



Investigating a non-destructive alternative for a preliminary evaluation of fungal growth in solid state fermentations



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ABSTRACT

Solid state fermentation (SSF) is an ancient technique which keeps attracting the attention of the food and biotechnology industries; however, a direct quantification of microbial biomass is still a fundamental challenge in this type of processes. Typically, growth is measured using indirect and destructive methods which do not allow a continuous evaluation of the evolution of microbial biomass within a single system. This article presents a non-destructive, quick and simple technique, based on digital imaging analysis (DIA) for the evaluation of growth in SSF laboratory experiments. DIA uses computational analysis of images from a SSF to measure areas and colour changes on a surface. The method can then be used to monitor microbial growth by assigning quantitative values for the growth of filamentous fungi. Firstly, studies on agar plates are used for the description of the method and to illustrate how it can be used to monitor fungal colony areas and densities. Following that, agro-industrial residues are used to demonstrate the application of the technique. DIA proved to be a practical and inexpensive tool to measure colony areas and densities. Furthermore, it is a non-destructive and non-intrusive method, which means that the evaluation of growth can be achieved within a single system.

1. Introduction

The intrinsic complexity of solid state fermentation (SSF) makes its control and application very difficult (Mitchell and Berovic, 2014) and, although experiencing growing interest, the evolution of SSF technologies has been rather slow. In the words of Mitchell et al. (1990) “Studies are hampered by the heterogeneity and complexity of the system and by problems in measuring fermentation parameters, the most critical of which is the biomass”. In SSF, the microorganism attaches to the solid substrate and, more specifically for the case of filamentous fungi, the mycelia penetrate the solid particles making an effective separation very difficult if not impossible (Pandey et al., 2008). The heterogeneity of the substrate and the substrate/fungal amalgam, together with the inability to perform the separation between the substrate and the microbe, make a direct quantification of microbial growth an unsolved fundamental problem for such processes (Manan and Webb, 2018a; Simeng et al., 2015; Steudler and Bley, 2015).

Since a direct quantification of growth is not possible some indirect methods, based on measuring biomass components, have been developed (Mitchell et al., 2000). Commonly, the estimation is performed by quantifying specific components of fungi such as ergosterol, glucosamine, nucleic acids, proteins, fungal specific phospholipid fatty acids

and spores (Steudler and Bley, 2015). However, one of the downsides of this type of measurement is that the relationship between cell components and fungal biomass may not be constant with time (Davey et al., 1991; Mitchell et al., 2000). Thus, although widely used in practice, results from such measurements only provide, at best, biomass estimates rather than absolute values of growth (Mitchell et al., 2004). Furthermore, such measurements require the homogenization of the samples during preliminary laboratory experiments. However, sacrificing whole cultures every time a measurement is required makes it impossible to follow the progress of a SSF in a single system and numerous replicates are necessary for the evaluation of growth, which makes the procedures destructive and impractical (Desgranges et al., 1991).

Colour measurement is widely used in many fields as a parameter that can be related to other properties of interest in a system. During growth on a solid substrate, a fungal colony expands while its mycelia density increases. As a result, there is an apparent variation in the colour of the substrate/fungal biomass mixture. Ramana Murthy et al. (1993), performed an investigation in which the change in colour of ground substrate, monitored using light reflectance, was an indicator of biomass presence. They compared the results from colour measurement to those from glucosamine and concluded that there was a good

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agreement between the different methods of quantification. Manan and Webb (2016, 2018b), reported a study based on spectrophotometer measurements of extracts from SSF systems to monitor the growth of fungi. They compared the results from the colour density measurements with those from enzyme production (as an indicator of growth) and concluded that there was good agreement between the methods. Nevertheless, as already mentioned, in addition to the length of the procedures, a drawback of both the above methods is still the need to sacrifice a plate every time a measurement is performed.

Colour measurement has also been studied as a method to identify fungal cultures on agar plates (Dörge et al., 2000), however, to the best of the authors' knowledge, direct measurement of colony colour for the evaluation of fungal growth in SSF has never been reported. The situation discussed above motivated the research reported in this article, to develop and investigate a quick and simple technique that could provide estimates of fungal biomass without the disadvantages of the more common alternatives. A novel idea based on the monitoring of colour variation in the SSF using digital images is presented.

1.1. Description of the method

Commonly, preliminary lab SSF experiments are carried out in petri dishes. In such cases, an alternative approach for the evaluation of growth is the measurement of colony areas in which larger areas indicate better growth (for cases where microbial growth is evaluated in agar media) (Brancato and Golding, 1953; Couri et al., 2006; Li and Wadsö, 2011; Mitchell et al., 1988). In this type of studies, a regular colony shape used to be critical to simplify the task of measuring areas (Reeslev and Kjølner, 1995). Nowadays, with the development of better software and digital imaging systems, it is possible to perform the measurement of the areas, regardless of their shape, in a quick and simple way (Li and Wadsö, 2011). Although a good indicator of growth, the measurement of area occupied by the colony is not sufficient to evaluate fungal biomass production (Bull and Trinci, 1977; Mitchell et al., 1988). It is possible that colonies of the same areas differ in fungal biomass, due to variations in their colony density.

The histogram (Fig. 1) is a fundamental component of any image manipulation software. It provides valuable information that allows the quantification of each colour in an image. In a histogram, the x-axis shows the intensity of the colour, which goes from 0 to 255, and the y-axis shows the number of pixels with that intensity. This feature can be used and exploited for the quantification of colour intensities of red, blue and green in an image. Computational software can calculate an average of the intensity of the colours and translate the final colour into a numerical value which corresponds to the mean.

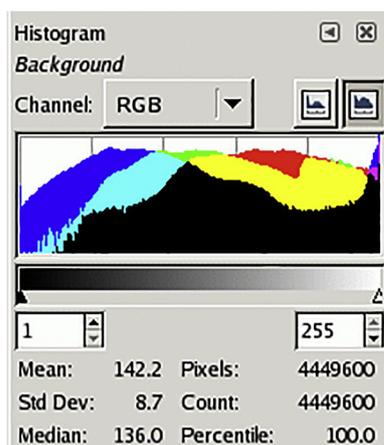


Fig. 1. Image of the histogram obtained in GIMP software. It can be obtained from any image (or part of it) and provides information of the number of pixels contained as well as the colour mean (mean) value.

In any plate, the growth of fungi would either reduce or increase the colour mean value. For the case of fungi with black spores, such as *A. awamori*, the colour mean should decrease as the SSF progresses. Conversely, fungi with a brighter colour, such as *Rhizopus oryzae* (white filamentous), would increase the colour mean values. The ease of selecting a specific range of colour and determining how many pixels match it, by just a couple of clicks, makes the method very quick and simple.

Fig. 2 exemplifies how digital imaging analysis could be used to evaluate fungal growth. The image simulates areas that, although having the same size, differ in fungal density. In this case, larger values represent a lesser dense colony than those from the lower values. In the example, a plate without growth would have a value of 255 and a plate completely covered by fungi, would have a value of 0.

In contrast to a greyscale image, in which a pixel can only acquire 256 values (corresponding to the intensity of the pixel with values from 0 to 255), a pixel in a colour image includes values for intensities of red, blue and green or $256 \times 256 \times 256$ which results in a total of 16,777,216 possible combinations, drastically increasing the sensitivity of the measurement. Due to their lower complexity and storage requirements, greyscale images tended to be preferred over colour images for solving mathematical models while saving space. Nonetheless, with the improvement in computing storage systems, the file sizes are not anymore a problem. Furthermore, in some cases, a measurement in colour or colour variations is indeed what really matters. The addition of an extra step to convert the colour image into grayscale does not bring any extra information but the opposite, reduces the info that we can extract from the photo. Due to these reasons, more and more scientific reports use colour images instead of greyscale (Dörge et al., 2000; Plavcan, 2004; Stevens et al., 2007).

2. Materials and methods

2.1. Inoculum preparation

The microorganism, *R. oryzae* NRRL 395 was obtained from LGC Standards, Teddington, United Kingdom. *A. awamori*, was obtained from the School of Chemical Engineering and Analytical Sciences, in the Faculty of Sciences & Engineering of The University of Manchester. The organisms were cultivated, following the method described in (López-Gómez et al., 2015), on petri dishes with potato dextrose agar (PDA) at 30 °C. After 6 days, spores of the fungi were collected with 10 mL of a sterile Tween 80 (0.01% v/v) solution. Spore suspensions, with approximately 2×10^6 spores/mL, were obtained, poured into universal bottles and stored at 4 °C.

2.2. Digital imaging analysis (DIA)

Images of the samples were taken using a NIKON D3200, the set up of the camera and samples is shown in Fig. 3. General Image Manipulation Program (GIMP), is a free and open-source raster graphics editing software and was used for the analysis of colony occupation areas and measurement of colour intensity in the images. Lighting conditions have an effect on the colour intensities of an image; therefore, it was necessary to take the images in a closed room with artificial light (without natural variations of light). Measurements of the colour mean using GIMP were performed without making any modification to the images.

2.3. Studies on agar plates

In a first step, studies on agar plates were carried out to evaluate the feasibility of measuring colour intensity. Potato dextrose agar plates were prepared, inoculated (at the centre of the plate) with 10 µL of a *R. oryzae* spore suspension (2×10^6 spores/mL) and incubated at 30 °C. Growth of the fungus was followed and images of the plates were taken

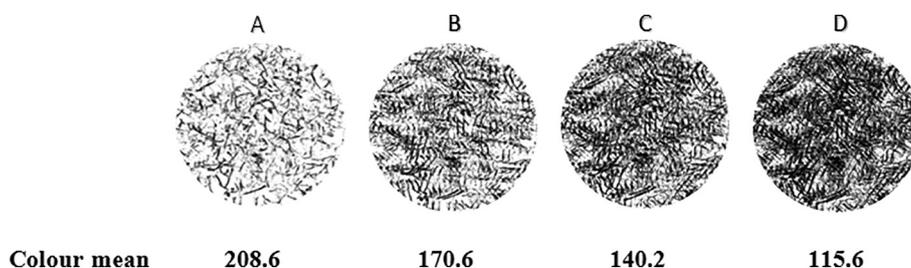


Fig. 2. Schematic representation of the variation of colony densities in areas of the same size. The values presented indicate the colour mean (measured using GIMP) for each plate. A value of 255 represents total brightness (white) and a value of 0 represents total darkness (black).

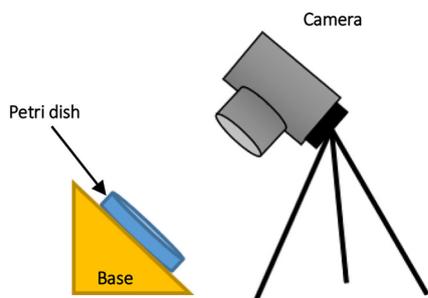


Fig. 3. Camera and sample set up for the capture of the images of growth in petri dishes during SSF.

at different time intervals.

Measurements of the colour mean were performed in three different delimited areas of the plate. The first one, the total area, corresponds to the full plate. The second, occupied area, corresponded to the section of the plate covered by the fungal mycelia. The third one, origin area, corresponded to a circular section (1.3 cm^2) with origin at the centre of the plate. Finally, a section from an image of a plate after 45 h of incubation was analysed. The section, which covered an area from the origin of the petri dish to its edge, was further divided into sub-sections and their colour mean values were measured.

2.4. Studies using sugarcane bagasse as a model solid support

Sugarcane bagasse was used as a typical natural solid substrate for experiments. It was obtained, vacuum packaged in plastic bags, from the School of Chemical Engineering and Analytical Science, Faculty of Science & Engineering, University of Manchester. Before the experiments, the substrate was ground using a kitchen blender. Following that, the solid particles were separated according to their size using a sieve. The particles with sizes in the range of $1400\text{--}850 \mu\text{m}$ were used for the experimentation. They were washed using copious amounts of tap water, to remove sugars and impurities, and after that placed in an oven at 60°C for 3 days. After drying, the material was stored in airtight plastic containers until used.

Before the cultivations, the moisture content of the bagasse was adjusted to 76% by mixing the dried substrate with sterile water or solutions containing different concentrations of yeast extract. The resulting sugarcane bagasse samples had a final concentration of 0, 10, 20, 25 and 40% (w/w) of yeast extract. Petri dishes were prepared (in triplicate) with 1.5 g of the samples.

Samples with concentrations of 0, 10 and 20% of yeast extract were inoculated by mixing $400 \mu\text{L}$ of an *A. awamori* spore suspension (2×10^6 spores/mL) into each sample. Samples with concentrations of 10, 25 and 40% were inoculated at the centre of the plate with same inoculum volume and concentration. After the inoculation, petri dishes were placed inside an incubator at 30°C . Finally, digital images were taken after 4 days of incubation.

2.5. Studies using an industrial residue

Industrial residues, namely, stillage and wheat bran, were obtained from the wheat based ethanol production plant of Cargill PLC., Trafford Park, Manchester, UK. In this plant, the residues are combined to produce moist feed for cattle. The stillage and the wheat bran were stored (for a maximum of 2 weeks) in sealed plastic bottles in a cold room at 4°C . The moist feed substrate was prepared by mixing wheat bran (34% w/w) and stillage (66% w/w). Initial water content of the moist feed was 50% w/w.

Samples of the moist feed with moisture contents of 45, 50, 55, 60 and 65% were prepared. The required amount of sterile distilled water was added to the stillage, before mixing it with the wheat bran, for the preparation of the samples at 55, 60 and 65% moisture content. For the case of the sample with 45% moisture content, the stillage was dried in an oven at 60°C until its moisture content was reduced from 70 to 63%, before being mixed with the wheat bran.

The inoculation was performed, using a spore suspension of *R. oryzae*, in the stillage before it was mixed in with the wheat bran, to achieve a spore concentration of approximately 10^4 spores/g_{moist feed}. Following that, triplicates containing approximately 20 g of the moist feed samples were prepared. The plates were incubated at 20°C and pictures were taken at different times for colour intensity measurement using GIMP.

3. Results and discussion

3.1. Studies on agar plates

Fig. 4 shows the average values for the colour mean from triplicates of the three different areas of the plates. At $t = 0$ h, right after the inoculation, the average colour mean of the plates was 50.1. At

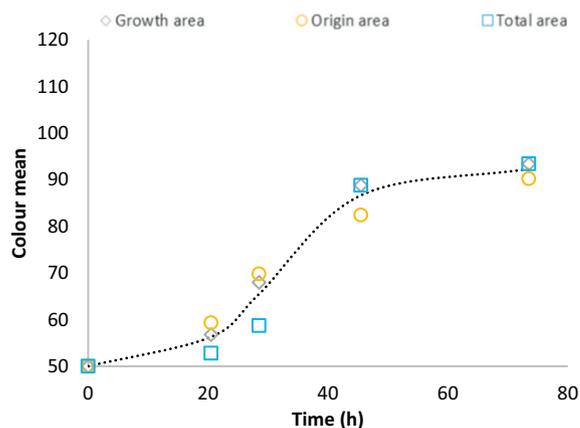


Fig. 4. Evolution of growth in a PDA plate inoculated with $10 \mu\text{L}$ of a *R. oryzae* spore suspension. Fungal density and colony occupation areas vary with time. The colour mean for all the sections increased with time resulting in a sigmoidal-shaped curve. The dotted line is the average of the three curves.

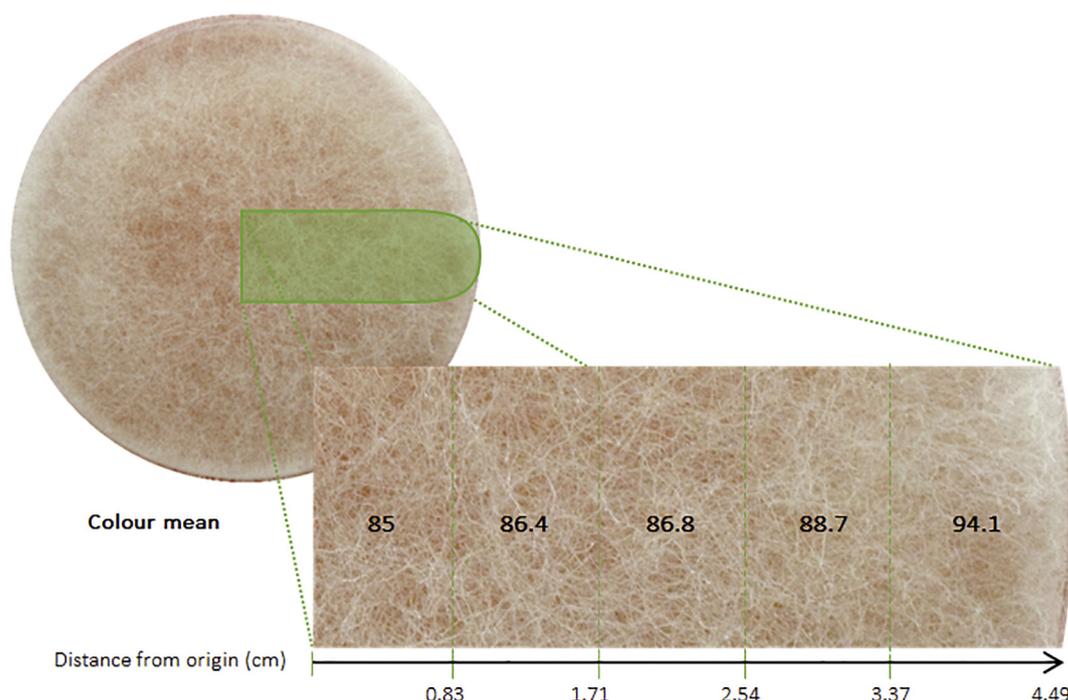


Fig. 5. Difference of colony density in terms of colour mean in a PDA plate inoculated with a *R. oryzae* spore suspension. Colour mean values suggest that sections of the colony closer to the edge of the plate showed a greater fungal density.

$t = 20.5$ h, the values for the colour mean of the origin area (1.3 cm^2) were larger than those of the total area. This difference indicates that the inoculum causes an increase in the colour mean value which shows that it is brighter than the background. In this particular case, an increase on the colour mean values over time indicates an increase in biomass.

Although all the colour mean values increased, as observed in the figure, there were differences between the origin area and the occupied area which indicated a difference in fungal density within the colony. By $t = 45.5$ h the colony already occupied all the plate hence the value for the occupied area remained constant (100%) after that time. Nevertheless, a change on the colour mean values could still be observed after that, indicating a change in fungal density.

Fig. 5, shows a close-up of a section of a plate at 45.5 h of incubation. As seen in the figure, there are colour variations within the colony.

As seen in Fig. 5, sections of the plate closer to the edge showed larger values for the colour mean than those near the centre of the plate. This difference was probably due to limitation of oxygen in the centre of the plate. Aeration in petri dishes occurs by diffusion and the oxygen that becomes available for the fungus enters the dish through the space between the dish and the lid. At $t = 0$, oxygen is readily available in all the plate and during the first 28.5 h the colour mean for the origin section goes from 50.1 to 69. However, after the colony occupied the outer sections of the petri dish, the variation in the colour mean for the origin section became slower, reaching 82.4 only after 45 h. In contrast, at the same time, ‘younger’ sections of the colony (nearer the edge of the plate) showed a colour mean value of already 94.1 and a maximum value of around 120 for some areas.

3.2. Combination of fungal density and occupation areas

A petri dish undergoes a change in its colour mean from the colour of the substrate (or background) at $t = 0$ to that of the microorganism as growth occurs and the colony becomes denser. When measuring colony densities there should be a maximum or minimum value for the colour of the colony which represents the maximum density of the colony, i.e. the actual value for the colour of the microorganism under

the specific lighting environment. In the given example this value is 120. Although in the colour histogram the scale goes from 0 to 255, there are certain colour values (x-axis) that are empty. In the example discussed above, the minimum value that the colour mean had was 50 (from the background) and the maximum value was 120.

The difference between the maximum and minimum colour mean values, i.e. the effective colour range of the image ($\Delta\bar{x}_{RGB}$) can be used as a reference to determine the relative change in colour of one sample.

$$\Delta\bar{x}_{RGB} = \bar{x}_{RGB,max} - \bar{x}_{RGB,min} \tag{1}$$

$$K_{RGB} = \frac{|\bar{x}_{RGB,i} - \bar{x}_{RGB,initial}|}{\Delta\bar{x}_{RGB}} \times 100 \tag{2}$$

$$\Delta\bar{x}_{RGB} = \text{colour range}$$

$$\bar{x}_{RGB,max} = \text{maximum colour mean value}$$

$$\bar{x}_{RGB,min} = \text{minimum colour mean value}$$

$$\bar{x}_{RGB,i} = \text{colour mean of the sample}$$

$$\bar{x}_{RGB,initial} = \text{initial colour mean of the sample}$$

$$K_{RGB} = \text{relative value of colour change (\%)}$$

The values for K_{RGB} can go from 0% (no growth) to 100% (maximum colony density). In the previous example, a sample with a colour mean value of 120 has a K_{RGB} equal to 100% which indicates that it has achieved the maximum density value possible for the colony. A sample with a colour mean value of 50 has a K_{RGB} equal to 0% which indicates that no growth has occurred. A comparison of the K_{RGB} and colour mean curves for the experiment is shown in Fig. 6.

The advantage of K_{RGB} is that it provides a clearer indication of how the process progresses. Since the values relate to the colour mean values that a colony has reached, and not to the full colour scale, it is possible to evaluate the growth.

3.3. Studies using sugarcane bagasse as a model solid support

Experimental work was performed to illustrate how DIA could be

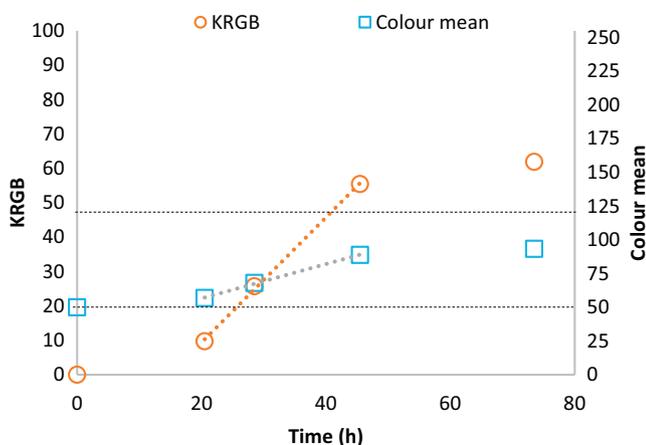


Fig. 6. Comparison between the colour mean and K_{RGB} curves. As observed, the curve for K_{RGB} shows a more pronounced change than the colour mean curve. The dashed lines are the upper and lower limits of the colour mean (120 and 50).

used for the evaluation of growth in SSF on natural substrates. In the experiments, sugarcane bagasse was used as the solid support and carbon source for the growth of *A. awamori*. The substrate was supplemented with a nitrogen source (yeast extract) in varying ratios and results after 4 days of incubation are shown in Fig. 7.

From the figure it would be possible, just visually, to define that growth in sample A is less than in sample B, which is less than in C. Although useful, that method of comparison does not give a clear indication of the degree of difference between samples. Differences in the values for the colour mean perhaps provide a better indicator of growth.

A similar experiment was performed in which the samples were first placed into petri dishes and then the inoculation was performed in the centre of the plate. Fig. 8 shows the results for the colour mean and colony occupation areas performed at 42, 66 and 91 h. The colour mean values are those corresponding only to the occupied area of each sample.

Fig. 8 shows the advantage of inoculating the plates in the centre rather than mixing the inoculum with the substrate. Even without colour intensity measurements it is clear the effect that the N source has on the growth in terms of radius of the colonies. The plate with lower concentration of N produced the smallest occupied area (18%) whereas the plate with more N showed the largest area (40%). However, the difference between fungal densities is not obvious, unlike in Fig. 9

where even without DIA it is possible to observe that growth density is greater as the yeast extract content increases.

Fig. 9a gives an indication of how fast the colonies expand on the plates. For example, in the figure, each sample contains a data point at around 40% colony occupation area. Although it is possible to state that as the yeast extract content increases the colony expansion rate increases, it is not possible to determine which colony is denser when they reach around 40% occupation area. Conversely, while the values for K_{RGB} (Fig. 9b) give an indication of colony density, they do not distinguish colony size. By combining both measurements a much more complete picture of growth can be obtained (Fig. 10).

Such a graph can be used to compare growth in samples taking into consideration how large and dense fungal colonies are. In the graph, data points that approximate 100% indicate better growth. Let us consider again the case of occupation areas of almost the same size (39.02%, 39.60%). Even though both values for the colour mean are similar, the data point for the sample with more yeast extract has a higher K_{RGB} . Similarly, two data points with the same K_{RGB} (65%) showed different occupation areas (47% and 63%) indicating better growth for the 40% yeast extract case.

Values from Fig. 10 can be used to calculate a ‘Digital imaging analysis coefficient of growth’ for the evaluation of growth:

$$K_{DIA} = \frac{a_g \times K_{RGB}}{100} \tag{3}$$

K_{DIA} = Digital Imaging Analysis coefficient of growth

a_g = area of growth

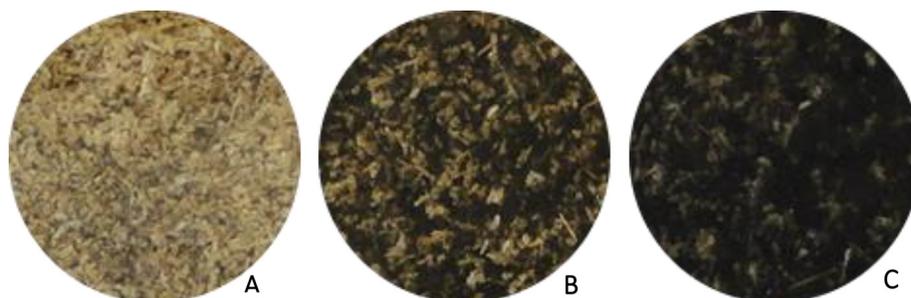
As observed, K_{DIA} is a relationship between the occupation areas and colour mean i.e. colony density. K_{DIA} values for the samples were calculated and the results are shown in Fig. 11.

3.4. Studies using an industrial residue

The positive effect that moisture content has on growth is clear in Fig. 12.

Images taken at 4 and 6 days show clearly that, for the samples tested, higher moisture contents resulted in more apparent growth. The standard sample (50% moisture content) was the second lowest with respect to growth. In addition to increasing the amount of growth, higher moisture contents also accelerated the appearance of visible colonies. Evidently, analysis of this type (qualitative) is very useful and can be further enhanced if DIA is applied as shown in Fig. 13 (Values of $\bar{x}_{RGB,max} = 160$ and $\bar{x}_{RGB,min} = 35$ were used for the calculation of K_{RGB}).

The graph shows that increasing the moisture content not only



Sample	A	B	C
Yeast extract	0 %	10 %	20 %
Colour mean	130	53.5	32.3

Fig. 7. Pictures taken after 4 days of a SSF on sugarcane bagasse mixed with yeast extract in different ratios. As observed, the quantification of colour mean values allows a rapid estimation of growth in the plates.

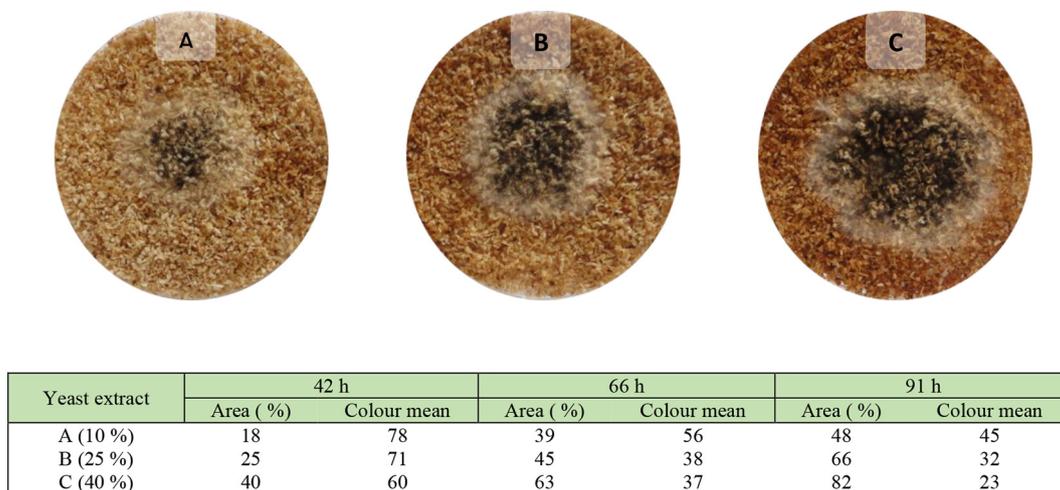


Fig. 8. Effect of yeast extract on the growth of *A. awamori* on sugarcane bagasse. Images of the systems after 42 h of incubation. As observed a higher yeast extract concentration has a positive effect on the colony size and on colour mean values.

benefits growth but also that there is an apparent linear relationship between these two variables for the samples tested. The sample with 65% moisture content had the highest K_{RGB} at every sample time. At 6 days, the sample with 65% moisture content reached a K_{RGB} of 98% while the sample with lowest moisture content (45%) barely reached 30%. Clearly, increasing the moisture content of the moist feed enhances the growth of *R. oryzae*.

4. Discussion

Digital imaging analysis (DIA) proved to be a practical tool that can be used to measure colony areas and densities in a quick and simple way. Besides, it is a non-destructive non-intrusive method, which means that estimation of growth can be followed in the same sample. In situations where there are many samples to analyse, DIA can be easier to implement than other more common methods to estimate growth. The technique provides values that allow an objective comparison between plates, making it of higher quality than just visual evaluation. The parameter K_{RGB} (relative value of colour intensity change) is a normalised value that allows the evaluation of growth on a surface based on its colour change. This value allows the comparison between samples and also to determine how far a colony is from reaching its maximum colour density i.e. colony density. A combination between colony occupation areas and K_{RGB} was used to create K_{DIA} (Digital imaging analysis coefficient of growth). This coefficient strengthens the applicability of colony occupation areas because it also includes the density of the colonies. Hence, it is possible to compare colonies of the

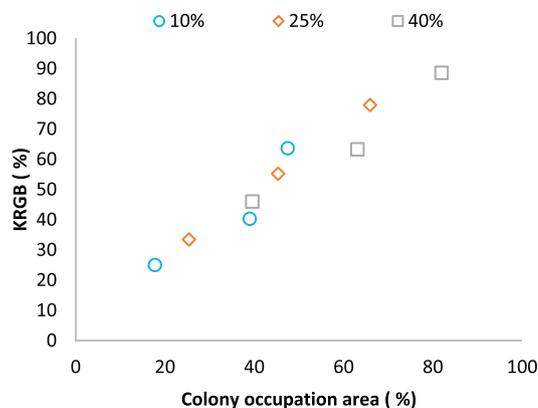


Fig. 10. Relationship between K_{RGB} and occupation areas. K_{RGB} values were calculated based on $\bar{x}_{RGB,min} = 13$ and $\bar{x}_{RGB,max} = 100$.

same occupation areas.

Evidently, DIA provides information only from the surface of a SSF system. Growth occurring in sections under the surface of the substrate cannot be monitored using this technique. This clearly is a limitation in systems where homogeneous growth throughout the substrate is desired. Nevertheless, because the surface is where oxygen is more readily available, growth on those areas should, in principle, occur at its best. Hence, DIA can be used in preliminary experiments in which the evaluation for the best conditions for growth is performed. Additionally, in

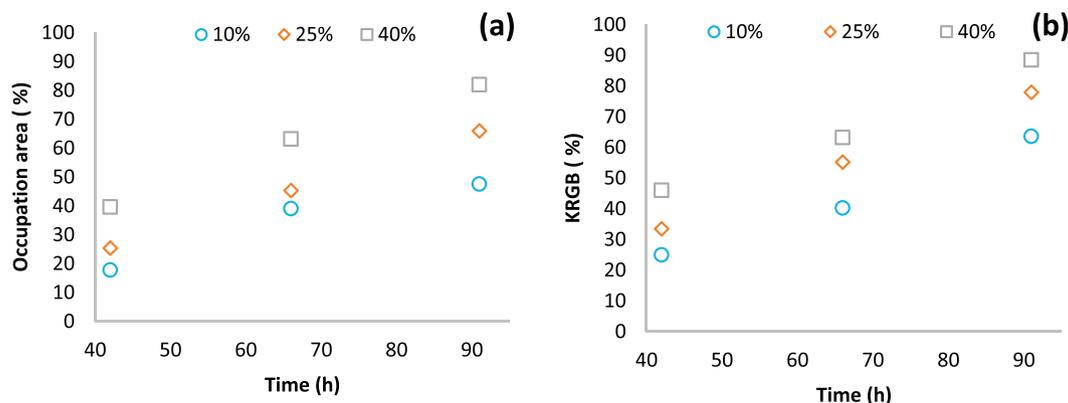


Fig. 9. Effect of yeast extract concentration on colony occupation areas (a) and K_{RGB} (b). K_{RGB} values were calculated based on $\bar{x}_{RGB,min} = 13$ and $\bar{x}_{RGB,max} = 100$.

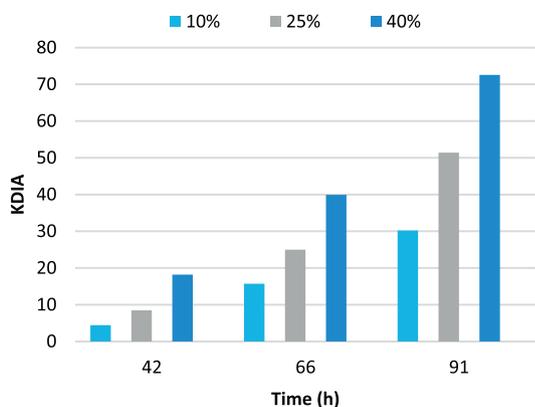


Fig. 11. K_{DIA} values for *A. awamori* grown on sugarcane bagasse supplemented with different concentrations of yeast extract.

systems where mixing occurs, microbial growth should produce colour intensity changes within the substrate. Therefore, in principle, the variation in colour intensity could be used also in that type of system. Probably, the analysis would include the determination of a maximum/minimum colour mean value (indicating best growth) which could be used for the evaluation of growth across the whole substrate bed.

DIA is based on colour intensity variations, thus only fungal colonies with different colour intensity from the substrate matrix would influence the change of colour intensity in an image. That being said, the software is highly sensitive, and it is capable of detecting even small differences. Further experimental work is necessary to determine the minimum difference in colour intensities, between the substrate and the microorganism, necessary to achieve an effective quantification. Future studies should focus on the applicability of DIA to more complex SSF.

Additionally, costs associated with the technique are just those related to the camera and digital imaging software making it a very cost effective method. Moreover, the standardisation of aspects such as lighting environment, geometry of the sample, distance from the sample to the camera, measurement of initial colour intensity of the

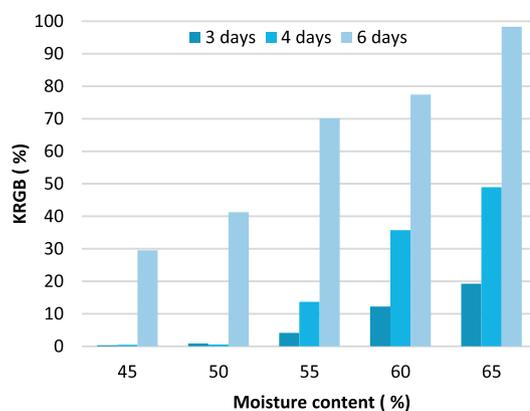


Fig. 13. Effect of the moisture content on SSF of stillage and wheat bran mixture samples on K_{RGB} . Plates with higher moisture content showed better growth of *R. oryzae* and higher K_{RGB} . K_{RGB} values were calculated based on $\bar{x}_{RGB,min} = 35$ and $\bar{x}_{RGB,max} = 160$.

sample and so on, would allow an objective comparison between experiments carried out anywhere in the world. The possibility of developing automated systems coupling SSF and DIA would further enhance the value of the method.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mimet.2019.03.021>.

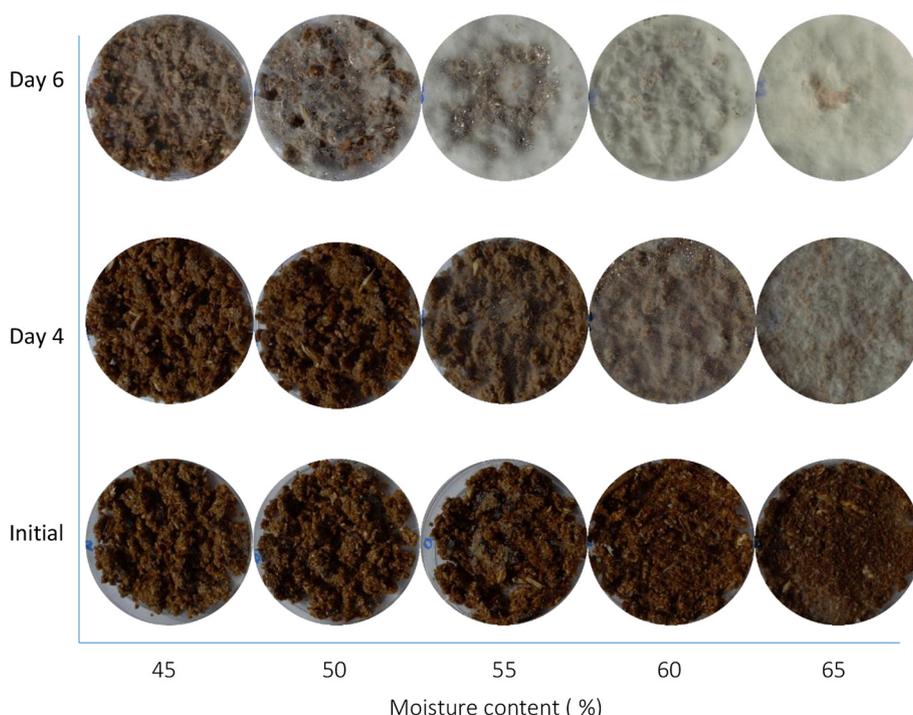


Fig. 12. Evolution of a SSF of stillage and wheat bran mixture samples with different moisture contents. Plates at 4 and 6 days show that growth was better in plates with higher moisture content.

References

- Brancato, F.P., Golding, N.S., 1953. The diameter of the Mold Colony as a reliable measure of growth. *Mycol. Soc. Am.* 45, 848–864.
- Bull, A.T., Trinci, P.J., 1977. The physiology and metabolic control of fungal growth. In: *Advance in Microbial Physiology*. Academic Press Inc, pp. 1–84.
- Couri, S., Mercês, E.P., Neves, B.C.V., Senna, L.F., 2006. Digital image processing as a tool to monitor biomass growth in *Aspergillus niger* 3T5B8 solid-state fermentation: preliminary results. *J. Microsc.* 224, 290–297. <https://doi.org/10.1111/j.1365-2818.2006.01699.x>.
- Davey, C.L., Peñalzo, W., Kell, D.B., Hedger, J.N., 1991. Real-time monitoring of the accretion of *Rhizopus oligosporus* biomass during the solid-substrate Tempe fermentation. *World J. Microbiol. Biotechnol.* 7, 248–259. <https://doi.org/10.1007/BF00328998>.
- Desgranges, C., Vergoignan, C., Georges, M., Durand, A., 1991. Biomass estimation in solid state fermentation I. Manual biochemical methods. *Appl. Microbiol. Biotechnol.* 35. <https://doi.org/10.1007/BF00184686>.
- Dörge, T., Carstensen, J.M., Frisvad, J.C., 2000. Direct identification of pure *Penicillium* species using image analysis. *J. Microbiol. Methods* 41, 121–133. [https://doi.org/10.1016/S0167-7012\(00\)00142-1](https://doi.org/10.1016/S0167-7012(00)00142-1).
- 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, 105–111. <https://doi.org/10.1007/s10973-010-1251-5>.
- López-Gómez, J.P., Blanco-Rosete, S., Webb, C., 2015. Extending shelf life of wheat based animal feed using solid state bioprocessing. *Chem. Eng. Res. Des.* <https://doi.org/10.1016/j.cherd.2015.10.049>.
- Manan, M.A., Webb, C., 2016. Extracted Substrate Colour as an Indicator of Fungal Growth in Solid State Fermentation 12. pp. 445–449.
- Manan, M.A., Webb, C., 2018a. Estimation of growth in solid state fermentation : a review. *Malays. J. Microbiol.* 14, 61–69.
- Manan, M.A., Webb, C., 2018b. Colour changes as an indicator for estimating fungal growth in solid state fermentation. *Malays. J. Microbiol.* 14, 254–264.
- Mitchell, D.A., Berovic, M., 2014. Solid state bioprocessing. In: *Principles of Bioprocessing Engineering*. University of Ljubljana, pp. 244–276.
- Mitchell, D.A., Docile, H.W., Greenfield, P.F., 1988. Agar plate growth studies of *Rhizopus oligosporus* and *Aspergillus oryzae* to determine their suitability for solid-state fermentation. *Appl. Microbiol. Biotechnol.* 598–602.
- Mitchell, D.A., Berovic, M., Krieger, N., 2000. Biochemical engineering aspects of solid state bioprocessing. *Adv. Biochem. Eng. Biotechnol.* 68, 61–138.
- Mitchell, D.A., von Meien, O.F., Krieger, N., Dalsenter, F.D.H., 2004. A review of recent developments in modeling of microbial growth kinetics and intraparticle phenomena in solid-state fermentation. *Biochem. Eng. J.* 17, 15–26. [https://doi.org/10.1016/S1369-703X\(03\)00120-7](https://doi.org/10.1016/S1369-703X(03)00120-7).
- Mitchell, D.A., Greenfield, P.F., Doelle, H.W., 1990. *World J Microbiol Biotechnol* 6, 201. <https://doi.org/10.1007/BF01200942>.
- Pandey, A., Soccol, C.R., Larroche, C., 2008. *Current Developments in Solid-State Fermentation*. Springer New York <https://doi.org/10.1007/978-0-387-75213-6>.
- Plavcan, J.M., 2004. A simple method for measuring colour in wild animals: validation and use on chest patch colour in geladas (*Theropithecus gelada*). *Sex. Sel. Primates New Comp. Perspect.* 94, 230–252. <https://doi.org/10.1111/j.1095-8312.2008.00981.x>.
- Ramana Murthy, M.V., Thakur, M.S., Karanth, N.G., 1993. Monitoring of biomass in solid state fermentation using light reflectance. *Biosens. Bioelectron.* 8, 59–63. [https://doi.org/10.1016/0956-5663\(93\)80044-P](https://doi.org/10.1016/0956-5663(93)80044-P).
- Reeslev, M., Kjølter, A., 1995. Comparison of biomass dry weights and radial growth rates of fungal colonies on media solidified with different gelling compounds. *Appl. Environ. Microbiol.* 61, 4236–4239.
- Simeng, Z., Sacha, G., Isabelle, H.-G., Marie-Noëlle, R., 2015. A PCR-based method to quantify fungal growth during pretreatment of lignocellulosic biomass. *J. Microbiol. Methods* 115, 67–70. <https://doi.org/10.1016/j.mimet.2015.05.024>.
- Stuedler, S., Bley, T., 2015. Biomass estimation during macro-scale solid-state fermentation of basidiomycetes using established and novel approaches. *Bioprocess Biosyst. Eng.* 38, 1313–1323. <https://doi.org/10.1007/s00449-015-1372-0>.
- Stevens, M., Párraga, C.A., Cuthill, I.C., Partridge, J.C., Troscianko, T., 2007. Using digital photography to study animal coloration. *Biol. J. Linn. Soc.* 90, 211–237. <https://doi.org/10.1111/j.1095-8312.2007.00725.x>.