Extracted substrate colour as an indicator of fungal growth in solid state fermentation

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ABSTRACT

Aims: In solid state fermentation (SSF), estimation of biomass is difficult as fungal mycelium penetrates deep and remains attached to the solid substrate particles. This study examines and evaluates a new technique based on colour changes of fermented substrates during SSF as an indicator for fungal growth.

Methodology and Results: SSF refers to microbial fermentation, which takes place in the absence or near absence of free water, thus being close to the natural environment in which the selected microorganisms, especially fungi, are naturally adapted. Although many promising methods are available, the evaluation of microbial growth in SSF may sometimes become difficult, impractical, and inaccurate. Essentially, this remains another critical issue for monitoring growth. In this study, measurements of colour changes of fermented substrates during SSF are used as indicators for growth and this technique has a potential to be used to quantify growth of microbes. For the growth of *Aspergillus awamori* and *A. oryzae* on wheat bran, soybean hulls, and rapeseed meal, it was confirmed that colour changes were directly proportional to the fungal growth. This new approach is an important complementation to the existing techniques, especially for basic studies. The advantages of this method are its ease of use, fast, non-destructive, cheap, and requires no special and expensive reagents. The key finding is that the colorimetric technique demonstrated in this study provides good means to estimate growth than that obtained by visual observation or spores counting.

Keywords: Biomass estimation, solid state fermentation, colour changes, *Aspergillus awamori, Aspergillus oryzae*

INTRODUCTION

Biomass is a fundamental parameter in the characterisation of microbial growth (Sonnleitner *et al.*, 1992). In solid state fermentation (SSF), estimation of biomass, which is essential for kinetic studies (Mitchell *et al.*, 2004), is difficult, as generally microbial cells remain attached to the substrate and it is difficult to separate them (Pandey *et al.*, 2010). In the case of fungal cultures, it is more difficult because fungal mycelia penetrates and tightly binds to the solid substrate particles. There are several direct or indirect techniques to estimate biomass as presented in open literatures; however, almost every method has its own drawbacks. Many efforts have been made to find a reliable and sensitive method that is convenient to use at every stage during SSF for biomass estimation that better describes the growth of microorganisms (Wei *et al.*, 2006). It is often desirable to know the current biomass or the quantity that will be present in the future if a product is subjected to particular treatments (Scotti *et al.*, 2001). Thus, by measuring fungal growth once using a desirable method, one could tell how a future population will develop under the same conditions (Cordova-Lopez *et al.*, 1996).

From the literatures, 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 at this phase, together with the physiological state of the microorganism, determines the later stages of development (Smits *et al.*, 1999). 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). Indirect estimation of loss of organic matter, dry weight (Hashemi *et al.*, 2011), spores count (Santa *et al.*, 2005), chitin or ergosterol (Feng *et al.*, 2005), glucosamine (Wei *et al.*, 2006), enzymes (Hashemi *et al.*, 2011) and proteins (Abd-Aziz *et al.*, 2008) content are made by taking samples intermittently and carrying out laboratory analyses. Direct estimation technique in SSF are done by measuring oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and heat evolved. According to Mitchell *et al.* (2004), OUR and and CER offer the advantage of fast response time and are directly linked to the metabolism of the

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microorganism. This is also true for the heat evolved during fermentation as a result of microbial metabolism (Harris and Kell, 1985).

This study examines and evaluates a new technique based on colour changes of fermented substrates during SSF as an indicator for fungal growth. Preliminary SSF using petri dishes was carried out in order to study the growth of *A. awamori* and *A. oryzae* as model microorganisms; whilst, wheat bran, soybean hulls, and rapeseed meal as model solid substrates. Thus, the main objective of this study is to evaluate a relatively simple colour technique to identify fungal growth phases in SSF processes. The data obtained will be discussed and compared in terms of ease and reliability to describe fungal growth.

MATERIALS AND METHODS

Microorganisms, preparation of the inoculum

The fungi *A. awamori* and *A. oryzae* were used to study colour changes. The spores were washed by lightly scrapping the spores with a wire loop and dipped in 10.0 mL of sterile 0.1% (v/v) Tween 80. 0.5 mL of the spore suspension was further transferred onto the surface of 100.0 mL of sporulation medium in a 500.0 mL Erlenmeyer flask and incubated for another 7 days at 30 °C. After the incubation period, 50.0 mL of sterile 0.1% (v/v) Tween 80 solution and several sterile glass beads (4-mm diameter) were added to the flask. The spores were suspended by shaking the flask gently and collected in one bottle as a spore suspension. The concentration of the spore suspension was quantified

using a haemocytometer. The volume of suspension needed for the inoculation of the solid substrate was calculated for each experiment to reach a concentration of around 1.0×0^6 spores/g solid substrate. Fungal spores in universal bottles were stored at 4 °C in agar slopes of solid sporulation medium, containing 5% (w/v) whole wheat flour and 2% (w/v) agar as a stock culture.

The substrates-solid state fermentation

The solid substrates: wheat bran, soybean hulls, and rapeseed meal, were used. Wheat bran was obtained from Cargill Wheat Processing Plant, Manchester, UK. Soybean hulls and rapeseed meal were obtained from Brocklebank Oilseed Processing Division, Cargill Wheat Processing Plant, Liverpool, UK. All three selected substrates were used without any treatment as solid media for growing *A. awamori* and *A. oryzae*. All experiments were carried out in 9.0 cm Petri dishes. 12.0 g of wheat bran and soybean hulls, and 15.0 g of rapeseed meal were used. These amounts were used to bring the height of the substrate bed in the petri dishes to 1.0 cm. After autoclaving, inoculum transfer was carried out aseptically, and sterile distilled water was used to moisten the solid substrates. The initial moisture content of the substrate was 65%. After the inoculation process, all the petri dishes were placed in a growth incubator with a strictly controlled temperature at 30 °C. At time intervals, one Petri dish was taken out. The performance of the system was evaluated in terms of the changes of colour in regards to fungal growth.
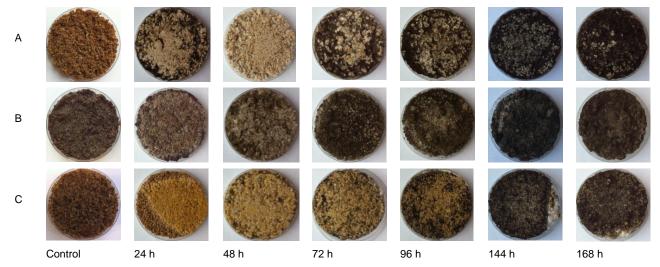

Figure 1: Visual observation of the colour changes during the course of the SSF of *A. awamori* on three different solid substrates. [A]: wheat bran; [B]: rapeseed meal and [C]: soybean hulls.
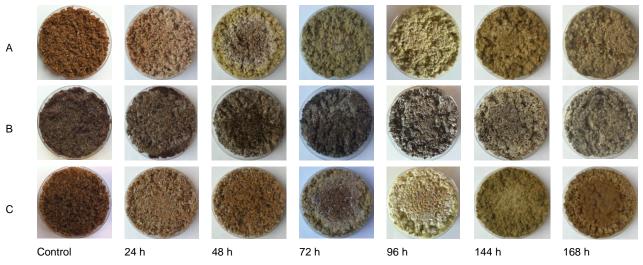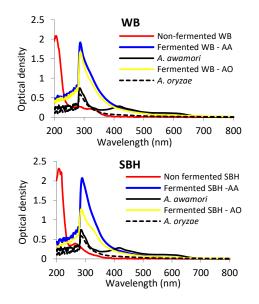
Figure 2: Visual observation of the colour changes during the course of the SSF of *A. oryzae* on three different solid substrates. [A]: wheat bran; [B]: rapeseed meal and [C]: soybean hulls.

Colorimetric technique-colour extraction

The whole fermented mass was blended using a food-processing blender. All the fermented samples were extracted with distilled water (40.0 mL) and shaken for 30 min on a rotary shaker (Infors A – CH 4103 Switzerland) at 250 rpm and 30 °C. Then the solid suspensions were centrifuged at 10,000 rpm for 10 min (4 °C). The clear supernatant was then used for determination of colour. After a suitable dilution, the absorbance of colour was then determined by UV-Vis specrophotometer at a wavelength of 300 nm. This was transformed into unit absorbance (UA_{300}/g) relative to the substrate dry mass by multiplying the absorbance by dilution factors and dividing by the substrate dry mass on the fermentation medium.
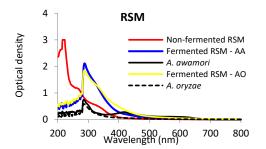

Figure 3: Comparison of typical normalised optical density spectra measured from colour solutions extracted from non-fermented substrate, fermented substrate with *A. awamori* [AA], fermented substrate with *A. oryzae* [AO]; fungus *A. awamori* and *A. oryzae* (7 days old, grown on whole wheat flour agar. [WB]: wheat bran; [SBH]: soybean and [RSM]: rapeseed meal.

RESULTS AND DISCUSSION

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 as shown in Figures 1 and 2. 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, which is known to be very challenging.

Preliminary Petri dish experiments

At first, the colour was extracted from fungi (spores and mycelia), non-fermented substrate, and fermented substrate using distilled water with shaking at 250 rpm for 1 h. This colourimetric technique explores the growth behaviour of *A. awamori* and *A. oryzae* from the

perspective of multiwavelength UV-VIS spectrophotemetry. The sample information is obtained from the spectrophotometric analysis of colour solution measured over a broad range of wavelengths (200 – 800 nm). Figure 3 shows typical normalised optical density spectra measured for non-fermented substrate, fermented substrate, and the fungi (extracted from spores and mycelia). The spectra for non-fermented substrate were different from those for fermented mass and those for fungi. These spectra clearly showed that there was a new component produced as a result of microbial activity of fungi during fermentation on the solid substrate. The peak around 300 nm is attributed to the maximum absorption of a new component from fermented substrate. This new component might be responsible for the colour characteristic reflected in the spectra.

Colour solutions extracted from fermented substrates at different times of fermentation were used to measure the abosorbance at different wavelengths; 260, 280, and 300 nm. Figure 4 shows the profiles of colour measurements at three different wavelenghts.This was carried out to decide which wavelength is more representative of the colour change. In Figure 4, the colour concentration gradually increased at the same rate when measured at 260, 280, and 300 nm.

The growth curve for a microorganism population is usually divided into four phases: lag phase, exponential phase or log phase, stationary phase, and death phase.

Notice that the measured optical density at 260, 280, and 300 nm, follows the expected behavioural growth curve. However, death phase was not clearly observed. This might be due to the SSF being carried out only for 7 days, which needs to be prolonged following the nature of SSF. Clearly, the changes in the colour of the fermented substrates during SSF can be related to the changes of the cells population

CONCLUSION

In this study, it is demonstarted that the colour changes observed during the growth of fungal SSF can be used to obtain quantitative information on their growth behavior. The increase in optical density and the subsequent scattering of light by microbial colour provide a good means to estimate fungal growth. This method is cost-effective, rapid, quantitative, and works well for colour in the logarithmic phase of growth. Effectively, extraction with distilled water, shaking at 250 rpm for 1 h at 30 °C was efficient to desorb all the colour fixed on the fungal cells, and the spectrophometric technique at wavelength of 300 nm was sensitive enough to estimate colour concentrations. Further studies were carried out to explore details of the phenomenon of colour changes during SSF and will be presented in the next report.
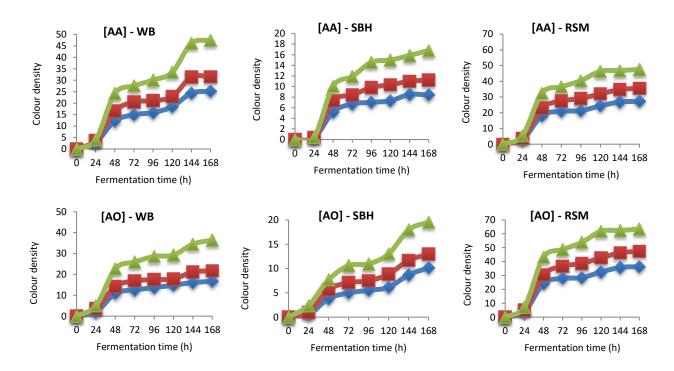

Figure 4: Growth profiles of *A awamori* [AA] and *A. oryzae* [AO] on three different solid substrates based on colour estimation at different wavelengths, expressed as unit absorbance (UA/g) [dry substrate]. [WB], wheat bran; [SBH], soybean hulls and [RSM], rapeseed (◆), 260 nm; (■), 280 nm and (▲): 300 nm.

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