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ORIGINAL PAPER

On-line biomass measurements in bioreactor cultivations: comparison study of two on-line probes

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Abstract Two on-line probes for biomass measurement in bioreactor cultivations were evaluated. One probe is based on near infrared (NIR) light absorption and the other on dielectric spectroscopy. The probes were used to monitor biomass production in cultivations of several different microorganisms. Differences in NIR probe response compared to off-line measurement methods revealed that the most significant factor affecting the response was cell shape. The NIR light absorption method is more developed and reliable for on-line in situ biomass estimation than dielectric spectroscopy. The NIR light absorption method is, however, of no significant use, when the cultivation medium is not clear, and especially in processes using adsorbents or solid matrix for the microorganism to grow on. The possibilities offered by dielectric spectroscopy are impressive, but the on-line probe technology needs to be improved.

Keywords Biomass · Cell density measurement · Dielectric spectroscopy · Near infrared light absorption · On-line · Viability

Introduction

Biomass measurement is one of the most challenging as well as important measurements in the bioreactor cultivation of live cells. All reactor measurements can be divided into three groups: on-line, at-line and off-line. On-line measurements can further be divided to in situ and ex situ measurements depending on whether the measurement takes place inside or outside the reactor [11]. Reliable on-line methods yield valuable knowledge of the process state and can facilitate process control [10]. Several methods have been developed for the measurement of biomass. They differ in the measured phenomena or correlating variable. An inevitable result of this is that all the different methods cannot be interrelated or applicable to all processes and organisms. Different methods emphasize different biomass properties, e.g. cell number, cell viability, metabolic state or total mass. Thus, it is also important to acknowledge the limitations of different measurement principles as well as the correlation of the method used to the variable that needs to be known.

Traditional optical methods are probably the easiest ways to estimate biomass concentration in a bioreactor. Currently the most common method for estimating biomass is undoubtedly the absorbance measurement. The development of optical methods into optical probes has already led to the in situ stage. However, optical methods also present challenges, as reliable measurement results are required regardless of stirring and aeration in the cultivation vessel. Another challenge arises, when a wide scale of correlation is required without the possibility of diluting the sample. The sensitivity of the measurement to differences in the cultivation medium and environmental conditions has been successfully reduced by using infrared (IR) as the light source [9]. Light scattering is another optical method for biomass measurement. More information can be obtained by applying a spectrum range, like in near-infrared spectroscopy (NIRS) [1]. This method, however, may require complex interpretation of the results. Fluorescence based methods are another type of optical technology. They have

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been used for biomass measurement both in situ and ex situ. Stirring, aeration, pH and viscosity of the cultivation broth may have cross-talk effects on the fluorescence methods, and thus its use in situ is rather complicated [11]. Measurement errors induced by process conditions can be minimized by proper placement of the measurement probe and by using calculation methods in data filtering [9]. Another method to overcome these difficulties is the use of 2D fluorescence spectroscopy. Lastly, in situ microscopic techniques have been developed for the image analysis of the cultivation broth [11].

Dielectric spectroscopy is one of the most promising on-line in situ technologies for the measurement of viable biomass. The method is based on the fact that intact cells become polarized when electric field is applied on them and thus behave like capacitors. The amount of this polarization can be measured as capacitance [5]. The magnitude of the measured capacitance is a function of volume fraction of the cells and cell size. This method measures only the viable cells, which have intact cell membranes [8]. The capacitance of a cell depends on the frequency of the applied electrical field. Under 0.1 MHz cells are fully polarized. The measurement is usually done with a frequency that results in half of the maximum polarization. Disturbance effects of medium components and the process environment can be overcome by using a reference frequency. This is usually a high (> 10 MHz) frequency and the response at this frequency is subtracted from the response at the measurement frequency [3]. When reactor probes are used, an internal compensation of culture broth conductivity is necessary [10]. Enhanced stability and precision of dielectric spectroscopy was obtained using galvanic separation, where the probe function was based on two nested coils [2].

Many biomass measurement methods are quite logical such as the automated wet weight determination [10]. In addition, acoustic methods such as ultrasound have been used in biomass estimation [10]. Computational methods are common in well-known process conditions, when the same process is repeated over and over again. These methods are usually based on the correlation of metabolic activity with biomass [11]. Correlations have been constructed of bioreactor off-gas measurements, need for reactor cooling, pH or consumption of pH control agent, and increase in viscosity and stirring power [10, 11]. Further extensions of computational methods, such as neural networks, have also been used in combining process variables with biomass estimates [4, 7].

This study presents measurement results and experiences in the use of two different on-line in situ cell density probes. We monitored growth in different yeast and bacterial cultivations. The morphologies of the microorganisms were rods, cocci, irregular, budding cells, streptomycetes and spore forming. Some of the studied bacteria usually form chains. The functioning of the two probe types was investigated in cultivations with significant chemical changes in the medium (conductivity change due to pH control) or strong physical disturbances inside the reactor (stirring, high rate aeration). We found that the NIR light absorption probe was more readily applied to different processes than the laborious dielectric spectroscopy probe.

Materials and methods

Bacterial strains, maintenance and media

Bacillus subtilis 168 with plasmid pKTH10 for α-amylase production was cultivated in media presented in Table 1. The inoculum was prepared at 37°C by incubating a 50 ml culture in a 250 ml shake flask at 300 rpm until the optical density ($\lambda = 600$ nm) reached 2.0.

Bifidobacterium longum ATCC 15707 and DSM 14583 were grown in a medium containing 30 g l^{-1} yeast extract (Lab M), 20 g l^{-1} glucose, 2 g l^{-1} K_2HPO_4, 1 g l^{-1} Tween 80 (Fluka), 0.8 g l^{-1} $MgSO_4 \cdot 6\ H_2O$ and 0.5 g l^{-1} L-cysteine-$HCl \cdot H_2O$ at 40°C. The inoculum was cultivated at 37°C. First, 15 ml MRS medium (Lab M) supplemented with 1 g l^{-1} L-cysteine-$HCl \cdot H_2O$ (MRSC) was inoculated and incubated for 10 h. This pre-culture was further diluted with fresh MRSC to 50 ml (4%) and incubated for another 10 h.

Escherichia coli XL-1 blue strains QMX-8b and TXC-1a were used in the study. *Actinoplanes missouriensis*

Table 1 Media compositions in the *Ba. subtilis* 168 cultivation

Component	Inoculum (g l^{-1})	Bioreactor (g l^{-1})	Feed (g l^{-1})
Glucose	4.5	10.0	130
Glutamine	1.0	5.7	21.7
$(NH_4)_2SO_4$	0.95	5.1	19.5
Sodium citrate	1.0	1.0	130
Phosphates	10	10.1	–
$MgSO_4 \cdot 7H_2O$	0.3	0.3	2.6
	Inoculum (mg l^{-1})	Bioreactor (mg l^{-1})	Feed (mg l^{-1})
Kanamycin	10	10.1	15
Tryptophan	120	1,300	–
$CaCl_2 \cdot 2H_2O$	9	30.3	240
$ZnCl_2$	0.45	1.52	12
$CuCl_2 \cdot 2H_2O$	0.12	0.40	3.2
$CoCl_2 \cdot 6H_2O$	0.18	0.61	4.8
$Na_2MoO_4 \cdot 2H_2O$	0.12	0.40	3.2
$FeCl_3 \cdot 6H_2O$	1.8	6.06	54
$MnSO_4 \cdot H_2O$	0.40	1.35	12

xylose isomerase was produced by the strain QMX-8b. A mutated *Nonomuraea flexuosa* xylanase II was produced by the strain TXC-1a. Both strains were cultivated in Luria-Bertani broth (LB, Pronadisa) with 125 mg l^{-1} ampicillin. Inoculum was prepared in 10 ml test tubes with 5 ml culture volume and incubated for 10 h. This was transferred to 250 ml shake flasks containing 50 ml culture broth and incubated for another 10 h. The incubations were done at 37°C and 200 rpm.

Lactobacillus plantarum BPT 197 was cultivated in MRS medium (Lab M) supplemented with 5 g l^{-1} L-arabinose at 30°C. *Lb. plantarum* BPT 186 was cultivated in slightly modified MRS; no sodium citrate and acetate were added. Arabinose was added as with the strain BPT 197 and the cultivation temperature was 30°C. The inoculum for both strains was prepared by incubating a 10 ml MRS inoculum for 12 h. A portion of this was diluted to 50 ml with fresh MRS and the incubation was continued for another 8 h.

Lactococcus lactis GRS71, NZ9010 and mutant (ptrfut5) were grown in M17 medium (Difco) with 20 g l^{-1} glucose and 60 g l^{-1} xylose at 30°C. *L. lactis* NZ9800 was cultivated in M17 supplemented with 5 g l^{-1} soy peptone (Lab M) and 50 g l^{-1} glucose at the same temperature. The inoculum was prepared by incubating a 15 ml M17 inoculum with 20 g l^{-1} glucose for 10 h. This was diluted with fresh MRS to 50 ml (4%) and incubated for another 10 h. Erythromycin (5 mg l^{-1}) was used with NZ9010 strains and chloramphenicol (8 ml l^{-1}) with the strain containing ptrfut5.

Probat 505 lactic acid bacteria mixture (Danisco) was cultivated in different media containing glucose 15–45 g l^{-1}, casitone 12.5 g l^{-1}, yeast extract 7.5 g l^{-1}, phosphates 3 g l^{-1} and $MgSO_4$ 0.05–0.35 g l^{-1} at temperatures 26–38°C. Inoculum was prepared in 50 ml M17 medium (Difco) at 30°C for 10 h.

Saccharomyces cerevisiae was cultivated in YM medium (Lab M) at 30°C. The 50 ml inoculum was incubated in a 250 ml shake flask at the same temperature with 150 rpm stirring. *Streptomyces peucetius* var. *caesius* N47 was cultivated as described earlier [6]. All strains were maintained at -80°C as glycerol stock (~13%).

Bioreactors and controls

Biostat MD (2 l, B. Braun Biotech International, Germany) and Biostat Q (1 l, B. Braun) reactors were used in the cultivations. The cultivation conditions of different microorganisms are shown in Table 2. In the *Ba. subtilis* cultivations the starting volume was 1.0 l. Glucose was measured at-line and controlled at 2.0 g l^{-1} using YSI 2700 Select analyzer (Yellow Springs Instruments, USA). The reactor was also equipped with three side baffles to enhance oxygen transfer. The *E. coli* cultivations were aerated at sequentially increasing rates from 0.3 to 1.0 l min^{-1} (QMX-8b) and from 0.3 to 1.2 l min^{-1} (TXC-1a). A measure of 1 mM IPTG was used for induction at 1 h cultivation time. The pH control agent was 3 M NaOH in all cultivations.

On-line cell density measurements

The NIR light absorption sensor used in this study was a TruCell cell density probe (Finesse Instruments, CA, USA). A laser was used as the NIR light source and two photo detectors were applied in the probe for measurement and reference. The probe response was transferred to computer database through 10-bit conversion of an analog 4–20 mA signal. The dielectric spectroscopy sensor used in this study was an AberProbe coupled with a Biomass Monitor 220 (Aber Instruments, UK). The AberProbe has four annular

Table 2 Bioreactor setup in different cultivations

Organism	Reactor	Cultivation	Aeration (l min^{-1})	Stirring (rpm)	T(°C)	pH
Ba. subtilis	Biostat MD	Fed-batch 1.0 l	3.0[a]	800[c]	37	7.0
B. longum	Biostat MD	Batch 1.0 l	–	100[d]	40	6.2
E. coli	Biostat MD	Batch 1.0 l	0.3–1.2[b]	500[d]	37	n.c.
Lb. plantarum	Biostat MD	Batch 1.5 l	–	200[d]	30	6.2
L. lactis	Biostat MD	Batch 1.0 l	–	100[d]	30	6.5
Probat 505	Biostat Q	Batch 0.8 l	–	300[e]	26–38	7.0
S. cerevisiae	Biostat MD	Batch 1.5 l	2.5	500[d]	30	5.5
St. peucetius	Biostat MD	Batch 1.5 l	1.0	600[d]	30	6.0

n.c. not controlled

[a] Dissolved oxygen control was applied by the addition of pure oxygen to the air flow while maintaining a constant aeration rate

[b] Sequential increase

[c] Three 6-blade Rushton turbine impellers

[d] Two 6-blade Rushton turbine impellers

[e] Magnetic stirrer

ring electrodes. The probe is capable of measuring the capacitance and conductivity of the culture broth at certain frequencies over a wide frequency range (0.1–20 MHz). The frequencies used are shown in Table 3. The probe response was transferred to the computer database via serial interface.

Analytical methods

Optical density (OD) of the cultivation was measured from samples diluted with H_2O to absorbance values ranging from 0.05 to 0.50. The measurement wavelength was 600 nm.

The cell dry weight (cdw) of *B. longum*, *E. coli*, *L. lactis* and *Lb. plantarum* cultivation samples was measured using pre-incubated glass tubes. A 4 ml sample was centrifuged at 8000 rpm for 5 min. The cell pellet was washed twice with 3 ml H_2O and dried overnight at 80°C. The cdw of *S. cerevisiae* and *St. peucetius* was measured using pre-incubated 0.2 µm membranes. The sample was vacuum-filtered and washed once with the sample volume of phosphate buffer (pH 6) and twice with the sample volume of H_2O. The membrane was dried overnight at 80°C.

Results

Cultivations were run mostly in duplicate. The analytical measurement results were compared with the on-line measurements. Correlations of analytical methods and TruCell measurements with different microorganisms are shown in Table 3. Figure 1 shows examples of TruCell probe and OD estimates of cdw for *S. cerevisiae*, *B. longum* ATCC 15707, *L. lactis* NZ9010, *E. coli* TXC-1a and *Lb. plantarum* BPT 197 together with measured cdw values. Differences between probe estimates and measurement data are largest with *E. coli* and *S. cerevisiae*, which were the aerated cultivation processes. Best results were obtained with all lactic acid bacteria.

Correlations of OD and Biomass Monitor measurements with different microorganisms are shown in Table 4. Examples of cdw estimates using the Biomass Monitor together with cdw estimates obtained from OD-values and the actually measured cdw values from *Ba. subtilis*, *S. cerevisiae*, *B. longum* ATCC 15707 and *Lb. plantarum* BPT 186 are shown in Fig. 2. The best biomass estimate was obtained with *Ba. subtilis*. The results obtained with *Lb. plantarum*

Table 3 Measurement correlations of TruCell probe with different microbial strains

Strain	OD	cdw
B. longum ATCC 15707	0.384 ± 0.023[b]	0.867 ± 0.170[b]
B. longum DSM 14583	0.474 ± 0.096[a]	1.242 ± 0.008[b]
E. coli QMX-8b	0.360[b]	–
E. coli TXC-1a	0.320[b]	–
Lb. plantarum BPT 197	0.296 ± 0.004[a]	1.241 ± 0.066[b]
Lb. plantarum BPT 186	0.288 ± 0.006[a]	1.128 ± 0.013[b]
L. lactis GRS71	0.414[b]	1.048[c]
L. lactis NZ9010	0.424 ± 0.000[b]	0.939 ± 0.013[b]
L. lactis NZ9010 ptrfut5	0.428 ± 0.021[a]	–
Probat 505	0.360 ± 0.023[b]	–
S. cerevisiae	0.223 ± 0.014[c]	1.077 ± 0.015[c]

The values show the increase in TruCell signal (mA) with a one unit increase of the measured variable. The deviations are standard deviations from replicate cultivations. The column OD designates the correlation slope of the TruCell signal with optical density and the column cdw with cell dry weight

[a] R^2 of linear fit over 0.995

[b] R^2 of linear fit over 0.950

[c] R^2 of linear fit over 0.900

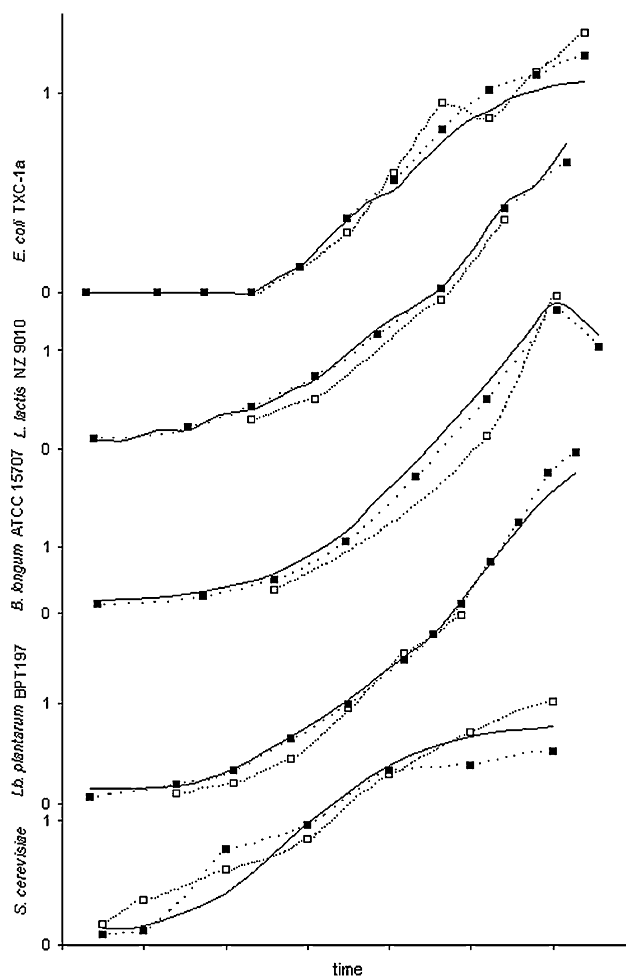

Fig. 1 Measured biomass concentration (cdw, *open squares*), biomass concentration calculated from optical density measurement results (*closed squares*) and biomass concentration calculated from TruCell probe measurement results (*lines*) from the cultivations of different microorganisms. The time-scale is proportional to cultivation length

Table 4 Measurement correlations of Biomass Monitor probe with different microbial strains

Strain	Frequency (kHz)	OD	R^2
Ba. subtilis 168	1000	0.13	0.996
B. longum ATCC 15707	1000	0.14	0.993
B. longum DSM 14583	1000	0.32	0.999
L. lactis NZ9010	2200	0.066	0.977
L. lactis NZ9010 ptrfut5	2200	0.056	0.966
L. lactis NZ9800	1000	0.13	0.980
Lb. plantarum BPT 197	2170	0.045	0.995
Lb. plantarum BPT 186	2200	0.056	0.926
S. cerevisiae	200	0.93	0.900

The values show the increase in capacitance with a one unit increase of the measured variable. The column OD designates the correlation slope of Biomass Monitor signal with optical density

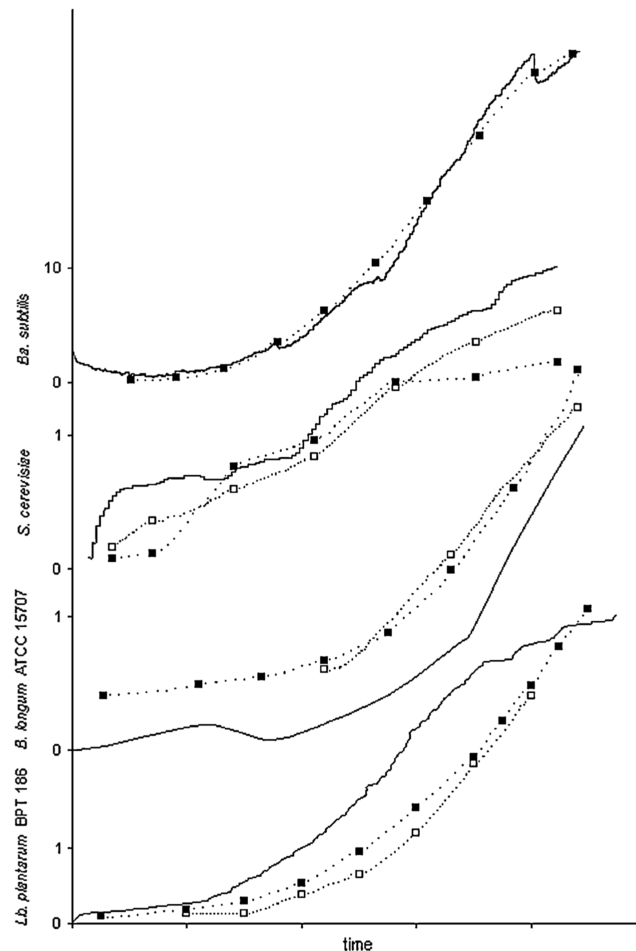

Fig. 2 Measured cdw (*open squares*), biomass concentration calculated from optical density measurement results (*closed squares*) and biomass concentration calculated from Biomass Monitor probe measurement results (*lines*) from the cultivations of different microorganisms. The time-scale is proportional to cultivation length

are logical: when the cultivation approaches the stationary phase the fraction of viable cells of the whole biomass decreases. Viable cell coefficients were determined for *Ba. subtilis* and *L. lactis* NZ9800. The linear correlations yielded slopes of 6.14×10^{-10} and 6.77×10^{-11} pF cm^{-1} cfu^{-1} with coefficients of determination 0.995 and 0.986 for *Ba. subtilis* and *L. lactis*, respectively. *St. peucetius* measurements correlated poorly with the probe response.

The results obtained with the *L. lactis* and *B. longum* were further analyzed. The slopes of the TruCell probe vs. optical density are shown in Fig. 3. Results obtained with the TruCell probe on different strains of the same species are generally not related to one another. However, the correlations of different metabolic mutants of the same strain were very close to one another. The difference in linear correlation slope between mutants of the same strain is less than 10%. The results obtained with the Biomass Monitor were less comparable.

Discussion

In this study, we used a linear correlation for the NIR light absorption probe. The manufacturer of the TruCell probe suggests that second order equations should be used. We found that when low cell densities are measured, the linear correlation is adequate. Problems arise in fed-batch cultivations, when the cell density rises high enough. The critical limit depends on the microorganism and cultivation type. The only cultivation type in this investigation requiring second order correlation was the *Ba. subtilis* fed-batch cultivation. Our results imply that the NIR light absorption method can be applied to different metabolic mutants directly (Fig. 3). Plasmid constructs have no effect on the probe response compared to the response with the native strain. Even a cocktail of different bacterial strains can be measured reliably and in different environmental conditions. The slope variation in the Probat 505 cultivations was only 6% although the glucose concentration and temperature variations in different cultivations were relatively large. This variation included measurements with four individual probes. No direct effects from glucose, temperature or individual probe were detected (results not shown).

Dielectric spectroscopy is complicated, and there are still problems when applying probes to the cultivation vessels. The method is laborious to apply to different microorganisms. Within the scope of this research, no suitable measurement parameters were found for the monitoring of *St. peucetius* cultivations. However, we believe that further research would yield successful on-line monitoring of this bacterium as well. According to the theory the best correlations with bacterial strains should at maximum be 1000 kHz. Our measurement frequencies were obtained

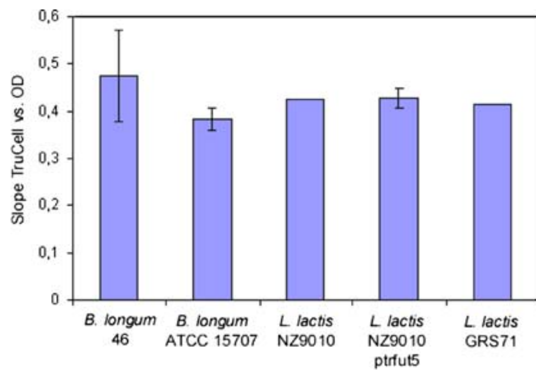

Fig. 3 Correlation slopes of TruCell probe and optical density (OD) measurements from *Bifidobacterium longum* and *Lactococcus lactis* cultivations

using the best correlations from scanning results (not shown). The best values obtained in this investigation are not consistent with the theory. This suggests that there are still development targets in the dielectric spectroscopy probe technology. At best the method is an excellent tool for process monitoring, and can yield valuable information on environmental or metabolic constraints that are applied to the organism. This is demonstrated in Fig. 2 with the *Ba. subtilis* fed-batch cultivation, when prior to the feeding phase a small decrease in probe response was observed. The decrease occurred simultaneously with a slight increase of pH in the cultivation medium. The long-term stability of the probes is unknown, as the two probes used in our study seemed to lose their accuracy and measurement capability while used in autoclaved bioreactors. The probe seems to lose its overall correlation capability and function over extended bioreactor use. During our 1-year research period with the equipment the two probes ceased working properly.

In conclusion, the NIR light absorption method is a more developed and reliable method for on-line in situ biomass estimation than dielectric spectroscopy. The NIR probe can be applied to the on-line estimation of biomass in various kinds of processes with various types of microorganisms. The probe is also suitable for monitoring different metabolic mutants of a specific bacterial strain without modifications to the parameters. The NIR light absorption method is, however, of no significant use, when the cultivation medium is not clear, and especially in processes using adsorbents or a solid matrix for the microorganism to grow on. The use of dielectric spectroscopy probes was less straight forward than the NIR light absorbance probe, but shows promise in determining small changes in the metabolic state of the cells. The possibilities offered by dielectric spectroscopy are impressive, but the on-line probe technology still requires improvement.

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