Estimation of growth in solid state fermentation: A review

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ABSTRACT

Carleysmith and Fox (1984) stated "without doubt, the single most vital yet most problematical value sought during fermentation is biomass estimation". Achieving a positive result in determining biomass remains a major challenge in solid state fermentation (SSF). Fungi are well-characterised microorganisms and are widely used in SSF due to their ability to colonise and penetrate into the solid substrate. The compressed structure of the mycelia and the solid substrate does not allow a complete recovery of the biomass, which may not be insurmountable. Since the use of a direct technique such as the dry weight method is impractical, the use of an indirect estimation technique is the only alternative. This review examines strategies that have been used to estimate biomass in SSF. Many promising indirect estimation techniques are available, which can be classified into six categories as follows: (i) measuring cell components not present in the substrate; (ii) measuring biomass component present in both substrate and biomass; (iii) measuring other secondary metabolites; (iv) measuring metabolic activity; (v) measuring images from direct microscopic observation and (vi) measuring biomass from the substrate matrix. New potential technique and future directions are also discussed in this review. Although significant advances have been made with the availability of various techniques; however, progress has been very unsatisfactory. The evaluation of microbial growth in SSF may sometimes become laborious, impractical and inaccurate. Essentially, this remains another critical issue for monitoring growth. The information of the profile of fungal biomass growth throughout any SSF process constitutes an essential parameter in estimation of kinetic variables and subsequently, scale-up of the process.

Keywords: Biomass estimation, fungal growth, solid state fermentation, filamentous fungi, solid substrate

INTRODUCTION

Solid state fermentation (SSF) has been defined in many ways. Many researchers in the field have introduced their own ways to define SSF. For example, Pandey et al. (2000) defined SSF as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can also be used as carbon and energy source. Mitchell et al. (2000) described SSF as any process in which substrates in a solid particulate state are utilised, while Viniegra-Gonzalez (1997) defined SSF as a microbial process occurring mostly on the surface of solid materials that have the property to absorb or contain water, with or without soluble nutrients. According to Rahardjo et al. (2006), SSF is the growth of microorganisms on moistened solid substrate, in which enough moisture is present to maintain microbial growth and metabolism, but where there is no free-moving water and air is the continuous phase. Rosales et al. (2007) gave a simple definition of SSF where the growth of microorganisms is on solid or semisolid substrates or support. In the latest definition, Mitchell et al. (2011) defined SSF as a process that involves the growth of microorganisms on moist particles of solid materials in beds in which the spaces between the particles are filled with a continuous gas phase. Whatever the definition, we can understand that SSF is referring to the microbial fermentation, which takes place in the absence or near absence of free water, thus being close to the natural environment to which the selected microorganisms, especially fungi, are naturally adapted.

SSF processes are clearly different from submerged fermentation (SmF). In most cases, it is soluble substrate supported on a solid insoluble matrix in an environment of low moisture content. In SSF, estimation of biomass presents difficulties as generally the fungal mycelium penetrates deep and remains attached with the solid substrate particles. The advantage of SSF comes from its...
simplicity and its closeness to the natural habitat of many microorganisms. Professor A.P.J. Trinci (Pandey et al., 2008) used the now frequently quoted phrase “God did not create filamentous fungi to grow in a fermenter”. He claimed that SmF is an artificial condition for filamentous fungi because they live in nature in a solid state (Pandey et al., 2008). Most microorganisms, especially filamentous fungi and many actinomycetes, primarily live and grow in nature in SSF conditions (Carlile and Watkinson, 1994). More than 98% of isolates from marine environment have been obtained from the underwater surfaces of solid substrates and less than 1% of all known fungi have been found in marine habitats (Kelecom, 2002). The morphology factor of the microorganisms plays an important role since it can affect many aspects of the process. Through modern biotechnology, there are new initiatives to improve and enhance the productivity of SSF (Dalsenter et al., 2005). Each microorganisms, solid substrate, biomass estimation and bioreactor system plays a major role in the success of SSF.

PROBLEM STATEMENT

Previous studies suggest that traditional methods for SSF are all very similar and often involve low technology. As a consequence, it is difficult to measure growth and difficult to control the environment in such systems. Therefore, it is necessary to significantly improve the ability to measure and control the physical environment in SSF systems in order to be able to develop new and effective processes. To confirm the particular end product, it is necessary to use analytical methods to evaluate the fermentation process. In contrast to conventional fermentation processes (e.g. SmF), SSF does not allow for accurate biomass estimation. Attempts to estimate biomass have been made using many indirect techniques. For example, in the case of bacteria, ATP or DNA can be extracted and measured or, in the case of fungi, enzyme activity or glucosamine. However, none of these methods give satisfactory/conclusive results. The main disadvantage of most indirect techniques is that they are off-line, usually apply under a limited range of conditions and are labour intensive. By contrast, on-line techniques that measure oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and heat evolution rate are the most likely to meet with success.

From the literature, it is clearly demonstrated that studying microorganisms in controlled, well-phased cultures is of great importance. Above all, studying the early developments of the culture is particularly important because it is this phase, together with the physiological state of the microorganism, that determines the later stages of development (Desgranges et al., 1993). Measuring growth through a direct or an indirect technique is also important to provide a better understanding of process control and also to give an idea regarding the productivity of a cell culture (Borzani, 2000) nor to determine the effectiveness of a biotechnological process (Kiviharju et al., 2008; Singh et al., 1994; Sonnleitner et al., 1992). Normally, estimation of organic matter loss, dry weight, spores count and glucosamine content are made by taking samples intermittently and carrying out laboratory analysis. Organic matter loss and dry weight reduction were successfully proven for biomass estimation. However, these techniques were only satisfactory in laboratory study and did not permit estimation in large scale SSF. Biomass estimation is of fundamental importance in many microbial fermentation processes (Zwietering et al., 1990). However, this estimation is very difficult to make when solid substrate particles are present and bind to the cells. This review examines and evaluates about six (6) common techniques that have been used to estimate biomass in SSF (or sometimes in SmF involves solid particles).

ESTIMATION OF GROWTH IN SOLID STATE FERMENTATION

It is difficult to estimate biomass in SSF directly because it is very difficult to separate the microorganisms and the solid particles. This is more the case for fermentations involving filamentous fungi because fungal mycelium penetrates deeply into the substrate and becomes inextricably entangled within the solids. For this reason, it is better to use indirect methodologies for biomass estimation and having information about the cell biomass in such systems is very important. Chattaway et al. (1992) state “in the case of biomass estimation, elemental balances are useful either for obtaining on-line estimates or for calculating biomass off-line, when direct measurement is difficult, during fermentations on solid-containing media”.

Although there are a large number of studies on biomass estimation in SSF, the available methods do not perform well. There are operational and sample preparation difficulties around these existing methods. For example, dry weigh measurements (including both cells and the solid particles), are used to estimate cell biomass concentration, resulting in accurate data. A range of indirect methods can be used to estimate biomass, which can be classified into six categories:

1. Measuring cell components not present in the substrate
2. Measuring biomass component present in both substrate and biomass
3. Measuring other secondary metabolites
4. Measuring metabolic activity
5. Measuring images from direct microscopic observation
6. Measuring biomass from the substrate matrix

Measuring cell components not present in the substrate

Cell components (which are products of microbial activity) can be measured to estimate the growth of biomass. To do this, it is necessary to identify and measure components of the cell or mycelium, which are not normally present in the solid substrate. Cell components which can be measured include: glucosamine (Swift,
1973; Sparringa and Owens, 1999), chitin or ergosterol (Feng et al., 2005), total sugar. DNA assay (nucleic acids) (Hashimoto et al., 1983; Solomon et al., 1983), ATP (Thierry and Chicheportiche, 1988), proteins (Raimbault and Alazard, 1980; Abd-Aziz et al., 2008), enzymes and others secondary metabolites. The most popular methods involve glucosamine estimation and profile production of enzymes (Ramesh et al., 1996). However, identifying cell components especially glucosamine, DNA and ergosterol is difficult and time-consuming due to tedious extraction procedures. For example, sample preparation for glucosamine and ergosterol measurement can take more than 24 h to complete. Measuring enzymatic activity or other secondary metabolites could be a reliable method for biomass estimation (Mitchell, 1992; Raimbault, 1997). A more practical logistic model developed by Ramesh et al. (1996) describes α-amylose and protease production, which are two non-growth associated enzymes produced by Bacillus licheniformis M27. However, it is not easy to perform sample preparation for the analytical assay.

**Measuring biomass components present in both substrate and biomass**

Protein content is related to cell biomass and is easy to measure when microorganisms are grown alongside non-protein solid substrates. Significant amounts of protein are usually found in solid materials. The microorganism will therefore hydrolyse the substrate protein and produce biomass protein. Therefore, it can be difficult to tell the difference between protein from the cells and solid substrate protein. Measurements can wrongly be taken of total protein from both cells and solids. This biomass measurement technique is only reliable if solid substrate has no protein. Recent works by Oto et al. (2012; 2013) and Oshita et al. (2011) resulted in a non-destructive and real-time estimation method for monitoring microbial contamination. The method used a UV-Vis reflectance spectrum to measure ATP and/or a plate count on the surface of pork meat.

**Measuring other secondary metabolites**

Microbial secondary metabolites are useful high value products because they undergo a large number of biological activities. Secondary metabolites produced by a SSF system such as penicillin, tetracyclin, cephalosporin, iturin A, cephamycin C, ergot, alkaloids, monacolin K, lovastatin and many others could be used to indicate growth in SSF. After microbial growth has occurred, secondary metabolites are synthesized in a fermentation medium (Krishna, 2005; Takashi et al., 2009). Pandey et al. (2001) argue that secondary metabolites build up near the end of fermentation, known as the idophase that follows the active growth phase called trophophase. Secondary metabolites produced in the idophase are independent of synthesised cell material and normal microorganism growth.

**Measuring metabolic activity**

During fermentation processes, carbon is aerobically transformed into biomass, primary or secondary metabolites, carbon dioxide and water (Cordova-Lopez et al., 1996). Growth happens because of the interactions of microorganisms and their environments. Besides product formation and nutrients consumed, other factors, which affect growth are: environmental conditions (oxygen and carbon dioxide level, temperature, pressure and pH) (Carrizalez et al., 1981). Continuously on-line monitoring of microbial growth could be performed by indirect methods measuring:

- Oxygen consumed (Cooney et al., 1969; Sato et al., 1983; Saucedo-Castaneda et al., 1994; Ikasari and Mitchell, 1998);
- Carbon dioxide produced (Carrizalez et al., 1981; Saucedo-Castaneda et al., 1994; Marsh et al., 1998; Assi et al., 2009);
- Metabolic heat (Cooney et al., 1969; Ratkowski et al., 1983; Sangsurasak and Mitchell, 1995; 1998; Dalsenter et al., 2005; Fanaei et al., 2009) and
- Pressure levels during fermentation (Auria et al., 1993; Auria and Revah, 1994 Auria et al., 1995)

The advantage of using this method is that there are no tedious and time consuming sampling processes. It is assumed that total carbon dioxide produced is proportional to the cumulative cell concentration (Terebiznik and Pilosof, 1999). The metabolic state of culture is estimated from the respiratory quotient (RQ). The RQ value is found by using carbon evolution and oxygen consumption rates (RQ = CER/OUR). This can give indication of how well the substrate is aerated as high RQ values indicate fermentative metabolism (Mitchell et al., 2002). This could be applied to other biological systems where carbon dioxide is a growth product. Heat is also produced by microbial activity and so could be used to measure biomass. Another reported method shows that growth and pressure drop at the same rate across an aerated SSF fermentation bed (Auria et al., 1993; Auria and Revah, 1994 Auria et al., 1995). Once technology becomes more advanced over time, on-line microbial metabolism could be suitable for determining growth in aerobic SSF, by measuring rates of oxygen uptake, carbon dioxide evolve, heat evolution and pressure changes. Facilities could be expensive but useful for gaining information on microbial growth yet very accurate. These techniques are suited for use on-line and so are most likely to meet success in the future.

Metabolic respiratory (OUR, CER) and temperature profiles during SSF are linearly related with biomass synthesis in an aerobic fermentation. They are considered to be the most accurate for determination of growth of the microorganisms in SSF. The technique is also suitable for large scale SSF due to its advantages such as overcoming the sampling process, avoiding damage of mycelium and the on-line and fast delivery nature of the
technique. OUR, CER and heat evolved are the most meaningful techniques to estimate biomass in SSF. The data obtained from OUR, CER and heat evolved allows the estimation of the kinetics of fungal growth. With the present advanced technology, solid state bioreactors should be designed with an on-line facility for monitoring of several parameters as well as studies that consider transport phenomena at micro-scale levels, primarily including heat and mass transfer in a system characterised by a gas-solid interface. Supervision is a higher-level automation, in which measurements are processed in real time to generate more useful information.

**Measuring images through direct microscopic observation**

Another method is image analysis through microscopic observation. This can be done using Scanning Electron Microscope or Confocal Microscopy. Growth patterns taken from digital image analysis (Yingyi et al., 2012; Barry and Williams, 2011; Feng et al., 2007; Couri et al., 2006) are interpreted by computer software, which calculates total length or volume mycelium growing in the SSF system (Raimbault, 1997). Osma et al. (2011) developed software using a Matlab platform to estimate fungal culture’s occupied area and volume. This allows high-definition images from scanning electron and environmental scanning electron microscope to be analysed. To monitor the biomass growth of *Aspergillus niger*, Dutra et al. (2008) measured the hyphae area in an image from stereomicroscope Carl Zeiss STEMI 2000-CS and found a correlation with lipase activity.

Another technique uses confocal scanning laser microscopy to measure concentration of penetrative biomass during growth (Nopharatana et al., 2003a; 2003b). However, this technique is only suitable if artificial solid substrate is used. Miri et al. (2003) studied fungal hyphae’s morphology and structure during SSF. They used manual image analysis after staining the sample with a fluorescent contrast agent. Afterwards, they used a fluorescence microscope to see the fibres. This method involves expensive equipment and specific software needs to be developed. With these above methods, there are disadvantages. Some need special processes with tedious procedures, the amount of sample needed varies and operator fatigue can decrease accuracy level. In all cases, the cells attach to the solid particles. Cells are scattered and not evenly distributed. Cell damages during sample processing are also high possibility. Despite clear developments in image analysis, predictive, easy to apply models have not been developed. Routine measurement of biomass using this method is not practical, despite great potential. Using haemocytometer under microscope to count spores or cells is usually a last resort.

**Measuring biomass from the solid matrix**

In recent times, direct evaluation of biomass using a membrane filter has become possible. This is because the whole fungal mycelium can be easily recovered by peeling it off the membrane and weighing it immediately after drying (Ooijkaas et al., 2000). However, this technique is only suited to small scale bioreactor or lab studies. Another disadvantage is that it is difficult for fungal mycelium to penetrate especially large and deep solid particles. Terebiznik and Pilosof (1999) grew *Aspergillus oryzae* NRRL 3458 in wheat bran and measured dry matter weight loss in terms of mycelia growth. Okazaki et al. (1980) used the mathematical model for estimating fungal growth in SSF. They estimated biomass growth from dry matter weight loss by incorporating with carbon dioxide evolution into a relationship. Wang et al. (2010) adapted the logistic model as originally proposed by Okazaki et al. (1980). They successfully applied a dry weight reduction ratio for *A. oryzae* fermentation using rapeseed meal.

**Other techniques**

Ramana Murthy et al. (1993) monitored biomass in SSF using light reflectance. They observed colour changes due to the growth of the fungus during SSF. They used CIE (Commission International de l’Eclairage) system based on the principles of additive colour mixing. The measurements taken were looked against glucosamine concentration. The authors concluded that colour and glucosamine were closely linked. This technique for biomass estimation in SSF could be developed but no follow-up work has been done.

There is potential for using Biomass Monitor to measure radio-frequency dielectric properties (Aber Instruments Ltd, Science Park, Cefn Llan, Aberystwyth, Dyfed SY23 3DA, UK). The online method involves taking measurements of the dielectric permittivity at low radio frequencies; by using biomass probe (the Bugmeter) which has four terminal sensors. Computerised system measures the capacitance at 0.3 MHz (in picofarads), which indicates of biomass. This technique was shown to measure biomass in SmF without presence of solid particles either using yeast, bacteria or even fungus (Harris and Kell, 1985; Harris et al., 1987).

Articles produced by Penaloza et al. (1991) and Davey et al. (1991); showed that the microbial biomass during SSF for tempe production could be estimated by the monitor measuring in capacitance (fungus *Rhizopus oligosporus* was used). It was found that this technique produced linearity during the growth phase, between the dielectric permittivity and the hyphae length (as determined microscopically). The finding was supported by other researchers (Fehrenbach et al., 1992) that biomass monitoring could be measured on-line by capacitance. According to Davey et al. 1991, the biomass monitor provides a reliable, one of a kind, easy to reproduce and on-line measurement of biomass in SSF. Botella (2007) also suggested that this technique has potential to monitor on-line fungal growth in SSF. As with light reflectance technique, there is no work reported after year 1991. Therefore, this technique shows potential for further development.
The online measurement of capacitance using the Biomass monitor technique seemed very promising but might not be approachable for SSF especially in large scale. However, it works excellently and should be explored further for fungal biomass estimation in large-scale SmF involving solid substrate particles. The measurement procedures can be done on-line without involving tedious sampling processes, although they are more expensive. If properly calibrated and compensated, these devices may be useful to measure the capacitance in SSF and SmF.

There are disadvantages to most of the categories listed in Table 1. A common problem for indirect methods of biomass estimation is that the relationship between biomass and the factor measured can change over time. No methods currently involve calibration, which could better measure the relationship with biomass. It is necessary to have a direct measurement of the biomass through calibration methods.

**New potential technique**

In general, colour of the fermenting solid substrate changes during the course of the SSF due to the growth of the fungus. During the course of the investigation of fungal growth on solid substrate, it was found that the colour of the solid substrate became darker with progressive growth. If this colour changes could be quantitatively related to fungal growth, it might become a new technique for the estimation of fungal growth in SSF.

The theory of measuring colour changes in SSF by UV-Vis spectroscopy demonstrates that this colourimetric technique provides valuable information. The key finding is that the colourimetric technique demonstrated and provided information of higher quality than that obtained by visual observation or spores counting (Musaabakri, 2014; Musaabakri and Webb 2016). For the growth of Aspergillus awamori and Aspergillus oryzae on wheat bran, soybean hulls and rapeseed meal, it was confirmed that colour production was directly proportional to fungal growth.

Colour from cell free extracts absorbs light in the region 260 – 300 nm that largely corresponds to pigments in the supernatant. Colour density measured for fungal SSF in cell free extracts can be determined quickly and accurately using absorbance measurement at the chosen wavelength of 300 nm. The advantages of this method include the fact that the procedure is cheap, fast, objective, simple to carry out, non-destructive and no special or expensive reagents are required. Making cell free extracts from fermented fungal biomass was carried only using distilled water and was performed in less than 2 h. Water was efficient in the recovery of colour. The results of this study indicated that the colourimetric technique could be used to monitor biomass growth in a SSF process and to correlate biomass growth and weight fungus, spores concentration, organic matter loss, glucosamine and enzyme activity. This colourimetric technique was also proved to be a feasible approach for fungal biomass estimation in SmF. This new approach is an important complementation to the existing techniques especially for basic studies.

Understanding the variations in biomass components and their interactions can enable development of new techniques for biomass estimation in SSF. Apart from being a potential technique to describe growth in fungal SSF, colour production measurement in fungi provides a very elegant technique to monitor the way fungi use their metabolism to survive and adapt to different environments. Overall, the estimation of colour density through UV-Vis spectrophotometry from SSF would be a favourable indirect method to describe the growth of fungi. A higher number of experiments and improvements on the colour changes technique in SSF using various microorganisms and solid substrates are needed to validate the technique. Further studies should be carried out to obtain more data to support the colourimetric technique using different fungi and bacteria, and also using different types of solid substrate whether in single form or mixed-solid substrate from different solid wastes.

**The future**

The promise of industrial biotechnology has been around since Chaim Weizmann developed the acetone-butanol-ethanol fermentation in the University of Manchester in 1917 and the prospects nowadays look brighter than ever. In the future, SSF may become more important as a technology to process alternative, bio-based feedstocks that will replace the declining petroleum resources. There will be efforts and ever-increasing pressure to move towards biorefineries for the production of industrial biochemicals (Mitchell et al., 2011). The large-scale cultivation of microorganisms will be an integral part of such biorefineries. Future biorefineries based on SSF aim to exploit the vast complexity of the technology to modify biomass produced by agriculture and food industry for valuable by-products through microbial bioconversion. This can be achieved since SSF technology offers the advantage and potential to minimise the addition of water and thus optimise process economics in biorefineries. On top of that, in order to meet the requirements of SSF as a potential technology, it is necessary to have effective methods to determine biomass growth for optimised performance. No method is ideally suited to all situations and hence the most appropriate method to a particular fermentation application must be chosen in each case based on the simplicity of the procedure, its cost and its accuracy.

**Concluding remarks**

Different techniques for biomass estimation are gathered, described and discussed. Several well-known indirect techniques to investigate biomass growth in simple petri dish and bioreactor cultures were reviewed. These were used to make biomass estimations, in order to carry out a critical informative comparison. Such techniques have the benefit of being practical, reliable and not too time-consuming in estimating fungal growth. It is about time...
now for research to be carried out in biosensor technology to design special electrodes for on-line pH measurement in solid substrate with the presence of little amount of water. Biosensors are compact and highly specific analytical instruments that comprise biological sensing components such as tissues, cells, enzymes or antibodies. To the best our knowledge, this type of instruments has not be evaluated in SSF so far.

Table 1: Summary of the categories of methods used to estimate biomass in SSF.

<table>
<thead>
<tr>
<th>Category</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measuring cell components that are not present within the solid substrate</td>
<td>Most of the cell components are not in constant proportion of the cell mass during all stages of development. Tedium extraction process, time-consuming. Expensive reagents/chemicals, strong chemicals, buffer preparation.</td>
</tr>
<tr>
<td>Measuring biomass components probably present in both solid substrate and biomass</td>
<td>Difficult to differentiate biomass components coming from either cells or solids – interference with a substrate rich in protein. Not accurate definition of biomass concentration. Involves tedious extraction process, time-consuming. Expensive reagents/chemicalskits, buffer preparation.</td>
</tr>
<tr>
<td>Measuring other secondary metabolite</td>
<td>Most of the cell components are not in a constant proportion of the cell mass during all stages of development. Not always related to growth. Tedium extraction process, time-consuming. Expensive reagents/chemicals, strong chemicals, buffer preparation.</td>
</tr>
<tr>
<td>Measuring metabolic activity</td>
<td>The oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and metabolic heat evolution rate techniques are the easiest to operate on-line. No regular sampling process. Shows a potential for further development. The metabolic production is proportional to biomass concentration but may vary with time during the fermentation. Always related to growth. Requires expensive equipment and dedicated software. Requires frequent calibration.</td>
</tr>
<tr>
<td>Measuring images through direct microscopic observation</td>
<td>Labour-intensive, time-consuming, cells adhering to solid surface causes damage problems, requires considerable time. Microscope has a higher resolution than the conventional light microscope. Suitable if using an artificial solid media.</td>
</tr>
<tr>
<td>Measuring biomass from the solid matrix</td>
<td>Adapted for fungi only. Suited only to a few cases, especially practical in lab scale studies. Complete separation is difficult.</td>
</tr>
</tbody>
</table>

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during solid-state fermentation in packed bed bioreactors. *Biotechnology and Bioengineering* 60(6), 739-749.


