Adv Biochem Eng Biotechnol (2015) 149: 223–252
DOI 10.1007/10_2014_300
© Springer International Publishing Switzerland 2015
Published Online: 10 April 2015

Better One-Eyed than Blind—Challenges and Opportunities of Biomass Measurement During Solid-State Fermentation of Basidiomycetes

Susanne Steudler and Thomas Bley

Abstract Filamentous fungi, especially basidiomycetes, produce a wide range of metabolites, many of which have potential biotechnological and industrial applications. Solid-state fermentation (SSF) is very suitable for the cultivation of basidiomycetes since it mimics the natural habitat of these fungi. Some of the major advantages of SSF are the robustness of the process, the use of low-cost residual materials as substrates, and the reduced usage of water. However, monitoring key variables is difficult, which makes process control a challenge. Specifically, it is very difficult to determine the biomass during SSF process involving basidiomycetes. This is problematic, as the biomass is normally a key variable in mass and energy balance equations. Further, the success of fungal SSF processes is often evaluated, in part, based on the growth of the fungus. Direct determination of the dry weight of biomass is impossible and indirect quantification techniques must be used. Over the years, various determination techniques have been developed for the quantification of fungal biomass in SSF processes. The current review gives an overview of various direct and indirect biomass determination methods, discussing their advantages and disadvantages.

Keywords Solid-state fermentation · Monitoring · Biomass determination · Basidiomycetes · Cell components · Microscopy · Metabolic activity

S. Steudler (✉) · T. Bley
Institut für Lebensmittel- und Bioverfahrenstechnik, Technische Universität Dresden, Bergstraße 120, 01069 Dresden, Germany
e-mail: Susanne.steudler@tu-dresden.de

Abbreviations

ATP	Adenosine triphosphate
B_f	Fungal biomass (g/g_{sample})
C	Carbon
CER	CO_2 evolution rate
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
e	Density of hypha (g/cm^3)
ELISA	Enzyme-linked immunosorbent assay
IR	Infrared
L	Length of hypha (cm/g_{sample})
Mbp	Mega base pairs
mRNA	Messenger ribonucleic acid
N	Nitrogen
O	Oxygen
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acids
qPCR	Quantitative polymerase chain reaction
r	Radius of hypha
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
S_c	Solid content
SIR	Substrate-induced respiration
SmF	Submerged fermentation
sRNA	Small ribonucleic acid
SSF	Solid-state fermentation
tRNA	Transfer ribonucleic acid

Contents

1 Introduction 225
2 Optical Determination Methods 226
2.1 Determination of the Diameter and Area of Fungal Colonies 227
2.2 Simple Optical Microscopy and Stereomicroscopy 228
2.3 Epifluorescence Microscopy 229
2.4 Confocal Laser Scanning Microscopy 230
2.5 Scanning Electron Microscopy 230
3 Measurement of Fungal Cell Components 231
3.1 Ergosterol 232
3.2 Glucosamine and Chitin 233
3.3 Nucleic Acids and Genomic DNA 234
3.4 Counting of Fungal Nuclei by Flow Cytometry 236
3.5 Lipids and Phospholipid Fatty Acids 237
3.6 Protein Content 238
3.7 Carbohydrate Content 239

4 Measurement of Metabolic Activity 239
4.1 Respiration and Gas Composition 239
4.2 Enzyme Activity 241
4.3 Nutrient Uptake 242
4.4 Production of Antigens 243
4.5 Primary Metabolites (ATP) 243
4.6 Changes of Dielectric Permittivity 244
4.7 Metabolic Heat Production 244
5 Other Determination Methods 245
5.1 Plate Count Technique 245
5.2 Agar Film Technique 245
5.3 Light Reflectance and IR-Spectroscopy 246
5.4 Substrate-Induced Respiration (SIR) 247
6 Conclusions 248
References 249

1 Introduction

For centuries, filamentous fungi have been used to produce enzymes, organic acids, antibiotics, pigments, and flavorings and have also been consumed as food, in the form of mushrooms and fermented foods [1]. This review focuses on one group of filamentous fungi, the basidiomycetes, which produce a wide range of metabolites, many of which have potential biotechnological and industrial applications, for example, lignocellulose-degrading enzymes, such as laccases, cellulases, and xylanases [1, 2]. Basidiomycetes are highly developed fungi and the second-largest group of fungi, after the ascomycetes [2].

Solid-state fermentation (SSF) is very suitable for the cultivation of basidiomycetes, since it reproduces the natural habitat of these fungi. In this fermentation technique, the microorganism is grown in a bed of solid particles, with the spaces between the particles forming a continuous gas phase. Although some droplets of free water can be present in the inter-particle spaces, the majority of the water in the system is bound within the matrix of the solid particles. This traditional fermentation technique is used at industrial scale in Asia for the production of fermented foods, such as miso or soy sauce [3]. In Western countries, SSF was used for the production of penicillin until 1940, when it was replaced by submerged fermentation (SmF). Over the last few decades, the potential and the possibilities of this fermentation method have been rediscovered. Some of the major advantages of the SSF are the robustness of the process, the use of low-cost residual materials as substrates, and the reduced usage of water [4].

Although SSF has been used for centuries, it still presents significant challenges with respect to process control and monitoring. One of the key variables in any fermentation process is the amount of microbial biomass, which not only appears in mass and energy balance equations but also is used as a criterion for evaluating the success of the process. Direct determination of the biomass of basidiomycetes in SSF by gravimetric techniques is impossible: The fungal hypha penetrate into the

matrix of the solid substrate, binding the mycelium so tightly to the solid particles that the two cannot be separated from each other. During growth, the overall mass of dry solids decreases, since the production of new fungal biomass is supported by the consumption of nutrients from the solid substrate. However, due to the complex range of nutrients present in most solid substrates, it is not possible to correlate the decrease in total dry weight directly with fungal growth.

As a result of the impossibility of the direct measurement of fungal biomass in SSF, indirect quantification techniques must be used. Various indirect determination techniques have been developed, which can be classified as follows: (I) optical detection using digital imaging or microscopy; (II) measurement of cell components (which might be specific to fungi or not), such as ergosterol, glucosamine or nucleic acids; and (III) measurement of metabolic activities, such as formation of carbon dioxide, release of metabolic heat, consumption of oxygen, or production of enzymes. Each different types of technique have its own advantages and disadvantages. Some problems that may be faced are masking, sorption, precipitation, inactivation of the compound that is to be measured, the presence of interfering substances, and poor extraction efficiencies [2]. This review evaluates the advantages and disadvantages of various indirect biomass quantification techniques that have been used in SSF, grouped according to the classification above.

2 Optical Determination Methods

The oldest and simplest method to assess the growth of basidiomycetes is direct optical detection. This can be carried out subjectively by direct observation with the naked eye or by digital imaging and analysis using a camera and appropriate software.

Optical determination methods need to take the filamentous mode of growth into account. Basidiomycetes are dikaryotic organisms with vegetative and reproductive growth stages. During the vegetative stage, they produce filamentous mycelia through the apical growth and branching of hyphae. During the reproductive stage, which is triggered by an acute lack of nutrients, they undergo sexual reproduction, forming fruiting bodies. The vegetative mycelium, which is usually of interest for the production of metabolites, consists of branched hypha, normally with a diameter of 2–7 μm, although there can also be microhyphae, from 0.1 to 0.4 μm in diameter or mycelia strands (cords) of up to 60 μm in diameter. The surface area covered by a mycelium can vary from a few mm^2 to several km^2. Depending on the limit of resolution that is desired or the accuracy that is required, the detection can be carried out solely by digital imaging, as is the case for the determination of colony diameters, or with the use of additional magnification, which allows investigations of samples at the hyphal level, as is the case with the various microscopy techniques that are described in detail below.

Optical methods are nondestructive direct techniques for biomass determination, but can only be used if observation is unhindered. In the case of transparent substrates such as agar-based media, the sample can be detected from all sides. In the

case of the nontransparent solids that are typically used in SSF systems, only the particle surface can be observed. Here, a good contrast between the background (usually the substrate) and the fungal mycelium is important.

2.1 Determination of the Diameter and Area of Fungal Colonies

The growth of fungal colonies on soft surfaces, such as agar, is often used for estimating growth rates and even for obtaining correlations that can be used later in measuring biomass in real SSF systems. A simple and fast method of optical determination of fungal biomass is the detection of colony diameter and area (see Fig. 1a). An increase in colony diameter correlates with the increase of fungal biomass, as more hyphae are present.

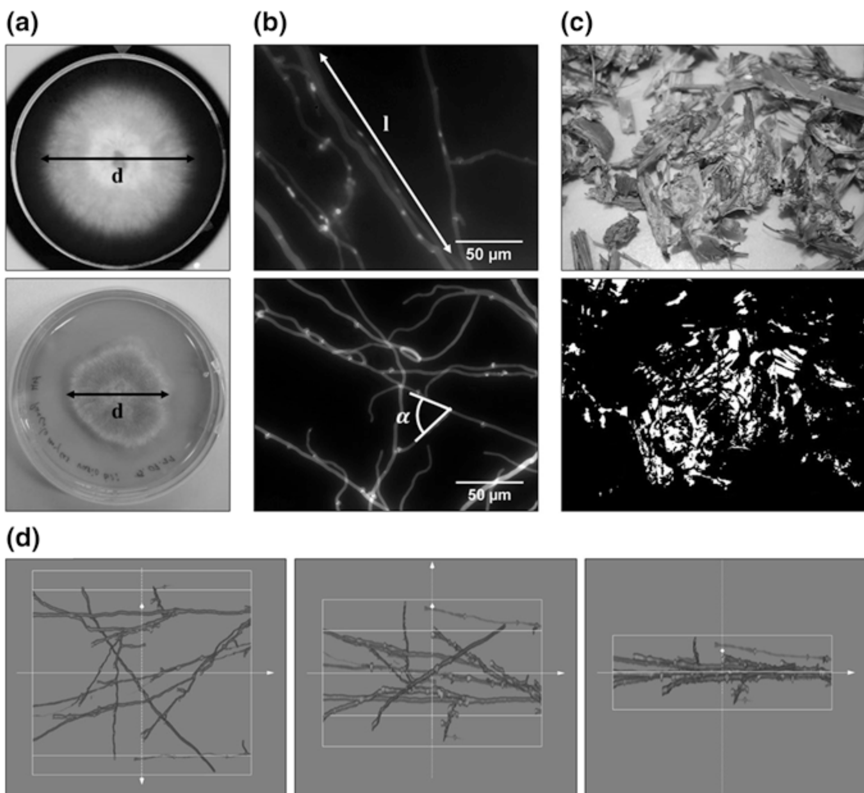

Fig. 1 Optical methods for biomass detection. Digital image of fungal colonies for diameter determination (**a**); Calculation of hyphal length and branching angle using a fluorescence microscopy image of *Trametes hirsuta* (**b**); Determination of fungal surface area of *Trametes hirsuta* on pine wood chips using a digital image (**c**); Three-dimensional image of hyphae of *Trametes hirsuta* obtained using confocal laser scanning microscopy (**d**)

A digital image of the corresponding surface is recorded for the determination of the biomass and converted to grayscale using an image editing software [5]. Subsequently, a fixed threshold value is selected to differentiate the biomass and the background from each other. Usually, the pixels are coded as 1 (white) for biomass and as 0 (black) for background, such that a clear black-and-white image is created for calculation of colony diameter and area. In order to follow colony growth, this can be done at several different times. Li and Wadsö [5] used this simple and non-destructive method to follow successfully the growth of the ascomycete *Penicillium brevicompactum* at various temperatures from 15 to 30 °C. This method can be easily adapted to basidiomycetes. However, Li and Wadsö [5] were not able to differentiate clearly between the growth phase and the stationary phase. The detection of the density of the mycelium within the colony and the height of the colony is not possible using this technique, which may lead to considerable errors in the biomass determination [5]. Also the reflection of incident light at the substrate surface diminishes the quality of the results and requires good image analysis software to minimize interferences. There are also problems in detecting the start of growth due to the poor contrast between the substrate and the young mycelium. Furthermore, it is not possible to differentiate between surface mycelium and hyphae penetrating into the substrate. For this reason, this simple method has been enhanced by various microscopy techniques, for example, stereomicroscopy and epifluorescence microscopy, that enable a more accurate determination of parameters, but require a lot of experience and effort to achieve reproducible results [2]. These techniques are time-consuming, difficult to automate, and susceptible to interference [6].

2.2 Simple Optical Microscopy and Stereomicroscopy

A stereomicroscope is a light microscope with separate beam paths for each eye. A spatial impression is created due to the different angles of the two beams. Stereomicroscopy can help in separating the overlying hyphae in a dense mycelium from each other, enabling determination of key parameters for modeling, such as hyphal length, hyphal diameter, branching points, tip number, branch angle and suchlike (see Fig. 1b), although a sparse mycelium is better suited for estimating key parameters.

Couri et al. [7] and Dutra et al. [8] used stereomicroscopy to determine the biomass of the ascomycete *Aspergillus niger* cultivated on wheat bran, using a procedure similar to that used by Li and Wadsö [5] to determine colony area. The samples were magnified tenfold using a Nikon SMZ 800 [7] or Carl Zeiss STEMI 2000-CS [8] stereomicroscope, and digital images were recorded. Each image was converted into a black-and-white image, as described above, and the fungal surface area was determined (see Fig. 1c). On such heterogeneous substrates, it is important not only have a good illumination of the sample, but also to obtain the right focus plane, in order to obtain a good contrast between background and mycelium [7].

Only surface mycelium is detected using stereomicroscopy. It is not possible to obtain a three-dimensional depiction of the complete mycelium, since hyphae

penetrating into opaque substrates are not visible. This makes it difficult to obtain an accurate determination of biomass.

An additional method for determining the fungal surface area is based on the estimation of fungal hyphal length used in the field of soil biology. In this method, the mycelium is stained and analyzed under a microscope with a suitable magnification. Stahl et al. [9] used a magnification of 600X (40X objective, 10X ocular, and 1.5X magnifier) and evaluated 25 randomly selected fields per object slide. The fungal biomass B_f (g/g_{sample}) was then calculated assuming a tubular structure, according to Paul and Clark [10].

$$B_f = \pi \times r^2 \times L \times e \times S_c \quad (1)$$

where r is the radius of the hypha (an average value of 1.5 μm was used), L is the observed total hyphal length in the sample (cm/g_{sample}), e is the density of the hypha (1.3 g/cm^3), and S_c is the solids content (reported to be 0.3) [9]. Observed hyphal length can be very different in different samples. For example, Stahl et al. [9] detected lengths of 146 m per gram of sample, corresponding to 0.06 mg fungal biomass per gram of sample, in an agricultural soil in Iowa, while Sönderström and Bååth [11] detected hyphal lengths of 66.9 km per gram of soil sample, with a resultant biomass of 35.1 mg per gram of sample, in a soil sample of a Swedish coniferous forest.

2.3 Epifluorescence Microscopy

In order to avoid the problem of the lack of contrast between background and mycelium, the hypha can be stained with dyes. One possibility is the use of fluorescent dyes.

Fluorescence microscopy has been used since 1970 [5] and is, like stereomicroscopy, a special form of light microscopy. The sample is stained with a fluorescent dye that binds specifically to fungal biomass. The stained sample is excited with light of a specific wavelength and emits light of a different wavelength. The emitted light is detected using special filters, so, in general, a good contrast is obtained between the bright sample and the dark background. Suitable and frequently used dyes are acridine orange, which has a high affinity for nucleic acids (under excitation with UV-light, acridine orange bound to DNA emits green light, while acridine orange bound to RNA emits orange light) [5, 6], 4′,6-diamidino-2-phenylindole (DAPI), the fluorescence of which correlated well with the fungal biomass according to Madrid and Felice [6], propidium iodide (PI), europium chelate and ethidium bromide, which stain DNA, and also fluorescein isothiocyanate (FITC), which stains proteins [5]. In the particular case of staining fungal hyphae, suitable fluorescent dyes include phenol blue [5], calcofluor-white (also called fluorescence brightener, FB) [9, 12] and Mykoval [13]. It is also possible to use so-called vital fluorescent stains, such as fluorescein diacetate (FDA). This compound does not fluoresce itself, but when cleaved by the fungus, producing

fluorescein and acetate, the fluorescein fluoresces and is therefore detectable [2]. In addition, the use of specific fluorescent antibodies (i.e., antibodies coupled with fluorescent dye) is possible [6]. Methods can be combined in order to obtain additional information: For example, if both a general stain and FDA are used, then, in addition to determining morphological parameters, it is possible to distinguish metabolically active and inactive hyphae from one another.

Additives, such as immersion oil, or the substrate should not autofluoresce, which would reduce the quality of the images [6]. In any case, the conversion of fluorescence microscopy data into fungal biomass remains problematic due to difficulties in estimating the hyphal volume [2]. This can be rectified by the use of confocal laser scanning microscopy.

2.4 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy involves pointwise scanning of the sample by a laser beam and scan mirrors. A three-dimensional image can be generated by assembling the various focus planes (see Fig. 1d). This enables the accurate detection of the three-dimensional hyphal geometry, and thus an accurate determination of the volume occupied by the fungal biomass. Nopharatana et al. [14] used this nondestructive microscopy technique to determine hyphal density within the mycelium of the zygomycete *Rhizopus oligosporus* on potato dextrose agar. Density profiles were obtained both for the growth of the aerial hyphae, as a function of height above the surface, and the growth of penetrative hyphae, as a function of depth below the surface. The conversion of the volume occupied by the biomass into an estimate of dry biomass of the fungus was successfully implemented. Nevertheless, this conversion can be problematic, because different parts of the mycelium can require different conversion factors, due to differences in water content, mycelium age, and hyphal cell wall thickness [14]. Also, it should be noted that this technique can only generate three-dimensional images of penetrative hyphae if the solid substrate is transparent.

2.5 Scanning Electron Microscopy

In order to improve the degree of magnification of the microscopy images, scanning electron microscopy can be used, although this method is expensive and time-consuming. Osma et al. [12] used this technique to quantify the biomass of the basidiomycete *Trametes pubescens* on banana peels and sunflower seed shells. The image analysis software is very important [12], because the software converts the image to gray scale and differentiates the fungal mycelium from the background based on a suitable threshold value. In order to calculate the area and volume, a comparison with structures of known height and width, for example defined tubular

structures, was used for the calibration of the height of the hyphae in the sample [12]. Nonetheless, the calculation of the biomass of complete colonies was not possible using this method: Due to limited ability to penetrate into the dense mycelium, the hyphae at the colony bottom were not visible. Furthermore, in order to obtain information that can be used in differentiating mycelium from the background, a position with low density is needed, usually in the peripheral region of the colony [12]. However, this noninvasive technique can be used for studies on agar with individual hypha or for simple detection of fungal contamination [15].

3 Measurement of Fungal Cell Components

It is possible to measure cell components that are found in the fungal biomass, such as ergosterol or glucosamine, but, unfortunately, sometimes also in the solid substrate, such as nucleic acids. However, these cell components can vary significantly, depending on the fungal species, the age of the mycelium, the cultivation temperature, and other growth conditions [16].

Figure 2 gives an overview of the structure of the mycelium of a basidiomycete, indicating the various cell components that can be used for the quantification of the fungal biomass.

The cell wall of the hypha is multilamellar. The inner layer consists mainly of chitin, which contains glucosamine residues, and has a thickness of 10–20 nm. The outer layer consists of a protein layer (thickness 10 nm), a glycoprotein layer (thickness 50 nm) and a 75–100-nm-thick slime layer, which contains a number of carbohydrates, fats, and proteins. The cytoplasm of the hypha contains not only nuclei and other organelles but also glycogen vesicles for storage of carbon, vacuoles for fat storage, as well as amino acids and proteins for nitrogen storage. This cytoplasm is enclosed by the plasma membrane. In order to quantify a particular

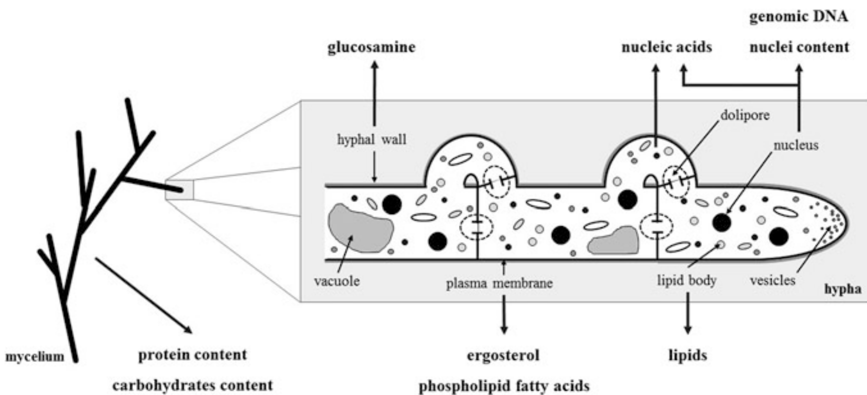

Fig. 2 Schematic depiction of the mycelium structure of a basidiomycete, indicating the various biomass components that can be measured

cell component, it is usually necessary to hydrolyze the sample and extract the target component [17]. The variety of detection methods presented in this section for different cellular components differ with respect to the time required, the cost of the measurement, as well as the interferences that occur [16].

3.1 Ergosterol

One of the most commonly used methods for the quantification of fungal biomass is the determination of the ergosterol content of the sample. Ergosterol is the most abundant sterol in the plasma membrane of hyphae and can represent from 0.7 to 1 % of the dry matter of the fungus [2, 18–21].

Ergosterol is not found in plants, animals, or other organisms and is found only in minimal amounts in some bacteria, protozoa, cyanobacteria, and other micro-algae [2, 18–22], so that no interference is expected during biomass determination [18]. The method for determining the ergosterol content is very accurate and reproducible, but it is also very time-consuming and labor-intensive [17, 23, 24]. The analysis is carried out in three steps: (I) hydrolysis and saponification of the sample to liberate the ergosterol from the plasma membrane, (II) extraction of the ergosterol, and (III) quantification of the ergosterol by HPLC [15–18, 20–22, 25], with detection at 282 nm, which is made possible by the shift of UV absorption due to the double bond of ergosterol [18].

There are efforts to reduce the time and cost of this method. In particular, accelerated extraction by microwave treatment gives good results [15, 23, 26]. With this treatment, Zhang et al. [23] obtained extraction efficiencies of over 99 %, while conventional extraction methods have lower efficiencies, ranging from 33 to 83 %. Similarly, Muniroh et al. [26] reduced the saponification and extraction time to 30 s (compared to a normal time of 30 min to 1 h) at 70 °C using microwave treatment during the biomass determination of the basidiomycete *Ganoderma boninense*.

Ergosterol has proven to be a suitable and sensitive indicator for the quantification of biomass of filamentous fungi, especially basidiomycetes [20–22, 25]. It is an indicator of living cells and is reduced relatively quickly (days to weeks) after the death of the cell [18, 27]. On the other hand, ergosterol is somewhat labile, being degraded by oxidizing substances and light [18]. Also, the content of ergosterol in the biomass varies, depending on the growth phase [16, 17, 22], the growth conditions (such as the cultivation temperature and the substrate used) [16, 17, 19], and the species [19, 22].

In our own experiments with the basidiomycete *Trametes hirsuta,* the ergosterol content depended on both the cultivation temperature and the mycelium age (see Fig. 3). In order to determine the extent of the influence of the cultivation temperature and the mycelium age, the biomass determined gravimetrically was compared to the biomass calculated from the ergosterol content using a constant conversion factor. The two biomass values converge with increasing temperature and mycelium age, so that a separate calibration curve must be prepared for each

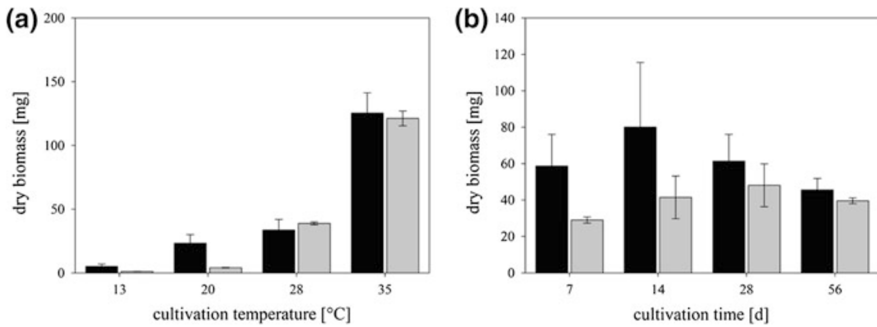

Fig. 3 Comparison of the biomass determined gravimetrically (*black*) and calculated by ergosterol content using a constant conversion factor (*gray*) of *Trametes hirsuta* during fermentation on malt extract agar at different temperatures for 7 days (**a**) and at 28 °C for different cultivation times (**b**) (n = 3)

combination of culture temperature and mycelium age. The calibration curves showed a very good correlation between the biomass and ergosterol content (e.g., calibration curve for 7 days and 28 °C: $R^2 = 0.98$).

An influence of the mycelium age on the ergosterol content was also observed for the basidiomycetes *Phanerochaete chrysosporium* and *Schizophyllum commune*; furthermore, the extent of the influence varied with the fungal species [22]. In studies with fungi of other divisions, different results have also been found. The ergosterol content did not vary with the mycelium age for the ascomycete *Phaeocryptopus gaeumannii* [19], but it did for the ascomycete *Beauveria bassiana* [17], various other ascomycetes [22], and the deuteromycete *Coniothyrium minitans* [16]. In the last of these cases, the ergosterol content increased, but then remained constant after the beginning of sporulation, so that the ergosterol content can be used as an indicator of conidia formation. For all these fungi (independent of the division), the ergosterol content of the biomass was affected by the nutritional composition of the medium used.

3.2 Glucosamine and Chitin

The measurement of glucosamine is also commonly used for estimating fungal biomass [7, 8]. Chitin, which contains residues of *N*-acetyl-β-D-glucosamine, is an essential cell wall component of most fungi, representing from 2 to 24 % of the dry weight of the biomass [28–30]. Glucosamine residues also occur in the murein of bacteria and in the chitin of insects and invertebrate soil animals (such as earthworms, nematodes, and snails) [2], but not in plant materials that are commonly used as substrates in SSF processes [21]. Chitin is much more stable than ergosterol and thus is an indicator of both living and dead biomass [30]. In fact, it remains in the cell walls of empty ghost hyphae [16].

The time that it takes to determine the glucosamine content varies, depending on the particular method that it used [20], but the methods tend to be labor-intensive and complex [17, 20, 21]. The analysis is carried out in two steps: (I) the hydrolysis and deacetylation of the chitin to liberate the glucosamine residues and (II) the detection of glucosamine, most commonly using the staining protocol of Ride and Drysdale [31]. Interference will occur if there are any other sources of glucosamine or other hexosamines [7, 20, 30].

As with the ergosterol content, the glucosamine content of the dry biomass may vary, depending on the cultivation conditions, the strain, and the physiological state of the culture [20, 21, 30]. Also, for the same sample of biomass (i.e., with the same underlying glucosamine content), different methods for glucosamine determination will give different results [20, 21, 30]. The glucosamine content of the biomass did not vary with mycelium age for the ascomycetes *B. bassiana* [17] and *A. niger* [29], as well as the zygomycete *Cunninghamella elegans* [32], although it did for the ascomycete *C. minitans* [16]. In the case of *C. elegans* [32], the glucosamine content of the biomass did not vary with cultivation type (SSF or SmF); however, it was influenced by the composition of the growth medium, especially the carbon source used. For the brown rot fungus *Neolentinus lepideus* (a basidiomycete) and the soft rot fungus *Phialophora* sp. (an ascomycete), Nilsson and Bjurman [30] showed that the glucosamine content was affected not only by the carbon and nitrogen source but also by the mycelium age, initial nutrient content, and temperature. Similarly, the glucosamine content of 11 *Ceuteromycotina* species was influenced by the nitrogen content of the substrate [33].

Although Matcham et al. [20] showed that the chitin content of the biomass of the basidiomycete *Agaricus bisporus* was constant, they found it impractical to use glucosamine determinations to estimate biomass in samples consisting of fungi growing on composted wheat straw, due to the high background levels caused by endogenous hexosamines in the compost. Also, in our own investigations with the basidiomycete *T. hirsuta*, a good correlation between biomass and glucosamine content was obtained ($R^2 = 0.98$); however, this method also proved unsuitable during the analysis of real samples obtained during the growth of this fungus on corn silage, pine wood chips, straw or orange peel, due to the poor reproducibility and sensitivity of the results.

3.3 Nucleic Acids and Genomic DNA

Nucleic acids and genomic DNA can be quantified rapidly, and it is possible to use high-throughput methods. Of course, DNA and RNA are not fungal-specific cell components [34]; they exist in all organic material and thus will be present in the solid substrates that are typically used in SSF processes. However, specific species can be detected using real-time PCR and specific primers.

Basidiomycetes contain the smallest genome of eukaryotes, with a genome size between 20 and 40 Mbp. In fact, the DNA content of the biomass of basidiomycetes

is a tenth of that found in bacteria and higher eukaryotes. Consequently, they also have a high ratio of RNA to DNA. Most of the RNA (approximately 80 %) is bound with ribosomes in the cytoplasm or is present in the form of ribonucleoprotein (RNP) or in small quantities in the form of sRNA. The remaining 20 % is also located in the cytoplasm: around 5 % as mRNA and 15 % as tRNA [28].

Determination of the total nucleic acid content is simple and rapid. The nucleic acids are extracted using hot and cold extraction with perchloric acid and then measured photometrically at 260 nm [16]. However, the nucleic acid content of the fungal biomass is not always constant. For example, with the ascomycete *C. minitans*, the content decreased with increasing cultivation time and depended strongly on the medium [16]. In our own investigations using the basidiomycete *T. hirsuta*, the content of nucleic acids in the fungal biomass was constant for biomass of the same mycelium age. As in the studies of Ooijkaas et al. [16], a decrease of the nucleic acid content with increasing mycelium age was observed. In addition, an independence of the cultivation temperature was found.

It is also possible to determine genomic DNA content specifically. Commercial extraction kits are now available for the extraction of genomic DNA, such as the FAST DNA® SPIN Kit for Soil [35] and the "NucleoSpin® Plant II Mini" extraction kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany), which we have used in our experiments. The extracted genomic DNA is measured at 260 nm and quantified. A second measurement is carried out, usually at 280 nm, in order to assess the purity of the DNA by calculation of the quotient "260 nm/280 nm." A ratio of 1.8 would be ideal, indicating pure DNA. A ratio of 2.0 implies the existence of pure RNA and a ratio smaller than 1.8 indicates a contamination with proteins. May et al. [36] used this method to determine the biomass of the oomycete *Lagenidium giganteum* on wheat bran at different temperatures (24–35 °C). A linear relationship was obtained between the concentration of dry biomass of the fungus, which was collected from liquid cultivation, and extracted DNA ($R^2 = 0.91$). This linear relationship was used to quantify the fungal biomass from solid cultures; however, representative sampling was problematic, so the dry biomass estimated by DNA measurement showed a high standard deviation. Further, there was an influence of the mycelium age, as well as interference from the medium. In our studies, we obtained a linear relationship between the dry biomass of the basidiomycete *T. hirsuta*, which was collected from agar culture, and the extracted genomic DNA ($R^2 = 0.93$), but the biomass values estimated by genomic DNA were far above those estimated using reference methods (e.g., ergosterol), probably due to the interference from the organic solid substrate. Also, Blagodatskaya et al. [34] showed that the genomic DNA content of fungal biomass is not constant and depends on the nitrogen content of the substrate.

Another method to quantify fungal biomass is PCR, especially real-time PCR. This technique requires not only appropriate equipment (a thermal cycler), but also a lot of know-how and optimization, as well as a good primer selection [37]. However, after successful establishment of this method, a species-specific quantification of fungal biomass is possible. In order to quantify the biomass of a specific fungus, it is important that the nuclei content of the target organism does not

change: Any change of the genomic DNA content with mycelium age or growth conditions will lead to an erroneous determination of the biomass content [38].

Voegele and Schmid [38] successfully used reverse transcription (RT) real-time PCR for rapid, specific, and highly sensitive quantification of the basidiomycete and plant pathogen *Uromyces fabae* within its host plant, using three constitutively overexpressed genes. Also, Tellenbach et al. [39] used locus-based quantitative PCR (qPCR) and obtained a strong linear relationship between the pure dry biomass of the fungus and the qPCR estimates (i.e., DNA amount) of the ascomycetes *Acephala applanata* (R^2 = 0.965), *Phialocephala fortinii* (R^2 = 0.963), and *Phialocephala subalpina* (R^2 = 0.936). For Tellenbach et al. [39], currently no better method of quantification exists, due to the speed and very high specificity.

The high sensitivity of quantitative real-time PCR was also confirmed by Pilgård et al. [40] during a study of the biodegradation of wood by basidiomycetes. As control methods, the ergosterol and glucosamine contents were determined and samples were also evaluated microscopically. In addition to the good correlation of biomass and quantified DNA content by qPCR, this method is much more sensitive than the determination of ergosterol and glucosamine and therefore is suitable for the determination of biomass in the early growth phase.

While the determination of nucleic acids and the genomic DNA using photometric detection is limited due to various interferences, quantitative real-time PCR, after optimization, is an appropriate and sensitive method for quantification of fungal biomass during SSF, provided the content of genomic DNA is independent of the culture conditions.

3.4 Counting of Fungal Nuclei by Flow Cytometry

Flow cytometry is a simple measurement technique that enables a quick quantification of cells, cell components, and particles. For this, the particles pass a laser beam in single file. In addition to pure counting, certain properties of the particles can also be detected, based on fluorescence and light scattering (forward and side scatter).

This method works well with unicellular organisms. Costa-de-Oliveira et al. [41] used it to quantify the glucosamine content of the yeast *Candida* spp. by staining the cells with calcofluor-white. In principle, this approach could be applied to basidiomycetes growing on solid substrates, after sample grinding, but dyes that stain chitin often also stain cellulose, which is present in large amounts in many SSF substrates and would therefore interfere with the measurement. Spores are easily counted by flow cytometry [42]. However, basidiomycetes only form spores in the fruiting bodies, while most SSF processes with basidiomycetes involve only pure mycelium.

In our own work, we have developed a flow cytometry method for quantifying fungal biomass by counting the fungal nuclei [43]. The sample is homogenized and the nuclei are extracted. The nuclei are then stained with a fluorescent dye (SYTOX® Green) and analyzed in the flow cytometer. A linear correlation between

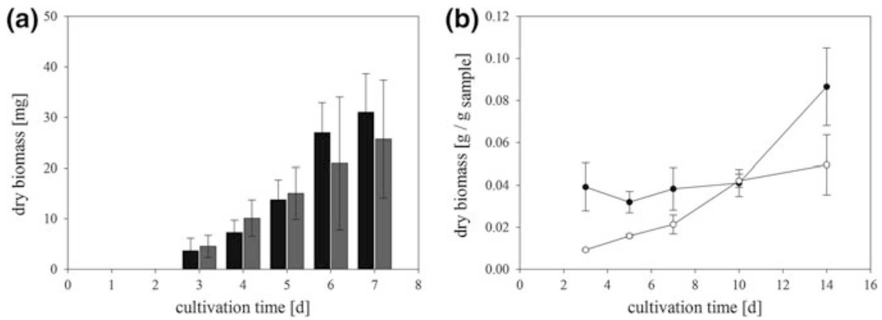

Fig. 4 Comparison of the biomass determined gravimetrically (*black*) and calculated by nuclei counting (*gray*) of *Trametes hirsuta* during fermentation on malt extract agar at 28 °C (**a**) ($n = 10$) and of the biomass determined by measurement of the ergosterol content (*white circles*) and by nuclei counting (*black circles*) during SSF of *Trametes hirsuta* at 28 °C on corn silage (**b**) ($n = 5$)

the pure dry biomass of the basidiomycete *T. hirsuta* and the counted nuclei was obtained ($R^2 = 0.93$). The content of nuclei within the biomass depended on both the culture conditions and the mycelium age. This variation was taken into account by the construction of different calibration curves for different cultivation temperatures and times.

With the use of different calibration curves, good agreement was found between the fungal biomass of *T. hirsuta* determined gravimetrically and biomass estimated by nuclei counting (see Fig. 4a) during a cultivation on malt extract agar at 28 °C for 7 days. The method was also used to monitor SSF of corn silage in a reactor with a working volume of 10 L (developed by the Research Center for Medical Technology and Biotechnology (fzmb GmbH)). Useful and reproducible results were obtained with this novel method. Furthermore, the accuracy of biomass values determined by flow cytometry was similar to that of the values obtained by the reference method (ergosterol content, see Fig. 4b).

The use of flow cytometry to quantify fungal biomass by nuclei counting represents a new, rapid, and simple method of biomass determination during SSF and, when used in combination with other methods, provides additional information about the physiology of the culture.

3.5 Lipids and Phospholipid Fatty Acids

The mycelium of basidiomycetes contains various glycerolipids, which, in turn, can contain both saturated and unsaturated fatty acids. These glycerolipids are located mostly in lipid bodies in the cytoplasm and in the plasma membrane and the membranes of organelles, in the form of acylglycerols and phosphoglycerides [44]. However, the lipid content of the biomass varies greatly, especially due to the fact that the amount of storage lipids varies greatly, depending on the growth phase [22]. Therefore, phospholipid fatty acids (PLFAs) can be used as a biomass

indicator. PLFAs are used for rapid and inexpensive determination of microbial diversity in soils [45], but can also be used during SSF. This method is sensitive and highly reproducible, and gives an indication of living biomass [45, 46].

In addition to determine the total PLFA content, it is possible to determine separately the contents of special PLFAs that are contained by certain fungi, such as the PLFA 18:2ω6,9 and the PLFA 18:2ω9 [22, 35, 45]. In basidiomycetes, the main PLFA, 18:2ω6,9, represents from 45 to 57 % of the total PLFA content [22]. This PLFA also occurs in small amounts in bacteria, as well as in plants and animals [22, 45, 46].

Frostegård and Bååth [46] correlated the content of PLFA 18:2ω6,9 in soil samples with the ergosterol content ($R = 0.92$), although influences of the substrate and cultivation conditions were observed [45]. A decrease of PLFA concentration in an SSF sample can imply either cell death or degradation of specific membrane phospholipids due to changes in environmental conditions [45].

It is problematic to use PLFA measurements to estimate fungal biomass since the conversion factor varies greatly. Despite this, Frostegård and Bååth [46] did use this method as a qualitative indicator of fungal biomass in 15 different soil samples, while Klamer and Bååth [22] used PLFA contents during an investigation of the levels of 11 fungi in compost. Klamer and Bååth [22] found a linear correlation between the content of the PLFA 18:2ω6,9 and the content of ergosterol within the samples ($R^2 = 0.782$), although both the ergosterol content and the PLFA 18:2ω6,9 content varied considerably among the tested species. The total PLFA content in fungal biomass grown on potato dextrose agar showed large interspecific variations. It does not depend significantly on the mycelium age, but does depend on the substrate and on the fermentation type (SSF and SmF). A perfect PLFA marker does not exist [45], but the determination of PLFA content (total and specific) is a suitable indicator of fungal biomass, so that this method is also suitable for the quantification of living biomass.

3.6 Protein Content

Proteins are ubiquitous in biomass and the protein content is often used as an indicator of fungal biomass. Protein measurement assays are usually sensitive, simple, and reproducible [29, 47]. In order to determine the insoluble protein in the fungal biomass, it is first necessary to disrupt the sample using, for example, phosphoric acid [47, 48] or sodium hydroxide [16, 29]. The protein that is released can then be determined by a colorimetric method, such as that of Bradford [47] or that of Lowry [29].

Protein determination can be useful when the solid substrate that is used in the process does not contain high protein levels. This method has been used to follow the growth of the ascomycete *A. niger* on various substrates, including sugar cane bagasse and palm kernel cake [29, 47, 48]. The protein content of the biomass of *A. niger* was found to remain constant [28].

Protein determination cannot be used for estimating fungal growth when the solid substrate that is used has a high protein content. This problem occurred in studies involving the ascomycete *C. minitans* [16] and in our studies with the basidiomycete *T. hirsuta*. In both cases, the protein content of early samples was high, but, due to consumption of the protein in the substrate, it decreased to a constant level in later samples, although a linear relationship between the pure fungal biomass and the protein content was found. However, the protein content depended on the substrate and the mycelium age [16].

3.7 Carbohydrate Content

Carbohydrates are located in fungi as sugars, sugar alcohols, polysaccharides, and derivatives. Pure sugars exist only in small amounts in hyphae and are present usually as phosphorylated derivatives [28].

Ooijkaas et al. [16] obtained a total carbohydrate content of up to 9.7 % of the dry biomass of the ascomycetes *C. minitans*. However, the content varied depending on the medium and mycelium age. Also Desgranges et al. [17] observed a variation in the sugar content of the biomass of the ascomycete *B. bassiana* depending on the medium and growth phase.

This method can be used only for special cases, because many substrates that will be used in SSF contain carbohydrates, so the carbohydrate content is inappropriate as an indicator of fungal biomass.

4 Measurement of Metabolic Activity

Normally, measurements of metabolic activity are simple and can be used for continuous monitoring of culture performance. As a result, they are suitable for on-line monitoring of mycelium growth in large-scale bioreactors. Figure 5 indicates the various techniques that will be discussed in this section.

4.1 Respiration and Gas Composition

The measurement of respiratory activity is simple to implement and is quite convenient for use at large scale. Determination of the oxygen (O_2) and carbon dioxide (CO_2) contents in the gas phase enables calculation of O_2 uptake rates and CO_2 evolution rates (CERs). It is also possible to integrate the values to obtain cumulative O_2 consumption and CO_2 formation. The measurement techniques are non-destructive, performable in real time and online [36, 49, 50]. An advantage of measuring both O_2 and CO_2 is that this gives additional information about the

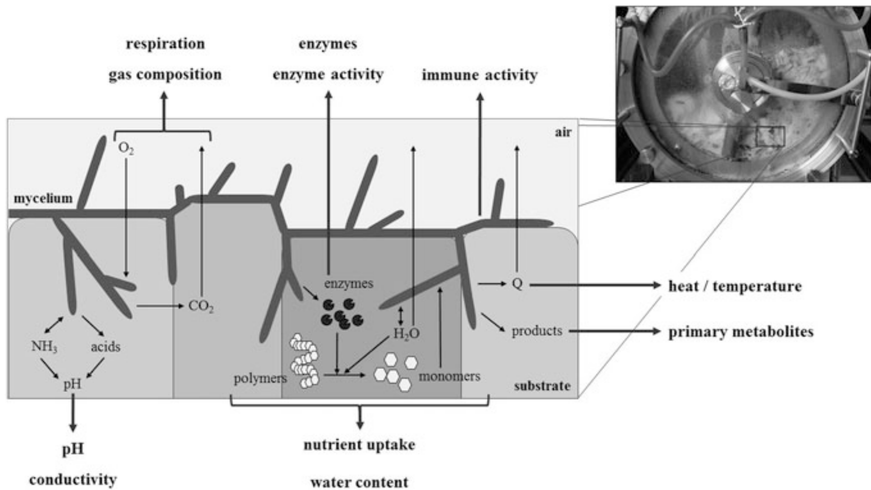

Fig. 5 Schematic depiction of the mycelium structure and metabolic activity of a mycelium, as well as the related determination methods

physiology and the state of the culture [49]. Fungi not only respire actively in the growth phase, but also in the stationary phase. Since it is impossible to differentiate the origin of the respiratory activity, the cultivation should be axenic.

CO_2 evolution was used to follow the growth of the ascomycetes *Penicillium roqueforti* [51] and *B. bassiana* [52] in SSF. A good correlation between the formation of CO_2 and the glucosamine content was obtained. However, although the method is sensitive, it is not suitable for comparing different processes since the relation of CO_2 evolution with growth depends on the medium [52].

May et al. [36] used the CER to follow growth of the oomycete *L. giganteum*. In SmF, the CER increased exponentially, similarly to the fungal biomass, until the stationary phase. Here, the fungal biomass leveled off at a constant value, while the CER peaked and then decreased. However, it is not always the case that CO_2 evolution and O_2 consumption are related to biomass production in a simple manner; therefore, the conversion into an amount of fungal biomass and thus the monitoring of the fungal biomass remain difficult [16, 50, 53]. In fact, Ikasari and Mitchell [50] noted that biomass estimation from O_2 uptake was problematic in fermentations involving the zygomycete *R. oligosporus*, since neither the yield coefficient for O_2 nor the maintenance coefficient for O_2 was constant. However, they did recommend the use of O_2 measurements for online monitoring of overall bioreactor performance, since O_2 consumption is a useful indicator of the metabolic activity of fungal biomass [50, 53].

In our own studies, the CO_2 content and the O_2 content in the gas phase were measured continuously during the fermentation of the basidiomycete *T. hirsuta* on corn silage. A linear relationship between the daily O_2 consumption and CO_2 formation and the fungal dry biomass was found (O_2: $R^2 = 0.68$; CO_2: $R^2 = 0.76$) and was used to convert the respiratory activity into fungal biomass during the SSF

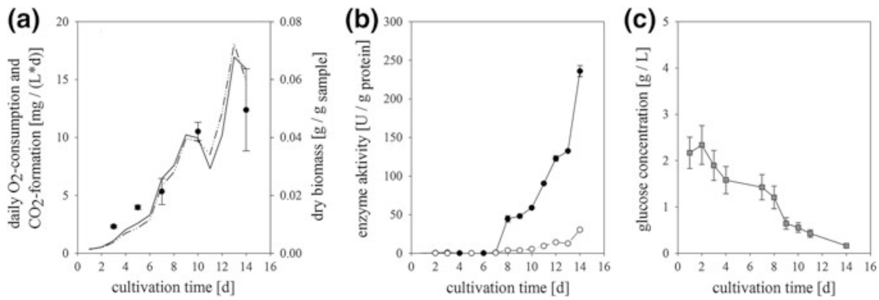

Fig. 6 Comparison of the biomass determined by the measurement of the ergosterol content (*black circles*, $n = 5$) and the corresponding daily O_2 consumption (*dotted line*) and CO_2 formation (*solid line*) (**a**), enzyme activity of laccase (*black circles*) and peroxidase (*white circles*) ($n = 3$) (**b**) and glucose content in the leached liquid phase obtained after extraction of the extracellular enzymes (*gray squares*, $n = 3$) (**c**) during SSF of *Trametes hirsuta* at 28 °C on corn silage

process. The biomass estimated from the daily O_2 consumption and CO_2 formation was quite similar to that estimated through measurement of the ergosterol content of the solids (see Fig. 6a).

According to our studies, the measurement of the respiratory activity is a simple and rapid method of determining the biomass of *T. hirsuta* until the stationary phase. Furthermore, it provides additional information about the physiological state of the culture, for example the type of metabolism (calculating respiratory quotient).

4.2 Enzyme Activity

The use of measurements of enzymatic activity to estimate fungal growth presupposes that the enzymes are fungal-specific, growth-associated, and, usually, extracellular. A few enzymes satisfy these criteria: Most enzymes are not suitable candidates for this approach, since their production is not related to growth in a simple manner; further, production levels can vary with the environmental conditions, such as pH, temperature, and water content [2]. Despite these potential problems, since determining enzyme activity is usually fast and easy, many workers have used this technique to estimate the biomass.

Dutra et al. [8] determined the activity of lipases of the ascomycete *A. niger* and correlated the results with glucosamine contents and microscopic observations. There was a good correlation between the area occupied by the hyphae, the glucosamine content of the solids and the lipase activity ($R > 0.9$). Similarly, for the growth of *A. niger* on palm kernel cake, the activity of β-mannanase correlated well with the biomass based on measuring the protein concentration ($R^2 = 0.941$) [29]. For the ascomycetes *Stachybotrys chartarum* and *Aspergillus versicolor* growing on gypsum board, the activity of β-*N*-acetylhexosaminidase correlated well with the biomass, which was estimated by measuring the ergosterol content, with R^2 values

of 0.935 and 0.968, respectively [24]. On the other hand, the activity of extracellular proteases of *Aspergillus tamarii* was unsuitable as a biomass indicator due to the strong influence of the substrate and physical factors, such as temperature, aeration, and hyphal density [54].

Basidiomycetes produce interesting extracellular enzymes such as laccases, peroxidases, cellulases, and xylanases. Matcham et al. [20] used the laccase activity of the basidiomycete *A. bisporus* to estimate the biomass during SSF on cereal grains. Although the laccase activity increased in direct proportion to the fungal biomass, it was problematic to determine the conversion factor, because the absolute enzyme activities were highly dependent on the substrate used. The determination of the enzyme activity was recommended as a qualitative indicator of biomass. In our studies of the SSF of the basidiomycete *T. hirsuta*, the maximum activities of laccases and peroxidases also varied with the substrate and cultivation conditions, but the activity profiles were proportional to the biomass level as determined using the ergosterol method (see Fig. 6b). On the other hand, the activities of xylanases and cellulases were unsuitable as indicators of biomass.

The usefulness of enzyme activity as a biomass indicator depends on the selected enzyme and the fermentation conditions. Enzyme activities can only be used as indicators under the same conditions as the correlation was originally determined.

4.3 Nutrient Uptake

The uptake of nutrients or the current nutrient concentration has been used in SmF as an indicator of growth and also has the potential to be used in SSF. Often the carbon source is used, such as glucose or other sugars.

Desgranges et al. [17] used the uptake of sucrose as an indicator of growth during the fermentation of the ascomycete *B. bassiana*. The amount of sucrose consumed correlated well with the content of ergosterol and glucosamine of the fungal biomass ($R > 0.81$) and was independent of the C/N ratio of the substrate. In contrast, the glucose uptake was not suitable for biomass estimation during the studies of Larroche and Gros [51] with the ascomycete *P. roqueforti.* They determined the glucose content of wheat starch after hydrolysis with amyloglucosidase using the dinitrosalicylate method. The glucose was taken up into both the mycelium and spores, so that only a portion of the glucose consumption was used for mycelium growth. Further, in studies undertaken with *A. niger,* Favela-Torres et al. [48] found that the amount of glucose taken up depended on the initial concentration in the medium and on the fermentation type (SmF or SSF). In SSF, higher initial glucose concentrations could be tolerated, and higher glucose uptake rates were obtained. In our own investigations with the basidiomycete *T. hirsuta*, extracellular enzymes were leached from the solid substrate daily with water. The glucose concentration of the leachate was also determined, in order to obtain an indication of growth. The amount of glucose extracted decreased as the fermentation progressed (see Fig. 6c). It also depended on the substrate that was used. For

example, over the first days of the SSF process, the amount of glucose extracted was around 2 g/L when corn silage was used, but around 0.3 g/L when pine wood chips were used.

It is usually fast and simple to determine the concentrations of sugars or other nutrients, enabling the use of nutrient uptake as a biomass indicator. However, the method must be carefully calibrated for each specific application.

4.4 Production of Antigens

Immunological methods for the detection of fungal antigens can be used to obtain an indication of growth. The enzyme-linked immunosorbent assay (ELISA) is often used. In this method, an antibody against a fungal antigen is added to a sample in a microtiter plate. A second antibody is then added; this antibody, which has an enzyme linked to it, reacts with the first antibody. Finally, a substrate for the enzyme is added, such that a colorogenic reaction occurs. The intensity of the color produced depends on the original amount of fungal antigen. The development of this method requires a lot of know-how, but, once it is optimized, it is very sensitive and quite specific.

Anand and Rati [55] used ELISA to quantify the biomass of the ascomycete *Aspergillus ochraceus* on coffee bean extract, chili extract, and poultry feed. The sensitivity was 0.2 mg biomass per gram substrate. The results were influenced by the particular substrate used and by the moisture content, such that a new calibration curve had to be established for each substrate. The background signal of the uninoculated substrate was much lower than the lower detection limit, so it did not cause any problems [55]. In a similar manner, Dubey et al. [56] used ELISA to monitor growth of the ascomycete *A. niger*. The suitable choice of a fungal cell wall antigen, which was not present in the substrate, meant that the biomass could be determined quickly in a substrate-independent manner. Furthermore, the fungus was detectable at all morphological stages.

Several factors make the use of ELISA to determine biomass interesting: not only is it rapid, reliable, and sensitive, but also it is inexpensive and requires only small amounts of substrate [56]. However, it does require a suitable antigen to be available, and the development of the assay requires a lot of know-how and optimization.

4.5 Primary Metabolites (ATP)

The quantification of fungal biomass based on primary metabolites, especially ATP, is often used in soil analysis. ATP is an indicator of living biomass. After sample drying, ATP is extracted and quantified by bioluminescence using the luciferin–luciferase system [57]. The method is sensitive, but reports about the contents of ATP in actively growing biomass and live but nongrowing biomass are inconsistent [57]. Also, the amount of ATP extracted is highly dependent on the extracting

agent. An internal standard is advisable, since a certain proportion of ATP is hydrolyzed during the extraction or adsorbed on the substrate matrix [57]. A further factor to consider is that the substrate can also contain ATP, which will interfere with the measurement of the ATP from microbial biomass [57, 58]. West et al. [58] used the determination of the ATP content for quantification of the microbial biomass in soil samples, but this method was only suitable for biomass estimation at equal cultivation conditions (e.g., same substrate, temperature, and cultivation time) due to the variable ATP content in the biomass.

The usefulness of ATP as a biomass indicator depends on many factors, including the physiological state of the culture. The determination is simple and fast; nevertheless, this method is unsuitable for the determination of fungal biomass during SSF due to the many influencing factors and fluctuations.

4.6 Changes of Dielectric Permittivity

A simple and rapid method for general biomass quantification is the measurement of the dielectric conductivity. This measurement is continuous, noninvasive and can be performed in situ and on-line [6, 59]. Living biomass is polarized by low radio frequencies with the degree of polarization depending on the cell structure in a phenomenon called beta-dispersion. The degree of beta-dispersion is linearly proportional to the volume of the biomass. Since the biomass volume changes during microbial growth, the dielectric conductivity will also change. It can be measured and subsequently correlated with the amount of biomass in the sample or bioreactor [6, 59].

Davey et al. [59] analyzed the change of the conductivity, capacitance, and pH during SSF of the zygomycete *R. oligosporus* on soy beans, lupine, and quinoa. The conductivity of the fungal biomass increased linearly with the total hyphal length, which was measured microscopically, during the growth phase and did not change during the death phase. The capacitance was also related to growth: It increased during the growth phase and dropped during cell death.

The measurement of the dielectric conductivity is simple and fast, which means that it has good potential to be used online for biomass quantification during SSF. However, this method requires a liquid film at the particle surface in order to ensure a proper contact with the electrode. Consequently, this method can only be used conditionally, due to the poor contact of the electrode with many of the porous substrates and substrate beds that are used in classical SSF.

4.7 Metabolic Heat Production

The release of waste metabolic heat from respiration and maintenance metabolism will affect the ambient temperature of the bed of solids within which the fungus is growing, and consequent temperature changes can be measured online in a

continuous and nondestructive manner. However, although it is theoretically possible to quantify biomass from measured temperature changes, such “calorimetry” is rarely used for biomass estimation in bioreactors [5] since the temperature is also affected by many other phenomena that occur in the bed (i.e., conduction, convection, and evaporation) [5, 54].

Li and Wadsö [5] used heat production, measured by isothermal calorimetry, as an indicator of the activity of the ascomycete *Penicillium brevicumpactum*. During the growth phase, the total heat produced correlated well with the colony area, which was recorded digitally [5]. According to Li and Wadsö [5], the method is suitable for all substrates and organisms and is usually very sensitive. Dhandapani et al. [54] used heat measurement to observe the growth of the ascomycete *A. tamarii* and described this method as a robust and suitable reference technique for the monitoring of fungal activity during SSF.

The measurement of heat production can be done rapid and continuously in a calorimeter. However, the quantification of the production of waste metabolic heat by calorimetry is more suitable for academic studies than for online monitoring of a bioreactor.

5 Other Determination Methods

There are other techniques that can be used to quantify fungal biomass in the laboratory or at large scale. They will briefly mention in this section.

5.1 Plate Count Technique

The plate count technique is a routine procedure in industry and research laboratories for determination of colony-forming units [2, 55]. However, for fungi the number of colony-forming units varies greatly depending on medium composition and cultivation conditions, which makes it difficult to compare the results with other studies. This method is only suitable for cultivable fungi, does not detect dead fungi, and is time-consuming [2, 55]. Only the level of sporulation is represented, instead of the current biomass. Therefore, this method is unsuitable for the determination of the biomass of basidiomycetes, as well as other filamentous fungi [12, 55].

5.2 Agar Film Technique

The agar film technique is an optical identification method that was developed in 1948 by Jones and Mollison [60]. The sample is mixed with agar after several washing steps, heated, and poured into a counting chamber (hemocytometer).

The piece of agar with uniform volume is released after solidification, dried, and analyzed after staining by microscopy. Subsequently, the biomass concentration is calculated using a conversion factor. Since 1948, the method has been further developed and optimized, conversion factors improved, and handling simplified, so that important biomass structures are not destroyed [61]. The conversion factor is influenced by the hyphal length, density, magnification, and substrate [62]. Usually, this method is used in parallel with other determination methods, because it is very complicated and requires expertise [63].

5.3 Light Reflectance and IR-Spectroscopy

Fungal biomass can be estimated using light reflectance and infrared spectroscopy (IR-spectroscopy).

Murthy et al. [64] used the light reflection method to measure the color change of the medium during production of *koji* with *A. niger*. The substrate (rice) took on a darker color during the fermentation. The principle of trichromatic generalization (i.e., that each color consists of 3 primary colors) was used to detect the color change. The reflection, absorption, and scattering of the light that is incident on the sample vary, depending on the color. Visible light (360–800 nm) was used as the incident light and the total color difference in the reflected light between the sample and the white standard (barium sulfate powder) was calculated. The total color difference correlated well with biomass in an experiment in which pure biomass was mixed with dried and autoclaved wheat bran in different proportions ($R = 0.98$), until a biomass concentration of 20 % in the sample.

IR-spectroscopy uses infrared light (800 nm–1 mm) and can be used to quantify known substances or for structural analysis. The IR-radiation is absorbed by certain bonds, which lead to a vibration of the bond. Different types of bonds give vibration peaks at different wavelengths in the IR spectrum. The interpretation and assignment of the individual spectra and absorption bands requires a lot of experience, but this method is an interesting technique for the determination of fungal biomass, because it is noninvasive and can also be used to quantify several components simultaneously.

Desgranges et al. [52] used IR-spectroscopy for the direct determination of the cell components, ergosterol and glucosamine, of the ascomycete *B. bassiana* and the medium components, sucrose and nitrogen. The values determined by IR-spectroscopy correlated well with contents determined in chemical assays. In the case of glucosamine, the nutrient medium interfered partially. The differentiation of the growth stages was possible. However, the accuracy of the measurement decreased with increasing biomass content in the sample being analyzed.

Zornoza et al. [65] used near-infrared (NIR) spectroscopy in an attempt to quantify fungal biomass, by detecting fungal-specific PLFAs in leaf litter. The values of the PLFAs predicted by NIR reflectance correlated well with the PLFA values that were determined by a standard chemical assay. However, the interpretation of the spectra

that was obtained during the measurement of the leaf litter, and thus, the estimation of the fungal biomass content was not directly possible due to interferences and superposition of spectra from various sample components.

In contrast to Zornoza et al. [65] and Desgranges et al. [52], Brandl [66] used IR-spectroscopy for qualitative determination of the ascomycete *Neotyphodium lolii* in perennial ryegrass and not for quantification. The detection limits were too high, so that small amounts of biomass remained undetected.

The use of IR-spectroscopy requires a lot of experience for proper interpretation of the spectra, and it is also time-consuming to calibrate the method properly. Additionally, the method does not differentiate between live and dead biomass. Nevertheless, it is an interesting alternative for the quantification of biomass during SSF since it is robust and nondestructive and the measurement itself is rapid. Also, sample preparation is easy and does not require the use of additional chemicals, thereby avoiding the generation of hazardous waste [52]. IR-spectroscopy is already used routinely in chemistry and can be used with relatively small samples. It might even be possible to use this method online for transparent reactors. On the other hand, the determination of the color change of the substrate as a biomass indicator is suitable only for special cases.

5.4 Substrate-Induced Respiration (SIR)

Biomass quantification using substrate-induced respiration (SIR) is a commonly used standardized method (ISO 14240-1) from soil biology and can be carried out with fresh samples. In this method, active biomass is estimated based on the determination of the CO_2 that is formed after the addition of a readily available carbon source, such as glucose. A conversion factor, obtained through calibration, is used to convert the CO_2 formed into biomass [57]. After optimization, this method is very sensitive, highly reproducible, automatable, and performable with large sample throughput [2, 67].

There are two general approaches. In the Heinemeyer approach, the CO_2 formation is measured continuously throughout the lag phase of the microbial growth using infrared gas analysis (measurement of the maximum initial respiratory response). In the Isermeyer approach, the CO_2 is absorbed in a sodium hydroxide solution. After 4 h of incubation, the carbonate is precipitated and the unused sodium hydroxide is determined by titration. The SIR method is commonly used and inexpensive, but only detects active fungi that are capable of metabolizing the added glucose. It is not suitable for samples with a high content of young cells due to the higher CO_2 formation compared to older cells, which leads to an overestimation of the fungal biomass [57]. Furthermore, this method is highly influenced by the humidity of the sample and the pH value [68]. When nonsterile samples are used, the percentage of fungal biomass can be quantified by the addition of streptomycin [67, 68]. However, the inhibitor concentration must be optimized for the particular conditions since it greatly affects the results.

6 Conclusions

Various biomass determination methods are available for the quantification of fungal biomass in SSF processes. Each method has its advantages and drawbacks. A perfect method does not yet exist, but some methods are more suitable than others.

Optical methods enable a deeper understanding of morphology and, with the use of appropriate staining techniques, a deeper understanding of the physiological state of the fungus. It is also possible to distinguish active and inactive hypha and quantify them separately. However, quantification of the biomass by imaging techniques is difficult during SSF, because usually only surface mycelium can be detected and the methods are very time-consuming.

The determination of cell components is commonly used to quantify fungal biomass. Fungal-specific components can be used in nonsterile processes, as well as in mixed cultures. New techniques, such as real-time PCR, enable a species-specific determination of the biomass. Most of these methods are very complicated and time-consuming, and require invasive sampling. However, the use of alternative measurement techniques, such as IR-spectroscopy, enables the nondestructive and online detection of certain components of the fungus and even of the substrate. Most methods involving measurement of cell components require careful calibration, and it is crucial to check whether the conversion factors depend on mycelium age and the cultivation conditions.

Measurement of the metabolic activity of the fungal biomass is usually suitable for continuous and online monitoring and is therefore appropriate for industrial bioreactors. However, it is usually difficult to convert the measured metabolic activities into reliable estimates of the biomass, so these methods should be used as qualitative indicators of the biomass and its physiological state. Also, species-specific differentiation of the biomass is usually not possible, so these methods are less suitable for nonsterile processes.

In most cases, it would be advantageous to use a combination of different methods. This has two advantages. Firstly, it can increase the reliability of the results. Secondly, it can provide additional information about the state of culture.

In our own work, we tested a variety of different methods to monitor growth of the basidiomycete *T. hirsuta* in cultivation systems that included a reactor of up to 10-L working volume. We obtained good results for the measurement of ergosterol content, the counting of cell nuclei, the measurement of respiratory activity, and the determination of the activity of lignolytic enzymes. The determination of the ergosterol content is expensive, but the results are accurate and reproducible. The specially developed method of counting the fungal cell nuclei by flow cytometry is influenced by many factors, but this can be resolved by the use of different calibration curves. The results correlated well with the biomass calculated by ergosterol measurement and were also reproducible. Furthermore, this method is characterized by rapidity, straightforward handling and enables additional information about the physiology of the culture. The measurement of respiratory activity was very suitable for continuous and online biomass estimation until the stationary phase.

The activities of lignolytic enzymes, such as laccases and peroxidases, were useful as qualitative indicators of biomass and gave additional information about the state of the culture. The enzyme activity assays were rapid and easy to perform.

To conclude, the most suitable methods for quantification of fungal biomass in SSF depend on the particular requirements for accuracy, cost, and throughput: We have given you some advice, now you must make your choice!

References

1. Krishna C (2005) Solid-state fermentation systems-an overview. Crit Rev Biotechnol 25(1–2):1–30
2. Ottow JCG (2011) Mikrobiologie von Böden. Springer, Berlin, 508 p
3. Böhmer U, Frömmel S, Bley T, Müller M, Frankenfeld K, Miethe P (2011) Solid-state fermentation of lignocellulotic materials for the production of enzymes by the white-rot fungus *Trametes hirsuta* in a modular bioreactor. Eng Life Sci 11(4):395–401
4. Mitchell DA, Krieger N, Berovic M (2006) Solid-state fermentation bioreactors: fundamentals of design and operation. Springer Science & Business Media, Berlin, 481 p
5. Li Y, Wadsö L (2011) Simultaneous measurements of colony size and heat production rate of a mould *Penicillium brevicompactum* growing on agar. J Therm Anal Calorim 104(1):105–111
6. Madrid RE, Felice CJ (2005) Microbial biomass estimation. Crit Rev Biotechnol 25(3):97–112
7. Couri S, Mercês EP, Neves BCV, Senna LF (2006) Digital image processing as a tool to monitor biomass growth in *Aspergillus niger* 3T5B8 solid-state fermentation: preliminary results. J Microsc 224(3):290–297
8. Dutra J, da C Terzi S, Bevilaqua J, Damaso M, Couri S, Langone M, Senna LF (2008) Lipase production in solid-state fermentation monitoring biomass growth of *Aspergillus niger* using digital image processing. Appl Biochem Biotechnol 147(1):63–75
9. Stahl PD, Parkin TB, Christensen M (1999) Fungal presence in paired cultivated and uncultivated soils in central Iowa. USA Biol Fertil Soils 29(1):92–97
10. Paul EA, Clark FE (1989) Soil microbiology and biochemistry. Academic Press, Waltham, 298 p
11. Söderström B, Bååth E (1979) Fungal biomass and fungal immobilization of plants nutrients in swedish coniferous forest soils. Rev Ecol Biol Sol 16(4):477–489
12. Osma JF, Toca-Herrera JL, Rodríguez-Couto S (2011) Environmental, scanning electron and optical microscope image analysis software for determining volume and occupied area of solid-state fermentation fungal cultures. Biotechnol J 6(1):45–55
13. Haus J (2014) Optische Mikroskopie: Funktionsweise und Kontrastierverfahren. Wiley, New York, 240 p
14. Nopharatana M, Mitchell DA, Howes T (2003) Use of confocal scanning laser microscopy to measure the concentrations of aerial and penetrative hyphae during growth of *Rhizopus oligosporus* on a solid surface. Biotechnol Bioeng 84(1):71–77
15. Horbik D, Łowińska-Kluge A, Górski Z, Stanisz E, Zgoła-Grześkowiak A (2013) Microwave-assisted extraction combined with HPLC-MS/MS for diagnosis of fungal contamination in building materials. J Braz Chem Soc 24(9):1478–1486
16. Ooijkaas LP, Tramper J, Buitelaar RM (1998) Biomass estimation of *Coniothyrium minitans* in solid-state fermentation. Enzyme Microb Technol 22(6):480–486
17. Desgranges C, Vergoignan C, Georges M, Durand A (1991) Biomass estimation in solid state fermentation I. Manual biochemical methods. Appl Microbiol Biotechnol 35(2):200–205
18. Gessner, Newell SY (2002) Manual of environmental microbiology, 2nd edn. ASM Press, Washington DC
19. Manter DK, Kelsey RG, Stone JK (2001) Quantification of *Phaeocryptopus gaeumannii* colonization in Douglas-fir needles by ergosterol analysis. For Pathol 31(4):229–240

20. Matcham SE, Jordan BR, Wood DA (1985) Estimation of fungal biomass in a solid substrate by three independent methods. Appl Microbiol Biotechnol 21(1):108–112
21. Zelles L, Hund K, Stepper K (1987) Methoden zur relativen Quantifizierung der pilzlichen Biomasse im Boden. Z Für Pflanzenernähr Bodenkd 150(4):249–252
22. Klamer M, Bååth E (2004) Estimation of conversion factors for fungal biomass determination in compost using ergosterol and PLFA 18:2ω6,9. Soil Biol Biochem 36(1):57–65
23. Zhang H, Wolf-Hall C, Hall C (2008) Modified microwave-assisted extraction of ergosterol for measuring fungal biomass in grain cultures. J Agric Food Chem 56(23):11077–11080
24. Reeslev M, Miller M, Nielsen KF (2003) Quantifying mold biomass on gypsum board: comparison of ergosterol and beta-N-acetylhexosaminidase as mold biomass parameters. Appl Environ Microbiol 69(7):3996–3998
25. Snajdr J, Valásková V, Merhautová V, Cajthaml T, Baldrian P (2008) Activity and spatial distribution of lignocellulose-degrading enzymes during forest soil colonization by saprotrophic basidiomycetes. Enzyme Microb Technol 43(2):186–192
26. Muniroh MS, Sariah M, Abidin MAZ, Lima N, Paterson RRM (2014) Rapid detection of *Ganoderrna*-infected oil palms by microwave ergosterol extraction with HPLC and TLC. J Microbiol Methods 100:143–147
27. Deng Z-L, Yuan J-P, Zhang Y, Xu X-M, Wu C-F, Peng J, Wang J-H (2013) Fatty acid composition in ergosteryl esters and triglycerides from the fungus *Ganoderma lucidum*. J Am Oil Chem Soc 90(10):1495–1502
28. Schmidt O (2006) Wood and tree fungi: biology, damage, protection, and use. Springer Science & Business Media, Berlin, 336 p
29. Abd-Aziz S, Hung GS, Hassan MA, Abdul Karim MI, Samat N (2008) Indirect method for quantification of cell biomass during solid-state fermentation of palm kernel cake based on protein content. Asian J Sci Res 1(4):385–393
30. Nilsson K, Bjurman J (1998) Chitin as an indicator of the biomass of two wood-decay fungi in relation to temperature, incubation time, and media composition. Can J Microbiol 44(6):575–581
31. Ride J, Drysdale R (1972) Rapid method for chemical estimation of filamentous fungi in plant-tissue. Physiol Plant Pathol 2(1):7–15
32. Scotti CT, Vergoignan C, Feron G, Durand A (2001) Glucosamine measurement as indirect method for biomass estimation of *Cunninghamella elegans* grown in solid state cultivation conditions. Biochem Eng J 7(1):1–5
33. Roche N, Venague A, Desgranges C, Durand A (1993) Use of chitin measurement to estimate fungal biomass in solid state fermentation. Biotechnol Adv 11(3):677–683
34. Blagodatskaya E, Blagodatsky S, Anderson T-H, Kuzyakov Y (2014) Microbial growth and carbon use efficiency in the rhizosphere and root-free soil. PLoS ONE 9(4):e93282
35. Jirout J, Šimek M, Elhottová D (2011) Inputs of nitrogen and organic matter govern the composition of fungal communities in soil disturbed by overwintering cattle. Soil Biol Biochem 43(3):647–656
36. May BA, VanderGheynst JS, Rumsey T (2006) The kinetics of *Lagenidium giganteum* growth in liquid and solid cultures. J Appl Microbiol 101(4):807–814
37. Knoll S, Mulfinger S, Niessen L, Vogel RF (2002) Rapid preparation of *Fusarium* DNA from cereals for diagnostic PCR using sonification and an extraction kit. Plant Pathol 51(6):728–734
38. Voegele RT, Schmid A (2011) RT real-time PCR-based quantification of *Uromyces fabae* in planta. FEMS Microbiol Lett 322(2):131–137
39. Tellenbach C, Grünig CR, Sieber TN (2010) Suitability of quantitative real-time PCR to estimate the biomass of fungal root endophytes. Appl Environ Microbiol 76(17):5764–5772
40. Pilgård A, Alfredsen G, Björdal CG, Fossdal CG, Børja I (2011) qPCR as a tool to study basidiomycete colonization in wooden field stakes. Holzforschung 65(6):889–895
41. Costa-de-Oliveira S, Silva AP, Miranda IM, Salvador A, Azevedo MM, Munro CA et al (2013) Determination of chitin content in fungal cell wall: an alternative flow cytometric method. Cytometry A 83(3):324–328

42. Throndset W, Kim S, Bower B, Lantz S, Kelemen B, Pepsin M et al (2010) Flow cytometric sorting of the filamentous fungus Trichoderma reesei for improved strains. Enzyme Microb Technol 47(7):335–341
43. Steudler S, Böhmer U, Weber J, Bley T (2014) Biomass measurement by flow cytometry during solid-state fermentation of basidiomycetes. Cytometry A. doi:10.1002/cyto.a.22592
44. Griffin DH (1996) Fungal physiology. Wiley, New York, 476 p
45. Frostegård A, Tunlid A, Bååth E (2011) Use and misuse of PLFA measurements in soils. Soil Biol Biochem 43(8):1621–1625
46. Frostegård A, Bååth E (1996) The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biol Fertil Soils 22(1):59–65
47. Córdova-López J, Gutiérrez-Rojas M, Huerta S, Saucedo-Castañeda G, Favela-Torres E (1996) Biomass estimation of *Aspergillus niger* growing on real and model supports in solid state fermentation. Biotechnol Tech 10(1):1–6
48. Favela-Torres E, Cordova-López J, García-Rivero M, Gutiérrez-Rojas M (1998) Kinetics of growth of *Aspergillus niger* during submerged, agar surface and solid state fermentations. Process Biochem 33(2):103–107
49. Saucedo-Castañeda G, Trejo-Hernández MR, Lonsane BK, Navarro JM, Roussos S, Dufour D et al (1994) On-line automated monitoring and control systems for CO_2 and O_2 in aerobic and anaerobic solid-state fermentations. Process Biochem 29(1):13–24
50. Ikasari L, Mitchell DA (1998) Oxygen uptake kinetics during solid state fermentation with *Rhizopus oligosporus*. Biotechnol Tech 12(2):171–175
51. Larroche C, Gros JB (1992) Characterization of the growth and sporulation behavior of *Penicillium roquefortii* in solid substrate fermentation by material and bioenergetic balances. Biotechnol Bioeng 39(8):815–827
52. Desgranges C, Georges M, Vergoignan C, Durand A (1991) Biomass estimation in solid state fermentation II. On-line measurements. Appl Microbiol Biotechnol 35(2):206–209
53. Sakurai Y, Misawa S, Shiota H (1985) Growth and respiratory activity of *Aspergillus oryzae* grown on solid state medium. Agric Biol Chem 49(3):745–750
54. Dhandapani B, Mahadevan S, Dhilipkumar SS, Rajkumar S, Mandal AB (2012) Impact of aeration and agitation on metabolic heat and protease secretion of *Aspergillus tamarii* in a real-time biological reaction calorimeter. Appl Microbiol Biotechnol 94(6):1533–1542
55. Anand S, Rati ER (2006) An enzyme-linked immunosorbent assay for monitoring of *Aspergillus ochraceus* growth in coffee powder, chilli powder and poultry feed. Lett Appl Microbiol 42(1):59–65
56. Dubey AK, Suresh C, Kumar SU, Karanth NG (1998) An enzyme-linked immunosorbent assay for the estimation of fungal biomass during solid-state fermentation. Appl Microbiol Biotechnol 50(3):299–302
57. Kaufmann K, Rossier N, Oberholzer H (2010) Niederhäusern A v. Vergleich dreier Methoden zur Langzeitbeobachtung der biologischen Bodenaktivität. Publ Bodenkd Ges Schweiz 30:75–80
58. West AW, Ross DJ, Cowling JC (1986) Changes in microbial C, N, P and ATP contents, numbers and respiration on storage of soil. Soil Biol Biochem 18(2):141–148
59. Davey CL, Peñaloza W, Kell DB, Hedger JN (1991) Real-time monitoring of the accretion of *Rhizopus oligosporus* biomass during the solid-substrate tempe fermentation. World J Microbiol Biotechnol 7(2):248–259
60. Jones PCT, Mollison JE, Quenouille MH (1948) A technique for the quantitative estimation of soil micro-organisms. J Gen Microbiol 2(1):54–69
61. Hill DR (1996) Thin agar film for enhanced fungal growth and microscopic viewing in a new sealable fungal culture case. J Clin Microbiol 34(9):2140–2142
62. Kasai K, Horikoshi T (1997) Estimation of fungal biomass in the decaying cones of Pinus densiflora. Mycoscience 38(3):313–322
63. Lodge DJ, Ingham ER (1991) A comparison of agar film techniques for estimating fungal biovolumes in litter and soil. Agric Ecosyst Environ 34(1):131–144
64. Ramana Murthy MV, Thakur MS, Karanth NG (1993) Monitoring of biomass in solid state fermentation using light reflectance. Biosens Bioelectron 8(1):59–63

65. Zornoza R, Guerrero C, Mataix-Solera J, Scow KM, Arcenegui V, Mataix-Beneyto J (2008) Near infrared spectroscopy for determination of various physical, chemical and biochemical properties in Mediterranean soils. Soil Biol Biochem 40(7):1923–1930
66. Brandl H (2013) Detection of fungal infection in *Lolium perenne* by fourier transform infrared spectroscopy. J Plant Ecol 6(4):265–269
67. Beare MH, Neely CL, Coleman DC, Hargrove WL (1990) A substrate-induced respiration (SIR) method for measurement of fungal and bacterial biomass on plant residues. Soil Biol Biochem 22(5):585–594
68. Lin Q, Brookes PC (1999) An evaluation of the substrate-induced respiration method. Soil Biol Biochem 31(14):1969–1983