CSL	-
Tripton	10 g/L	-	-
Meat extract	5 g/L	-	-
KH_2PO_4	2 g/L	6 g/L	-
Na-citrate	2,815 g/L	-	-
NH_4Cl	0,54 g/L	-	-
Na-acetate	5 g/L	-	-
$MgSO_4 \cdot 7\ H_2O$	0,1 g/L	3 g/L	75 mg/l
$MnSO_4$	0,05 g/L	-	-
$FeSO_4 \cdot 7H_2O$	-	1 mg/l	-
$ZnSO_4 \cdot 7\ H_2O$	-	10 mg/L	trace
$CuSO_4 \cdot 5\ H_2O$	-	0,2 mg/L	trace
$NaNO_3$	-	7 g/L	1,5 g/l
$Na_2MgEDTA$	-	-	1 mg/l
$FeNH_4SO_4$	-	-	6 mg/l
Citricacid	-	-	6 mg/l
$CaCl_2 \cdot 2\ H_2O$	-	-	36 mg/l
$K_2HPO_4 \cdot 3\ H_2O$	-	-	40 mg/l
Na_2CO_3	-	-	20 mg/l
A5 trace solution	-	-	1 ml/l
*Agar	15 g/L	-	-

A5 trace element solution contained 2,86 g/L H_3BO_3, 1,81 g/L $MnCl_2 \cdot 4H_2O$, 0,222 g/L $ZnSO_4$, 0,079 g/L $CuSO_4 \cdot 5H_2O$, 0,05

[1] Cell Density Monitoring Systems – Operating Instructions (Hamilton Bonaduz AG)

g/L $CoCl_2 \cdot 6H_2O$ and 0,391 g/L $NaMoO_4 \cdot 2H_2O$. BG-11 culture medium was prepared with deionised water (Simplicity®, EMD Millipore, Darmstadt, Germany). The reference off-line cell concentration measurement was to determine sample's optical density on 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). Every measurement was done in three triplicate. We used for the fermentations Biostat Q (B. Braun, Melsungen, Germany) bench-top fermenters with magnetic stirrer, Mettler pH and Ingold oxygen electrodes. The glass reactors had jacket wall. We sterilized the culture media and the fermenters at 121 °C for 20 minutes in an autoclave (Tuttnauer ELV 3870). The Hamilton InCyte online viable cell count (OVCC) monitoring system consisted of an InCyte sensor (Hamilton), a Pre-Amp (Fogale) signal transmitter and an iBiomass (Fogale) controller (touchscreen computer + software). Off-line chemical sample analysis (glucose, lactic acid) were done by Waters Breeze HPLC system with RI detection after separation on BioRad Aminex HPX87H 65°C at 0,5ml/min.

RESULTS

Lactobacillus sp. MKT878

During the fermentation, the semi-industrial Lac-2 medium (Hetényi *et al*. 2011) was used with $CaCO_3$ for pH regulation at 37°C and 500rpm. The suspended limestone made it very difficult to determine classical off-line OD_{600} for biomass concentration (the only way is to dilute samples with 1N HCl, but this can damage the cells, too). Therefore it would be advisable to apply online measurement for viable cell count determination. Since lactic acid bacteria are prokaryotes having less membrane-bounded compartments, we expected lower polarization than in case of yeasts, i.e. higher LOD. Since the measured and calculated 3 standard parameters (α, Fc, $\Delta\varepsilon$) of the β-dispersion curves showed no significant changes during the fermentation, but the conductivity increased, we assumed, that it was caused by the lactic acid formation (more exactly, the formed lactic acid was neutralized by $CaCO_3$, thus Ca-lactate was causing the increase in conductivity). After the fermentation, we done a re-evaluation of the acquired data to answer the question, why InCyte could not detect cell growth. Fig. 2A shows the time curve of permittivity differences ($\Delta\varepsilon$) calculated from each measured LFP frequencies. We could observe, that 300 kHz should be applied instead of factory default 2000 kHz for bacteria. Thus on Fig. 2B the offline measured OD_{600} values and the online measured OVCC calculated from $\Delta\varepsilon_{300KHz}$ are presented versus fermentation time.

Furthermore Fig. 2B shows the off-line measured glucose, lactic acid concentrations (i.e. product concentration) and calculated lactic acid concentrations on the basis of measured conductivity values, too. Unfortunately the exponential phase of the cultivation was in the night when we were not able to take samples and make off-line sample analysis, thus the plot of permittivity against OD_{600} (i.e. calibration curve) could not be established. However, the online sensor could provide some information on that period, too. This fact reinforced also the importance of an online viable cell count (OVCC) measurement. In summary, Hamilton's InCyte sensor was able to follow both product and biomass formation, furthermore providing continuous monitoring of the bacteria fermentation as well.

Saccharomyces cerevisiae

Before fermentation test of *S. cerevisiae* commercially baker's yeast (Le Safre, Budafok, Hungary) was suspended in water. InCyte sensor was immersed into pure tap-water and high cell density suspension added slowly. While online data were monitored with the sensor, samples were withdrawn in every 2 minutes for OD_{600} off-line determination. Fig. 3A represents the measured data vs time, i.e. optical density of samples, permittivity differences and calculated biomass, the latter calculated with factory default slope and offset values. After plotting $\Delta\varepsilon$ vs OD_{600} we obtained Fig. 3B, which clearly shows the calibration curve, on which the online permittivity measurements start to correlate with offline OD_{600} data at higher cell concentration, and above OD_{600}=15 the correlation is really linear. For high cell density *S.cerevisiae* fermentation we have run a fed-batch cultivation of 0,8/1L, in which oxygen controlled molasses fed was applied into inoculated tap-water to keep sugar concentration as low as possible. Through this way Pasteur and Crabtree (or reverse Pasteur) effects (De Deken 1966) could be avoided. Fig. 4 shows that the 1% ammonium-hydroxide added to the molasses provided not only N-source but alkaline too for pH controlling at the same time. Thus the pH and the conductivity of InCyte sensor stepped if the feeding pump was turned on by the increasing oxygen signal (pO_2). When fresh molasses were introduced into the culture, oxygen value fell back, and feeding pump turned off with some delay. While only the beginning of the experiment was monitored at low level of biomass, the capacitance sensor still showed increasing permittivity ($\Delta\varepsilon$) suggesting the growth of biomass.

Cholorella vulgaris

Chlorella vulgaris was grown in standing flask on BG-11 medium (Feng *et al*., 2011) and then concentrated by decanting. The sensor was zero calibrated with pure BG-11 media. Then we pumped (constant flow rate) the concentrated biomass into the empty media. While sensor parameters were recorded online, we took samples in every 4 minutes for off line OD measurements on 560 nm. Fig. 5A. Shows that the eukaryotic algae produces a measurable signal ($\Delta\varepsilon$) already at much lower biomass concentration (offline OD_{560}=2) than prokaryotic cells did (Fig.3B LOD of offline OD_{600}=15). With the help of the above calibration, we started a fermentation with *C.vulgaris*. According to Chisti (2007), the illumination was periodically changed (in our case: 16 hours lighting and 8 hours dark period) beside low level of aeration (0,2 L/min). The obtained online permittivity against off line measured OD_{560} (Fig.5B.) verified the pre-calibration curve (Fig.5A.) with almost the same slope. Online and off line measured biomass results are presented on Fig. 6. While the biomass data calculated from online permittivity measurement (OVCC=3,1*$\Delta\varepsilon$-2,95) scatter very much, its tendency is the same as the offline measured OD_{560}. These results reinforced, that the preliminary calibration and sensor test is really useful during real cultivations.

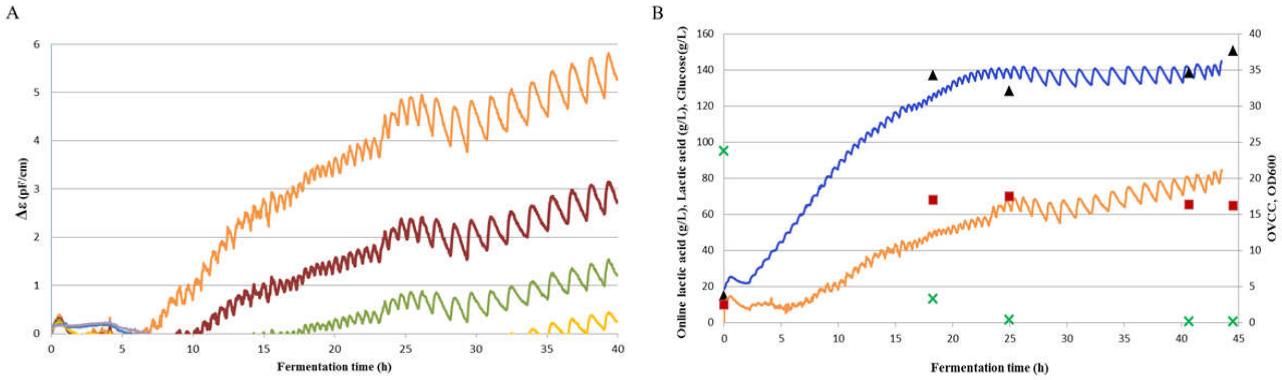

Figure 2. The measured parameters during the *Lactobacillus sp.* fermentation: A: Various permittivity (Δε) calculated at different LFP frequencies, —300 kHz —373 kHz —465 kHz —578 kHz —1732 kHz —2156 kHz; B: Lactic acid fermentation: ■ OD_{600} — OVCC(=3*$Permittivity_{300\ kHz}$[pF/cm]+2,5), — Online lactic acid (=9,8*Conductivity [mS/cm]-45), ▲ Lactic acid [g/L], □ Glucose [g/L]

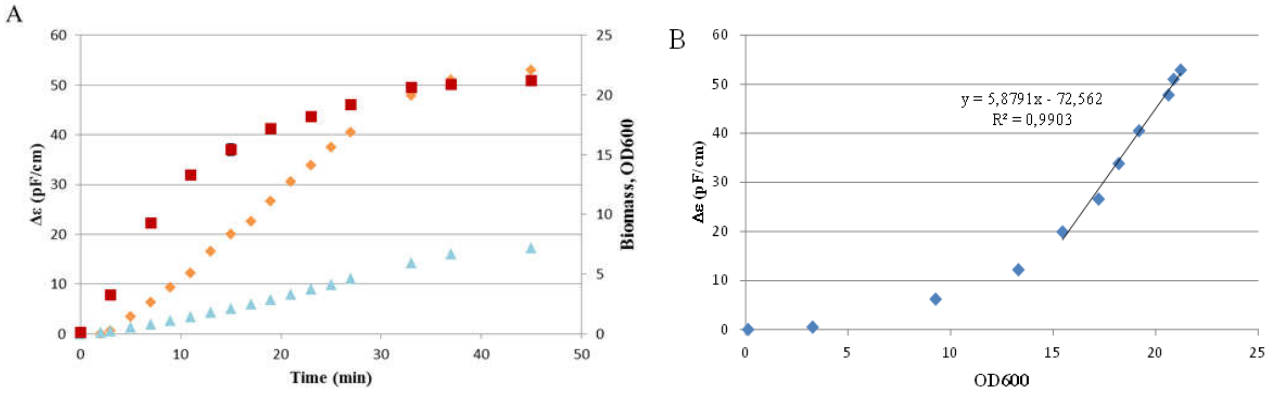

Figure 3. OVCC sensor calibration for baker's yeast A: *S. cerevisiae* calibration: ■ OD_{600}, ◆ Δε (2000 kHz) [pF/cm], ▲ OVCC with factory default Slope and Offset (=$Permittivity_{2000\ kHz}$[pF/cm]-2,49) B: pre-calibration curve: offline measured OD_{600} vs. ⊗ε [pF/cm]

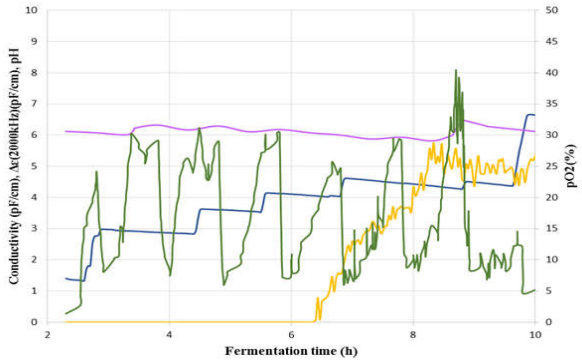

Figure 4. *S. cerevisiae* fermentation online parameters: — Conductivity [mS/cm] — Δε (2000 kHz) [pF/cm], — pH — pO_2[%]

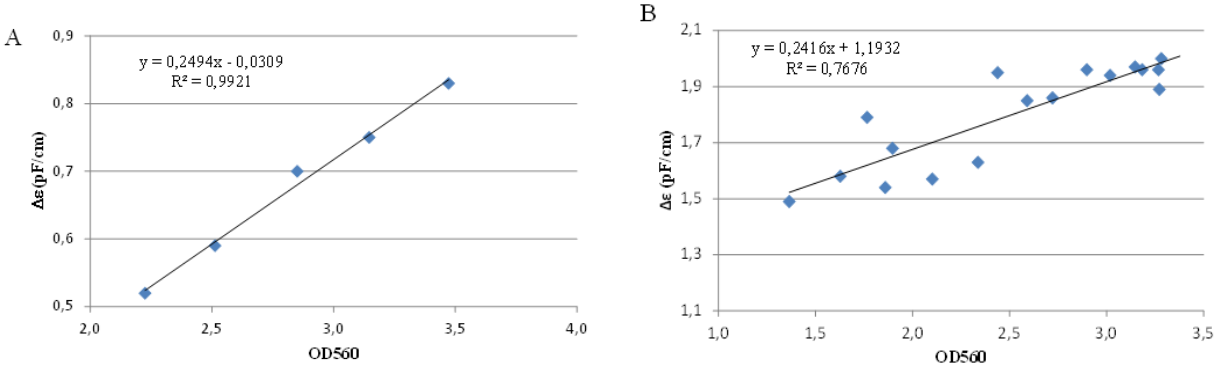

Figure 5. Sensor calibration for *C. vulgaris* A: pre-calibration: offline measured OD_{560} vs. Δε [pF/cm]
B: verification of calibration with fermentation data

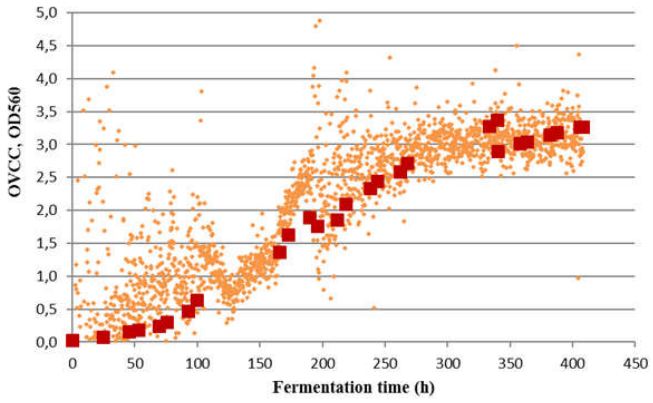

Figure 6. Results of different biomass determinations of *Chlorella vulgaris* during fermentation ■ OD_{560}, ◆ OVCC=3,1*Δε-2,95

Summary

The tested InCyte (Hamilton Bondauz AG) sensor was found to be appropriate for viable cell count monitoring for wide range of microorganism (prokaryotic bacteria, eukaryotic yeast and algae) out of the envisaged culture of cell tissues like CHO. The elaborated calibration method give a rapid and reliable forecast on 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, every fermenter and culture differ in size of air bubble, turbulence of stirrer etc. Since the tendency of the growth is clearly indicated by the sensor, a potential solution could be a noise filtration algorithm to improve results on the presented fermentations. We continue this work with filamentous fungi pellets and filamentous bacteria, too.

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