Microbial Biomass Estimation

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Critical Reviews in Biotechnology, 25:1–16, 2005
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ISSN: 0738-8551 print / 1549-7801 online
DOI: 10.1080/07388550500248563

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Microbial Biomass Estimation

R. E. Madrid and C. J. Felice
Departamento de Bioingeniería, FACET/INSIBIO, Universidad Nacional de Tucumán, Consejo Nacional de Investigaciones Científicas y Técnicas, Tucuman, Argentina

ABSTRACT The development of a fully automated on-line monitoring and control system is very important in bioprocesses. One of the most important parameters in these processes is biomass. This review discusses different methods for biomass quantification. A general definition of biomass and biovolume are presented. Interesting concepts about active but not culturable cells considerations are included as well as concepts that must be taken into account when selecting biomass quantification technology. Chemical methods have had few applications in biomass measurement to date; however, bioluminescence can selectively enumerate viable cells. Photometric methods including fluorescence and scattered light measurements are presented. Reference methods including dry and wet weight, viable counts and direct counts are discussed, as well as the physical methods of flow cytometry and impedancimetric and dielectric techniques.

KEYWORDS biomass, biovolume, bioprocess control, physical methods, chemical methods, photometric methods, classical methods.

I. INTRODUCTION

A. General Concepts

The development of a fully automated on-line monitoring and control system is very important in bioprocesses. Biotechnology development has been very vertiginous and required new probes and sensors for optimal control of these processes (Schügerl, 2001; Locher *et al.*, 1992; Liu *et al.*, 2001).

Sensors of different types may be used, such as amperometric, potentiometric, fluorescence and chemiluminescence detectors as well as photometers. Miniaturized sensors for the *in-situ* measurement of pH, pO_2 are well developed for biotechnological measurements (Steenkiste *et al.*, 1997; Voigt *et al.*, 1997). In recent years, the number of research groups dealing with sensors for on-line bioprocess control has increased dramatically and many groups have developed biosensors for this specific purpose.

When designing sensors, it is important to fully exploit the potential of modern measurement instrumentation and advanced control methods. It is very important to classify and to properly process the great amount of information given by the sensors and control devices of a bioreactor. Some papers integrate all of this information for monitoring and controlling a bioprocess through an expert system in real time (Cimander *et al.*, 2003) or special control software (Zelić *et al.*, 2004; Liu *et al.*, 2001; Turner *et al.*, 1994).

Address correspondence to R. E. Madrid, FACET/INSIBIO, UNT, CONICET, CC 327—Correo Central, 4000—Tucuman, Argentina. E-mail: rmadrid@herrera.unt.edu.ar

25 Biomass is a critical parameter in the fermentation process, and it is difficult to measure. It is important because it is a key variable to optimize a specific process, or to reach a maximum efficiency to obtain a certain product, i.e. an antibiotic.

30 Through the years, a number of methods have been developed to detect and quantify biomass, which are useful in different cases, depending on the application. At present maximum possible automation is desired in every bioprocess, so that this can be carried out more efficiently and, at the same time, monotonous and boring 35 tasks are avoided (Sonnleitner, 1997). Automation allows us to control processes safely and reliably, 24 hours a day and 365 days a year, with minimum errors and maximum safety. The processes turn out to be quite reproducible and the products obtained observe quality 40 and standard regulations. But such an automatic control requires devices for on-line monitoring of different variables needed in every process, such as temperature, pH, aeration or biomass (Locher *et al.*, 1992). This is why a large number of researchers are continuously looking 45 for methods and devices that can provide fast *on-line* and *in-situ* results. Generally, these devices take part in control loops, and the faster and more accurate they are, the more efficient the control performed in the bioreactor will be. There are control programs (Liu *et al.*, 2001) and 50 even expert systems (Cimander *et al.*, 2003), or neural nets (Leal Ascencio and Aguilera Galicia, 2000; Vaněk *et al.*, 2004) for process regulation.

In the case of biomass measurement, there are only a few sensors, generally based on the measurement of 55 physical, chemical and photometrical variables and occasionally on biological ones (Sonnleitner, 1999). In other cases, the measurement of other variables might drive to the measurement of biomass (Hrdlicka *et al.*, 2004; Couriol *et al.*, 2001; Bai *et al.*, 2005). Couriol *et al.* 60 (2005) estimated biomass concentrations from the measurement of CO_2 production during batch cultivation of *Geotrichum candidum* (Couriol *et al.*, 2001). The work of Bai *et al.* is particularly interesting. They estimated floc biomass concentrations on-line using an optical 65 detecting technique to measure floc chord length distribution (Bai *et al.*, 2005). This technique was useful for flocculating microorganisms, but presented problems due to disturbances resulting from CO_2 in the case of ethanol fermentation and air bubbles in the case of aer- 70 obic cultivations.

Methods that measure physical variables are generally better adapted for on-line, *in-situ* biomass measurements (Harris and Kell, 1985; Vicente *et al.*, 1998; Hoffmann *et al.*, 2000; Neves *et al.*, 2000; Arnold *et al.*, 2002). One of these is dielectric spectroscopy, which 75 is a direct technique useful only with high cell concentrations, i.e., higher than $2–5 \cdot 10^5$ cell/ml for yeast or animal cells (Ducommun *et al.*, 2002; Guan and Kemp, 1998). The minimum measurable concentration is higher for bacteria, typically 10^{10} (Harris *et al.*, 1987). 80

Until now, the most universally applicable *in-situ* devices for *on-line* biomass monitoring were the photometric methods, including optical probes such as turbidimetric or nephelometric sensors (Stärk *et al.*, 2002). For example, a sensor for on-line determination 85 of biomass in microalgae bioreactor was reported by Meireles *et al.* (2002). This measured optical density of the culture using a Flow Injection Analysis (FIA) system with two loops to provide two dilution factors, coupled with a spectrophotometric detector. They obtained 90 good results with a simple and economic system, which could be applied to unicellular microorganisms such as bacteria.

There are a few chemical methods to measure biomass. One of them, bioluminescence, is applied in 95 biotechnology to monitor a bioprocess product, by introducing a reporter gene which codifies for this product (Roda *et al.*, 2004). In microbiology, bioluminescence is a very rapid and sensitive method for detection and quantification of bacteria (Hobson *et al.*, 1996). 100

Biomass is defined below from the microbiological and biotechnological point of view. Furthermore, biovolume is a more useful way of quantifying biomass.

B. Biomass in Microbiology and Biotechnology 105

Biomass *sensu stricto* is the amount of cell material that is able to grow and multiply, and this quality distinguishes it from necromass (Postgate, 1969). However, this definition is old and does not include metabolically active microorganisms, which are unable to grow 110 *in-vitro* under ordinary culture conditions and methods.

The existence of "viable but not culturable" microorganisms (VBNC) made several authors analyze the subject in depth to try to clarify this concept. The need arose as the expression "viable but not cultivable" is con- 115 tradictory since that definition of viability states that a bacteria is "viable" if it is able to grow and proliferate. Some authors define the term VBNC as an oxymoron or a misnomer (Bloomfield *et al.*, 1998; Kell *et al.*, 1998;

Barer and Hardwood, 1999), because these cells are not non-culturable since we fail to provide the adequate culture conditions (Bloomfield *et al.*, 1998).

In 1998, Kell *et al.* published a paper where they analyzed this subject and they clarified the concepts. They proposed *operative definitions* of viability and culturability instead of *conceptual ones.*

According to the conceptual definition, a viable cell is *an organism able to grow and multiply.* This definition does not consider that there are cells able *to grow and multiply*, but do not grow axenically, because we cannot provide them the proper growth environment.

The operative definition considers that all metabolically active cells are viable. This definition includes as viable cells the ones excluded in the previous definition.

Kell *et al.* proposed that the cells can be characterized in four main categories: i) culturable, ii) nonculturable, iii) (metabolically) active, and iv) (metabolically) inactive. In Figure 1, the different categories are presented.

They proposed that the terms viability (viable) and culturability (culturable) are operative synonym, and that nonviable = nonculturable. They also proposed that the term "active but not culturable" (ABNC) should be used instead of VBNC. "Dormant" cells are metabolically inactive but capable of making a transition to a growing state.

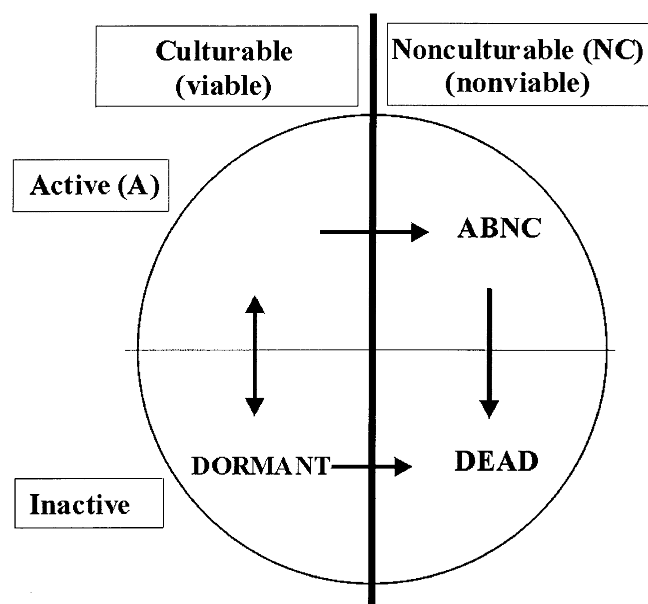


FIGURE 1 **Major physiological states of non-growing microorganisms and their interrelationships (From Kell *et al.*, 1998, reproduced by permission of Kluwer Academic Publishers.)**

The term "dormancy" refers to cells with negligible activity but which are ultimately culturable. The VBNC cells have exactly the opposite properties: they are (metabolically) active but "nonculturable" (ABNC). Examples where the terms VBNC and dormant cells are used as synonymous can be found in the literature despite the fact that they are exactly the opposite (Steinert, 1997). The concept of VBNC state arises from the existence of bacteria capable of causing diseases but that can not be quantified through ordinary culture procedures.

ABNC cells can become a major public health problem since they cannot be detected by traditional culture methods. That is why several cases of pathogenic bacteria in the ABNC state have been studied. Whether or not all human pathogens remain virulent when entering the ABNC state has not been definitively demonstrated. Besnard *et al.* determined environmental and physico-chemical factors, that induce the ABNC state in a food-borne pathogen of public concern such as *Listeria monocytogenes* (Besnard *et al.*, 2002). In order to evaluate the cell activity, they used the Direct Viable Count technique and CTC-DAPI double staining.

Wang and Doyle (1998) studied the survival of enterohemorrhagic *Escherichia coli* O157:H7 in water and proved that they can survive in water in the ABNC state, but they have not studied the possible pathogenic activity of this state, or after resuscitation. Another investigator who studied the pathogenic activity of bacteria in the ABNC state, suggested with his data that cells of *V. vulnificus* (an estuarine bacterium responsible for 95% of all seafood-related deaths in the United States) remained virulent, at least for some time, when present in the ABNC state and are capable of causing fatal infections following in vivo resuscitation (Oliver and Bockian, 1995).

These are only a few examples, but there are numerous research groups working on this subject, and in almost all of the cases the methods used for the determination of microorganisms in the ABNC state are being carried out through molecular methods, fluorescent labelling (AODC, DAPI, DTAF double staining), epifluorescence microscopy or flow cytometry (Steinert *et al.*, 1997; Bunthof *et al.*, 2001; Chaveerach *et al.*, 2003; Kaeberlein *et al.*, 2002; Keer and Birch, 2003).

Daugelavičius *et al.* (2001) provided a method to distinguish "viables" from "dead" based on the amount of lipophilic anions (PCB^-) taken up by the cells. The cell membrane is permeable to lipophilic ions due to

the ability of these ions to bind and even translocate the lipid membrane. The amount of ions taken up by the cell allows the quantification of cells in a sample with a previous calibration measurement. Biomass can be monitored *in-situ* by this method, and only an ionometer (pH meter), a PCB^- sensitive electrode (commercially available) and the addition of PCB^- salts are needed. One problem with this technique is that it is not sensitive enough for the detection of a low concentration cells.

The limit of detection is 6×10^6 cell/ml for *S. cerevisiae*, and approximately 10^7 cell/ml for bacteria such as *E. coli* or *B. subtilis*. The method is repeatable and the results are obtained in 10 minutes.

Direct microscopic methods are adequate to show the total number of cells (non-viables and ABNC) and they can be differentiated by epifluorescence microscopy. The latter may be considered adequate to quantify microorganisms in samples where the presence of cells in the ABNC state is suspected.

Molecular methods are also used to determine ABNC bacteria. These methods offer velocity, sensitivity and specificity with respect to the classic methods. They determine DNA or RNA by staining or molecular amplification, since any method which requires microorganism growth will fail to provide accurate results. Polymerase chain reaction (PCR), reverse transcriptase (RT-PCR), and nucleic acid sequence-based amplification (NASBA) are the most frequently used. Keer and Birch performed a comparative study of these methods for the assessment of bacterial viability (2003).

The determination of ABNC microorganisms is highly relevant in clinical and/or environmental samples since their presence may be potentially dangerous.

However, these dormant or ABNC states are not common in biotechnology, where the cultures generally have the proper nutrients and the cells are not harvested. The exception to this rule are high density cultures where the main cell state is ABNC (Andersson *et al.*, 1996); in other words, the bacteria maintain their metabolic activity but they do not multiply.

In the previous paragraphs, the ABNC microorganisms were not included in the biomass definition. In 1990, Harris *et al.* introduced a new operational definition, the *biovolume*, which includes ABNC microorganisms. This is the volume fraction enclosed into the cell cytoplasmic membrane in a suspension (Harris and Kell, 1985; Harris *et al.*, 1987). This is a definition of biomass to be used strictly in fermentations.

C. Biovolume as a Biomass Estimator

Biovolume is defined as the product between the cell volume and the total number of cells. This value can be converted to biomass using a proper conversion factor.

On the assumption that the microorganism is a sphere, ellipsoid or cylinder, its volume can be estimated by measuring its radius, diameter or length with a microscope. This estimation includes the volume enclosed by the cytoplasmic membrane (Kell *et al.*, 1990).

This measurement can be carried out through different methods, including electronic sizing, flow cytometry (Bouvier *et al.*, 2001), and different microscopic techniques (Bratbak, 1985). The main disadvantage of the methods that enumerate and measure cell length to estimate microbial volume is their deficiency to differentiate active microorganisms from inactive ones (Bölter *et al.*, 2002). This deficiency becomes even more significant when Kell and Young's classification is used, where microorganisms are considered as *active and culturable, metabolically active and not culturable*, or *metabolically inactive and not culturable* cells (Kell and Young, 2000).

Complementary methods can be used to overcome the limitations mentioned in the previous paragraph. These methods include activity measurements (e.g. respiration, growth velocity) or biomass (e.g. ATP, ergosterol). This is particularly important for nutrient-poor environments such as soils and habitats for oligotrophic organisms (Bölter *et al.*, 2002).

Biomass in biotechnology is referred to cell material, which is able to grow and multiply. This condition is not applied in real time applications because of the problem of having to wait until cells grow and multiply.

The real-time alternative for biomass estimation is *biovolume* measurement, which can be carried out through physical methods such as dielectric spectroscopy (DE) (Harris *et al.*, 1987; Kell *et al.*, 1990). This technique measures the dielectric permittivity variation of a cell suspension in the radio frequency range of the electromagnetic spectrum. This variation is related to the biological volume fraction present in a fermenter (Pauly and Schwan, 1959).

The main advantage of using biovolume to estimate biomass in fermenters is that it allows the measurement of the content of cells with an intact membrane in *real time*, making use of dielectric measurements of the

suspension. It also allows one to differentiate these cells from those with a lysed membrane (Davey *et al.*, 1993).

295 The disadvantage of using dielectric estimation is that the biovolume is always lower than the total volume fraction, because the cell wall is not included in the theoretical basis of the method. It can represent from 25% to 50% of the total cell volume (Orlean, 1997). If 300 DE measurements are calibrated against methods that include the cell wall, this underestimation will not be a problem.

Another subject to be considered is that the biovolume estimated by DE does not differentiate among 305 the four main categories proposed by Kell (Kell *et al.*, 1998). Nevertheless, in a review (Yardley *et al.*, 2000) it is stated that this underestimation is not significant under fermentation conditions. Whether this may become a problem or not depends on the analysis of each particu- 310 lar situation, such as in high cell density fermentations, where the *active but non culturable* cells may represent a significant part of the cell population (Andersson *et al.*, 1996).

II. METHODS FOR THE 315 QUANTIFICATION OF BIOMASS

There are diverse methods for the quantification of biomass. These methods must be fast, and if it is possible, *in-situ*, in order to control the systems in real-time. The measurements in a reactor may be as follows 320 (Sonnleitner, 1999):

- *off-line*: when the result is manually obtained and cannot be automated. The time delay of the results impedes a real-time control of the system.
- *on-line*: when the result is obtained immediately after 325 the measurement. These kinds of measurements are fully automatic, and they allow a real-time control of the system.
- *in-situ*: when the sensors are located inside the system to be measured.
- 330 *ex-situ*: when the sensors are located in a bypass or in an exit line.
- *continuous*: when the measurements are continuous in time.
- *discrete*: when the measurements are performed at reg- 335 ular intervals.

These classifications may be used in reactors for the determination of biomass. In the next sections some examples of these methods can be found, such as a capacitive biomass measurement (*in-situ*, on-line, continuous), sensors in FIA systems (*ex-situ*, on-line, discrete), 340 or dry weight (*ex-situ*, off-line, discrete).

The quantification of biomass in samples of environmental and clinical origin, as well as in reactors, where a determined product is expected to be obtained, is of great concern. The term is generally associated with 345 samples that have a high cell concentration such as reactors, where the concentration may be higher than 10^7 cell/ml.

The microorganism concentration expected in samples coming from a clinical environment or the food 350 industry is low ($<10^7$) but the measurement is of great significance, since certain microorganisms are likely to produce diseases or to alter organoleptic, physical or chemical properties of the medium.

The following five sections describe different meth- 355 ods for biomass quantification, including reference, physical, chemical, photometric, and non-conventional methods. Both in chemical and physical methods, only the most common techniques are taken into account.

A. Reference Methods 360

1. Dry and Wet Weight

The dry weight method is the most widely applied for biomass estimation. It is also used as a reference method.

The cell density can be quantified in two basic ways: 365 as grams of dry or wet weight per liter of sample, or as a number of viable/dead cells per ml. The cells in a sample can be separated from the broth and weighed while they are wet, or the cells may be thoroughly dried before weighing. The dry weight measurement usually gives a 370 much more consistent result than the wet weight and is usually used as a reference method. It is simple but laborious and takes a lot of time. Although it is a widely used method it can be erroneous if the broth contains other insoluble material as is commonly found in a 375 practical fermenter. In addition, these methods cannot distinguish viable cells from dead cells.

2. Viable Counts

Another way to quantify microorganisms is concentration measurement, defined as the number of viable 380 cells per volume unit. The *Plate Count*, the *Membrane Filtration* and the *Most Probable Number Method* (MPN) are examples of these techniques.

In the plate count method, the number of cells can be counted by successively diluting the original sample and plating on a Petri dish. This plating method detects only the viable cells. This technique requires elaborate preparations, is laborious, and takes 24–72 hours for the cells to be incubated and counted. Therefore, it is useless as a feedback control of a fermentation process and is used mainly industrially to countercheck other measurements, especially optical density. Despite the fact that other more recent techniques have surpassed it, the plate count method is still one of the basic techniques in microbial quantification.

The filter membrane method simply consists of collecting bacteria from a sample on a highly porous cellulose acetate membrane of different pore sizes, with high volumes of water passing through under pressure. The membranes generally employed have pores of 0.45 μm. This membrane is placed on a plate with growth medium, incubated at an appropriate temperature and the number of colonies counted.

When the number of microorganisms is low, this method is used to concentrate the sample, and it is generally applied for the quantification of water samples. The versatility of this conventional method also allows the membrane to be incubated in the presence of different compounds in order to quantify specific microorganisms, such as coliforms. This technique can also be combined with other techniques such as epifluorescent microscopy (previously staining the sample with an appropriate stain) and using the epifluorescence filter technique.

The MPN method allows for the estimation of the number of viable microorganisms in a sample, capable of growing in liquid growth medium. A selective growth medium is usually employed. It is based on a 10-fold dilution series and a calculation is made of the number of bacteria present in the highest dilution. These procedures can be carried out with 3 or 5 tubes. The number of dilutions required for the sample and the number of tubes that show positive growth (tubes that shows turbidity) are required for the determination of the most probable number of microorganisms, and these numbers are referred to probability tables (Hobson *et al.*, 1996). This method is used to quantify coliforms and sulphate reducing bacteria (SRB) and the main disadvantage is the time required to obtain results. SRB may take up to 28 days. It is not a very accurate method, but it could be improved by increasing the number of replicates, but this also increases the material used and handling difficulties.

B. Direct Count Methods

1. Epifluorescence Microscopy

This is one of the Direct Count (DC) methods. Direct Count is a general term that involves all the direct microscopic methods for enumerating bacteria. It is based on the same optical principles of common microscopy, but differs in sample handling and in the design and operation of the microscopes used.

When designing the generation systems and wave transmissions of these microscopes, adequate wavelengths for the fluorochromes to be visualized must be taken into account. A fluorochrome is a fluorescent dye used to label biological material. The excitation processes generally require short wavelengths, in the near UV (halogen-quartz lamps, mercury arc lamps, etc.). The lens must be made of a special material (generally fluorite) that is able to transmit these wavelengths. The immersion oil must be non-fluorescent.

McFeters *et al.* (1999) made a useful revision of rapid direct methods for enumeration of bacteria in water and biofilm. Fluorescence microscopy has provided a very rapid method for microbial enumeration, and one which does not require an incubation period. It allows the direct observation and total enumeration of viable and non-viable organisms in less than 30 minutes as compared to traditional culturing methods that may require incubation times of up to 72 hours. Traditional culturing methods used with environmental samples also underestimate the total number of microorganisms due to the selective nature of the media employed, lack of detection of ABNC microorganisms, and failure to count microorganisms that are present as aggregates or associated with particles. Acridine orange, the most commonly used fluorochrome, has a high affinity for nucleic acids, and is used as a viable cell stain, since nucleic acids are rapidly degraded upon cell death. Thus a total bacterial count can be accomplished using this method. Under UV light, acridine orange stains deoxyribonucleic acid (DNA) green and ribonucleic acid (RNA) is stained orange. Actively growing bacteria can therefore be distinguished from inactive bacteria on the basis of their higher RNA content (Hobson *at al.*, 1996).

The most successful technique of this nature is the epifluorescence filter technique (DEFT) (Hobson *et al.*, 1996). By using this technique, bacteria are filtered

to be retained in an appropriate membrane. The filter is treated with detergents to destroy the somatic cells which may be retained together with the microorganisms, and afterwards the fluorescent agent is added (e.g., acridine orange or diamidino-2-phenylindole) in order to stain the bacterial cells. Microorganism detection is carried out by fluorescent microscopy or by other methods capable of measuring epifluorescence. In some cases, the membranes are incubated to produce colonies, which are more easily detectable. The detection limit of the method is $5 \cdot 10^3$ microorganism/ml.

Solera *et al.* (2001) described the determination of a microbial population in a thermophilic anaerobic reactor using different counting methods. This case is different from the environmental samples. Cells are growing inside the reactor and therefore conclusions are in accordance with what is expected (a good correlation between direct counts by DAPI (4′,6-diamidine-2-phenylindole) epifluorescence microscopy and viable plate counts). They also found that it was equivalent in this case to measure microorganisms and biomass because they obtained a high correlation between DAPI epifluorescence microscopy and biomass. This biomass was determined by measuring the volatile suspended solids contained in the digester medium according to “Standard Methods” (Solera *et al.*, 2001).

Another extremely sensitive cytochemical staining method, is the fluorescent antibody (FA) technique. This method consists of labeling an antibody with a fluorescent molecule and incubating it with cells in the sample. Cells to which the FA is attached are detected by epifluorescence microscopy. This is a widely used technique to enumerate bacteria in public health, water microbiology, microbial ecology and environmental biotechnology. The result was a successful on-line estimation of bacterial or yeast biomass in aerobic fermenter cultures (Hobson *et al.*, 1996; Beyeler *et al.*, 1981).

In practice all these techniques are labor intensive, difficult to automate and susceptible to various forms of interference, which may cause errors.

2. *In-Situ Microscopy*

In-situ microscopy (ISM) is another method reported for the on-line estimation of biomass but it is not widely used for the determination of biomass in fermenters. In a paper by Bittner *et al.* (1998), a measurement system is reported. It consists of a direct-light microscope with a measuring chamber, integrated in a 25-mm stainless steel tube, two CCD-cameras and two frame-grabbers. The data obtained are processed by an automatic image analysis system.

They applied this system for the estimation of *Saccharomyces cerevisiae* biomass concentration in a reactor, within a measurement range of 10^6–10^9 cells/mL (equivalent to a biomass of 0.01 g/L to 12 g/L). They concluded that biomass concentrations up to 80 g/L can be determined with the ISM from the image area occupied by the cells. The calibration curve obtained between the cell volume per image and biomass had a good correlation. The system was simple and made with components commercially available, but it was necessary to adapt the optic for other types of cells such as bacteria or animal cells. Nevertheless, the bonus was that one could examine the cells microscopically during the process.

C. Physical Methods

1. *Flow Cytometry*

Cytometry refers to the measurement of the physical and chemical characteristics of cells. The technique is based on a procedure where the cells pass one by one from a suspension through a detection system, which may be a detector or a group of detectors that are able to measure different parameters (different types of fluorescence, absorbance, light dispersion, etc). This allows one to identify and characterize bacteria while they are passing through the detector. This is the most important characteristic of this method, since it allows the rapid measurement of individual cells and gives a population distribution for each of the characters of interest. This information is useful to quantify the heterogeneity of a population (Davey and Kell, 1996).

Throughout the last 20 years a great number of papers have been published describing various applications of flow cytometry in the field of microbiology (Betz *et al.*, 1984; Steen, 2000; Winson and Davey, 2000; Malacrino *et al.*, 2001; Bradner *et al.*, 2003).

Several attempts to assemble flow cytometers for on-line determinations in fermenters have been made. But the handling of the samples and waste and the qualified staff required has limited the use of flow cytometry to research laboratories (Bittner *et al.*, 1998).

Flow cytometry is a widely popular method and it allows one to carry out on-line biomass estimation as well as revealing addition data about the population.

Zhao *et al.* (1999) have designed a flow injection flow cytometry system for on-line monitoring of bioreactors. The system includes a specially designed microchamber, which allows not only an accurate on-line dilution but also on-line cell fixation, staining, and washing. These features allow the system to measure a wide range of cellular components after appropriate sample processing. The system can also measure biomass on-line by automatically carrying out dilutions of the sample to keep absorbance readings in the linear range using a spectrophotometer (Zhao *et al.*, 1999). It is an interesting method, but it is complex technology when compared to technologies such as dielectric spectroscopy or turbidity.

2. Impedancimetric Methods

In this section the basis of impedance microbiology is presented as well as typical applications in low, medium and high microorganism concentrations. Conductivity measurements for monitoring biomass in fermenters are included.

Electric impedance as a transduction principle (Geddes and Baker, 1989) is a tool provided by electrical engineering and is widely applied to study biological materials (Valentinuzzi *et al.*, 1996). It has been used for the detection and quantification of bacteria, yeast, animal and vegetal cells, as well as in clinical, industrial and research fields (Alexandrou *et al.*, 1995; Dang *et al.*, 2003; Duran and Marshall, 2002; Felice and Valentinuzzi, 1999; Felice *et al.*, 1999; Glassmoyer and Russell, 2001; Moore and Madden, 2002; Owens *et al.*, 1992; Ramalho *et al.*, 2001).

The impedancimetric technique has been particularly successful in microbial quality control of foods and has been reviewed in detail by other authors (Wawerla *et al.*, 1999; Silley and Forsythe, 1996). An interesting application was the monitoring of conductivity changes of soil solutions as a method for detecting extraterrestrial life (Silverman and Muñoz, 1974). Electrical impedance is complex, composed of a resistive and a reactive part. The inverse values of both parts are generally measured, that is the conductance and susceptance (or capacity if constant frequency is used). When the interface component is not included, the conductance and capacity are respectively physically associated to the suspension media and the measurement electrodes.

Impedance Microbiology (IM) is defined as the collection of methods applied in microbiology, which use impedance measurements to detect, monitor and quantify *active* and *viable* microorganisms (Kell *et al.*, 1998). Capacity and permittivity measurements of cellular suspensions are not included in IM, but they are described in another section of this paper (*Dielectric Spectroscopy*).

The methods used in IM can reflect different phenomena depending on the concentration of the microorganisms present. When the concentration is under 10^7 cells/ml, the impedance or its components reflect *only* the effect of the microbial metabolic activity in the culture media and/or the measurement electrodes (Noble *et al.*, 1999; Felice and Valentinuzzi, 1999; Firstenberg-Eden and Eden, 1984). These methods are considered *classic* in IM.

On the other hand, when the cell concentration exceeds 10^7 cells/ml (Davey *et al.*, 1992), the conductance measurements of a cell suspension can reflect the biomass *and* its metabolic activity simultaneously and *instantaneously* or on-line (Yardley *et al.*, 2000; Fehrenbach *et al.*, 1992; Harris *et al.*, 1987).

This ability to reflect two phenomena without discrimination makes this application one not commonly used in biotechnology. In addition, the measured value is affected by the presence of bubbles (Connolly *et al.*, 1988).

The concentration that produces a detectable change in the impedance curves is defined as the concentration threshold (CT). It is 10^7 cells/ml for yeast and 10^8 cells/ml for bacteria such as *Micrococcus luteus* (Davey *et al.*, 1992). The conductance signal is only incorporated as a bonus in the Biomass Monitor™ (BM, Aber Instruments Ltd., Science Park, Cefn Llan, Aberystwyth, SY23 3AH), an instrument that allows one to estimate biomass *on-line*, and *in-situ* using dielectric permittivity measurements (Yardley *et al.*, 2000).

The classical techniques used in IM require that the microorganisms grow, multiply and reach a concentration of approximately 10^7 CFU/ml to produce a detectable impedance signal (Firstenberg-Eden and Eden, 1984). The estimation of *viable* biomass using these techniques for concentrations lower than 10^7 cells/ml is deduced indirectly from the relationship between the *threshold detection time (TDT)* and the initial cell concentration present in the inoculated culture media. In these cases, the lower the initial concentration, the higher the *TDT*, since the microorganisms need to grow for a longer time to produce significant changes in the initial ionic content of the culture media. In practice, a time measurement in an impedance curve replaces the concentration measurement made with a classical

method such as a plate count (Felice *et al.*, 1999). By using these techniques, concentrations between 10^1 and 10^7 CFU/ml can be quantified without any additional special processes (Noble *et al.*, 1999).

Impedance Microbiology has two main areas of application. The first one is *direct impedance* technology, where the change in the conductivity of the liquid culture medium or the change in the impedance of the measurement electrodes serves as measuring parameters. The other one is *indirect impedancemetry*, where the change in the electrical conductivity of a reaction solution is measured. This change occurs through the absorption of gases originating in the inoculated microbial culture.

There are several microbiological applications using *direct impedance* measurement. The factors for medium optimization are analyzed in these applications to obtain better quality growth curves (Glassmoyer and Russel, 2001; Edmiston and Russell, 2000; Colquhoun *et al.*, 1995).

There is less information in the literature about *indirect impedancemetry*, since it is a more recent technique. Devices for indirect monitoring of microbial growth are still appearing. The technique consists of measuring the impedance in one of two linked and hermetically sealed cells. In the first one there is an inoculated medium without electrodes and the second one contains potassium hydroxide with a pair of electrodes. When microorganisms grow, they produce CO_2, which is absorbed by the potassium hydroxide, lowering the interelectrode impedance. This method is used when culture media with high concentrations of salts are needed as commercial equipment measurements may be out of range (Sawai and Yoshikawa, 2003; Riveiro *et al.*, 2003; Owens *et al.*, 1989).

Both techniques, *direct or indirect impedancemetry*, are faster for the quantification of microorganisms than the classical methods such as CFU counts. They can also be implemented for massive analysis of samples. For example, some of the commercial technologies available can simultaneously evaluate up to 512 samples.

In order to distinguish which technology could be employed in the quantification of biomass, the concentration range to be measured must be taken into account. There are also some questions to be answered. Which microorganisms are of interest? Viable or ABNC? Are the concentrations to be measured lower or higher than 10^7 CFU/ml? It is very important to answer these questions before a method is selected.

3. Dielectric Methods

The dielectric method is the only one which allows a continuous, on-line and in-situ biomass measurement. The next paragraphs describe dielectric spectroscopy fundamentals, the relationship with biomass, and some applications thereof.

The electrical properties (or dielectric) of cell suspensions are given by conductivity and permittivity (dielectric constant ε). They change with the frequency of the applied electric field, where each frequency region is characterized by a specific mechanism, such as cell membranes, organelles inside cells, double layer counterions relaxation, etc. These regions correspond to the α, β, δ and γ dispersions (Schwan, 1957). They are typical for the majority of tissues and suspensions, but despite that the magnitude and frequencies may vary (Foster and Schwan, 1989; Schwan, 1957; Schanne and Ruiz–Ceretti, 1978). Dispersion is generally shown as a sigmoid change between a low and a high value or vice versa and with a determined time constant.

β dispersion is of general concern for biomass determination, since its amplitude is intimately related to the volume fraction occupied by the cells in a suspension. It is in the range of the radiofrequencies and its origin is still a matter of opinion.

The following equations are those proposed by Schwan (1957) for high and low concentrations, and they are the ones which are generally used in biomass determination:

$$\text{High concentration: } \Delta\varepsilon_\beta = \frac{9}{4}\frac{pRC_m}{\varepsilon_0(1+p/2)^2} \quad (3)$$

$$\text{Low concentration: } \Delta\varepsilon_\beta = \frac{9}{4}\frac{pRC_m}{\varepsilon_0} \quad (4)$$

where $\Delta\varepsilon_\beta$ is the β dispersion of the permittivity, p is the cell volume fraction, R is the cell radius, ε_0 is the absolute vacuum permittivity and C_m is the cellular membrane capacitance.

One useful example to make clear the information given by the dielectric permittivity and the conductance of a cell suspension is the real-time monitoring of the accretion of *Rhizopus oligosporus* biomass during a solid-substrate tempe fermentation (Davey *et al.*, 1991). In this case, capacity and permittivity provided the same information.

Figure 2 shows the time course of the fermentation of lupins, where two phases can be clearly distinguished: a growing phase, in which capacitance

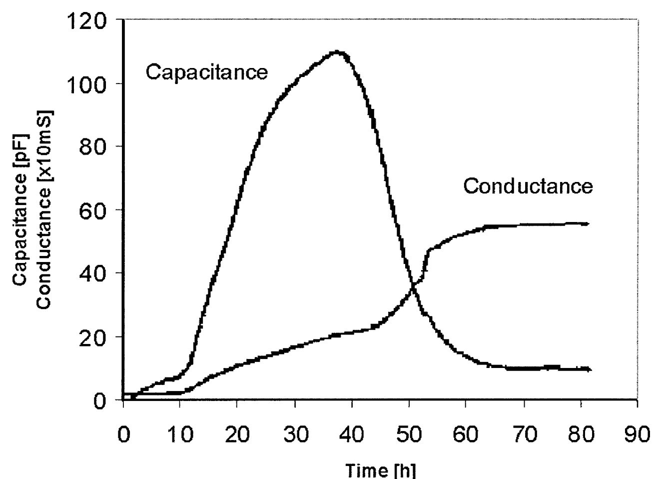


FIGURE 2 **Time course of lupin tempe fermentation. Y axis: Capacitance C of culture broth, Conductance Ge of culture broth. X axis: time in hours. C= ($\varepsilon . \varepsilon_0$. A/d) where ε: permittivity, ε_0: vacuum permittivity. A: transversal area of the sample, and d: distance between electrodes. (Adapted from Davey *et al.*, 1991, by permission of Kluwer Academic Publishers.)**

increases monotonically; and a second one, a non-growing or lytic phase, characterized by a continuous decrease of capacitance with time. At the same time, the conductance increases slowly during the growing phase and sharply in the lytic phase, when cytoplasmic material is released into the medium thereby changing its conductivity.

Up to now, a great number of papers have reported on the use of the dielectric permittivity methods to measure biomass. There are two commercial systems using dielectric spectroscopy to measure biomass: the Biomass System^{TM} (Fogale Nanotech, Nimes, France) and the Biomass Monitor^{TM} (Aber Instruments Ltd., Aberystwyth, U.K.), which is available in different models. The former is a three-frequency capacitance analyzer that computes the biomass concentration (Sarrafzadeh *et al.*, 2005) from the difference between measurements made at two frequencies. The latter is suitable for precise on-line monitoring of homogeneous (unicellular) and heterogeneous (mycelial) cultures in bioreactors as reported by Fehrenbach *et al.* (1992). The measurements were performed on bioreactors of up to 2000 liters on cultures of *Saccharomyces cerevisiae*, *Pichia pastoris* and the filamentous bacterium *Streptomyces virginiae*. The capacitance signal measured was stable, with a slight influence of external parameters such as agitation speed and strongly influenced by medium conductance above 20 mS. However, they concluded that this instrument was useful for monitoring mycelial and yeast growth under industrial conditions.

Recently, a new probe has been described. The prototype was an annular probe, which was evaluated in real-time monitoring of the concentration of viable cells during an industrial pilot-scale fermentation to produce an active pharmaceutical ingredient (Ferreira *et al.*, 2005). The new probe used the same measurement principle as the four-pin probe of the Biomass Monitor^{TM}, but was reported to be easier to handle and more robust than the latter.

Mishima *et al.* (1991a) studied other kind of cells such as *E. coli*, *Aspergillus niger*, human leukemia (K562) cells and a culture of Mardin-Darby bovine kidney (MDBK) cells. They were cultured in different ways, immobilized, aggregated or suspended. Rather than the Biomass Monitor^{TM} they used a measuring chamber that was a parallel plate condenser with two electrodes. The dielectric measurements were made using a Hewlett-Packard 4194A Impedance/Gain Phase Analyzer controlled by a microcomputer. They have obtained very good results with very high correlation coefficients, more than 0.99 for all the cells and type of growth tested (Mishima *et al.*, 1991a). In other work of the same group, they developed two types of electrodes for measuring the dielectric properties of a suspending medium of *S. cerevisiae,* obtaining the same results (Mishima *et al.*, 1991b).

Using a HP4192A impedance analyzer, Bragós *et al.* (1999) presented two biomass estimators derived from a generic cell suspension model and parameters from the Cole-Cole impedance model (Davey *et al.*, 1992). They also obtained good correlation coefficients for all cells tested and found that the concentration threshold of *E. coli* was higher than the concentration threshold of yeast, or inclusive of other bacteria such as *R. capsulata* (Bragós *et al.*, 1999).

a. Permittivity Measurement Using the Inductive Method

A relatively recent technological development, which avoids all the obstacles generated by electrode polarization, was the design of a biomasss sensor based on the indirect determination of permittivity of a biological suspension by measuring inductance. The HP inductive permittivity probe E5050A was presented in the market in 1995 by Hewlett-Packard. It was then applied to dielectric spectroscopy of colloidal suspensions, including the biomass measurements of yeast, bacteria and mammalian cells (Wakamatsu, 1997; Asami *et al.*, 1996; Siano *et al.*, 1996). The E5050A was also applied to the dielectric monitoring of cell growth in whisky

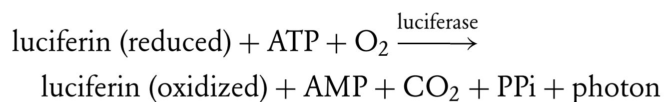
and beer fermentations (Asami *et al.*, 1996; Asami and Yonezawa, 1995).

The E5050 sensor consists of two coaxial toroidal coils, between which the microbial suspension is located. Both coils are covered with an epoxy resin. When voltage is applied to the primary coil, a current is induced in the secondary one, which depends on the sample admittance, that is, on the conductivity and permittivity of the microbial suspension. Admittance is determined by the rate between the voltage applied to the primary coil and the current in the secondary one.

This sensor avoids the use of electrodes and therefore eliminates all the polarization impedances in the measurement. The probe was not sterilizable and requires the HP4285A precision LCR meter to function. At present, the E5050A colloid dielectric probe is no longer being sold or supported by Hewlett-Packard.

D. Chemical Methods: Bioluminescence

Chemiluminescence occurs when a chemical reaction produces an electronically excited species, which emits a photon in order to reach the ground state. These reactions are encountered in biological systems and the effect is called *bioluminescence*. Bioluminescence is a very rapid and sensitive method for bacterial detection. Assuming that living cells of a given type, contain a reasonable constant amount of adenosine 5'triphosphate (ATP), which is lost rapidly upon cell death, it can be a good parameter to measure to quantify cells. The reaction of ATP with luciferin catalyzed by the luciferase enzyme is the principle of the *bioluminescence* method.



$$\text{luciferin (reduced)} + \text{ATP} + O_2 \xrightarrow{\text{luciferase}} \text{luciferin (oxidized)} + \text{AMP} + CO_2 + \text{PPi} + \text{photon}$$

One photon of light is produced per molecule of hydrolyzed ATP and this can be measured using a photometer (Hobson *et al.*, 1996), giving a sensitivity of about 10^{-4} mol of ATP. The light emitted is proportional to the amount of ATP present. By knowing the concentration of ATP in the sample, an estimation of microbial content can be made.

In some cases, when a variation of cellular ATP content occurs, the measurement of the total concentration of adenine nucleotides (ATP, ADP, and AMP) is used.

These variations are due to incomplete extraction, activity of ATPases or kinases, or variation in physiological conditions, among others. The total concentration of these three nucleotides remains essentially constant. It is not possible to measure biomass on-line with this method because it is necessary to remove the sample from the fermenter with the corresponding delay before the results are available (Harris and Kell, 1985).

Billard and DuBow (1998) considered that luminescence-based assays to detect bacteria were better compared to traditional methods such as microscopy, immunological and nucleic acid-base detection assays because the method uses reporter genes that enable selective viable cell enumeration. That is, the use of molecular marked cells offers more versatility to the assays because of the number and types of genes that can be introduced.

There are many papers dealing with bioluminescent biosensors in the literature. Most of them were developed to study the toxicity of different chemical compounds or heavy metals (Kim and Gu, 2003; Kim *et al.*, 2003; Horsburgh *et al.*, 2002; Billard and DuBow, 1998; Premkumar *et al.*, 2002). Other biosensors were developed to be incorporated inside a FIA system for the determination of ATP and NADH (Blum *et al.*, 1993). However, it was not stated that these biosensors could serve as biomass determinators, although if a calibration through any software or expert system were made, ATP concentration could be related to biomass.

E. Photometric Methods

1. Fluorescence

Fluorescence is the result of a three-stage process that occurs in certain molecules (generally polyaromatic hydrocarbons or heterocycles) called fluorophores or fluorescent dyes. The process responsible for the fluorescence of fluorescent probes and other fluorophores is related to the levels of energy of a molecule and the jump of an electron from a high energy state to a lower one, emitting a photon at the same time.

Absorption of UV radiation by a molecule excites the electron from a vibrational level in the electronic ground state to one of the many vibrational levels in the electronic excited state. This excited state is usually the first excited *singlet* state (all electrons in the molecule are spin-paired). A molecule in a high vibrational level of the excited state will quickly fall to the lowest vibrational level of this state by losing energy to other molecules through collision. The molecule

will also partition the excess energy to other possible modes of vibration and rotation. Fluorescence occurs when the molecule returns to the *electronic* ground state, from the excited singlet state, by emission of a photon. This process distinguishes fluorescence from chemiluminescence, in which the excited state is populated by a chemical reaction.

If a molecule which absorbs UV radiation does not fluoresce it means that it must have lost its energy. These processes are called *radiationless transfer of energy* and may be collisional quenching, Fluorescence Resonance Energy Transfer (FRET) and intersystem crossing.

Four essential elements of fluorescence detection systems are identified from the preceding discussion: 1) an excitation source, 2) a fluorophore, 3) wavelength filters to isolate emission photons from excitation photons, and 4) a detector that registers emission photons and produces a recordable output, usually as an electrical signal or a photographic image. Regardless of the application, compatibility of these four elements is essential for optimizing fluorescence detection (Abelson *et al.*, 1997).

Fluorescence is the most widely used method for on-line biomass determination in a bioprocess. The intensity of the fluorescence is affected by the amount of viable biomass concentration and by abiotic factors such as air bubbles or other fluorescent components in the medium. Different complicated fluorometer devices were developed over the years to be used in fermenters, and based on these devices, different biomass estimation experiments were performed (Stärk *et al.*, 2002; Parésys *et al.*, 2005). Zabriskie and Humphrey (1978) applied for the first time culture fluorescence for the estimation of biomass in fermenters. The logarithm of fluorescence was found to be generally linear with the one of the biomass, but the relationship depended on the culture environment, making the technique slightly reliable for *in situ* monitoring of biomass. This is a good technique only under strictly controlled cultivation conditions.

Honraet *et al.* (2005) obtained good results with fluorescence measurements with different dyes for the quantification of *Candida* biomass in suspension. This technique could be applied for the quantification of biomass in fermenters. However it requires extraction of the sample from the reactor making the method less practical.

Recently, different spectroscopic techniques have been studied intensively for potential application in bioprocess monitoring (Wolfbeiss, 2002). Optical techniques using fiber technology are of particular interest. They provide the possibility for non-invasive monitoring of bioprocesses. Fluorescence spectroscopy is one of these techniques and offers the possibility to directly follow different analyte concentrations and even the metabolic state of the biomass.

Fluorescent spectroscopy and chemometric methods are able to monitor on-line bioprocesses parameters, including biomass, with good results (Boehl *et al.*, 2003).

2. Scattered Light Measurement

Biomass estimation based on the optical properties of the medium is traditionally used in fermenters. The Huygens basic measurement principle says: "When the light energy cannot be absorbed, a light quantum of the same energy (color) must be re-radiated. This light can emerge in any direction. This means that all atoms in a physical body serve as secondary sources of light."

The light scatter measurement methods can be classified according to the position of the light detector as:

- *Turbidimetry*–measures the primary beam of light that passes into the sample without deviation to reach the detector. Not recommended when used in fermenters in a conventional way, since the signal-to-noise ratio is too low.
- *Nephelometry*–measurement of the scattered light. Recommended for low biomass concentration. The signal is directly proportional to biomass. Typically nephelometers detect light scattered usually at 90° to the incident beam. Backscattering optical probes use light scattered at 180° and are useful for high cell concentrations.

Detectors with optimized path lengths are available to improve the signal-to-noise ratio and they are used in extremely dense microbial cultures.

III. CONCLUSIONS

Biomass measurement continues to be a critical parameter in bioprocess control. The methods now need to be faster, on-line and in-situ, to yield greater technological progress. The physical methods for biomass determination prevail in terms of practicality. These methods are closer to the ideal, but they still need to solve problems such as biofouling or the electrode-electrolyte interface interference. In the case of

1025 dielectric spectroscopy specifically, it is necessary to achieve a lower resolution when bacterial cultures are measured.

Fluorescence is the most widely used method and it is a good technique only under strictly controlled 1030 cultivation conditions, because it depends on the culture conditions. In practical terms, fluorescence sensors for biomass determination are well developed. Another widespread physical method is dielectric spectroscopy, but although it is an efficient method, the necessary 1035 equipment is onerous. Flow cytometry is very promising but too complex at this time. Other methods are successful only in specific cases.

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