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REVIEW

Biomass measurement online: the performance of in situ measurements and software sensors

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Abstract Biomass measurement is one of the most critical measurements in biotechnological processes. The technologies developed for the measurement of biomass in situ have developed over the years. Because it has been over 10 years since the last review concentrating on practical issues concerning biomass measurements, it is time to evaluate recent developments in the field. This review concentrates on the applications of dielectric spectroscopy, optical density, infrared spectroscopy, and fluorescence for in situ measurement of biomass. The advantages offered by these methods and an economic way of estimating biomass concentration, the software sensors, are considered.

Keywords Biomass · In situ · Online measurement · Probe · Software sensor

Introduction

The reliable in situ measurement of microbial biomass has been a challenge for decades. Many methods have been designed over the years and they have ended up as prototypes, one-time applications or rarely even as industrially applicable probes. The general requirements for a reliable probe are the possibility of calibration, linear dependency, and precision at both low and high cell densities [46]. However, in bioreactors the probes (Fig. 1) have a few other requirements—they should be sterilizable, they should endure temperature and pressure, and they should be corrosion stabile and biologically inactive [17]. Furthermore, for industrial applications the probe calibration should also be stabile over multiple fermentations, sterilizations, and cleaning cycles.

Many reviews have been written on biomass measurements from bioreactors over the years, some concentrating on general analysis methods, others on online technologies. The practical aspects of online in situ measurement methods have been reviewed 10 years ago by Olsson and Nielsen [46] and a good applicability study was written by Sonnleitner [55]. This review concentrates on updates and recent developments in the technologies and presents the current industrially applicable probes. More specifically, the probe technologies under review are dielectric spectroscopy, optical density, infrared spectroscopy, and fluorescence. Furthermore, calculation methods in biomass estimation are briefly summarized in this review.

Dielectric spectroscopy

Dielectric spectroscopy was developed in the beginning of the twentieth century [66], but its first applications to biological materials were published as late as the 1950s [51]. The theory of dielectric spectroscopy has been excellently reviewed at the turn of the century [37, 70]. Simplified dielectric spectroscopy utilizes measurements of conductance and capacitance of the cultivation broth. The most used indicators of cell measurement are the change in capacitance (ΔC) or the relative permittivity (ε). The relative permittivity is calculated from the capacitance measurement results and physical constants related to the probe. The probe signal is usually so weak that a pre-amplifier of considerable size is necessary near the probe.

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Fig. 1 Two different biomass monitoring probe types connected to a bioreactor

Capacitance can be measured at different frequencies, thus generating a dielectric spectrum of the cell suspension. The dielectric spectrum is affected by both cell concentration and media components. Several dispersion areas (α, β, δ, and γ) can be observed from the shape of the dielectric spectrum [37]. The β-dispersion of the measurement gives a suggestion of a suitable measurement frequency according to cell morphology, size, and type. Usually the suitable measurement frequency is at 0.5–3.0 MHz radio frequency range [17].

Applications of dielectric spectroscopy are reviewed in Table 1. By far the biggest industrial application area of dielectric spectroscopy is currently in breweries. Table 1 shows that the Aber Instruments probes have been the most popular in academic research. A major disadvantage is that most of the publications are either directly or indirectly related to Aber Instruments Ltd.

In low conductivity medium with plant cell concentrations up to 15 g l^{-1}, the accuracy of the Aber Instruments Bugmeter was 0.5 g l^{-1} [38]. Stirring and aeration caused highly noisy results with just one measurement frequency using the Model 214 Aber Instruments probe [44]. Nevertheless, one of the best correlations of ΔC to biomass was obtained with the same probe type for *Streptomyces clavuligerus* while a reference frequency was used [0.46 ± 0.005 pF cm^{-1} (g l^{-1})$^{-1}$, $R^2 = 0.998$] [43]. However, in two distinct studies on *Candida utilis* by the same study group yielded two quite different calibration equations for the Model 214 Aber Instruments probe. In 2000 the instrument calibration equation was $\Delta C = 0.3509X + 0.1901$ ($R^2 = 0.978$) [44], and in 2002 the instrument calibration equation was $\Delta C = 0.2746X + 0.0015$ ($R^2 = 0.932$) [45]. This implies that

Table 1 Dielectric spectroscopy probes and applications

Instrument	Species	Range	Frequency	Conductivity	Reference
Biomass System[a]	*Lactobacillus casei*	Over 0.2 g l^{-1}	5.7 MHz	15–75 mS cm^{-1}	[4]
Biomass System[a]	*Bacillus thuringiensis*		Low, mid, and high	15–40 mS cm^{-1}	[49, 50]
Biomass System[a]	*Saccharomyces cerevisiae*	10–100 g l^{-1}	Dual frequency		[41]
HP E5050A[b]	Yeast	Over 10^7 cells ml^{-1}	At-line		[20]
HP 4194A[b]	*Enterococcus hirae* and *Klebsiella oxytoca*	1–10 and 3–15 g l^{-1}	At-line 0.1 MHz		[7]
HP E5050A[b]	*Escherichia coli* and *S. cerevisiae*	OD 92 and 100 g l^{-1}			[52]
Model 214[c]	*S. uvarum* (*carlsbergensis*)	0.5–3.5 g l^{-1} (poor R^2 also for viable)	0.3 MHz		[5]
Model 214[c]	*Candida utilis*	Up to 10 g l^{-1}	0.8 MHz	Max 16 mS	[44, 45]
Model 214[c]	*Streptomyces clavuligerus*	Up to 20 g l^{-1}	0.4 and 10 MHz		[43]
24CT35 and 24CT38[c]	*S. cerevisiae*, *Pichia pastoris*, and *St. virginiae*	Up to 106 g l^{-1}, 89 g l^{-1} and 30 g l^{-1}	Not reported	Max 20 mS	[19]
Viable Cell Monitor[c]	CHO 320	Over 1.4×10^5 ml^{-1}	0.5 MHz		[23]
Bugmeter[c]	*Festuca arundinacea*	3–15 g l^{-1}	0.4 MHz	1–2 mS cm^{-1}	[38]
Model 214M[c]	CHO SSF3	10^7 ml^{-1}	0.8–1.0 MHz	16 mS cm^{-1}	[12]
Model 214M[c]	*Spodoptera frugiperda* and *Trichopulsia ni*	5×10^7 ml^{-1}	Not reported	Not reported	[71]
BM 216[c]	Yeast strains		0.3 MHz		[32]
BM 220[c]	*B. subtilis*, lactic acid bacteria, yeast, *E. coli*	Batch and fed-batch concentrations	Various	Variable	[31]

[a] Fogale Nanotech, France

[b] Hewlett-Packard, USA

[c] Aber Instruments, UK

the calibration equation might be subject to change over extended use, and that the correlation of the signal to biomass could become weaker. However, the change in the calibration equation is expected, if the individual probe was changed between the experiments or the probe geometry changed. The Biomass System by Fogale Nanotech yielded an offline calibration of 0.096 ± 0.008 pF cm^{-1} (g l^{-1})$^{-1}$ and an online correlation of 0.090 ± 0.005 pF cm^{-1} (g l^{-1})$^{-1}$ for *Lactobacillus casei* [4], but the measurements were done at a frequency so high (5.7 MHz) that the β-dispersion theory does not support the reliability of the results.

Commercial electrodes have exhibited polarization problems, when the biomass concentration is low and when the conductivity is high [70]. Thus, a different measurement technology was developed in order to overcome the polarization problems. This novelty was the inductive dielectric spectroscopy, where the electrodes are assembled as rings on the electrode surface (Fig. 2).

Fehrenbach et al. [19] found no significant difference in the relative capacitance measurements from two distinct Aber Instruments probes at higher biomass concentrations. However, with a Model 220 inductive dielectric spectroscopy probe by Aber Instruments the differences of relative capacitance measurements with two individual probes were quite distinct. The calibration equation was also found unstable for various bacterial strains [31]. Nevertheless, the older, direct Aber Instruments probe has proved extremely reliable in yeast monitoring at breweries, but calibration is always necessary when the probe geometry changes, and differences between individual probes are frequent (personal communication, Jukka Kronlöf).

Optical probes

Optical methods are probably the easiest methods for biomass measurement (Fig. 3). Thus optical density probes are the most commonly used in situ devices for online biomass estimation [40]. The measured object determines the wavelength area which should be used in the measurement. If the objects are smaller than 3 μm, visible wavelength

Fig. 2 The tip of the annular type dielectric probe

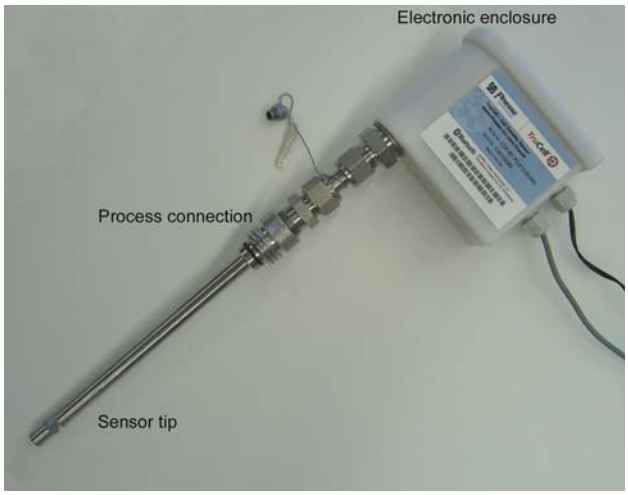

Fig. 3 Optical cell density probe

should be used. Larger objects are best detected in the near infrared area, where most media have low absorbance [46]. Measurement response is affected by different morphologies and different cell types [40]. Agitation and aeration affect the size and the amount of air bubbles, which may cause remarkable error in biomass estimation with optical probes. This is circumvented by certain models (e.g., Cerex and ASR) by passing the sample into a degassed measurement chamber, but this arrangement may affect CIP results [46].

Applications of optical biomass measurement probes are reviewed in Table 2. The applicability of six different optical cell density probes was compared in continuous monitoring of mammalian cell cultivations [69]. All tested probes were turbidimeters in which measurement is based on scattering and/or light transmittance: two backscattering probes (Aquasant and Ingold), two laser probes (ASR and Cerex), and two transmission probes (Wedgewood and Monitek). The Cerex probe was omitted during the study due to operational difficulties. Emitting/receiving wavelengths varied between 780 and 1,100 nm, thus being in the near infrared (NIR) area. Two bioreactors were equipped with three or four probes, so that Aquasant probes were used in both reactors as a means of cross-comparison. Murine hybridoma cells were cultivated for 46 days, the measurement interval was 30 s and cell densities varied from 1×10^6 to 20×10^6 cells ml^{-1}. Calibration with known cell concentrations revealed that backscattering probes gave the most linear and transmission probes the least linear responses. This does not indicate better performance as long as reproducibility is at the same level. It was not possible to say that one of the probes was best for all reactor systems. Cell density, cell stickiness, and reactor geometry affected the probe choice. Transmission probes with a wide or long detection zone may have higher

Table 2 Optical density probes in biomass estimations

Instrument	Manufacturer	Principle	Wavelength (nm)	Species	Range	Light source	Reference
653/BT65	Wedgewood, USA	Transmission	950–1,100	*Lb. casei*	Linear to 1 g l^{-1}	Filtered light	[4]
BT/65	Wedgewood, USA	Transmission	950–1,100	*B. thuringiensis*	Nonlinear	Filtered light	[49, 50]
MAX	Cerex, USA	Transmission	820–850	*E. coli*	Up to 40 g l^{-1}	Laser diode	[16]
Ingold FSC 402	Mettler-Toledo, Switzerland	Back scattering	880	Murine hybridoma		LED	[69]
LT-300	ASR, Japan	Transmission	780	Murine hybridoma		Laser	[69]
FT3	Monitek, USA	Transmission	800–1,100	Murine hybridoma		Filtered light	[69]
650	Wedgewood, USA	Transmission	950–1,100	Murine hybridoma		Filtered light	[69]
AF 44 S/R	Aquasant, Switzerland	Back scattering	940	Murine hybridoma		LED	[69]
Cerex 910-0006	Cerex, USA	90° scattering and absorbance	820–850	Murine hybridoma		Laser	[69]
LA 300LT	ASR, Japan	Transmission		*S. cerevisiae*	Up to 5 g l^{-1}	Laser	[28]
TruCell	Finesse Instruments, USA	Transmission	850	*E. coli*, lactic acid bacteria		Laser	[31]
	FiberTech, Germany	Back scattering	400–700	*S. cerevisiae*	2–20 g l^{-1}	Halogen	[26]

sensitivities at low cell densities than backscattering probes. However, at higher cell densities the backscattering probe performs better than the transmission probe. A narrower gap, which was available for both transmission probes, would give a more linear response for the whole range, but also increase risks of probe clogging and fouling. Probe fouling was not a problem in this study, but it was noted that probes should always be placed to a high shear position to avoid fouling. As a practical perspective it was also mentioned that backscattering probes require the shortest insertion length. On the other hand, the probe also requires an unobstructed view deep into the cultivation broth. Transmission probes sense the cells only inside their detection zone, but require a longer insertion length.

The Biomass System (Fogale Nanotech) was compared with the Wedgewood BT/65 optical density probe with two different microorganisms: *Lb. casei* and *Bacillus thuringiensis* [4, 49, 50]. The *Lb. casei* investigation showed fairly linear correlation of both probe responses up to an optical density of 1.5 [4]. The correlation was not linear in the *B. thuringiensis* process, where maximum optical density was around 20 [49, 50]. The Biomass Monitor (Aber Instruments) was compared with the TruCell probe (Finesse Instruments) with many different microorganisms [31]. The age of the Biomass Monitor probe affected the linearity of the correlation with the TruCell probe. When compared to dry cell weight measurements the TruCell probe linearity range (up to 3 g l^{-1}) was higher than the Biomass Monitor linearity range (less than 2 g l^{-1}), although some investigations have reported linearity of the Biomass Monitor measurements up to 100 g l^{-1} (Table 1).

Infrared spectroscopy

The advantage of spectroscopy techniques (scanning absorbance or transmittance measurement) while comparing to simple OD measurements is the possibility of obtaining more process component information besides biomass concentration. Infrared spectroscopy has been utilized in different ways: near infrared (NIR), mid-infrared (MIR) and the whole infrared range. Data preprocessing and advanced analysis algorithms such as derivatives, PLS models, or Fourier transformations are required when true analytical information is obtained from the analyte's absorption bands.

The use of infrared spectroscopy technologies in biomass estimation is reviewed in Table 3. Infrared spectroscopy is typically applied to bioprocesses at-line or online through medium circulation systems. The best biomass correlating wavelength areas have been around 2,300 nm in at-line systems. The online medium circulation NIR spectroscopy has been applied to fully automated process control in lactic acid production by *Lb. casei* subsp. *casei* [21]. The NIR spectrum yielded online information on biomass, glucose, and lactic acid.

NIR spectroscopy has recently been applied to in situ process monitoring via fiber optic probes [3, 58]. Process interferences at wavelengths over 2,000 nm are too large with fiber optic probes and a suitable process monitoring wavelength area is around 1,500 nm in these applications [3, 58]. Probe path length is a critical factor influencing the accuracy of the fiber optics probe [3], and agitation and aeration can have profound effects on the measurement baseline [58].

Table 3 Infrared spectroscopy in biomass measurements

Instrument	Operation mode	Wavelength (nm)	Species	Accuracy	Calculation	Reference
6500[a]	At-line	1,600–2,350	*St. fradiae*		PCA morphology study	[62]
6500[a]	In situ (fiber optics)	700–1,800	*Staphylococcus* and *Lactobacillus*	S: SEP 0.85, L: SEP 0.55	PLS calibration from cultivation samples	[58]
InfraAnalyzer 450[b]	At-line		*Lb. casei* subsp. *casei*			[60]
6500[a]	In situ (fiber optics)	1,500–1,800	*E. coli*	SEP 1.39	PLS from second derivative	[3]
6500[a]	At-line	1,600–1,800	*Penicillium chysogenum*	SEP 0.74	PLS from second derivative	[61]
6500[a]	In situ (fiber optics)	904–1,406	*Vibrio cholerae*	SEP 0.20	PLS	[42]
FTS 6000[c]	At-line	IR	*S. cerevisiae*	SEP 0.55[f]	Fourier transformation, PLS + PCA	[65]
870[d]	At-line	MIR	*Lb. casei*	SEP 0.37[f]	Fourier transformation	[53]
DA 7000[e]	At-line	NIR	*Lb. casei*	SEP 0.39[f]		[53]
870 + 32B[d]	At-line	Raman	*Lb. casei*	SEP 0.85[f]	Fourier transformation	[53]

SEP Standard error of prediction

[a] Foss NIRSystem, USA

[b] Bran & Luebbe, Germany

[c] Bio-Rad, USA

[d] Nicolet, USA

[e] Perten Instruments, USA

[f] Presented in OD units

Fluorescence

Culture fluorescence was applied for biomass monitoring already in the 1950s [46]. The first application of online systems was published in 1970 [34]. The method is based on excitation of UV light at one wavelength and measuring the emission of culture components at another wavelength. This only works for viable cells [34] and is applicable only if NAD(P)H amount per cell remains constant [40, 46]. Biomass estimations using one wavelength for excitation and another for emission (1D fluorescence) have been brought in situ already in the 1970s. A good list of applications has been published elsewhere [56]. Changes in culture conditions and background fluorescence interfere with 1D fluorescence biomass estimations. Major problems result from medium components that absorb light at excitation wavelength or at the emission wavelength and from other possible fluorophores, like penicillin [46].

Some interference problems can be solved using multiple excitation–fluorescence measurement. This is called 2D fluorescence spectroscopy. This enables detection of several fluorophores (e.g., tryptophan, pyridoxine, FAD, FMN, NAD(P)H, and riboflavin) that can be correlated with biomass [34, 46]. BioView and Hitachi provide equipment for 2D fluorescence spectroscopy. The method requires use of chemometric tools, such as PLS, PCA, or neural networks, for calculation. There is also considerable delay in measurement, as one measurement takes around 1 min to complete. Excitation wavelengths are usually around 250–550 nm and emission wavelengths around 300–600 nm. Subtraction spectra are required when process conditions vary during cultivations, but this is often compensated in commercial software that accompanies products [57]. 2D spectroscopy has been applied to *Saccharomyces cerevisiae* [24, 39, 59], *Escherichia coli* [39, 59], *Claviceps purpurea* [10, 39], and *Sphingomonas yanoikuyae* [39]. Biomass estimate errors were around 10%, when reported [10, 24].

Calculation methods

Correlation methods

A simple but often case-specific method for the estimation of biomass concentration is the use of correlation methods. Correlations of biomass are sought from variables that are commonly measured online from bioreactors. Stoichiometric coefficients and modeling skills are also often required while using correlation methods. The most popular correlation method is the use of off-gas analysis in biomass estimation [11, 15, 18, 48, 64]. Measurements of chemical properties of the medium such as pH, conductivity, or the demand of pH control agents have also been used in estimating biomass concentrations [1, 25, 67]. Also physical properties of the culture such as broth viscosity have been combined with biomass measurements [6, 68]. Heat balances in bioreactors can also be used in estimating biomass concentration [22, 29, 33, 36]. All of these are no doubt

useful and readily available to all industrial applications, where the process is similar in every case and when adequate measurements and process knowledge are available.

Software sensors

Typical software sensors are mathematical models based on growth kinetics or statistical analysis [such as multilinear regression (MLR) or principal component analysis (PCA)], neural networks, or combinations of all of these. Readily available online variables are inputs of the software sensors in various combinations; some are based on simple off-gas or base consumption results, others rely on a more holistic variety of online process data. All software sensors are relatively economic, as they can be constructed on simple PCs using common bioreactor measurements. The only necessary item is the interface between a commercial process monitoring database and the software making the estimates.

Model-based biomass observers have been built based on microbial growth kinetics or simple first order rate equations. The most used growth kinetics equation is the Monod equation [13], but also Haldane kinetics [54] and the logistic equation [47] have been successfully used for biomass estimations. Another popular way for biomass estimation has emerged from state estimation and process control theory in the form of adaptive state estimators. These models are often based on simple first-order rate equations and continuous parameter estimation (or tuning) protocols [35, 67]. A common drawback with kinetics-based biomass estimators is the assumption of constant coefficients. This assumption is often non-valid in bioprocesses, as the characteristics of the microbial strain are not constant during changing environmental conditions. Despite the advantages, adaptive state estimators are not a common choice for biologists, as the mathematics behind the system is still perceived as complex and intimidating.

Environmental conditions and vast amounts of process measurements are linked to biomass estimates via multivariate statistical analysis or artificial neural networks (ANN). In principle, the former methods are linear and the latter non-linear, in relation to their parameters. Multivariate linear regression (MLR) models have been used for this purpose in combination with growth kinetics [47]. PCA models are also useful, particularly because they provide a simple variable for fault detection and quality control [72]. Neural networks have been used for biomass estimation on their own [2, 14, 27, 30, 47] or in combination with other modeling techniques [9].

A comparative study using different software sensor types was conducted on *E. coli* [27]. Biomass estimators were formed and tested on the basis of 20 cultivations, and their performance was evaluated using root mean square error. All software sensor types used off-gas CO_2 and O_2, and base consumption as inputs. The results revealed the following performance order, from best to worst: feed forward ANN, polynomial regression model, auto associative ANN, Luedeking–Piret based model, PCA model, and MLR model using cumulative inputs. The authors found that although the PCA model was more inaccurate than the simple ANN, the performance of the PCA model was least disturbed by unexpected process conditions. Thus the suggestion of this study was a combinatory use of ANN and PCA, where the PCA model is used as a quality control measure, which yields information on whether or not to rely on the ANN biomass estimates.

Conclusions

The methods of biomass measurement that have developed into probe technologies are mainly dielectric spectroscopy and various optical methods. Both technologies have advantages and disadvantages. These were reviewed over 15 years ago [55], but developed probe technologies have since overcome some of the obstacles. A brief summary is presented in Table 4. The main differences today compared to the situation in 1992 are that the optical sensor technology

Table 4 Probe technologies in different application areas

Method	Interference				Application		
	Solids	Gas bubbles	Conductivity	Medium composition	Single cell suspension	Filamentous	Immobilized
Optical density	+	t[a]	–	t	+	t	–
Dielectric spectroscopy	–	–[b]	–[a]	t	t	+	+
Infrared spectroscopy	+	+[c]	–	t	+	t	–
Fluorescence spectroscopy	t	t	–	t	+	+	+
Software sensors	–	–	–	–	+	+	+

+ Significant interference or applicability possible, – no significant interference or not applicable, *t* testing required for every case specifically

[a] Depends on the probe type used

[b] No interference of gas bubbles when dual frequency measurement is used

[c] With fiber optic probes path length affects the amount of interference

has developed probes that are less disturbed by reactor conditions, and that some dielectric spectroscopy probes have evolved past the previous problems with gas bubbles and conductivity. Dielectric spectroscopy is now applicable also to immobilized cells and solid-state cultivations. The prospects offered by infrared spectroscopy were effectively displayed in the utilization of NIR in full scale automation of lactic acid production [21].

In search of a perfect biomass measurement system to a specific application, one needs to bear in mind a few critical questions. Is the cultivation medium transparent and is it free of insoluble particles other than cells? If not, a suitable probe could be fluorescence or dielectric spectroscopy. Is the amount of viable cells a critical factor? If yes, optical and infrared probes probably offer less to the process than fluorescence and dielectric spectroscopy. Is the price of the measurement equipment a critical issue? If yes, calculation methods are the cheapest to apply to a relatively well-known and much repeated process. Of the probe technologies, the simple optical density probes presented in Table 2 also provide some cheap solutions. Is additional information about your process of greater value than instrument cost? If yes, infrared spectroscopy and 2D fluorescence offer insight to the changes in culture medium during cultivation. Detection of contaminants is an application field that could also be useful. The only methods offering promise to this aspect are currently software sensors.

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